Advances in Experimental Medicine and Biology 850

Stephen P. Schoenberger
Peter D. Katsikis
Bali Pulendran *Editors*

Crossroads Between Innate and Adaptive Immunity V

Aegean conferences

Springer

Advances in Experimental Medicine and Biology

Volume 850

Advances in Experimental Medicine and Biology presents multidisciplinary and dynamic findings in the broad fields of experimental medicine and biology. The wide variety in topics it presents offers readers multiple perspectives on a variety of disciplines including neuroscience, microbiology, immunology, biochemistry, biomedical engineering and cancer research. Advances in Experimental Medicine and Biology has been publishing exceptional works in the field for over 30 years and is indexed in Medline, Scopus, EMBASE, BIOSIS, Biological Abstracts, CSA, Biological Sciences and Living Resources (ASFA-1), and Biological Sciences. The series also provides scientists with up to date information on emerging topics and techniques. 2014 Impact Factor: 1.958

More information about this series at http://www.springer.com/series/5584

Stephen P. Schoenberger · Peter D. Katsikis ·
Bali Pulendran
Editors

Crossroads Between Innate and Adaptive Immunity V

Springer

Editors
Stephen P. Schoenberger
Laboratory of Cellular Immunology
La Jolla Institute for Allergy and Immun
La Jolla
USA

Peter D. Katsikis
Department Microbiology & Immunology
Drexel University College of Medicine
Philadelphia
Pennsylvania
USA

Bali Pulendran
Yerkes National Primate Research Center
Emory University Emory Vaccine Center &
Atlanta
Georgia
USA

ISSN 0065-2598 ISSN 2214-8019 (electronic)
Advances in Experimental Medicine and Biology
ISBN 978-3-319-15773-3 ISBN 978-3-319-15774-0 (eBook)
DOI 10.1007/978-3-319-15774-0

Library of Congress Control Number: 2015948173

Springer Cham Heidelberg New York Dordrecht London

Printed on acid-free paper

Springer International Publishing AG Switzerland is part of Springer Science+Business Media (www.springer.com)

Contents

Contributors

Evangelos Andreakos Department of Immunology, Center for Translational and Clinical Research, Biomedical Research Foundation, Academy of Athens, Athens, Greece

Vincenzo Barnaba Dipartimento di Medicina Interna e Specialità Mediche, Sapienza Università di Roma, Rome, Italy

Istituto Pasteur -Fondazione Cenci Bolognetti, Rome, Italy

Aimee M. Beaulieu Memorial Sloan Kettering Cancer Center, New York, NY, USA

Ioanna E. Galani Department of Immunology, Center for Translational and Clinical Research, Biomedical Research Foundation, Academy of Athens, Athens, Greece

John T. Harty Interdisciplinary Graduate Program in Immunology, University of Iowa, Iowa City, IA, USA

Department of Microbiology, University of Iowa, Iowa City, IA, USA

Katharina Hochheiser Institute of Experimental Immunology(IMMEI), Rheinische Friedrich-Wilhelms University, Bonn, Germany

Kwang Soon Kim Academy of Immunology and Microbiology (AIM), Institute for Basic Science (IBS), Pohang, Republic of Korea

Department of Integrative Biosciences and Biotechnology, Pohang University of Science and Technology (POSTECH), Pohang, Republic of Korea

Eynav Klechevsky Washington University School of Medicine, St. Louis, USA

Ourania Koltsida Department of Immunology, Center for Translational and Clinical Research, Biomedical Research Foundation, Academy of Athens, Athens, Greece

Christian Kurts Institute of Experimental Immunology(IMMEI), Rheinische Friedrich-Wilhelms University, Bonn, Germany

Grégoire Lauvau Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY, USA

Sharline Madera Memorial Sloan Kettering Cancer Center, New York, NY, USA

Rebecca Newman Laboratory of Lymphocyte Signalling and Development, The Babraham Institute, Babraham, UK

Silvia Piconese Dipartimento di Medicina Interna e Specialità Mediche, Sapienza Università di Roma, Rome, Italy

Saïdi M'Homa Soudja Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY, USA

Gabriel R. Starbeck-Miller Interdisciplinary Graduate Program in Immunology, University of Iowa, Iowa City, IA, USA

Joseph C. Sun Memorial Sloan Kettering Cancer Center, New York, NY, USA

Charles D. Surh Academy of Immunology and Microbiology (AIM), Institute for Basic Science (IBS), Pohang, Republic of Korea

Department of Integrative Biosciences and Biotechnology, Pohang University of Science and Technology (POSTECH), Pohang, Republic of Korea

Division of Developmental Immunology, La Jolla institute for Allergy and Immunology, La Jolla, CA, USA

Martin Turner Laboratory of Lymphocyte Signalling and Development, The Babraham Institute, Babraham, UK

E. J. Wherry Department of Microbiology, Institute for Immunology, Philadelphia, PA, USA

D. Zehn Division of Immunology and Allergy, Lausanne University Hospital, Lausanne, Switzerland

Chapter 1
Type III interferons (IFNs): Emerging Master Regulators of Immunity

Ioanna E. Galani, Ourania Koltsida and Evangelos Andreakos

Abstract Lambda interferons (IFN-λs), type III interferons or interleukins 28 and 29 are the latest addition to the class II cytokine family. They share low homology with the interferon (IFN) and IL-10 cytokine families, yet they exhibit common and unique activities, the full spectrum of which still remains incompletely understood. Although initially described for their antiviral functions, it is now appreciated that IFN-λs also mediate diverse antitumor and immune-modulatory effects, and are key determinants of innate immunity at mucosal sites such as the gastrointestinal and respiratory tracks. Here, we are reviewing the biological functions of IFN-λs, the mechanisms controlling their expression, their downstream effects and their role in the maintenance of homeostasis and disease. We are also exploring the potential application of IFN-λs as novel therapeutics.

Keywords Lambda interferons (IFN-λs) · Type III interferons · Interleukin 28 · Interleukin 29 · Antiviral activity · Mucosal immunity

Type III IFNs or Lambda IFNs: A Unique Interferon Family

Interferons (IFNs) were originally described over 50 years ago as anti-viral proteins. However, they have since been shown to regulate multiple other biological processes including cell proliferation and survival, inflammation and immunity. They have also been implicated in many pathological situations including infections, cancer and autoimmunity. The diversity and functional organization of this family became obvious in the 1980s with the cloning of the IFN-β gene and several isoforms of IFN-α. This led to the realization that most of these molecules are part of a single family, the type I IFN or IFN-α/β family, and signal through the same heterodimeric receptor comprising the IFNαR1 and IFNαR2 chains. In humans,

E. Andreakos (✉) · I. E. Galani · O. Koltsida
Department of Immunology, Center for Translational and Clinical Research, Biomedical Research Foundation, Academy of Athens, Athens 11527, Greece
e-mail: vandreakos@bioacademy.gr

S. P. Schoenberger et al. (eds.), *Crossroads Between Innate and Adaptive Immunity V,*
Advances in Experimental Medicine and Biology 850, DOI 10.1007/978-3-319-15774-0_1

the type I IFN family includes 13 IFN-α subtypes, IFN-β and IFN-ω, -κ, and -ε. In addition, there is a second family, the type II IFN family that has a sole member, IFN-γ. Although IFN-γ is not directly induced by viruses, it also has antiviral activity (Young and Bream 2007).

More recently, a third family of IFNs, termed as type III or IFN-λs was described (Kotenko et al. 2003; Sheppard et al. 2003). Type III IFNs consist of three members in humans, IFN-λ1, -λ2 and -λ3, which are also known as IL-29, IL28A and IL-28B, respectively. All these genes are closely positioned on human chromosome 19. In mice, a similar gene organization is found on chromosome 7, although IFN-λ1 is a pseudogene; there is a stop codon in the first exon that prevents the full-length transcript from being expressed (Lasfar et al. 2006). Notably, a fourth member of the IFN-λ family, IFN-λ4, has also been described in a subfraction of the human population. The gene for IFN-λ4 is found upstream of IFN-λ3 but in the majority of individuals this is out of frame and thus non-functional. A functional version of IFN-λ4 can only be found in humans bearing a specific genetic polymorphism that introduces a frameshift mutation that enables transcription (Prokunina-Olsson et al. 2013). The genomic organization of IFN-λ genes is shown in Fig. 1.1.

As expected, IFN-λs share homology with type I and type II IFNs. However, they also share homology with the IL-10 superfamily and are structurally more

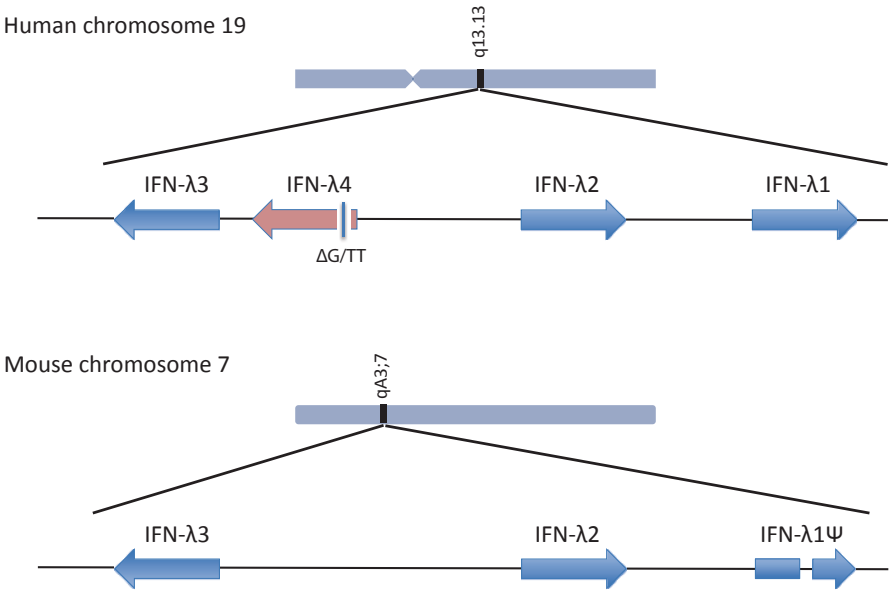

Fig. 1.1 Schematic representation of the genomic organization of IFN-λ genes in human chromosome 19 and mouse chromosome 7. There are three functional IFN-λ genes in humans, as well as the newly characterized IFN-λ4 gene in individuals bearing the ΔG genotype, whereas only two genes in the mouse genome encode functional proteins, IFN-λ2 and IFN-λ3; IFN-λ1 is a pseudogene in mouse, due to a stop codon in the first exon and lack of exon 2. The genes are transcribed in the direction indicated by the *arrows*

similar to IL-10 family members than to type I IFNs (Gad et al. 2009). At all cases, this homology is low: 15–19% in amino acid identity with IFN-α and IL-22, 11–13% in amino acid identity with IL-10 (Sheppard et al. 2003). Among the IFN-λ family, IFN-λ2 and -λ3 are more closely related to one another than either of them is to IFN-λ1: IFN-λ1 and IFN-λ2 share 81% amino acid identity, while IFN-λ2 and IFN-λ3 are almost indistinguishable, with 96% amino acid identity (Sheppard et al. 2003). This is because IFN-λ2 and -λ3 have occurred via a more recent duplication event during evolution. Noteworthy, in contrast to type I IFNs that completely lack introns, IFN-λ genes have a similar organization to the IL-10 gene family, with multiple exons and introns (Sheppard et al. 2003).

IFN-λs signal via a distinct receptor complex, named IL-28R/LICR2, which includes a unique alpha chain, the IFN-λR1/IL28Rα/CRF2-12/LICR2 chain that has the higher affinity for the ligand and confers ligand specificity, and the IL-10Rβ/CRF2-4 chain common to all IL-10 superfamily members (IL-10, IL-19, IL-20, IL-22, IL-24 and IL-26). IL-10Rβ/CRF2-4 has a relatively long intracellular domain and comprises a docking site for downstream signaling (Kotenko et al. 2003; Sheppard et al. 2003). A secreted soluble IFN-λR1 splice variant lacking exon 6 has also been reported and potentially mediates inhibitory effects to the action of IFN-λs (Witte et al. 2009). Of interest, identification of type III IFN-like genes in the zebrafish genome suggests that IFN-λ genes have preceded type I IFNs during evolution, and may constitute a more ancestral antiviral protection system (Levraud et al. 2007). In agreement with that, genomic analysis has identified IFN-λ genes is several mammalian species (Fox et al. 2009) and birds (Karpala et al. 2008).

Regulation of IFN-λ Expression

IFN-λs were initially described to be induced in cell lines in response to diverse DNA and RNA viruses including the double–stranded DNA virus (dsDNA) herpes simplex virus type 2 (HSV-2), the double-stranded RNA (dsRNA) Reovirus (Reo), the positive single-stranded RNA (+ssRNA) viruses Sindbis virus (SV), Dengue virus 2 (DV) and encephalomyocarditis virus (EMCV), and the negative single-stranded RNA (-ssRNA) viruses vesicular stomatitis virus (VSV), Sendai Virus (SeV) and Influenza A virus (IAV) (Ank et al. 2006; Kotenko et al. 2003; Sheppard et al. 2003). They were subsequently shown to be triggered in primary cells such as human monocyte-derived dendritic cells (MDDCs) and plasmacytoid DCs (pDCs) upon IAV and SeV infection (Coccia et al. 2004; Osterlund et al. 2005) or TLR stimulation (Coccia et al. 2004; Kotenko et al. 2003; Sheppard et al. 2003), primary human monocytes, macrophages and bronchial epithelial cells upon rhinovirus (RV) infection (Contoli et al. 2006), and primary type II alveloar epithelial cells upon IAV infection (Wang et al. 2009). TLR-3 stimulation can also induce IFN-λs in human neuronal cells (Zhou et al. 2009), and airway epithelial cells (Ioannidis et al. 2013). More recently, Lauterbach et al. demonstrated that among various DC subsets, mouse $CD8\alpha^+$ DCs, and their human equivalent $BDCA3^+$ DCs, were

the main producers of IFN-λs in response to poly (I:C) administration (Lauterbach et al. 2010). Of interest, IFN-λs were additionally shown to be induced by Gram$^+$ bacterial infections of epithelial cells (Bierne et al. 2012; Lebreton et al. 2011).

IFN-λs are regulated at the level of transcription and depend on intracellular sensors of viral infection and downstream molecules such as TLR3, retinoic acid-inducible gene I (RIG-I), interferon-β promoter stimulator 1 (IPS-1), TANK-binding kinase 1 (TBK1), and interferon-regulated factors (IRFs), which also control type I IFN production (Onoguchi et al. 2007). Accordingly, several IRF and NF-κB binding sites have been identified in the promoter regions of the human IFN-λ genes on the basis of computational approaches, which may be used differentially to drive their expression in different cells and in response to different stimuli (Onoguchi et al. 2007; Osterlund et al. 2007). Thus, IFN-λ1 is mostly regulated by virus-activated IRF3 and IRF7, similar to the IFN-β gene, whereas IFN-λ2/3 gene expression is mainly controlled by IRF7, resembling the induction of IFN-α genes (Osterlund et al. 2007). As IRF7 is induced via the Jak-Stat pathway in most cells, this suggests the existence of a positive feedback loop that regulates IFN-λs (Marie et al. 1998; Sato et al. 1998). This may also explain why pDCs that constitutively possess high levels of IRF7 are the abundant sources of type I IFNs following viral infection (Fitzgerald-Bocarsly 1993; Siegal et al. 1999). IRF1 is also critical for IFN-λ1 mRNA induction in certain experimental settings such as SeV infection of Huh7 hepatocytes (Odendall et al. 2014) and is triggered by RIG-I like receptors (RLRs) and their adaptor protein MAVS located on peroxisomes. In human epithelial cells, this seems to be the predominant pathway induced by intracellular pathogens including viruses (such as reovirus, SeV and DV) and bacterial pathogens (such as Listeria monocytogenes) that drives IFN-λ1 production (Odendall et al. 2014).

NF-κB is also involved in the induction of IFN-λs. A cluster of NF-κB-binding sites distal to the IFN-λ1 promoter has been found that is required for maximal IFN-λ1 production in human MDDCs following LPS stimulation (Thomson et al. 2009). Nevertheless, although disruption of both IRF and NF-κB sites significantly reduced transcription of IFN-λs, residual activation could still be detected, suggesting yet unidentified cis-regulatory elements that guide IFN-λ expression (Onoguchi et al. 2007). Furthermore, the organization of IFN-λ genes with multiple exons and introns suggests additional post-transcriptional regulation, absent from type I IFN genes, which may be crucial for IFN-λ production. Therefore, it appears that although type I and type III IFNs share many of their functions, they are not controlled through identical mechanisms but rather involve distinct regulators that enable their fine-tuning during inflammation and immunity.

IFN-λ Receptor Distribution and Downstream Signaling

As opposed to IFNAR that is ubiquitously found in all nucleated cells, IFNλR1 has a more restricted pattern of expression. It is mostly expressed by cells of epithelial origin including respiratory, intestinal and reproductive tract epithelial cells,

hepatocytes and keratinocytes (Mordstein et al. 2010; Sommereyns et al. 2008; Witte et al. 2009), although cells of the myeloid lineage such as cDCs (Mennechet and Uze 2006; Koltsida et al. 2011) and pDCs (Megjugorac et al. 2009; Yin et al. 2012) also express the receptor. Downstream signaling through IFN-λR follows a pattern common to the class II cytokine receptor family. IFN-λs initially bind to IFNλR1/IL-28Rα, their specific ligand-binding chain, and the IL-10Rβ chain is then recruited to the complex. The IFNλR complex then signals through the Janus kinases-signal transducers and activators of transcription (Jak-STAT) pathway in a cascade of events that resembles that triggered by type I IFNs (Kotenko et al. 2003). This leads to the phosphorylation of several STATs, predominantly STAT1 and STAT2 (Donnelly et al. 2004) and the formation of STAT dimers that combine with the cytosolic protein IRF-9 (p48) to form the IFN-stimulated gene factor 3 (ISGF3). ISGF3 translocates to the nucleus to guide the transcription of IFN-regulated genes (ISGs) that function to inhibit viral replication, thus inducing an anti-viral state in these cells (Ank et al. 2006, 2008) (Fig. 1.2). Among the ISGs induced, Myxovirus resistance gene A (MxA) and 2',5'-oligoadenylate synthetase 1 (OAS1) are shared by type I and III IFNs and are considered to mediate a significant part of the anti-viral activities of IFNs (Kotenko et al. 2003). IFN-λ signaling can be blocked by

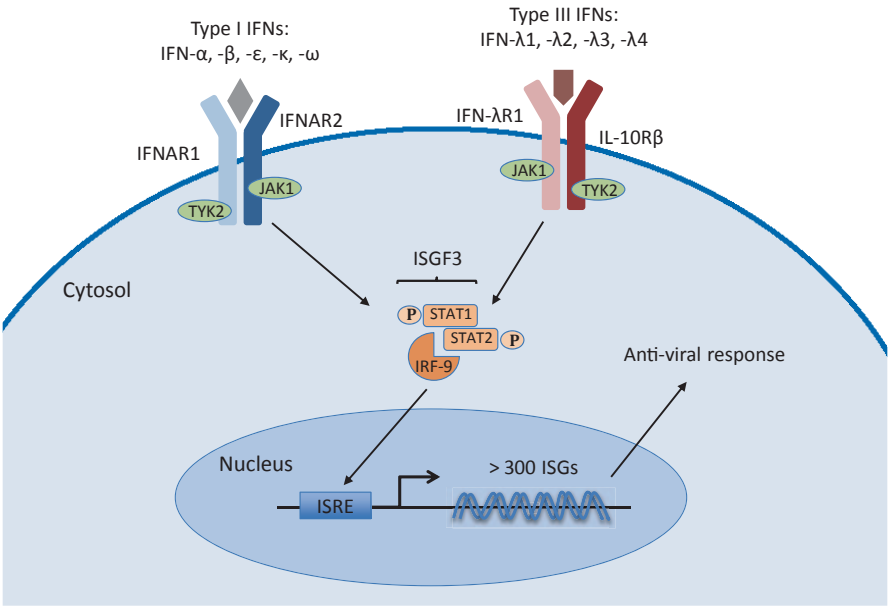

Fig. 1.2 Type I and type III IFNs signal through distinct heterodimeric receptors. Type I IFN receptor (*IFNAR*) is composed of IFNAR1 and IFNAR2, while IFN-λ receptor is composed by the unique IFN-λR1 chain and the IL-10Rβ chain, common to all IL-10 superfamily members. A common intracellular pathway is induced by ligand binding of IFNs to their receptors that leads to activation of the JAK-STAT pathway and the formation of ISGF3, consisting of phosphorylated STAT1-STAT2 heterodimers and IRF-9/p48. ISGF3 translocates to the nucleus where it binds to the promoter region of hundreds of IFN-stimulated genes (*ISGs*) leading to their transcription.

SOCS 1, a known inhibitor of type I IFNs as well (Brand et al. 2005b; Wei et al. 2014). The large amount of data on gene expression regulated by IFNs, including IFN-λs, is organized on a database made available on the web, the Interferome 2.0 (Rusinova et al. 2013).

Of interest, several reports indicate significant differences in the pattern of ISGs induced by type I and III IFNs. One study investigating type I and type III IFN-dependent ISG induction following HCV infection found that IFN-α led to an early peak of ISGs that declined rapidly, while IFN-λs induced steady increases in the corresponding genes that were also sustained (Marcello et al. 2006). Another study examining the induction of ISGs following systemic IFN-λ versus IFN-α administration observed differential responses in various organs (Sommereyns et al. 2008). This points to distinct biological effects of IFN-λs that cannot be substituted by the type I IFN system.

Anti-Viral Actions

The main characteristic of IFNs is their production during viral infection and their potency in inducing an anti-viral state to the cells in their vicinity. Following this paradigm, IFN-λs were initially described for their anti-viral activity both in cells in culture and experimental animals. Specifically, exogenously administered IFN-λs were shown to inhibit the replication of a wide variety of viruses *in vitro*, including VSV (Kotenko et al. 2003), EMCV (Kotenko et al. 2003; Sheppard et al. 2003), hepatitis B and C viruses (Robek et al. 2005) and human cytomegalovirus (CMV) (Brand et al. 2005a), and *in vivo* including vaccinia virus (VACV) (Bartlett et al. 2005) and herpes simplex virus type 2 (HSV-2) (Ank et al. 2006). Thus, although IFN-λ genes have a distinct evolutionary origin and use a distinct receptor complex, they exhibit similar antiviral activity to type I IFNs, (Kotenko et al. 2003; Sheppard et al. 2003). This indicates the existence of a second anti-viral defense mechanism that is distinct from that of type I IFNs.

The generation of IFNλR1/IL-28Rα deficient mice made possible the dissection of the relative contribution of endogenously produced IFN-λs in antiviral immunity (Ank et al. 2008). Although these cytokines were first considered to be dispensable in the defense against viruses, with several studies on systemic or topical infections of HSV-2, lymphocytic choriomeningitis virus (LCMV), VSV or hepatotropic viruses failing to find an effect (Ank et al. 2008; Mordstein et al. 2008), it was later realized that IFN-λs may play a more restricted role, especially in specific settings and mucosal sites as discussed below. Thus, several reports demonstrated that IFN-λs are needed for antiviral immunity against IAV and rotavirus infections, while type I IFNs cannot substitute for their actions (Jewell et al. 2010; Mordstein et al. 2008, 2010; Pott et al. 2011). In addition, another report showed that IFN-λs are required for optimal TLR-activated immunity (Ank et al. 2008), in line with the ability of TLRs to induce type III IFNs in DCs and macrophages (Coccia et al. 2004).

Link between Innate and Adaptive Immunity

In addition to inhibiting viral replication, type III IFNs may also influence the innate and adaptive immune response. Initial experiments with IFN-λs showed that these cytokines can up-regulate MHC class I expression comparably to type I IFNs (Kotenko et al. 2003). High expression of MHC class I and II molecules on antigen presenting cells, tumor cells or infected epithelial cells is generally associated with induction of more effective host immunity. Subsequent studies suggested that IFN-λ1 can up-regulate IL-6, -8 and -10 cytokine production in human monocytes (Jordan et al. 2007a) and induce MIG/CXCL9, IP-10/CXCL10 and I-TAC/CXCL11, chemokines typically triggered by IFN-γ (Pekarek et al. 2007). The caveat in these studies, however, has been that they were all performed in mixed human peripheral blood mononuclear cell cultures, leaving open the possibility that many of these effects are indirect. Several reports have also proposed a role of IFN-λs in the regulation of DC function. Megjugorac et al. and Yin et al. indicated that human pDCs produce IFN-λs and respond to them by upregulating CD80 and ICOS-L expression (Megjugorac et al. 2009; Yin et al. 2012). Mennechet et al. showed that IFN-λ treatment of human conventional DCs (cDCs) induced the proliferation of $Foxp3^+$ suppressor T cells, and proposed an immunoregulatory function of type III IFNs (Mennechet and Uze 2006). Finally, Koltsida et al. demonstrated that IFN-λs signal on cDCs to down-regulate OX40L, up-regulate IL-12 and mediate Th1 polarization in the context of respiratory inflammation (Koltsida et al. 2011). Other studies *in vitro*, have also suggested a role of IFN-λs in the modulation of the Th1/Th2 response through the reduction of GATA3 and IL-13, and possibly the increase of IFN-γ (Dai et al. 2009; Jordan et al. 2007b). However, whether IFN-λs can directly act on human $CD4^+$ T cells, or whether this is mediated through professional antigen presenting cells such as DCs has remained controversial.

IFN-λs in Mucosal Immunity

Accumulating reports in the literature describe a prominent role of IFN-λs in host defense at mucosal sites. Viruses that enter the body via the respiratory and gastrointestinal tracts most commonly target the epithelial lining layer to induce IFN-λs, which act in turn to establish an antiviral state in the nearby tissue (Sommereyns et al. 2008; Ank et al. 2008). IFN-λs were therefore shown to be the predominant IFNs produced in the mouse lung after IAV infection, and to be involved in mediating anti-viral protection irrespectively of type I IFNs (Jewell et al. 2010). Occasionally, $CD11c^+$ DCs can also express IFN-λs, e.g. during infection with HSV-2, and become an additional source of type III IFNs (Iversen et al. 2010). The crucial role of IFN-λs in mucosal immunity is indicated by the fact that several respiratory pathogens have evolved strategies to evade the immune system through the suppression of IFN-λ production or the inhibition of its downstream signaling. IAV and RSV are two such common human pathogens that target the lung epithelium and are

associated with severe disease and mortality. RSV proteins NS1 and NS2 have been shown to account for the poor production of IFN-λs upon RSV infection, as IFN-λ production can be restored upon deletion of these viral regions (Spann et al. 2004). Similarly, the NS1 region of IAV has been shown to inhibit type I IFN production (Garcia-Sastre et al. 1998) and is also likely to do so for IFN-λs. IFN-λ antiviral activity can also be inhibited by the orthopoxvirus protein VACV E3L (Bandi et al. 2010), and the NS1 and NS2 proteins of pneumonia virus of mice (Heinze et al. 2011). The secreted glycoprotein Y136 from Yaba-like disease virus, with tropism for the skin, has additionally been shown to directly inhibit both type I and type III IFN signaling and biological activities (Huang et al. 2007).

In addition to anti-viral immunity, two important studies hinted to a role of IFN-λs in allergic airway disease (Contoli et al. 2006; Bullens et al. 2008). Contoli et al. reported an impaired production of IFN-λs by primary bronchial epithelial cells and alveolar macrophages during allergic asthma exacerbations in patients infected with RV. IFN-λ levels were inversely correlated to viral load and disease severity (Contoli et al. 2006). Bullens et al. detected increased IFN-λ mRNA in the sputum of asthmatics versus healthy individuals, in the absence of evidence of viral infection, and these correlated with milder asthma symptoms in steroid-naïve patients (Bullens et al. 2008). Yet, an immunoprotective role of IFN-λs in asthma was demonstrated only later on by the study of Koltsida et al. This provided *in vivo* evidence that IFN-λs can up-regulate IL-12, induce Th1 immunity and suppress pathogenic Th2 mediated immune responses that drive asthma (Koltsida et al. 2011). These concerted antiviral and anti-inflammatory actions of IFN-λs in the lung establish them as attractive immunotherapeutic compounds for the treatment of asthma exacerbations which are commonly triggered by viruses and mediated by heightened Th2 responses (Andreakos and Papadopoulos 2014) (Fig. 1.3). Overall, these data suggest that the IFN-λ system may have evolved as a specific protection mechanism of epithelial surfaces against viral invasion.

Other Functions of IFN-λs

Type III IFNs were also shown to exhibit anti-tumor activity. *In vitro*, IFN-λs exerted anti-proliferative effects in the pancreatic neuroendocrine cell line BON-1 (Zitzmann et al. 2006) and the human keratinocyte cell line HaCaT (Maher et al. 2008), and induced apoptosis in HT29 colorectal adenocarcinoma cells (Li et al. 2008). B16 melanoma cells engineered to constitutively express mouse IFN-λ2 were less tumorigenic in mice *in vivo*, an effect that was mediated via the action of IFN-λ2 on host immune cells rather than directly on tumor cells (Lasfar et al. 2006). Similarly, Numasaki et al. documented reduced tumor growth and fibrosarcoma metastases in the lung of mice treated with IFN-λ, in a process that involved the action of immune cells (Numasaki et al. 2007). In a mouse model of hepatocellular carcinoma, IFN-λ acted on DCs to potentiate the anti-tumor action of NK cells (Abushahba et al. 2010). To the contrary, Sato et al. showed that the anti-tumor

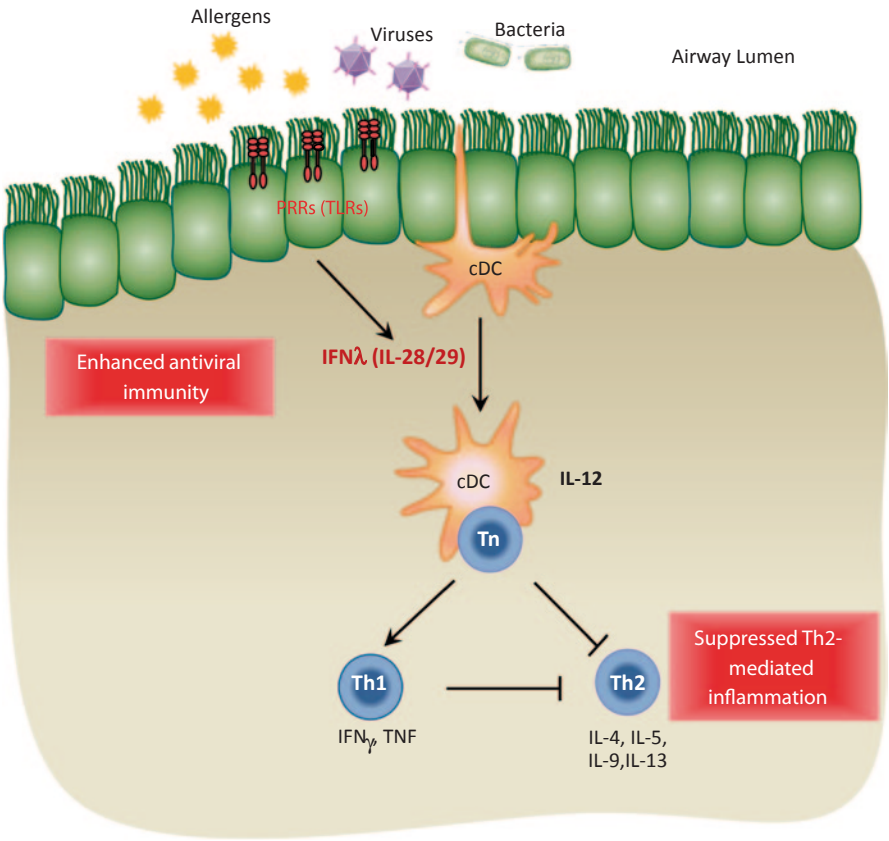

Fig. 1.3 Immunomodulatory mechanism of action of type III IFNs in asthma exacerbations. Induced by viruses, allergens or bacteria, IFN-λs act on cDCs to up-regulate IL-12 and drive Th1 cell differentiation. This in turn inhibits Th2-driven allergic responses in the lung. In parallel, IFN-λs mediate direct inhibitory effects to viral replication, reducing viral load and preventing further tissue damage

effect of IFN-λs in murine models of B16 melanoma and Colon26 cancer cells was exerted by IFN-λ through both direct and indirect effects; inhibition of tumor growth and induction of NK/NKT cell cytotoxic activity *in vivo* (Sato et al. 2006).

Therapeutic Opportunities Using IFN-λs

Soon after their discovery, IFN-λs have attracted great interest in the treatment of viral hepatitis. IFN-λs were shown to inhibit hepatitis B and C replication *in vitro* in hepatocyte cell lines. Inhibition was equally efficient as that of type I IFNs (Robek et al. 2005) which are currently used in combination with the antiviral compound

ribavirin as the standard method of care for hepatitis C patients. However, as type I IFNs are toxic leading to several adverse effects including flu-like disease and neurological as well as neuropsychiatric manifestations (Aspinall and Pockros 2004), IFN-λs have drawn attention as a safer alternative. Thus, a pegylated form of IFN-λ1 (ZymoGenetics Inc/Bristol Myers Squibb) has already reached phase 3 trials for the treatment of hepatitis C infection. Data so far report a positive outcome of the therapy which is advantageous over IFN-α treatments, with fewer side effects and good clinical response. This is likely to be due to the more restricted pattern of expression of the IFN-λR, which is absent from hematopoietic progenitor cells and the CNS (Sommereyns et al. 2008), and thus does not provoke cytopenia or neurological side effects commonly seen following IFN-α treatment (Ramos 2010). Recently, the discovery of a fourth member of the type III IFN family, IFN-λ4, has introduced a new level of complexity in IFN therapy of hepatitis C. Originally described in 2009, as a genetic polymorphism upstream of the IFN-λ3 gene associated with poor response to hepatitis C treatment (Ge et al. 2009; Thomas et al. 2009), IFN-λ4 was later shown to constitute a new IFN-λ family member expressed in carriers of the genetic variation ss469415590 TT/ΔG which was also linked with a poor response to IFN-α treatment (Prokunina-Olsson et al. 2013). Although IFN-λ4 was initially proposed to be inhibitory to other IFNs, this has been challenged (Hamming et al. 2013) and the underlying basis of the poor response of ss469415590 TT/ΔG polymorphism carriers to treatment remains unknown.

In the respiratory system, deficient IFN-λ production has been linked to asthma severity and disease exacerbations due to higher viral load and airway inflammation (Bullens et al. 2008; Contoli et al. 2006; Koltsida et al. 2011). In experimental models of asthma, IFN-λ administration has been further shown to suppress respiratory viral infections and inhibit allergic airway inflammation and disease (Koltsida et al. 2011). This provides a strong rationale for the therapeutic administration of recombinant IFN-λs in asthma exacerbations with the aim to reduce viral load while at the same time inhibiting the underlying immunological basis of the disease. Clinical trials in that respect are therefore eagerly awaited.

Finally, IFN-λs are promising therapeutics for the treatment of diverse viral infections and cancer. For example, as keratinocytes and melanocytes express IFN-λR and respond to IFN-λs (Witte et al. 2009), several skin viral infections and carcinomas may be treatable through the application of these cytokines. In addition, several gastrointestinal and systemic infections may also be confronted through the administration of IFN-λs. Noteworthy, IFN-λs can instruct adaptive immunity and potentiate $CD8^+$ T cell cytotoxic functions in vivo in mice (Hamming et al. 2013) and HIV-infected rhesus macaques (Morrow et al. 2010). They are therefore attractive candidates for boosting anti-microbial immune defenses and enhancing the efficacy of vaccines. As our knowledge of the homeostatic, antiviral and immunoinflammatory functions of IFN-λs expands, so will do the therapeutic opportunities offered by this new but highly promising class II cytokine family.

References

Abushahba, W., Balan, M., Castaneda, I., Yuan, Y., Reuhl, K., Raveche, E., De La Torre, A., Lasfar, A., & Kotenko, S. V. (2010). Antitumor activity of type I and type III interferons in BNL hepatoma model. *Cancer Immunology, Immunotherapy, 59,* 1059–1071.

Andreakos, E., & Papadopoulos, N. G. (2014). IL-25: The missing link between allergy, viral infection, and asthma? *Science Translational Medicine, 6,* 256fs38.

Ank, N., West, H., Bartholdy, C., Eriksson, K., Thomsen, A. R., & Paludan, S. R. (2006). Lambda interferon (IFN-lambda), a type III IFN, is induced by viruses and IFNs and displays potent antiviral activity against select virus infections in vivo. *Journal of Virology, 80,* 4501–4509.

Ank, N., Iversen, M. B., Bartholdy, C., Staeheli, P., Hartmann, R., Jensen, U. B., Dagnaes-Hansen, F., Thomsen, A. R., Chen, Z., Haugen, H., Klucher, K., & Paludan, S. R. (2008). An important role for type III interferon (IFN-lambda/IL-28) in TLR-induced antiviral activity. *Journal of Immunology, 180,* 2474–2485.

Aspinall, R. J., & Pockros, P. J. (2004). The management of side-effects during therapy for hepatitis C. *Alimentary Pharmacology and Therapeutics, 20,* 917–929.

Bandi, P., Pagliaccetti, N. E., & Robek, M. D. (2010). Inhibition of type III interferon activity by orthopoxvirus immunomodulatory proteins. *Journal of interferon and cytokine research, 30,* 123–134.

Bartlett, N. W., Buttigieg, K., Kotenko, S. V., & Smith, G. L. (2005). Murine interferon lambdas (type III interferons) exhibit potent antiviral activity in vivo in a poxvirus infection model. *Journal of General Virology, 86,* 1589–1596.

Bierne, H., Travier, L., Mahlakoiv, T., Tailleux, L., Subtil, A., Lebreton, A., Paliwal, A., Gicquel, B., Staeheli, P., Lecuit, M., & Cossart, P. (2012). Activation of type III interferon genes by pathogenic bacteria in infected epithelial cells and mouse placenta. *PLoS ONE, 7,* e39080.

Brand, S., Beigel, F., Olszak, T., Zitzmann, K., Eichhorst, S. T., Otte, J. M., Diebold, J., Diepolder, H., Adler, B., Auernhammer, C. J., Goke, B., & Dambacher, J. (2005a). IL-28A and IL-29 mediate antiproliferative and antiviral signals in intestinal epithelial cells and murine CMV infection increases colonic IL-28A expression. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 289,* G960-8.

Brand, S., Zitzmann, K., Dambacher, J., Beigel, F., Olszak, T., Vlotides, G., Eichhorst, S. T., Goke, B., Diepolder, H., & Auernhammer, C. J. (2005b). SOCS-1 inhibits expression of the antiviral proteins 2',5'-OAS and MxA induced by the novel interferon-lambdas IL-28A and IL-29. *Biochemical and Biophysical Research Communications, 331,* 543–548.

Bullens, D. M., Decraene, A., Dilissen, E., Meyts, I., De Boeck, K., Dupont, L. J., & Ceuppens, J. L. (2008). Type III IFN-lambda mRNA expression in sputum of adult and school-aged asthmatics. *Clinical and Experimental Allergy, 38,* 1459–1467.

Coccia, E. M., Severa, M., Giacomini, E., Monneron, D., Remoli, M. E., Julkunen, I., Cella, M., Lande, R., & Uze, G. (2004). Viral infection and Toll-like receptor agonists induce a differential expression of type I and lambda interferons in human plasmacytoid and monocyte-derived dendritic cells. *European Journal of Immunology, 34,* 796–805.

Contoli, M., Message, S. D., Laza-Stanca, V., Edwards, M. R., Wark, P. A., Bartlett, N. W., Kebadze, T., Mallia, P., Stanciu, L. A., Parker, H. L., Slater, L., Lewis-Antes, A., Kon, O. M., Holgate, S. T., Davies, D. E., Kotenko, S. V., Papi, A., & Johnston, S. L. (2006). Role of deficient type III interferon-lambda production in asthma exacerbations. *Nature Medicine, 12,* 1023–1026.

Dai, J., Megjugorac, N. J., Gallagher, G. E., YU, R. Y., & Gallagher, G. (2009). IFN-lambda1 (IL-29) inhibits GATA3 expression and suppresses Th2 responses in human naive and memory T cells. *Blood, 113,* 5829–5838.

Donnelly, R. P., Sheikh, F., Kotenko, S. V., & Dickensheets, H. (2004). The expanded family of class II cytokines that share the IL-10 receptor-2 (IL-10R2) chain. *Journal of Leukocyte Biology, 76,* 314–321.

Fitzgerald-Bocarsly, P. (1993). Human natural interferon-alpha producing cells. *Pharmacology and Therapeutics, 60,* 39–62.

Fox, B. A., Sheppard, P. O., & O'hara, P. J. (2009). The role of genomic data in the discovery, annotation and evolutionary interpretation of the interferon-lambda family. *PLoS One,* 4*,* e4933.

Gad, H. H., Dellgren, C., Hamming, O. J., Vends, S., Paludan, S. R., & Hartmann, R. (2009). Interferon-lambda is functionally an interferon but structurally related to the interleukin-10 family. *Journal of Biological Chemistry,* 284*,* 20869–20875.

Garcia-Sastre, A., Egorov, A., Matassov, D., Brandt, S., Levy, D. E., Durbin, J. E., Palese, P., & Muster, T. (1998). Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. *Virology, 252,* 324–330.

Ge, D., Fellay, J., Thompson, A. J., Simon, J. S., Shianna, K. V., Urban, T. J., Heinzen, E. L., Qiu, P., Bertelsen, A. H., Muir, A. J., Sulkowski, M., Mchutchison, J. G., & Goldstein, D. B. (2009). Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature, 461,* 399–401.

Hamming, O. J., Terczynska-Dyla, E., Vieyres, G., Dijkman, R., Jorgensen, S. E., Akhtar, H., Siupka, P., Pietschmann, T., Thiel, V., & Hartmann, R. (2013). Interferon lambda 4 signals via the IFNlambda receptor to regulate antiviral activity against HCV and coronaviruses. *EMBO Journal, 32,* 3055–3065.

Heinze, B., Frey, S., Mordstein, M., Schmitt-Graff, A., Ehl, S., Buchholz, U. J., Collins, P. L., Staeheli, P., & Krempl, C. D. (2011). Both nonstructural proteins NS1 and NS2 of pneumonia virus of mice are inhibitors of the interferon type I and type III responses in vivo. *Journal of Virology, 85,* 4071–4084.

Huang, J., Smirnov, S. V., Lewis-Antes, A., Balan, M., LI, W., Tang, S., Silke, G. V., Putz, M. M., Smith, G. L., & Kotenko, S. V. (2007). Inhibition of type I and type III interferons by a secreted glycoprotein from Yaba-like disease virus. *Proceedings of the National Academy of Sciences of the United States of America, 104,* 9822–9827.

Ioannidis, I., YE, F., Mcnally, B., Willette, M., & Flano, E. (2013). Toll-like receptor expression and induction of type I and type III interferons in primary airway epithelial cells. *Journal of Virology, 87,* 3261–3270.

Iversen, M. B., Ank, N., Melchjorsen, J., & Paludan, S. R. (2010). Expression of type III interferon (IFN) in the vaginal mucosa is mediated primarily by dendritic cells and displays stronger dependence on NF-kappaB than type I IFNs. *Journal of Virology,* 84*,* 4579–4586.

Jewell, N. A., Cline, T., Mertz, S. E., Smirnov, S. V., Flano, E., Schindler, C., Grieves, J. L., Durbin, R. K., Kotenko, S. V., & Durbin, J. E. (2010). Lambda interferon is the predominant interferon induced by influenza A virus infection in vivo. *Journal of Virology, 84,* 11515–11522.

Jordan, W. J., Eskdale, J., Boniotto, M., Rodia, M., Kellner, D., & Gallagher, G. (2007a). Modulation of the human cytokine response by interferon lambda-1 (IFN-lambda1/IL-29). *Genes and Immunity, 8,* 13–20.

Jordan, W. J., Eskdale, J., Srinivas, S., Pekarek, V., Kelner, D., Rodia, M., & Gallagher, G. (2007b). Human interferon lambda-1 (IFN-lambda1/IL-29) modulates the Th1/Th2 response. *Genes and Immunity, 8,* 254–261.

Karpala, A. J., Morris, K. R., Broadway, M. M., Mcwaters, P. G., O'neil, T. E., Goossens, K. E., Lowenthal, J. W., & Bean, A. G. (2008). Molecular cloning, expression, and characterization of chicken IFN -lambda. *Journal of Interferon and Cytokine Research, 28,* 341–350.

Koltsida, O., Hausding, M., Stavropoulos, A., Koch, S., Tzelepis, G., Ubel, C., Kotenko, S. V., Sideras, P., Lehr, H. A., Tepe, M., Klucher, K. M., Doyle, S. E., Neurath, M. F., Finotto, S., & Andreakos, E. (2011). IL-28A (IFN-lambda2) modulates lung DC function to promote Th1 immune skewing and suppress allergic airway disease. *EMBO Molecular Medicine, 3,* 348–361.

Kotenko, S. V., Gallagher, G., Baurin, V. V., Lewis-Antes, A., Shen, M., Shah, N. K., Langer, J. A., Sheikh, F., Dickensheets, H., & Donnelly, R. P. (2003). IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nature Immunology, 4,* 69–77.

Lasfar, A., Lewis-Antes, A., Smirnov, S. V., Anantha, S., Abushahba, W., Tian, B., Reuhl, K., Dickensheets, H., Sheikh, F., Donnelly, R. P., Raveche, E., & Kotenko, S. V. (2006). Characterization of the mouse IFN-lambda ligand-receptor system: IFN-lambdas exhibit antitumor activity against B16 melanoma. *Cancer Research, 66,* 4468–4477.

Lauterbach, H., Bathke, B., Gilles, S., Traidl-Hoffmann, C., Luber, C. A., Fejer, G., Freudenberg, M. A., Davey, G. M., Vremec, D., Kallies, A., WU, L., Shortman, K., Chaplin, P., Suter, M., O'keeffe, M., & Hochrein, H. (2010). Mouse CD8alpha+ DCs and human BDCA3+ DCs are major producers of IFN-lambda in response to poly IC. *Journal of Experimental Medicine, 207,* 2703–2717.

Lebreton, A., Lakisic, G., Job, V., Fritsch, L., Tham, T. N., Camejo, A., Mattei, P. J., Regnault, B., Nahori, M. A., Cabanes, D., Gautreau, A., Ait-Si-Ali, S., Dessen, A., Cossart, P., & Bierne, H. (2011). A bacterial protein targets the BAHD1 chromatin complex to stimulate type III interferon response. *Science, 331,* 1319–1321.

Levraud, J. P., Boudinot, P., Colin, I., Benmansour, A., Peyrieras, N., Herbomel, P., & Lutfalla, G. (2007). Identification of the zebrafish IFN receptor: implications for the origin of the vertebrate IFN system. *Journal of Immunology, 178,* 4385–4394.

Li, W., Lewis-Antes, A., Huang, J., Balan, M., & Kotenko, S. V. (2008). Regulation of apoptosis by type III interferons. *Cell Proliferation, 41,* 960–979.

Maher, S. G., Sheikh, F., Scarzello, A. J., Romero-Weaver, A. L., Baker, D. P., Donnelly, R. P., & Gamero, A. M. (2008). IFNalpha and IFNlambda differ in their antiproliferative effects and duration of JAK/STAT signaling activity. *Cancer Biology and Therapy, 7,* 1109–1115.

Marcello, T., Grakoui, A., Barba-Spaeth, G., Machlin, E. S., Kotenko, S. V., Macdonald, M. R., & Rice, C. M. (2006). Interferons alpha and lambda inhibit hepatitis C virus replication with distinct signal transduction and gene regulation kinetics. *Gastroenterology, 131,* 1887–1898.

Marie, I., Durbin, J. E., & LEVY, D. E. (1998). Differential viral induction of distinct interferon-alpha genes by positive feedback through interferon regulatory factor-7. *EMBO Journal, 17,* 6660–6669.

Megjugorac, N. J., Gallagher, G. E., & Gallagher, G. (2009). Modulation of human plasmacytoid DC function by IFN-lambda1 (IL-29). *Journal of Leukocyte Biology, 86,* 1359–1363.

Mennechet, F. J., & Uze, G. (2006). Interferon-lambda-treated dendritic cells specifically induce proliferation of FOXP3-expressing suppressor T cells. *Blood, 107,* 4417–4423.

Mordstein, M., Kochs, G., Dumoutier, L., Renauld, J. C., Paludan, S. R., Klucher, K., & Staeheli, P. (2008). Interferon-lambda contributes to innate immunity of mice against influenza A virus but not against hepatotropic viruses. *PLoS Pathogens, 4,* e1000151.

Mordstein, M., Neugebauer, E., Ditt, V., Jessen, B., Rieger, T., Falcone, V., Sorgeloos, F., Ehl, S., Mayer, D., Kochs, G., Schwemmle, M., Gunther, S., Drosten, C., Michiels, T., & Staeheli, P. (2010). Lambda interferon renders epithelial cells of the respiratory and gastrointestinal tracts resistant to viral infections. *Journal of Virology, 84,* 5670–5677.

Morrow, M. P., Yan, J., Pankhong, P., Shedlock, D. J., Lewis, M. G., Talbott, K., Toporovski, R., Khan, A. S., Sardesai, N. Y., & Weiner, D. B. (2010). IL-28B/IFN-lambda 3 drives granzyme B loading and significantly increases CTL killing activity in macaques. *Molecular Therapy, 18,* 1714–1723.

Numasaki, M., Tagawa, M., Iwata, F., Suzuki, T., Nakamura, A., Okada, M., Iwakura, Y., Aiba, S., & Yamaya, M. (2007). IL-28 elicits antitumor responses against murine fibrosarcoma. *Journal of Immunology, 178,* 5086–5098.

Odendall, C., Dixit, E., Stavru, F., Bierne, H., Franz, K. M., Durbin, A. F., Boulant, S., Gehrke, L., Cossart, P., & Kagan, J. C. (2014). Diverse intracellular pathogens activate type III interferon expression from peroxisomes. *Nature Immunology, 15,* 717–726.

Onoguchi, K., Yoneyama, M., Takemura, A., Akira, S., Taniguchi, T., Namiki, H., & Fujita, T. (2007). Viral infections activate types I and III interferon genes through a common mechanism. *Journal of Biological Chemistry, 282,* 7576–7581.

Osterlund, P., Veckman, V., Siren, J., Klucher, K. M., Hiscott, J., Matikainen, S., & Julkunen, I. (2005). Gene expression and antiviral activity of alpha/beta interferons and interleukin-29 in virus-infected human myeloid dendritic cells. *Journal of Virology, 79,* 9608–9617.

Osterlund, P. I., Pietila, T. E., Veckman, V., Kotenko, S. V., & Julkunen, I. (2007). IFN regulatory factor family members differentially regulate the expression of type III IFN (IFN-lambda) genes. *Journal of Immunology, 179,* 3434–3442.

Pekarek, V., Srinivas, S., Eskdale, J., & Gallagher, G. (2007). Interferon lambda-1 (IFN-lambda1/IL-29) induces ELR(-) CXC chemokine mRNA in human peripheral blood mononuclear cells, in an IFN-gamma-independent manner. *Genes and Immunity, 8,* 177–180.

Pott, J., Mahlakoiv, T., Mordstein, M., Duerr, C. U., Michiels, T., Stockinger, S., Staeheli, P., & Hornef, M. W. (2011). IFN-lambda determines the intestinal epithelial antiviral host defense. *Proceedings of the National Academy of Sciences of the United States of America, 108,* 7944–7949.

Prokunina-Olsson, L., Muchmore, B., Tang, W., Pfeiffer, R. M., Park, H., Dickensheets, H., Hergott, D., Porter-Gill, P., Mumy, A., Kohaar, I., Chen, S., Brand, N., Tarway, M., Liu, L., Sheikh, F., Astemborski, J., Bonkovsky, H. L., Edlin, B. R., Howell, C. D., Morgan, T. R., Thomas, D. L., Rehermann, B., Donnelly, R. P., & O'brien, T. R. (2013). A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. *Nature Genetics, 45,* 164–171.

Ramos, E. L. (2010). Preclinical and clinical development of pegylated interferon-lambda 1 in chronic hepatitis C. *Journal of Interferon and Cytokine Research, 30,* 591–595.

Robek, M. D., Boyd, B. S., & Chisari, F. V. (2005). Lambda interferon inhibits hepatitis B and C virus replication. *Journal of Virology, 79,* 3851–3854.

Rusinova, I., Forster, S., YU, S., Kannan, A., Masse, M., Cumming, H., Chapman, R., & Hertzog, P. J. (2013). Interferome v2.0: an updated database of annotated interferon-regulated genes. *Nucleic Acids Research, 41,* D1040-6.

Sato, M., Hata, N., Asagiri, M., Nakaya, T., Taniguchi, T., & Tanaka, N. (1998). Positive feedback regulation of type I IFN genes by the IFN-inducible transcription factor IRF-7. *FEBS Letters, 441,* 106–110.

Sato, A., Ohtsuki, M., Hata, M., Kobayashi, E., & Murakami, T. (2006). Antitumor activity of IFN-lambda in murine tumor models. *Journal of Immunology, 176,* 7686–7694.

Sheppard, P., Kindsvogel, W., Xu, W., Henderson, K., Schlutsmeyer, S., Whitmore, T. E., Kuestner, R., Garrigues, U., Birks, C., Roraback, J., Ostrander, C., Dong, D., Shin, J., Presnell, S., Fox, B., Haldeman, B., Cooper, E., Taft, D., Gilbert, T., Grant, F. J., Tackett, M., Krivan, W., Mcknight, G., Clegg, C., Foster, D., & Klucher, K. M. (2003). IL-28, IL-29 and their class II cytokine receptor IL-28R. *Nature Immunology, 4,* 63–68.

Siegal, F. P., Kadowaki, N., Shodell, M., Fitzgerald-Bocarsly, P. A., Shah, K., Ho, S., Antonenko, S., & Liu, Y. J. (1999). The nature of the principal type 1 interferon-producing cells in human blood. *Science, 284,* 1835–1837.

Sommereyns, C., Paul, S., Staeheli, P., & Michiels, T. (2008). IFN-lambda (IFN-lambda) is expressed in a tissue-dependent fashion and primarily acts on epithelial cells in vivo. *PLoS Pathogens, 4,* e1000017.

Spann, K. M., Tran, K. C., Chi, B., Rabin, R. L., & Collins, P. L. (2004). Suppression of the induction of alpha, beta, and lambda interferons by the NS1 and NS2 proteins of human respiratory syncytial virus in human epithelial cells and macrophages [corrected]. *Journal of Virology, 78,* 4363–4369.

Thomas, D. L., Thio, C. L., Martin, M. P., QI, Y., GE, D., O'huigin, C., Kidd, J., Kidd, K., Khakoo, S. I., Alexander, G., Goedert, J. J., Kirk, G. D., Donfield, S. M., Rosen, H. R., Tobler, L. H., Busch, M. P., Mchutchison, J. G., Goldstein, D. B., & Carrington, M. (2009). Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature, 461,* 798–801.

Thomson, S. J., Goh, F. G., Banks, H., Krausgruber, T., Kotenko, S. V., Foxwell, B. M., & Udalova, I. A. (2009). The role of transposable elements in the regulation of IFN-lambda1 gene expression. *Proceedings of the National Academy of Sciences of the United States of America, 106,* 11564–11569.

Wang, J., Oberley-Deegan, R., Wang, S., Nikrad, M., Funk, C. J., Hartshorn, K. L., & Mason, R. J. (2009). Differentiated human alveolar type II cells secrete antiviral IL-29 (IFN-lambda 1) in response to influenza A infection. *Journal of Immunology, 182,* 1296–1304.

Wei, H., Wang, S., Chen, Q., Chen, Y., Chi, X., Zhang, L., Huang, S., Gao, G. F., & Chen, J. L. (2014). Suppression of interferon lambda signaling by SOCS-1 results in their excessive production during influenza virus infection. *PLoS Pathogens, 10,* e1003845.

Witte, K., Gruetz, G., Volk, H. D., Looman, A. C., Asadullah, K., Sterry, W., Sabat, R., & Wolk, K. (2009). Despite IFN-lambda receptor expression, blood immune cells, but not keratinocytes or melanocytes, have an impaired response to type III interferons: implications for therapeutic applications of these cytokines. *Genes and Immunity, 10,* 702–714.

Yin, Z., Dai, J., Deng, J., Sheikh, F., Natalia, M., Shih, T., Lewis-Antes, A., Amrute, S. B., Garrigues, U., Doyle, S., Donnelly, R. P., Kotenko, S. V., & Fitzgerald-Bocarsly, P. (2012). Type III IFNs are produced by and stimulate human plasmacytoid dendritic cells. *Journal of Immunology, 189,* 2735–2745.

Young, H. A., & Bream, J. H. (2007). IFN-gamma: recent advances in understanding regulation of expression, biological functions, and clinical applications. *Current Topics in Microbiology and Immunology, 316,* 97–117.

Zhou, L., Wang, X., Wang, Y. J., Zhou, Y., Hu, S., Ye, L., Hou, W., Li, H., & Ho, W. Z. (2009). Activation of toll-like receptor-3 induces interferon-lambda expression in human neuronal cells. *Neuroscience, 159,* 629–637.

Zitzmann, K., Brand, S., Baehs, S., Goke, B., Meinecke, J., Spottl, G., Meyer, H., & Auernhammer, C. J. (2006). Novel interferon-lambdas induce antiproliferative effects in neuroendocrine tumor cells. *Biochemical and Biophysical Research Communications, 344,* 1334–1341.

Chapter 2
Stability of Regulatory T Cells Undermined or Endorsed by Different Type-1 Cytokines.

Silvia Piconese and Vincenzo Barnaba

Abstract Regulatory T cells (Tregs) encompass an array of immunosuppressive cells responsible for the protection against exacerbated immune responses and the maintenance of tissue homeostasis. Various Treg subtypes, normally resident within distinct lymphoid and non-lymphoid tissues, can be recruited and expanded during inflammation, possibly undergoing functional and molecular re-programming. Generally, two processes have been reported in different settings of type-1 response: *i)* Treg subpopulations acquiring the ability to specifically suppress Th1 cells (called Th1-suppressing Tregs), and *ii)* Treg subsets rather polarizing into IFN-γ-producing (called Th1-like) Tregs.

Along the development of type-1 responses, Tregs are exposed to a variety of cytokines and other signals, exerting disparate activities. The combinatorial effects of typical Th1-driving cytokines, such as IL-12 (mostly produced by antigen-presenting cells during Th1 priming) and IFN-γ (mostly produced by pre-existing NK cells) lead to inhibition of Treg expansion and function, while promoting Th1-like Treg polarization. Conversely, cytokines produced at more advanced phases by Th1 effectors, such as IL-2, TNF-α and IFN-γ, promote Treg proliferation and/or Th1-suppressing Treg specialization. Some controversy exists around IL-27 and IFN-α, cytokines possibly released during bacterial or viral infections. Furthermore, cytokine signals can be finely tuned by the concomitant stimulation of costimulatory or coinhibitory receptors, such as OX40 and PD-1 respectively, within inflamed tissues.

A model may be envisaged of an alternate Treg response to type-1 cytokines, being hampered or boosted by early or late phase cytokines, respectively. Such regulation would unleash the development of protective type-1 immunity while constraining exacerbated Th1 responses, possibly causing immunopathology.

V. Barnaba (✉)
Istituto Pasteur -Fondazione Cenci Bolognetti, Rome, Italy
e-mail: vincenzo.barnaba@uniroma1.it

V. Barnaba · S. Piconese
Dipartimento di Medicina Interna e Specialità Mediche, Sapienza Università di Roma, Policlinico Umberto I, Viale del Policlinico 155, I-00161 Rome, Italy
e-mail: silvia.piconese@uniroma1.it

S. P. Schoenberger et al. (eds.), *Crossroads Between Innate and Adaptive Immunity V,*
Advances in Experimental Medicine and Biology 850, DOI 10.1007/978-3-319-15774-0_2

Keywords Th1 cells · Regulatory T cells (Tregs) · Th1-suppressing · Th1-like · Cytokine signals · Type-1 cytokines · CD4 T cells

Introduction

Most of the information about regulatory T cell (Treg) biology derives from the analysis of peripheral cells in the circulation and in lymphoid organs. While such data have provided important clues for the understanding of the crucial immune suppressive activity of Tregs in many settings, they may not recapitulate the complexity of Treg behavior within disparate non-lymphoid tissues. Many recent data, mostly obtained in experimental models, indicate a fine adaptation of Treg molecular program and suppressive functions in distinct tissues even under physiological conditions. For instance, the visceral adipose tissue and the skeletal muscle constitutively contain pools of resident Tregs characterized by PPARγ (Cipolletta et al. 2012) and amphiregulin (Burzyn et al. 2013b) overexpression respectively. Such molecules drive peculiar suppressive programs in the respective tissue-resident Tregs, shaped on the prevalent inflammatory mechanisms that need to be moldered to maintain or rescue homeostasis in each tissue (Burzyn et al. 2013a). Indeed, while visceral fat Tregs are particularly devoted to suppress metabolic inflammation, muscular Tregs are specialized in promoting muscle repair via the amphiregulin/epithelial growth factor receptor pathway.

In conditions of tissue injury, the development of immune and inflammatory responses is accompanied by, and possibly drives, the expansion of tissue-resident Tregs and the accrual of circulating Tregs, in an attempt to restore tissue homeostasis (Burzyn et al. 2013a). However, Treg suppression needs to be carefully regulated in the different phases of an inflammatory response, especially during chronic viral infections, such to achieve a compromise between pathogen containment and maintenance of tissue integrity and performance (Barnaba 2010).

Within tissues, Tregs are overwhelmed by a plethora of extracellular signals, such as cytokines, growth factors, chemokines and membrane-bound ligands, which finely and concurrently re-shape their functions, in line with the requirements of tissue preservation and immune response development (Smigiel et al. 2014). Cytokines represent crucial mediators in dictating and arranging the dominant type of immune response. At early phases of immune responses to virus-derived and also to other danger signals, both tissue and immune cells produce cytokines such as type I IFNs that orchestrate an innate defense program. Afterwards, appropriately stimulated antigen-presenting cells (APCs) release T helper 1 (Th1)-polarizing cytokines such as interleukin (IL)-12, IL-18 and IL-27, which drive Th1 differentiation and cytotoxic T lymphocyte (CTL) activation. At later phases, Th1- and CTL-derived cytokines, such as IL-2, interferon (IFN)-γ and tumor necrosis factor (TNF)-α, propagate inflammation and protective immunity. When recruited and/or expanded into inflamed tissues dominated by type-1 responses, Tregs may be exposed to combinations of APC- or T cell-derived type-1 cytokines, and differentially affected by them depending on cytokine receptor expression and sensitivity.

Treg Subsets Play Divergent Roles in Type-1 Inflammation

In the context of type-1 inflammation, two facets of Treg behavior have been described: some reports have underscored the existence of a Treg subset specialized in the suppression of Th1 responses (hereafter called "Th1-suppressing"), while others have identified subpopulations of Tregs competent for IFN-γ production thus resembling Th1 cells (hence called "Th1-like"). Different type-1 cytokines may shift the Treg balance between these two extremities, determining whether suppression or inflammation will prevail.

Specialized Th1-Suppressing Treg

A subpopulation of murine Tregs constitutively expresses CXCR3, the chemokine receptor typically associated to Th1 effector cells. Alike classical Th1 cells, CXCR3 expression in Tregs is strictly dependent on T-bet, the transcription factor orchestrating and stabilizing Th1 polarization (Koch et al. 2009). T-bet is markedly up-regulated in Tregs during type-1 inflammation and drives expansion and migration of Tregs specifically devoted to the regulation of type-1 responses (Koch et al. 2009). Contrary to classical Th1 cells, such Th1-suppressing Tregs are unable to secrete IFN-γ despite T-bet expression, a defect dependent on the low susceptibility of Tregs to IL-12, which is due to an epigenetic constrain in the expression of IL-12R-β2, the inducible subunit of IL-12 receptor (Koch et al. 2012). Expansion of Th1 suppressing Tregs has been observed not only in experimental models of type-1 inflammation (Koch et al. 2012; Koch et al. 2009) but also in human ovarian (Redjimi et al. 2012) and hepatic (Piconese et al. 2014) cancer, where the anti-tumor type-1 immune response cannot succeed in tumor eradication, being kept under strict control by populations of specialized Tregs.

Plastic Th1-Like Treg

In tissues characterized by acute, exacerbated and/or prolonged type-1 responses, it was possible to identify subsets of Tregs not only expressing T-bet but also producing detectable amounts of IFN-γ. Such Th1-like Tregs were observed in a variety of pathological conditions including graft-versus-host disease (Koenecke et al. 2012), viral infection (Zhao et al. 2011), parasite infection (Oldenhove et al. 2009), multiple sclerosis (Dominguez-Villar et al. 2011) and diabetes (Du et al. 2013; McClymont et al. 2011). Conceivably, the competence for IFN-γ production may render those Tregs more prone to contribute to, rather than suppress, inflammatory responses. In line with this possibility, IFN-γ-producing Tregs display reduced suppressive function *in vitro* (Dominguez-Villar et al. 2011), and conditions associated to Th1-like Treg polarization are also characterized by uncontrolled immunopathology (Lu et al. 2010; Oldenhove et al. 2009).

Type-1 Cytokines Have Disparate Effects on Tregs

IL-12 Counteracts Treg Suppression

Tregs do not constitutively express IL-12R-β2, but IL-12-responsive Tregs can be found within tissues characterized by type-1 inflammation, and IL-12 susceptibility can be induced *in vitro* upon prolonged exposure to IFN-γ, in both murine (Koch et al. 2012) and human (Piconese et al. 2014) Tregs. In the resulting IFN-γ-sensitized, IL-12-responsive Tregs, IL-12 seems to exert a variety of functions aimed at destabilizing Treg suppression. At late phases of *Mycobacterium tuberculosis* infection, pathogen-specific highly activated Tregs undergo an IL-12-dependent contraction, thus unleashing protective immune responses (Shafiani et al. 2013). *In vitro,* IL-12 paralyzes Treg activity at different levels, inhibiting their proliferation, suppressive function, Foxp3 and CD25 expression (Dominguez-Villar et al. 2011; Zhao and Perlman 2012). Specifically, IL-12 produced by CD16-positive monocytes inhibits the proliferation of the Helios^{high} Treg subpopulation, characterized by epigenetic stability and increased suppressive function (Zhong and Yazdanbakhsh 2013). Importantly, IL-12 appears as a pivotal signal in driving the polarization of Th1-like Tregs, both directly through IL-12R-β2/STAT4-mediated signaling pathway (Koch et al. 2012; Piconese et al. 2014), and indirectly by restraining IL-2 production in T cells and blocking CD25 expression on Tregs (Zhao and Perlman 2012). As discussed below, IL-2 is instead considered as one of the most relevant trophic factors for Treg maintenance and stability.

IL-2, IFN-γ and TNF-α Favor Treg Activation and Suppression

Under physiological conditions, IL-2 is released at the steady-state level mostly by T cells, and Tregs constitutively and highly express CD25, the high-affinity alpha-subunit of the IL-2 receptor (Boyman and Sprent 2012). Initially thought to be crucial for T cell clonal proliferation, IL-2 was soon recognized as the cytokine playing pivotal and non-redundant role in preserving tolerance, rather than immunity, through exerting a variety of functions on Treg homeostasis and activation (Malek and Bayer 2004). Indeed, IL-2 or IL-2 receptor deficiency or blockade has proven to severely impair Treg development and suppressive function, leading to lethal lymphoproliferation and autoimmunity, in a variety of experimental settings (Cheng et al. 2011). IL-2 determines not only the development of thymus-derived (previously called “natural”) Tregs, but also the homeostatic maintenance and survival of peripheral Tregs. IL-2 promotes the *in vitro* differentiation of induced Tregs (iTregs) and maintains their stability in opposition to other polarizing cytokines. Through STAT5 signaling, IL-2 directly induces and sustains Foxp3 expression, thus driving the molecular program of Treg suppressive function (Cheng et al. 2011).

During immune responses, antigen-activated T cells (especially CD4) early release huge amounts of IL-2 and up-regulate high-affinity IL-2 receptor. IL-2 is captured by CD4 T cells, fostering their proliferation and directing their Th1 polarization, and by CD8 cells, optimizing their primary expansion and the development of long-lived memory cells (Boyman and Sprent 2012). A self-amplifying loop is established, with IL-2 expanding T cells that in turn further increase IL-2 amounts. To interrupt such circle turning from virtuous into vicious, Treg suppression intervenes: indeed Tregs, constitutively expressing CD25, promptly respond to IL-2 and proliferate concomitantly to activated T cells, until balancing and even outcompeting them.

At later phases of immune responses, CD4 T cells fully polarized into Th1 effectors, expressing high levels of T-bet, partially lose their competence for IL-2 production (Lazarevic et al. 2013). Therefore, it may be argued that control mechanisms other than IL-2-mediated Treg expansion may take place during an ongoing type-1 response. Self-limiting processes have been discovered that protect from collateral damage possibly triggered by excessive Th1 responses, such as IL-10 production (O'Garra and Vieira 2007) or, more recently, Twist expression (Niesner et al. 2008). However, also Th1-extrinsic mechanisms may contribute to the regulation of type-1 responses, again involving Tregs. Indeed, two cytokines abundantly released by Th1 effectors, namely TNF-α and IFN-γ, may replace IL-2 in activating Treg suppression at later phases of Th1 responses.

TNF-α may play crucial, possibly underestimated so far, roles in promoting Treg suppression not only in physiological conditions but also, and more importantly, in type-1 responses. At steady-state conditions, a subset of both human and mouse Tregs, constitutively expressing at high level the type-2 receptor for TNF-α (TNFR2), displays a more potent suppressive function (Chen et al. 2008; Chen et al. 2010). Tumor necrosis factor receptor (TNFR) 2, together with other members of the TNFR superfamily, plays non-redundant function in Treg thymic development (Mahmud et al. 2014). TNF-α stimulation promotes an activation program in Tregs, which is potently amplified by the induction of other members of the TNFR superfamily exerting similar functions (Chen et al. 2007; Hamano et al. 2011; Nagar et al. 2010). We have recently underscored the relevance of OX40, a receptor belonging to the same family, in fostering Treg activation and suppressive function (Piconese et al. 2014). OX40 was highly expressed in Tregs infiltrating tumor and pre-tumor liver tissues in chronic hepatitis C patients, in direct correlation with the high Treg frequency at those sites. OX40-expressing Tregs were mostly included among Helios^{high} Tregs, characterized by signs of epigenetic and functional stability and by markers of operational immune suppression. *In vitro*, OX40 stimulation, by a soluble agonist or by monocytes expressing OX40 L, promoted Treg proliferation and stability (Piconese et al. 2014). Interestingly, TNF-α promptly induced OX40 up-regulation on Tregs and strongly enhanced Treg suppressive function *in vitro* (Piconese et al. 2014). In line with these data, others have shown TNF-α inhibiting preferentially Helios^{low} Tregs (Zhong and Yazdanbakhsh 2013).

This effect may acquire utmost importance during type-1 inflammatory responses, in which not only Th1 and CTL but also other cells such as M1 macrophages produce vast amounts of TNF-α. In a mouse model of colitis, TNFR2-deficient Tregs failed to maintain Foxp3 expression within the inflamed tissue and could not suppress colitogenic T cells (Chen et al. 2013). In an experimental model of type 1 diabetes, diabetogenic effector cells boosted the expansion of islet-specific and polyclonal Tregs, in an IL-2-independent and rather TNF-α-dependent fashion (Grinberg-Bleyer et al. 2010). Of note, in that model, Tregs up-regulated CXCR3, a marker of the Th1-suppressing specialization program, when boosted by effector T cells-derived TNF-α (Grinberg-Bleyer et al. 2010). Therefore, the effector T cell response may exploit TNF-α to activate an immunoregulatory feedback loop when a potentially dangerous response needs to be moldered. In an experimental setting of human Th1-like Treg polarization *in vitro,* we have recently demonstrated that TNF-α completely counteracts the polarizing activity of IL-12, and rather stabilizes the suppressive Treg phenotype (Piconese et al. 2014). It should be noted, however, that some studies have reported a negative effect of TNF-α on Treg stability and function. For instance, TNF-α in the synovium of rheumatoid arthritis patients was shown to reverse Treg suppressive function and destabilize FOXP3 expression by decreasing its phosphorylation (Nie et al. 2013), and therapies with TNF-α antagonists/inhibitors have shown to recover high proportions of Tregs in several autoimmune diseases (Di Sabatino et al. 2010; Nadkarni et al. 2007). Such controversy may arise from the intrinsic duality of TNF-α activities, pro-inflammatory (through the activation of innate cells) and anti-inflammatory (through the expansion of Tregs). Indeed, Treg rescue following TNF-α blockade approaches may be secondary to indirect effects on other inflammatory pathways depressing Treg expansion, rather than attributable to direct effects of TNF-α on Tregs.

IFN-γ is considered the prototypical cytokine released by NK cells during early innate responses and by Th1 cells and CTL at more advanced phases, thus conceivably abundant in microenvironments characterized by type-1 inflammation. IFN-γ has been recognized to directly induce (via STAT1 phosphorylation) T-bet expression that in turn promotes IL-12R-β2 transcription (Koch et al. 2012). Therefore, at early moments of type-1 responses, IFN-γ derived from innate cells can sensitize Tregs to IL-12, thus rendering them susceptible to Th1-like polarization. However, along the development the adaptive type-1 immunity, IFN-γ, mostly derived from T cells, paradoxically drives the expansion of Th1-suppressing Tregs (Koch et al. 2009). This likely occurs in advanced phases of Th1-responses, in which the depletion of IL-12 curtails the full differentiation into Th1-like Tregs and favors the establishment of Th1-suppressing Tregs producing low amount of IFN-γ. Such activity contributes to the regulation of immunopathology in a mouse model of toxoplasmosis (Hall et al. 2012). Similarly, we have shown that IFN-γ induces T-bet up-regulation in human Tregs (Piconese et al. 2014). Therefore, like IL-2 and TNF-α, also IFN-γ produced by Th1 effector cells can initiate a

feedback regulatory loop through the expansion of Tregs specially addressed to controlling type-1 response. However, IFN-γ signaling should be finely regulated to prevent the conversion of Th1-suppressing into Th1-like Tregs. Indeed, a prolonged IFN-γ/STAT1 signaling, otherwise normally controlled by miR146, can paralyze Th1-suppressing Tregs and promote the polarization of IFN-γ producing Th1-like cells, disrupting Treg-mediated regulation of type-1 inflammation (Lu et al. 2010).

Controversies About IL-27 and IFN-α

IL-27, belonging to the same family of IL-12, is released by APCs during type-1 responses and contributes to Th1 cell polarization by activating STAT1 and T-bet. However, contrary to IL-12, IL-27 also displays some immunoregulatory properties, promoting the induction of suppressive cytokines, such as IL-10, in a variety of immune cell types (Hunter and Kastelein 2012). With respect to Tregs, and again unlike IL-12, IL-27 did not down-regulate Foxp3 expression or molder their suppressive function (Hunter and Kastelein 2012). Rather, and similarly to IFN-γ, IL-27 induced Th1-suppressing Tregs, expressing T-bet and CXCR3, in experimental models of bacterial infection (Hall et al. 2012). Interestingly, the two cytokines showed a preferential anatomical competence, with IFN-γ being more relevant in lymphoid tissue and IL-27 particularly prominent at sites of inflammation (Hall et al. 2012).

Controversy also exists about the role of type I IFNs, especially IFN-α, if predominantly pro-inflammatory or rather immunomodulatory cytokines in type-1 responses (Gonzalez-Navajas et al. 2012; Trinchieri 2010). Indeed, while on the one side IFN-α may induce IFN-γ in T and NK cells via STAT4 phosphorylation (which is anyway unstable), in some conditions it rather antagonizes IL-12 production and signaling (Trinchieri 2010). Controversy also exists about the effects of IFN-α in Treg expansion and function. Some reports assert that IFN-α promotes Treg-mediated suppression in mouse models of cancer (Stewart et al. 2013) and colitis (Lee et al. 2012). Other studies have instead underscored a negative effect of IFN-α on both murine (Pace et al. 2010) and human (Bacher et al. 2013; Le Buanec et al. 2011) Treg suppressive function. A recent report has clearly shown that, during an acute viral infection in mice, type I IFNs inhibited costimulation-dependent Treg proliferation thus unleashing the emergence of an optimal antiviral adaptive immunity (Srivastava et al. 2014). In line with this data, we could observe that IFN-α strongly suppressed the proliferation of human Tregs *in vitro,* and significantly decreased Treg frequency *in vivo* in patients with chronic hepatitis C undergoing PEG-interferon/ribavirin therapy (unpublished data). It may be argued that different aspects, such as local dosage of IFN-α, timing of IFN-α release and concomitant presence of other cytokines may determine the prevalent effect of this cytokine on Treg functions.

Interplay Between Costimulatory/Coinhibitory Receptors and Cytokine Signals

Several examples can be quoted from the literature of the interplay between cytokine signaling pathways and costimulatory or coinhibitory receptors. Tregs may constitutively express some of these receptors in the peripheral lymphoid organs, and many of them have been shown to be strongly induced or up-regulated within inflamed tissues.

Tregs infiltrating human liver affected by chronic hepatitis C significantly up-regulate PD-1 (Franceschini et al. 2009; Piconese et al. 2014), a receptor belonging to the CD28/CTLA-4 family and recognized as a co-inhibitory molecule. Programmed cell death (PD)-1 was shown to temper the expansion of a peculiar Treg subtype known as T follicular regulatory cells, specialized in the suppression of T follicular helper cells that arrange humoral responses (Sage et al. 2013). Within the liver, PD-1 signal dampened Treg proliferation mainly through the inhibition of IL-2/STAT5 axis. Indeed, PD-1 blockade rescued Treg proliferation and STAT5 phosphorylation *ex vivo* (Franceschini et al. 2009). Such PD-1-mediated constraining of Treg expansion may contribute to mitigate immune suppression, allowing an immunological compromise between anti-viral immunity and immunopathology in chronic infections. Therefore, PD-1 seems to provide opposite signals to Tregs according to the phase of T cell activation: they contribute to Treg conversion from naïve Tconvs (induction signal), on the one hand, and constrain experienced Treg expansion and functions (inhibition signal), on the other hand (Barnaba and Schinzari 2013).

Contrary to PD-1, the costimulatory receptor OX40 seems to rather sustain immunoregulatory Treg activities in HCV-related cirrhosis and cancer (Piconese et al. 2014). OX40 may result in opposite effects depending on the cytokine contexts, whether non-inflammatory or pro-inflammatory. Indeed, in a mouse model of autoimmune disease, an OX40 agonist promoted the expansion of protective Tregs only when administered at priming, and not after disease onset (Ruby et al. 2009). This data suggests that the OX40 pathway may cooperate with steady-state homeostatic cytokines, mostly IL-2, and rather work in opposition to inflammatory cytokines. In line with this hypothesis, a defective Treg homeostasis and competitive fitness was observed in OX40-null mice, which was attributable to an impaired ability to optimally utilize IL-2 (Piconese et al. 2010). OX40-null Tregs showed reduced STAT5 phosphorylation in response to IL-2, an event possibly linked to an overexpression of the STAT5 inhibitor SOCS1, in turn sustained by low levels of miR155 (Piconese et al. 2010). Of note, such defect impaired Treg expansion *in vivo* not only in conditions of homeostatic proliferation but also during inflammatory responses in mouse models of colitis (Griseri et al. 2010; Piconese et al. 2010).

OX40 engagement may promote Treg proliferation partly through the interaction with cytokine signaling. In mice, OX40 ligation synergized with IL-2 administration in promoting STAT5 phosphorylation and the expansion of fully suppressive Tregs (Xiao et al. 2012). Using human cells, others and we have

demonstrated that OX40 stimulation promotes Treg proliferation (Hippen et al. 2008; Piconese et al. 2014). Of note, OX40 was up-regulated on human Treg surface by IL-2 and more massively by IL-2 and TNF-α co-exposure (Nagar et al. 2010; Piconese et al. 2010). The OX40/OX40 L axis may then represent an amplification loop of TNF-α signal towards Treg expansion and stabilization. In line with this idea, Tregs highly expressing OX40 in human hepatic cancer and cirrhosis were preferentially contained within the committed (Helios^{high}) and specialized (Th1-suppressing) subpopulation, rather than in the unstable Th1-like counterpart. Supporting OX40 as a Treg-stabilizing signal, an OX40 agonist inhibited Th1-like Treg polarization *in vitro* (Piconese et al. 2014). It may be suggested that OX40- and TNF-α-initiated signaling pathways, mostly mediated by NF-kB activation (Nagar et al. 2010), may directly antagonize IL-12 axis by still unknown mechanism, possibly mediated by SOCS molecules. However, the antagonism between OX40/TNF-α and IL-12-mediated Th1-like polarization may also be explained taking into account the heterogeneity of Tregs: indeed, OX40 and TNF-α may preferentially promote the proliferation of those Treg subsets which are less susceptible to IL-12-mediated diversion. Supporting this view, we could observe a pattern of mutually exclusive expression between OX40 and IL-12R-β2 in human Tregs *ex vivo* (Piconese et al. 2014).

Conclusions

Tregs reside in lymphoid and non-lymphoid tissues, hence being possibly exposed to distinct type-1 cytokines at different sites and phases of a type-1 response. Furthermore, the concomitant stimulation of some surface receptors may finely modulate Treg response to many cytokines, amplifying or antagonizing cytokine signals. From the above overview, a duality of Treg response to type-1 cytokines may be broadly delineated: on the one side, IL-12, mostly produced by APCs at the initial stages of Th1 priming in lymphoid organs, seems to antagonize Treg suppression and rather promote Th1-like Treg polarization; on the other side, cytokines released by proliferating T cells, such as IL-2, or by already differentiated Th1 cells, such as TNF-α and IFN-γ, at later phases of type-1 immunity mainly in inflamed tissues, seem to rather promote Treg proliferation, suppressive function and specialization into Th1-suppressing cells (Fig. 2.1). Of note, Tregs only inducibly express IL-12R-β2, while constitutively expressing at high levels the receptors for IL-2 and TNF-α. Similarly to IL-12, IFN-α is released by plasmacytoid dendritic cells and tissue cells relatively early during viral infections, and it may contribute to the initiation of innate and adaptive anti-viral responses also by antagonizing Tregs. We are tempted to speculate that such alternate processes may have evolved to ensure a transient Treg deactivation when type-1 responses need to be initiated, while promoting the expansion of specialized Tregs when type-1 inflammation should undergo resolution, to prevent collateral tissue damage.

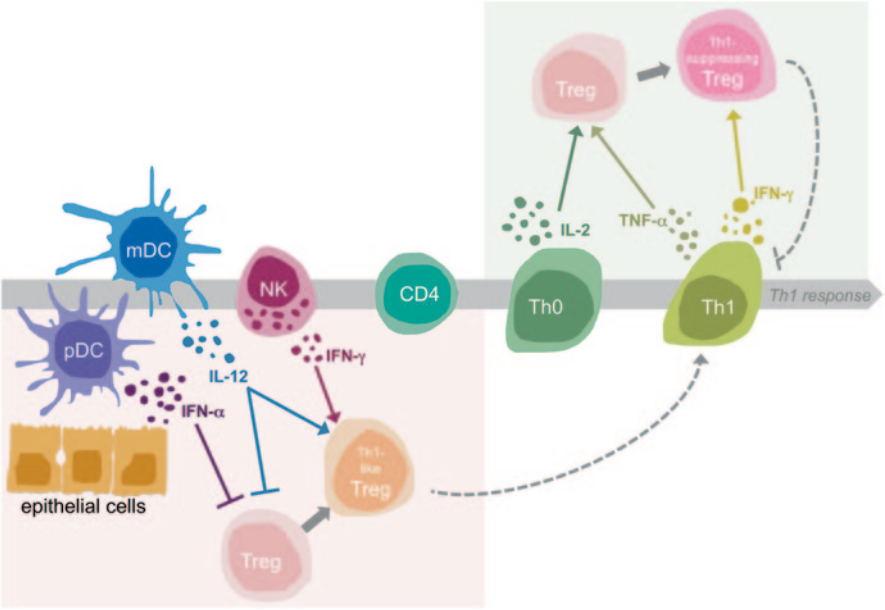

Fig. 2.1 Type-1 cytokines differentially affect Treg functions at distinct stages of type-1 responses. Cytokines produced during priming or effector phase of type-1 response may respectively antagonize or promote Treg-mediated suppression. At the initiation of type-1 responses (*red area on the left*), IFN-γ produced by innate lymphocytes (i.e., NK cells) induces (via STAT1/T-bet axis) IL-12R-β2 expression on Tregs making them susceptible to IL-12, which is mostly produced by myeloid dendritic cells (*mDC*). IL-12, together with IFN-α released by plasmacytoid dendritic cells (*pDC*) and epithelial cells, inhibits Treg proliferation and suppression; IL-12 even promotes the polarization of Th1-like Tregs, which possibly contribute to inflammation. At later phases of type-1 responses (*green area on the right*), Th0- and Th1-derived cytokines such as IL-2, TNF-α and IFN-γ foster Treg expansion and inhibitory function and promote their specialization into Th1-suppressing cells. Such alternate modulation would allow the development of type-1 responses while ensuring proper resolution of inflammation.

References

Bacher, N., Raker, V., Hofmann, C., Graulich, E., Schwenk, M., Baumgrass, R., Bopp, T., Zechner, U., Merten, L., Becker, C., & Steinbrink, K. (2013). Interferon-alpha suppresses cAMP to disarm human regulatory T cells. *Cancer Research, 73*(18), 5647–5656. doi:10.1158/0008–5472.CAN-12-3788.

Barnaba, V. (2010). Hepatitis C virus infection: A "liaison a trois" amongst the virus, the host, and chronic low-level inflammation for human survival. *Journal of Hepatology, 53*(4), 752–761. doi:10.1016/j.jhep.2010.06.003.

Barnaba, V., & Schinzari, V. (2013). Induction, control, and plasticity of Treg cells: The immune regulatory network revised? *European Journal of Immunology, 43*(2), 318–322. doi:10.1002/eji.201243265.

Boyman, O., & Sprent, J. (2012). The role of interleukin-2 during homeostasis and activation of the immune system. *Nature Reviews Immunology, 12*(3), 180–190. doi:10.1038/nri3156.

Burzyn, D., Benoist, C., & Mathis, D. (2013a). Regulatory T cells in nonlymphoid tissues. *Nature Immunology, 14*(10), 1007–1013. doi:10.1038/ni.2683.

Burzyn, D., Kuswanto, W., Kolodin, D., Shadrach, J. L., Cerletti, M., Jang, Y., Sefik, E., Tan, T. G., Wagers, A. J., Benoist, C., & Mathis, D. (2013b). A special population of regulatory T cells potentiates muscle repair. *Cell, 155*(6), 1282–1295. doi:10.1016/j.cell.2013.10.054.

Chen, X., Baumel, M., Mannel, D. N., Howard, O. M., & Oppenheim, J. J. (2007). Interaction of TNF with TNF receptor type 2 promotes expansion and function of mouse CD4+ CD25+ T regulatory cells. *The Journal of Immunology, 179*(1), 154–161.

Chen, X., Subleski, J. J., Kopf, H., Howard, O. M., Mannel, D. N., & Oppenheim, J. J. (2008). Cutting edge: Expression of TNFR2 defines a maximally suppressive subset of mouse CD4+CD25+FoxP3+ T regulatory cells: Applicability to tumor-infiltrating T regulatory cells. *The Journal of Immunology, 180*(10), 6467–6471.

Chen, X., Subleski, J. J., Hamano, R., Howard, O. M., Wiltrout, R. H., & Oppenheim, J. J. (2010). Co-expression of TNFR2 and CD25 identifies more of the functional CD4+FOXP3+ regulatory T cells in human peripheral blood. *European Journal of Immunology, 40*(4), 1099–1106. doi:10.1002/eji.200940022.

Chen, X., Wu, X., Zhou, Q., Howard, O. M., Netea, M. G., & Oppenheim, J. J. (2013). TNFR2 is critical for the stabilization of the CD4+Foxp3+ regulatory T. cell phenotype in the inflammatory environment. *The Journal of Immunology, 190*(3), 1076–1084. doi:10.4049/jimmunol.1202659.

Cheng, G., Yu, A., & Malek, T. R. (2011). T-cell tolerance and the multi-functional role of IL-2.R signaling in T-regulatory cells. *Immunological Reviews, 241*(1), 63–76. doi:10.1111/j.1600–065X.2011.01004.x.

Cipolletta, D., Feuerer, M., Li, A., Kamei, N., Lee, J., Shoelson, S. E., Benoist, C., & Mathis, D. (2012). PPAR-gamma is a major driver of the accumulation and phenotype of adipose tissue Treg cells. *Nature, 486*(7404), 549–553. doi:10.1038/nature11132.

Di Sabatino, A., Biancheri, P., Piconese, S., Rosado, M. M., Ardizzone, S., Rovedatti, L., Ubezio, C., Massari, A., Sampietro, G. M., Foschi, D., Porro, G. B., Colombo, M. P., Carsetti, R., MacDonald, T. T., & Corazza, G. R. (2010). Peripheral regulatory T cells and serum transforming growth factor-beta: Relationship with clinical response to infliximab in Crohn's disease. *Inflammatory Bowel Diseases, 16*(11), 1891–1897. doi:10.1002/ibd.21271.

Dominguez-Villar, M., Baecher-Allan, C. M., & Hafler, D. A. (2011). Identification of T helper type 1-like, Foxp3+ regulatory T cells in human autoimmune disease. *Nature Medicine, 17*(6), 673–675. doi:10.1038/nm.2389.

Du, W., Shen, Y. W., Lee, W. H., Wang, D., Paz, S., Kandeel, F., & Liu, C. P. (2013). Foxp3+ Treg expanded from patients with established diabetes reduce Helios expression while retaining normal function compared to healthy individuals. *PLos One, 8*(2), e56209. doi:10.1371/journal.pone.0056209.

Franceschini, D., Paroli, M., Francavilla, V., Videtta, M., Morrone, S., Labbadia, G., Cerino, A., Mondelli, M. U., & Barnaba, V. (2009). PD-L1 negatively regulates CD4+CD25+Foxp3+ Tregs by limiting STAT-5 phosphorylation in patients chronically infected with HCV. *Journal of Clinical Investigation, 119*(3), 551–564. doi:10.1172/JCI36604.

Gonzalez-Navajas, J. M., Lee, J., David, M., & Raz, E. (2012). Immunomodulatory functions of type I interferons. *Nature Reviews Immunology, 12*(2), 125–135. doi:10.1038/nri3133.

Grinberg-Bleyer, Y., Saadoun, D., Baeyens, A., Billiard, F., Goldstein, J. D., Gregoire, S., Martin, G. H., Elhage, R., Derian, N., Carpentier, W., Marodon, G., Klatzmann, D., Piaggio, E., & Salomon, B. L. (2010). Pathogenic T cells have a paradoxical protective effect in murine autoimmune diabetes by boosting Tregs. *Journal of Clinical Investigation, 120*(12), 4558–4568. doi:10.1172/JCI42945.

Griseri, T., Asquith, M., Thompson, C., & Powrie, F. (2010). OX40 is required for regulatory T cell-mediated control of colitis. *The Journal of Experimental Medicine, 207*(4), 699–709. doi:10.1084/jem.20091618.

Hall, A. O., Beiting, D. P., Tato, C., John, B., Oldenhove, G., Lombana, C. G., Pritchard, G. H., Silver, J. S., Bouladoux, N., Stumhofer, J. S., Harris, T. H., Grainger, J., Wojno, E. D., Wagage, S., Roos, D. S., Scott, P., Turka, L. A., Cherry, S., Reiner, S. L., Cua, D., Belkaid, Y., Elloso, M. M., & Hunter, C. A. (2012). The cytokines interleukin 27 and interferon-gamma promote

distinct Treg cell populations required to limit infection-induced pathology. *Immunity, 37*(3), 511–523. doi:10.1016/j.immuni.2012.06.014.

Hamano, R., Huang, J., Yoshimura, T., Oppenheim, J. J., Chen, X. (2011). TNF optimally activatives regulatory T cells by inducing TNF receptor superfamily members TNFR2, 4-1BB and OX40. *European Journal of Immunology, 41*(7), 2010–2020. doi:10.1002/eji.201041205.

Hippen, K. L., Harker-Murray, P., Porter, S. B., Merkel, S. C., Londer, A., Taylor, D. K., Bina, M., Panoskaltsis-Mortari, A., Rubinstein, P., Van Rooijen, N., Golovina, T. N., Suhoski, M. M., Miller, J. S., Wagner, J. E., June, C. H., Riley, J. L., & Blazar, B. R. (2008). Umbilical cord blood regulatory T-cell expansion and functional effects of tumor necrosis factor receptor family members OX40 and 4-1BB expressed on artificial antigen-presenting cells. *Blood, 112*(7), 2847–2857. doi:10.1182/blood-2008-01-132951.

Hunter, C. A., & Kastelein, R. (2012). Interleukin-27: Balancing protective and pathological immunity. *Immunity, 37*(6), 960–969. doi:10.1016/j.immuni.2012.11.003.

Koch, M. A., Tucker-Heard, G., Perdue, N. R., Killebrew, J. R., Urdahl, K. B, & Campbell, D. J. (2009). The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nature Immunology, 10*(6), 595–602. doi:10.1038/ni.1731.

Koch, M. A., Thomas, K. R., Perdue, N. R., Smigiel, K. S., Srivastava, S., & Campbell, D. J. (2012). T-bet(+) Treg cells undergo abortive Th1 cell differentiation due to impaired expression of IL-12 receptor beta2. *Immunity, 37*(3), 501–510. doi:10.1016/j.immuni.2012.05.031.

Koenecke, C., Lee, C. W., Thamm, K., Fohse, L., Schafferus, M., Mittrucker, H. W., Floess, S., Huehn, J., Ganser, A., Forster, R., & Prinz, I. (2012). IFN-gamma production by allogeneic Foxp3+ regulatory T cells is essential for preventing experimental graft-versus-host disease. *The Journal of Immunology, 189*(6), 2890–2896. doi:10.4049/jimmunol.1200413.

Lazarevic, V., Glimcher, L. H., & Lord, G. M. (2013). T-bet: A bridge between innate and adaptive immunity. *Nature Reviews Immunology, 13*(11), 777–789. doi:10.1038/nri3536.

Le Buanec, H., Gougeon, M. L., Mathian, A., Lebon, P., Dupont, J. M., Peltre, G., Hemon, P., Schmid, M., Bizzini, B., Kunding, T., Burny, A., Bensussan, A., Amoura, Z., Gallo, R. C., & Zagury, D. (2011). IFN-alpha and CD46 stimulation are associated with active lupus and skew natural T regulatory cell differentiation to type 1 regulatory T (Tr1) cells. *Proceedings of the National Academy of Sciences of the United States of America, 108*(47), 18995–19000. doi:10.1073/pnas.1113301108.

Lee, S. E., Li, X., Kim, J. C., Lee, J., Gonzalez-Navajas, J. M., Hong, S. H., Park, I. K., Rhee, J. H., & Raz, E. (2012). Type I interferons maintain Foxp3 expression and T-regulatory cell functions under inflammatory conditions in mice. *Gastroenterology, 143*(1), 145–154. doi:10.1053/j.gastro.2012.03.042.

Lu, L. F., Boldin, M. P., Chaudhry, A., Lin, L. L., Taganov, K. D., Hanada, T., Yoshimura, A., Baltimore, D., & Rudensky, A. Y. (2010). Function of miR-146a in controlling Treg cell-mediated regulation of Th1 responses. *Cell, 142*(6), 914–929. doi:10.1016/j.cell.2010.08.012.

Mahmud, S. A., Manlove, L. S., Schmitz, H. M., Xing, Y., Wang, Y., Owen, D. L., Schenkel, J. M., Boomer, J. S., Green, J. M., Yagita, H., Chi, H., Hogquist, K. A., & Farrar, M. A. (2014). Costimulation via the tumor-necrosis factor receptor superfamily couples TCR signal strength to the thymic differentiation of regulatory T cells. *Nature Immunology, 15*(5), 473–481. doi:10.1038/ni.2849.

Malek, T. R., & Bayer, A. L. (2004). Tolerance, not immunity, crucially depends on IL-2. *Nature Reviews Immunology, 4*(9), 665–674. doi:10.1038/nri1435.

McClymont, S. A., Putnam, A. L., Lee, M. R., Esensten, J. H., Liu, W., Hulme, M. A., Hoffmuller, U., Baron, U., Olek, S., Bluestone, J. A., Brusko, T. M. (2011). Plasticity of human regulatory T cells in healthy subjects and patients with type 1 diabetes. *The Journal of Immunology, 186*(7), 3918–3926. doi:10.4049/jimmunol.1003099.

Nadkarni, S., Mauri, C., & Ehrenstein, M. R. (2007). Anti-TNF-alpha therapy induces a distinct regulatory T cell population in patients with rheumatoid arthritis via TGF-beta. *The Journal of Experimental Medicine, 204*(1), 33–39. doi:10.1084/jem.20061531.

Nagar, M., Jacob-Hirsch, J., Vernitsky, H., Berkun, Y., Ben-Horin, S., Amariglio, N., Bank, I., Kloog, Y., Rechavi, G., & Goldstein, I. (2010). TNF activates a NF-kappaBregulated cellular

program in human CD45RA-regulatory T cells that modulates their suppressive function. *The Journal of Immunology, 184*(7), 3570–3581. doi:10.4049/jimmunol.0902070.

Nie, H., Zheng, Y., Li, R., Guo, T. B., He, D., Fang, L., Liu, X., Xiao, L., Chen, X., Wan, B., Chin, Y. E., & Zhang, J. Z. (2013). Phosphorylation of FOXP3 controls regulatory T cell function and is inhibited by TNF-alpha in rheumatoid arthritis. *Nature Medicine, 19*(3), 322–328. doi:10.1038/nm.3085.

Niesner, U., Albrecht, I., Janke, M., Doebis, C., Loddenkemper, C., Lexberg, M. H., Eulenburg, K., Kreher, S., Koeck, J., Baumgrass, R., Bonhagen, K., Kamradt, T., Enghard, P., Humrich, J. Y., Rutz, S., Schulze-Topphoff, U., Aktas, O., Bartfeld, S., Radbruch, H., Hegazy, A. N., Lohning, M., Baumgart, D. C., Duchmann, R., Rudwaleit, M., Haupl, T., Gitelman, I., Krenn, V., Gruen, J., Sieper, J., Zeitz, M., Wiedenmann, B., Zipp, F., Hamann, A., Janitz, M., Scheffold, A., Burmester, G. R., Chang, H. D., & Radbruch, A. (2008). Autoregulation of Th1-mediated inflammation by twist1. *The Journal of Experimental Medicine, 205*(8), 1889–1901. doi:10.1084/jem.20072468.

O'Garra, A., & Vieira, P. (2007). T(H)1 cells control themselves by producing interleukin 10. *Nature Reviews Immunology, 7*(6), 425–428. doi:10.1038/nri2097.

Oldenhove, G., Bouladoux, N., Wohlfert, E. A., Hall, J. A., Chou, D., Dos Santos, L., O'Brien, S., Blank, R., Lamb, E., Natarajan, S., Kastenmayer, R., Hunter, C., Grigg, M. E., & Belkaid, Y. (2009). Decrease of Foxp3+ Treg cell number and acquisition of effector cell phenotype during lethal infection. *Immunity, 31*(5), 772–786. doi:10.1016/j.immuni.2009.10.001.

Pace, L., Vitale, S., Dettori, B., Palombi, C., La Sorsa, V., Belardelli, F., Proietti, E., & Doria, G. (2010). APC activation by IFN-alpha decreases regulatory T cell and enhances Th cell functions. *The Journal of Immunology, 184*(11), 5969–5979. doi:10.4049/jimmunol.0900526.

Piconese, S., Pittoni, P., Burocchi, A., Gorzanelli, A., Care, A., Tripodo, C., & Colombo, M. P. (2010). A non-redundant role for OX40 in the competitive fitness of Treg in response to IL-2. *European Journal of Immunology, 40*(10), 2902–2913. doi:10.1002/eji.201040505.

Piconese, S., Timperi, E., Pacella, I., Schinzari, V., Tripodo, C., Rossi, M., Guglielmo, N., Mennini, G., Grazi, G. L., Di Filippo, S., Brozzetti, S., Fazzi, K., Antonelli, G., Lozzi, M. A., Sanchez, M., & Barnaba, V. (2014). Human OX40 tunes the function of regulatory T cells in tumor and non-tumor areas of HCV-infected liver tissue. Hepatology. doi:10.1002/hep.27188.

Redjimi, N., Raffin, C., Raimbaud, I., Pignon, P., Matsuzaki, J., Odunsi, K., Valmori, D., & Ayyoub, M. (2012). CXCR3+ T regulatory cells selectively accumulate in human ovarian carcinomas to limit type I immunity. *Cancer Research, 72*(17), 43514360. doi:10.1158/0008-5472. CAN-12-0579.

Ruby, C. E., Yates, M. A., Hirschhorn-Cymerman, D., Chlebeck, P., Wolchok, J. D., Houghton, A. N., Offner, H., & Weinberg, A. D. (2009). Cutting Edge: OX40 agonists can drive regulatory T cell expansion if the cytokine milieu is right. *The Journal of Immunology, 183*(8), 4853–4857. doi:10.4049/jimmunol.0901112.

Sage, P. T., Francisco, L. M., Carman, C. V., & Sharpe, A. H. (2013). The receptor PD-1 controls follicular regulatory T cells in the lymph nodes and blood. *Nature Immunology, 14*(2), 152–161. doi:10.1038/ni.2496.

Shafiani, S., Dinh, C., Ertelt, J. M., Moguche, A. O., Siddiqui, I., Smigiel, K. S., Sharma, P., Campbell, D. J., Way, S. S., & Urdahl, K. B. (2013). Pathogen-specific Treg cells expand early during mycobacterium tuberculosis infection but are later eliminated in response to Interleukin-12. I*mmunity, 38*(6), 1261–1270. doi:10.1016/j.immuni.2013.06.003.

Smigiel, K. S., Srivastava, S., Stolley, J. M., & Campbell, D. J. (2014). Regulatory T-cell homeostasis: Steady-state maintenance and modulation during inflammation. *Immunological Reviews, 259*(1), 40–59. doi:10.1111/imr.12170.

Srivastava, S., Koch, M. A., Pepper, M., & Campbell, D. J. (2014). Type I interferons directly inhibit regulatory T cells to allow optimal antiviral T cell responses during acute LCMV infection. The *Journal of Experimental Medicine, 211*(5), 961–974. doi:10.1084/jem.20131556.

Stewart, C. A., Metheny, H., Iida, N., Smith, L., Hanson, M., Steinhagen, F., Leighty, R. M., Roers, A., Karp, C. L., Muller, W., & Trinchieri, G. (2013). Interferon-dependent IL-10 production by Tregs limits tumor Th17 inflammation. *Journal of Clinical Investigation, 123*(11), 4859–4874. doi:10.1172/JCI65180.

Trinchieri, G. (2010). Type I interferon: Friend or foe? *The Journal of Experimental Medicine, 207*(10), 2053–2063. doi:10.1084/jem.20101664.

Xiao, X., Gong, W., Demirci, G., Liu, W., Spoerl, S., Chu, X., Bishop, D. K., Turka, L. A., & Li, X. C. (2012). New insights on OX40 in the control of T cell immunity and immune tolerance in vivo. *The Journal of Immunology, 188*(2), 892–901. doi:10.4049/jimmunol.1101373.

Zhao, J., & Perlman, S. (2012). Differential effects of IL-12 on Tregs and non-Treg T cells: Roles of IFN-gamma, IL-2 and IL-2R. *PLos One, 7*(9), e46241. doi:10.1371/journal.pone.0046241.

Zhao, J., Fett, C., Trandem, K., Fleming, E., & Perlman, S. (2011). IFN-gamma-and IL-10expressing virus epitope-specific Foxp3(+) T reg cells in the central nervous system during encephalomyelitis. *The Journal of Experimental Medicine, 208*(8), 1571–1577. doi:10.1084/jem.20110236.

Zhong, H., & Yazdanbakhsh, K. (2013). Differential control of Helios(+/-) Treg development by monocyte subsets through disparate inflammatory cytokines. *Blood, 121*(13), 2494–2502. doi:10.1182/blood-2012-11-469122.

Chapter 3
The Role of Il-12 and Type I Interferon in Governing the Magnitude of CD8 T Cell Responses

Gabriel R. Starbeck-Miller and John T. Harty

Abstract Antigen-specific CD8 T cells provide an important protective role in response to infection by viruses, intracellular bacteria, and parasites. Pathogen-specific CD8 T cells render this protection by undergoing robust expansion in numbers while gaining the ability to produce cytokines and cytolytic machinery. Creating optimal CD8 T cell responses to infection can be critical for raising sufficient armament to provide protection against invading intracellular pathogens. Although CD8 T cells have protective value, many vaccine strategies tend to focus on creating productive B cell antibody responses to promote immunological protection. Even though antibody responses can be highly protective, coupling optimal CD8 T cell responses with suboptimal B cell responses could provide higher orders of protection than either one on their own. Therefore, a deeper understanding of the pathways that ultimately guide the magnitude of CD8 T cell responses is required to explore this potential therapeutic benefit. The following chapter highlights our current understanding of how inflammatory cytokines regulate the magnitude of CD8 T cell responses.

Keywords CD8 T cells · Inflammatory cytokines · Interleukin-12 · Interleukin-2 · Type I interferon · Signal 3

J. T. Harty (✉)
Department of Microbiology, University of Iowa, Iowa City, IA 52242, USA
e-mail: john-harty@uiowa.edu

Department of Pathalogy, University of Iowa, Iowa City, IA 52242, USA

G. R. Starbeck-Miller
Interdisciplinary Graduate Program in Immunology, University of Iowa,
Iowa City, IA 52242, USA
e-mail: gabriel.starbeck.miller@gmail.com

S. P. Schoenberger et al. (eds.), *Crossroads Between Innate and Adaptive Immunity V,*
Advances in Experimental Medicine and Biology 850, DOI 10.1007/978-3-319-15774-0_3

Initiating a CD8 T Cell Response

CD8 T cells can provide a valuable adaptive immune response to help reduce and control intracellular pathogens. In order to initiate an optimal CD8 T cell response, naïve CD8 T cells ultimately require cognate antigen stimulation in the context of MHC I presentation by professional antigen presenting cells (APC) such as dendritic cells (DC). APC have developed several pathways to efficiently find and display cognate antigen to T cells. During homeostasis and infection, DC are constantly breaking down endogenous proteins with cytosolic peptidases and transporting these peptides to the endoplasmic reticulum (ER) using the MHC locus-encoded peptide transporter associated with antigen presentation (TAP) (Rock and Shen 2005). After being further processed by ER aminopeptidases, remnants of these endogenous proteins are broken down into 8- and 9-mer peptides that are then able to interact with MHC I molecules (York et al. 2002; Serwold et al. 2002). If a particular peptide contains an appropriate amino acid sequence that enables it to bind to and stabilize the interactions between MHC I complex proteins, it can then be transported from the ER and presented on the surface of the APC (Joffre et al. 2012). This classical MHC I presentation pathway enables DC to present pathogen-associated peptides to CD8 T cells when directly infected by an intracellular pathogen.

Since the cellular tropism of infection can vary depending on the nature of the intracellular pathogen, DC may never become directly infected. However, DC maintain highly active endocytotic and phagocytotic pathways thereby enabling them to constantly engulf and clear nearby cell debris (Serwold et al. 2002). Similar to endogenously expressed proteins, engulfed proteins can also be broken down and presented in complex with MHC I on the surface of the DC by a process called cross-presentation (Joffre et al. 2012; Bevan 1976). Although it is not completely understood how internalized proteins are processed and loaded onto MHC I complexes, studies have demonstrated that the endosomal peptidase insulin-responsive aminopeptidase (IRAP) is important for cross-presentation (Saveanu et al. 2009). Moreover, endocytic and phagocytic compartments have also been shown to co-localize with TAP/MHC I-loading molecules (Houde et al. 2003; Guermonprez et al. 2003), or, in some instances, directly fuse with the ER (Saveanu et al. 2009). Regardless of the particular pathway, DC are well adapted to process and present antigen during infection. The existence of both classical MHC I presentation and cross-presentation enables DC to initiate robust antigen-specific CD8 T cell responses to a wide variety of infections, regardless of cellular tropism.

The Expansion and Contraction of the CD8 T Cell Response

Antigen processing and presentation is just the first step for initiating a CD8 T cell response. Stimulating DC with inflammatory cytokines (Trombetta and Mellman 2005) and pathogen-associated molecular patterns (PAMPs) (Medzhitov 2001) greatly enhances their ability to prime naïve CD8 T cells as a result of increased

MHC I complex surface expression and increased expression of co-stimulatory molecules (e.g. B7 ligands). This maturation process also promotes DC migration toward secondary lymphoid tissue (Randolph et al. 2008) wherein many naïve CD8 T cells wait to become activated. If a single naïve CD8 T cell is sufficiently activated by matured DC presenting cognate peptide, it will soon divide and produce as many as 10,000 daughter cells within 5–7 days (Kaech et al. 2002). In addition to its division, activated CD8 T cells will also gain effector functions that include the ability to produce cytokines (such as IFNγ and TNFα) while also gaining cytolytic machinery (such as Granzyme B and Perforin expression) (Harty and Badovinac 2008). After a brief period (typically 5–8 days) of expansion, approximately 90–98 % of the effector CD8 T cells will undergo apoptosis and contract down to a small pool of memory CD8 T cells (Badovinac and Harty 2006).

Signals that Guide Optimal Primary CD8 T Cell Responses

During infection, the initiation of CD8 T cell responses is ultimately dependent on TCR ligation by antigen in the context of MHC I presentation. TCR ligation induces the activation of Src family kinases (such as Lck and Fyn) that are then able to phosphorylate the immunoreceptor tyrosine-based activation motifs (ITAMs) of CD3. CD3 ITAM phosphorylation promotes the recruitment and activation of zeta-chain-associated protein kinase of 70 kDA (ZAP-70) (Smith-Garvin et al. 2009) that is then able to phosphorylate the adaptor molecules linker for the activation of T cells (LAT) and the cytosolic adaptor protein Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDA (SLP-76) (Smith-Garvin et al. 2009). Activated SLP-76 and LAT mediate complex protein-protein interactions that are critical for perpetuating the TCR signal via multiple signaling pathways (including Phospholipase C, Phosphoinositide 3-kinase, and Ras (Koretzky et al. 2006; Zhang et al. 1999; Sommers et al. 2004)) that promote T cell proliferation and effector protein expression. Although TCR stimulation is important for initiating the activation of naïve CD8 T cells, a second signal (i.e. co-stimulation) is crucial for full T cell activation. Co-stimulatory interactions, such as CD28 ligation on the surface of a responding CD8 T cells by B7 ligands (CD80/86) on the surface of APC, co-localize with TCR components and amplify TCR signaling (Saito et al. 2010; Ledbetter et al. 1987). In turn, cross-linking of both TCR and CD28 leads to enhanced T cell expansion and effector cytokine production relative to TCR ligation alone (Acuto and Michel 2003). Thus, TCR ligation and co-stimulation work together in order to promote the full activation of naïve CD8 T cells.

"Signal 3": Inflammatory Cytokines

Although providing high levels of "signal 1" (TCR stimulation) and "signal 2" (co-stimulation) to naive T cells induces their activation, a third signal (i.e. inflammatory cytokine stimulation) is important for optimal expansion. Early studies had

indicated that cytokines such as IFNγ (Simon et al. 1986), IL-12 (Gately et al. 1992; Mehrotra et al. 1993), IL-1 (Renauld et al. 1989), and IL-6 (Renauld et al. 1989) can substantially enhance activated CD8 T cell cytotoxicity and expansion *in vitro*, as well as during *in vivo* peptide immunization (Schmidt and Mescher 1999) and allogeneic challenge (Gately et al. 1994). However, the direct role of these inflammatory cytokines on activated CD8 T cells was unclear since many of these studies were conducted using mixed populations of lymphocytes or whole-body KO approaches. By a reductionist approach, Curtsinger et al. used a purified population of naïve TCR-transgenic CD8 T cells, cultured them *in vitro* with artificial APC (microspheres bound to antigenic-peptide/MHC protein complexes and B7 ligands) in the presence or absence of several candidate cytokines, and evaluated the expansion (Curtsinger et al. 1999). In this study, exogenous IL-12 stimulation significantly improved the expansion of CD8 T cells within 3 days post-activation *in vitro* while IL-1, IL-6, TNFα, and α-IFNγ antibody treatment did not have a significant impact (Curtsinger et al. 1999). However, IL-12 was only able to enhance the expansion of aAPC-stimulated CD8 T cells when exogenous IL-2 was present (Curtsinger et al. 1999). Several years later, direct Type I IFN stimulation was also shown to increase the accumulation of purified CD8 T cells following 3 days of *in vitro* aAPC/IL-2 stimulation (Curtsinger et al. 2005). These and data from other studies verified that direct cytokine stimulation can bolster the expansion of CD8 T cells following *in vitro* activation.

The importance of direct inflammatory cytokine signaling for optimal CD8 T cell accumulation was also established *in vivo*. In response to LCMV Armstrong infection, the accumulation of adoptively transferred Type I Interferon (IFN) receptor deficient (IFNARKO) P14 TCR transgenic (GP_{33}-specific) CD8 T cells was drastically reduced relative to WT controls (Aichele et al. 2006; Kolumam et al. 2005). Lack of IL-12β2-receptor expression also significantly reduced P14 CD8 T cell expansion in response to GP_{33}-expressing *Listeria monocytogenes* (LM-GP33) infection (Keppler et al. 2012; Keppler et al. 2009). Interestingly, the importance of IL-12 or Type I IFN was pathogen-dependent. Adoptively transferred-IFNAR-KO P14 CD8 T cell only displayed a slight accumulation impairment in hosts that had been infected with GP_{33}-expressing vaccinia virus (Vac-GP33) (Aichele et al. 2006). Similarly, IL-12RKO P14 CD8 T cell responses to LCMV Armstrong and Vac-GP33 were not significantly altered compared to WT controls (Keppler et al. 2009). The varying dependency of CD8 T cell responses on particular inflammatory cytokine is probably a result of the inflammatory milieu associated with each specific infection. It is known that high levels of Type I IFN can be detected in the serum of LCMV Armstrong infected hosts while IL-12 is predominantly found in the serum of LM-GP33 infected mice (Keppler et al. 2012). These data suggest that the effect of "signal 3" is not dependent on a particular cytokine to promote the optimal accumulation of responding CD8 T cells. Thus, direct inflammatory cytokine signaling may promote the "signal 3" effect by either acting alone or in combination to sufficiently regulate convergent and/or parallel pathways. As a result of this interplay, IL-12 and Type I IFN enable robust CD8 T cell responses regardless of the unique inflammatory environment elicited by a particular infection.

The Mechanisms Utilized by IL-12 and Type I IFN to Promote Optimal CD8 T Cell Accumulation

Although it is has been well established that "signal 3" plays an important role in regulating the magnitude of CD8 T cell responses, the exact mechanism by which inflammatory cytokines increase cellular accumulation is not clear. Previously, two models were used to explain how "signal 3" increases CD8 T cell accumulation. The first model states that "signal 3" does not alter the division of activated CD8 T cells, but instead enhances early survival. This conclusion is most often supported by indirect evidence. After 72 h culture, the presence of IL-12 during *in vitro* activation improved the maintenance of OT-I CD8 T cell numbers following transfer into antigen/cytokine barren environment without altering (if any) subsequent division (Valenzuela et al. 2005). It has also been reported that WT P14 CD8 T cells significantly outnumbered IL-12RKO P14 CD8 T cells on day 5–6 following LM-GP33 infection while exhibiting similar BrdU incorporation and, perplexingly, similar Annexin V staining (Keppler et al. 2009). Interestingly, other studies have demonstrated that IFNARKO and WT P14 CD8 T cells maintain similar rates of division around 3, 5, and day 8 post-LCMV Armstrong infection even though they have significantly different accumulation as early as day 5 post-infection (Aichele et al. 2006; Kolumam et al. 2005). Since the presence or absence of either IL-12 or Type I IFN signaling did not alter in division of activated CD8 T cells in these studies, many authors assumed that "signal 3" promotes activated CD8 T cell survival (Mescher et al. 2006).

Direct evidence for "signal 3" in changing the survival of activated CD8 T cells *in vivo* is minimal (Aichele et al. 2006; Keppler et al. 2012; Kolumam et al. 2005; Mescher et al. 2006). One study indicated that when activated CD8 T cells are harvested from mice given SEB superantigen and plated *in vitro*, exogenous Type I IFN stimulation can substantially decrease the frequency of propidium iodide-positive cells within several hours (Marrack et al. 1999). Another study has shown that when splenocytes are harvested from mice at day 5 post-LCMV Armstrong infection and incubated *in vitro* in the absence of antigen/cytokine for 24 h, IFNARKO P14 CD8 T cells were highly susceptible to gaining an apoptotic phenotype as detected by Forward/Side Scatter FACS profile (Kolumam et al. 2005). These same authors were also able to detect similar differences in freshly harvested splenocytes as early as 66 h post-LCMV Armstrong infection (Kolumam et al. 2005). Interestingly, one study indicated that *in vitro* IL-12 stimulation promotes the expression of Bcl-3 by activated CD8 T cells (Valenzuela et al. 2005), a factor that has been suggested to increase the survival of expanding CD8 T cells (Mitchell et al. 2001). Although these studies have suggested that "signal 3" increases the accumulation of activated CD8 T cells by enhancing their survival as early as 66 h post-activation, the molecular mechanisms by which "signal 3" utilizes to promote the survival of activated CD8 T cells *in vivo* are largely unknown.

A second and less experimentally supported "signal 3" model indicates that IL-12 signaling can provide an early proliferative advantage for activated CD8 T

cells. Using *in vitro* culture conditions, a study by Valenzuela et al. indicated that the presence of IL-12 transiently increases the expression of the high-affinity IL-2 receptor α-chain (IL-2Rα, CD25) and IL-2 receptor β-chain (CD122), while also enhancing proliferation of activated CD8 T cells within 48 h following stimulation when exogenous IL-2 is present (Valenzuela et al. 2002). Interestingly, activated CD8 T cells stimulated with IL-12/IL-2 were not absolutely dependent on CD25 for this window of enhanced proliferation (Valenzuela et al. 2002). Unfortunately, this study did not evaluate the contribution of IL-12/IL-2 stimulation in regulating activated CD8 T cell survival, making it difficult to compare these results to the other studies that support the early survival model. Regardless, it was unknown whether CD25 expression by activated CD8 T cells is primarily regulated by IL-12 and/or other "signal 3" cytokines *in vivo*, or if Type I IFN signaling promoted CD8 T cell accumulation by a similar or different pathway *in vivo*.

A recent *in vivo* study by our lab has helped to resolve these two "signal 3" models *in vivo*. Using a DC immunization approach (DC), we were able to initiate *in vivo* CD8 T cell responses in the presence of low (DC alone) or high inflammation (DC coupled with CpG treatment) while keeping the level of antigen presentation and co-stimulatory molecule expression by APC constant (Starbeck-Miller et al. 2014; Pham et al. 2009). In this study, we demonstrated that the presence of CpG-induced inflammation during activation does not alter CD8 T cell survival or proliferation early after activation. Instead, direct IL-12 and Type I IFN stimulation promoted the maintenance of CD25 (while not altering CD122) expression, thereby extending activated CD8 T cell division (Starbeck-Miller et al. 2014) (Fig. 3.1). Prolonged IL-2/PI3K signaling was important for maintaining the expression of FoxM1 [a critical transcription factor required for division by *in vitro* activated CD8 T cells (Xue et al. 2010)], and for bolstering robust CD8 T cell responses initiated in a high inflammatory environment. In this study, we also determined whether extended high-affinity IL-2 signaling was sufficient to help prolong the division of CD8 T cells in mice given DC immunization alone. Since the administration of IL-2 in complex with S4B6 α-IL-2 antibody (IL-2/S4B6) treatment has been previously shown to induce high-affinity IL-2 signaling in the absence of CD25 on the surface of CD122-expressing cells (Boyman and Sprent 2012), we treated DC alone immunized mice with IL-2/S4B6 complex during the period when curtailing of CD25 expression was observed. With this approach, we were able to demonstrate that extended high-affinity IL-2 signaling was sufficient to help maintain the expression of FoxM1 and division of CD8 T cells activated in a low inflammatory context (Starbeck-Miller et al. 2014).

Our results demonstrated that IL-12 and Type I IFN work by a common mechanism to extend the window of division by activated CD8 T cells in order to bolster the magnitude of the response. It is unclear from our study if these seemingly disparate cytokines promote the maintenance of CD25 expression through a common molecular pathway. Interestingly, Gil et al. have demonstrated that Type I IFN stimulation primarily activates STAT4 signaling in CD8 T cells responding to LCMV Armstrong infection, rather than by the canonical STAT1 pathway (Gil et al. 2012). Since IL-12 stimulation also induces STAT4 activation (Vignali and Kuchroo 2012)

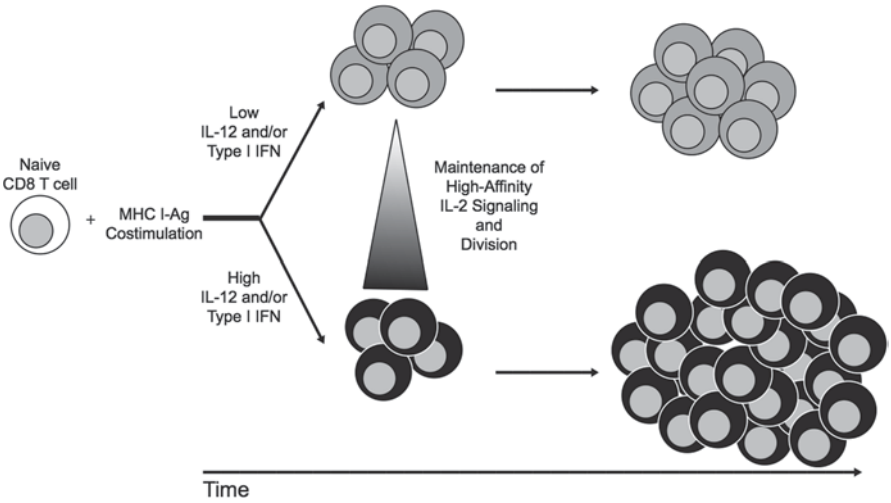

Fig. 3.1 IL-12 and Type I IFN Cooperatively Promote the Maintenance of High-Affinity IL-2 Signaling in Order to Extend the Proliferation of Activated CD8 T cells. Naïve CD8 T cells can become activated and proliferate in response to infection after interacting with APC bearing cognate antigen (MHC I-Ag) and co-stimulatory ligands. The presence of low or high levels of IL-12 and Type I IFN during activation does not affect the initial division or survival of responding CD8 T cells, but extends the window of CD8 T cell division by prolonging the expression of the high-affinity component (CD25) of the IL-2 receptor. By improving the maintenance of CD25 expression, IL-12 and Type I IFN, alone or in cooperation, prolong high-affinity IL-2 signaling and permit optimal accumulation of activated CD8 T cells

it is possible that this common signaling pathway permits cooperative maintenance of CD25 expression. This cooperative mechanism may exist to ensure support for CD8 T cell responses that are initiated in a variety of inflammatory contexts.

Results from our study also highlight the context-dependent effects of high-affinity IL-2 signaling on primary CD8 T cell responses. In other studies, IL-2 signaling has been shown to be important for promoting effector differentiation (Boyman and Sprent 2012; Pipkin et al. 2010). Other studies have indicated that the constitutive ablation of CD25 on CD8 T cells resulted in decreased expansion and increased the rate of memory differentiation in response to LCMV Armstrong or *Listeria monocytogenes* infection (Williams et al. 2006; Obar et al. 2010). In addition, Williams et al. indicated that CD25 expression during primary CD8 T cell responses to LCMV Armstrong infection were required for robust secondary expansion (Williams et al. 2006). Interestingly, our study indicated that late/persistent high-affinity IL-2 signaling specifically affects the accumulation of CD8 T cells without altering differentiation, as indicated by surface phenotype (Starbeck-Miller et al. 2014). Together, these results indicate that the effect of high-affinity IL-2 signaling on CD8 T cell biology may be temporally dependent; in that ‘early’ high-affinity IL-2 signaling affects differentiation while ‘late’ high-affinity IL-2 signaling affects division and accumulation.

Improving Current Adoptive Cellular Immunotherapy

Looking beyond the context of acute infection, controlling the magnitude of CD8 T cell responses can also be beneficial for combating tumor burden. In some clinical cases, adoptive cellular immunotherapy (ACT) is a current avenue of research that shows promise for those afflicted by certain cancers that are unaffected by current treatment regimens (Rosenberg et al. 2011; Crompton et al. 2014). This immunotherapy typically involves either the harvesting and autologous transfer of tumor-infiltrating lymphocytes (TILs) (Restifo et al. 2012) or the transfer of genetically modified T cells in combination with IL-2 therapy (Rosenberg and Dudley 2009) to treat patients with advanced hematologic or solid cancers. The success of this therapies correlate with the absolute number of transferred cells (Besser et al. 2010; Radvanyi et al. 2012), and the potential for cells to expand and persist following transfer (Robbins et al. 2004). Unfortunately, inducing robust expansion of CD8 T cells is often coupled with strong effector differentiation and decreased memory potential (Crompton et al. 2014). Thus, it will be imperative for future studies to identify mechanisms that can be exploited to specifically enhance the magnitude of CD8 T cell responses without altering differentiation. Since our study demonstrated that therapies like IL-2/S4B6 treatment in mice affects the accumulation and not the differentiation of CD8 T cells when given distal to activation, this type of therapy could serve as a useful supplement to ACT. Interestingly, Levin et al. have developed a version of human IL-2 (IL-2 superkine) that has significantly increased binding affinity for CD122, mimicking the effect of IL-2/S4B6 treatment in mice (Levin et al. 2012). This treatment elicited robust CD8 T cell and NK responses that were able to significantly reduce tumor burden in mice (Levin et al. 2012). Based on these results, treating CD8 T cells with IL-2 superkine after *in vitro* activation may help bolster the success of ACT and other related therapies.

References

Acuto, O., & Michel, F. (2003). CD28-mediated co-stimulation: A quantitative support for TCR signalling. *Nature Reviews Immunology, 3,* 939–951.

Aichele, P., Unsoeld, H., Koschella, M., Schweier, O., Kalinke, U., & Vucikuja, S. (2006). CD8 T cells specific for lymphocytic choriomeningitis virus require type I IFN receptor for clonal expansion. *Journal of Immunology, 176,* 4525–4529.

Badovinac, V. P., & Harty, J. T. (2006). Programming, demarcating, and manipulating CD8+ T-cell memory. *Immunological Reviews, 211,* 67–80.

Besser, M. J., Shapira-Frommer, R., Treves, A. J., Zippel, D., Itzhaki, O., Hershkovitz, L., Levy, D., Kubi, A., Hovav, E., Chermoshniuk, N., Shalmon, B., Hardan, I., Catane, R., Markel, G., Apter, S., Ben-Nun, A., Kuchuk, I., Shimoni, A., Nagler, A., & Schachter, J. (2010). Clinical responses in a phase II study using adoptive transfer of short-term cultured tumor infiltration lymphocytes in metastatic melanoma patients. *Clinical Cancer Research, 16,* 2646–2655.

Bevan, M. J. (1976). Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *Journal of Experimental Medicine, 143,* 1283–1288.

Boyman, O., & Sprent, J. (2012). The role of interleukin-2 during homeostasis and activation of the immune system. *Nature Reviews Immunology, 12,* 180–190.

Crompton, J. G., Sukumar, M., & Restifo, N. P. (2014). Uncoupling T-cell expansion from effector differentiation in cell-based immunotherapy. *Immunological Reviews, 257,* 264–276.

Curtsinger, J. M., Schmidt, C. S., Mondino, A., Lins, D. C., Kedl, R. M., Jenkins, M. K., & Mescher, M. F. (1999). Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. *Journal of Immunology, 162,* 3256–3262.

Curtsinger, J. M., Valenzuela, J. O., Agarwal, P., Lins, D., & Mescher, M. F. (2005). Type I IFNs provide a third signal to CD8 T cells to stimulate clonal expansion and differentiation. *Journal of Immunology, 174,* 4465–4469.

Gately, M. K., Wolitzky, A. G., Quinn, P. M., & Chizzonite, R. (1992). Regulation of human cytolytic lymphocyte responses by interleukin-12. *Cellular Immunology, 143,* 127–142.

Gately, M. K., Warrier, R. R., Honasoge, S., Carvajal, D. M., Faherty, D. A., Connaughton, S. E., Anderson, T. D., Sarmiento, U., Hubbard, B. R., & Murphy, M. (1994). Administration of recombinant IL-12 to normal mice enhances cytolytic lymphocyte activity and induces production of IFN-gamma in vivo. *International Immunology, 6,* 157–167.

Gil, M. P., Ploquin, M. J., Watford, W. T., Lee, S. H., Kim, K., Wang, X., Kanno, Y., O'shea, J. J., & Biron, C. A. (2012). Regulating type 1 IFN effects in CD8 T cells during viral infections: Changing STAT4 and STAT1 expression for function. *Blood, 120,* 3718–3728.

Guermonprez, P., Saveanu, L., Kleijmeer, M., Davoust, J., Van Endert, P., & Amigorena, S. (2003). ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. *Nature, 425,* 397–402.

Harty, J. T., & Badovinac, V. P. (2008). Shaping and reshaping CD8+ T-cell memory. *Nature Reviews Immunology, 8,* 107–119.

Houde, M., Bertholet, S., Gagnon, E., Brunet, S., Goyette, G., Laplante, A., Princiotta, M. F., Thibault, P., Sacks, D., & Desjardins, M. (2003). Phagosomes are competent organelles for antigen cross-presentation. *Nature, 425,* 402–406.

Joffre, O. P., Segura, E., Savina, A., & Amigorena, S. (2012). Cross-presentation by dendritic cells. *Nature Reviews Immunology, 12,* 557–569.

Kaech, S. M., Wherry, E. J., & Ahmed, R. (2002). Effector and memory T-cell differentiation: Implications for vaccine development. *Nature Reviews Immunology, 2,* 251–262.

Keppler, S. J., Theil, K., Vucikuja, S., & Aichele, P. (2009). Effector T-cell differentiation during viral and bacterial infections: Role of direct IL-12 signals for cell fate decision of CD8(+) T cells. *European Journal of Immunology, 39,* 1774–1783.

Keppler, S. J., Rosenits, K., Koegl, T., Vucikuja, S., & Aichele, P. (2012). Signal 3 cytokines as modulators of primary immune responses during infections: The interplay of type I IFN and IL-12 in CD8 T cell responses. *PLoS ONE, 7,* e40865.

Kolumam, G. A., Thomas, S., Thompson, L. J., Sprent, J., & Murali-Krishna, K. (2005). Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. *Journal of Experimental Medicine, 202,* 637–650.

Koretzky, G. A., Abtahian, F., & Silverman, M. A. (2006). SLP76 and SLP65: complex regulation of signalling in lymphocytes and beyond. *Nature Reviews Immunology, 6,* 67–78.

Ledbetter, J. A., Gentry, L. E., June, C. H., Rabinovitch, P. S., & Purchio, A. F. (1987). Stimulation of T cells through the CD3/T-cell receptor complex: Role of cytoplasmic calcium, protein kinase C translocation, and phosphorylation of pp60c-src in the activation pathway. *Molecular and Cellular Biology, 7,* 650–656.

Levin, A. M., Bates, D. L., Ring, A. M., Krieg, C., Lin, J. T., SU, L., Moraga, I., Raeber, M. E., Bowman, G. R., Novick, P., Pande, V. S., Fathman, C. G., Boyman, O., & Garcia, K. C. (2012). Exploiting a natural conformational switch to engineer an interleukin-2 'superkine'. *Nature, 484,* 529–33.

Marrack, P., Kappler, J., & Mitchell, T. (1999). Type I interferons keep activated T cells alive. *Journal of Experimental Medicine, 189,* 521–530.

Medzhitov, R. (2001). Toll-like receptors and innate immunity. *Nature Reviews Immunology, 1,* 135–145.

Mehrotra, P. T., Wu, D., Crim, J. A., Mostowski, H. S., & Siegel, J. P. (1993). Effects of IL-12 on the generation of cytotoxic activity in human CD8+ T lymphocytes. *Journal of Immunology, 151,* 2444–2452.

Mescher, M. F., Curtsinger, J. M., Agarwal, P., Casey, K. A., Gerner, M., Hammerbeck, C. D., Popescu, F., & Xiao, Z. (2006). Signals required for programming effector and memory development by CD8+ T cells. *Immunological Reviews, 211,* 81–92.

Mitchell, T. C., Hildeman, D., Kedl, R. M., Teague, T. K., Schaefer, B. C., White, J., Zhu, Y., Kappler, J., & Marrack, P. (2001). Immunological adjuvants promote activated T cell survival via induction of Bcl-3. *Nature Immunology, 2,* 397–402.

Obar, J. J., Molloy, M. J., Jellison, E. R., Stoklasek, T. A., Zhang, W., Usherwood, E. J., & Lefrancois, L. (2010). CD4+ T cell regulation of CD25 expression controls development of short-lived effector CD8+ T cells in primary and secondary responses. *Proceedings of the National Academy of Sciences of the United States of America, 107,* 193–198.

Pham, N. L., Badovinac, V. P., & Harty, J. T. (2009). A default pathway of memory CD8 T cell differentiation after dendritic cell immunization is deflected by encounter with inflammatory cytokines during antigen-driven proliferation. *Journal of Immunology, 183,* 2337–2348.

Pipkin, M. E., Sacks, J. A., Cruz-Guilloty, F., Lichtenheld, M. G., Bevan, M. J., & Rao, A. (2010). Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells. *Immunity, 32,* 79–90.

Radvanyi, L. G., Bernatchez, C., Zhang, M., Fox, P. S., Miller, P., Chacon, J., Wu, R., Lizee, G., Mahoney, S., Alvarado, G., Glass, M., Johnson, V. E., Mcmannis, J. D., Shpall, E., Prieto, V., Papadopoulos, N., Kim, K., Homsi, J., Bedikian, A., Hwu, W. J., Patel, S., Ross, M. I., Lee, J. E., Gershenwald, J. E., Lucci, A., Royal, R., Cormier, J. N., Davies, M. A., Mansaray, R., Fulbright, O. J., Toth, C., Ramachandran, R., Wardell, S., Gonzalez, A., & Hwu, P. (2012). Specific lymphocyte subsets predict response to adoptive cell therapy using expanded autologous tumor-infiltrating lymphocytes in metastatic melanoma patients. *Clinical Cancer Research, 18,* 6758–6770.

Randolph, G. J., Ochando, J., & Partida-Sanchez, S. (2008). Migration of dendritic cell subsets and their precursors. *Annual Review of Immunology, 26,* 293–316.

Renauld, J. C., Vink, A., & Van Snick, J. (1989). Accessory signals in murine cytolytic T cell responses. Dual requirement for IL-1 and IL-6. *Journal of Immunology, 143,* 1894–1898.

Restifo, N. P., Dudley, M. E., & Rosenberg, S. A. (2012). Adoptive immunotherapy for cancer: Harnessing the T cell response. *Nature Reviews Immunology, 12,* 269–281.

Robbins, P. F., Dudley, M. E., Wunderlich, J., El-Gamil, M., LI, Y. F., Zhou, J., Huang, J., Powell, D. J., Jr., & Rosenberg, S. A. (2004). Cutting edge: Persistence of transferred lymphocyte clonotypes correlates with cancer regression in patients receiving cell transfer therapy. *Journal of Immunology, 173,* 7125–7130.

Rock, K. L., & Shen, L. (2005). Cross-presentation: underlying mechanisms and role in immune surveillance. *Immunological Reviews, 207,* 166–183.

Rosenberg, S. A., & Dudley, M. E. (2009). Adoptive cell therapy for the treatment of patients with metastatic melanoma. *Current Opinion in Immunology, 21,* 233–240.

Rosenberg, S. A., Yang, J. C., Sherry, R. M., Kammula, U. S., Hughes, M. S., Phan, G. Q., Citrin, D. E., Restifo, N. P., Robbins, P. F., Wunderlich, J. R., Morton, K. E., Laurencot, C. M., Steinberg, S. M., White, D. E., & Dudley, M. E. (2011). Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. *Clinical Cancer Research, 17,* 4550–4557.

Saito, T., Yokosuka, T., & Hashimoto-Tane, A. (2010). Dynamic regulation of T cell activation and co-stimulation through TCR-microclusters. *FEBS Letters, 584,* 4865–4871.

Saveanu, L., Carroll, O., Weimershaus, M., Guermonprez, P., Firat, E., Lindo, V., Greer, F., Davoust, J., Kratzer, R., Keller, S. R., Niedermann, G., & Van Endert, P. (2009). IRAP identifies an endosomal compartment required for MHC class I cross-presentation. *Science, 325,* 213–217.

Schmidt, C. S., & Mescher, M. F. (1999). Adjuvant effect of IL-12: Conversion of peptide antigen administration from tolerizing to immunizing for CD8+ T cells in vivo. *Journal of Immunology, 163,* 2561–2567.

Serwold, T., Gonzalez, F., Kim, J., Jacob, R., & Shastri, N. (2002). ERAAP customizes peptides for MHC class I molecules in the endoplasmic reticulum. *Nature, 419,* 480–483.

Simon, M. M., Landolfo, S., Diamantstein, T., & Hochgeschwender, U. (1986). Antigen- and lectin-sensitized murine cytolytic T lymphocyte-precursors require both interleukin 2 and endogenously produced immune (gamma) interferon for their growth and differentiation into effector cells. *Current Topics in Microbiology and Immunology, 126,* 173–185.

Smith-Garvin, J. E., Koretzky, G. A., & Jordan, M. S. (2009). T cell activation. *Annual Review of Immunology, 27,* 591–619.

Sommers, C. L., Samelson, L. E., & Love, P. E. (2004). LAT: A T lymphocyte adapter protein that couples the antigen receptor to downstream signaling pathways. *Bioessays, 26,* 61–67.

Starbeck-Miller, G. R., Xue, H. H., & Harty, J. T. (2014). IL-12 and type I interferon prolong the division of activated CD8 T cells by maintaining high-affinity IL-2 signaling in vivo. *Journal of Experimental Medicine, 211,* 105–120.

Trombetta, E. S., & Mellman, I. (2005). Cell biology of antigen processing in vitro and in vivo. *Annual Review of Immunology, 23,* 975–1028.

Valenzuela, J., Schmidt, C., & Mescher, M. (2002). The roles of IL-12 in providing a third signal for clonal expansion of naive CD8 T cells. *Journal of Immunology, 169,* 6842–6849.

Valenzuela, J. O., Hammerbeck, C. D., & Mescher, M. F. (2005). Cutting edge: Bcl-3 up-regulation by signal 3 cytokine (IL-12) prolongs survival of antigen-activated CD8 T cells. *Journal of Immunology, 174,* 600–604.

Vignali, D. A., & Kuchroo, V. K. (2012). IL-12 family cytokines: Immunological playmakers. *Nature Immunology, 13,* 722–728.

Williams, M. A., Tyznik, A. J., & Bevan, M. J. (2006). Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells. *Nature, 441,* 890–893.

Xue, L., Chiang, L., HE, B., Zhao, Y. Y., & Winoto, A. (2010). FoxM1, a forkhead transcription factor is a master cell cycle regulator for mouse mature T cells but not double positive thymocytes. *PLoS ONE, 5,* e9229.

York, I. A., Chang, S. C., Saric, T., Keys, J. A., Favreau, J. M., Goldberg, A. L., & Rock, K. L. (2002). The ER aminopeptidase ERAP1 enhances or limits antigen presentation by trimming epitopes to 8–9 residues. *Nature Immunology, 3,* 1177–1184.

Zhang, W., Sommers, C. L., Burshtyn, D. N., Stebbins, C. C., Dejarnette, J. B., Trible, R. P., Grinberg, A., Tsay, H. C., Jacobs, H. M., Kessler, C. M., Long, E. O., Love, P. E., & Samelson, L. E. (1999). Essential role of LAT in T cell development. *Immunity, 10,* 323–332.

Chapter 4
Functional Diversity of Human Dendritic Cells

Eynav Klechevsky

Abstract At the crossroad between innate and adaptive immunity are the dendritic Cells (DCs), a "novel cell type." discovered in 1973 by Ralph Steinman. Although not entirely appreciated at first, it is clear that they play a critical role as specialized antigen-presenting cells and essential mediators in shaping immune reactivity and tolerance. Dendritic cells are now recognized as a heterogeneous group of cells in terms of cell-surface markers, anatomic location, and function adapted to protect against an array of pathogens and conditions. Importantly, these subsets are also unique to each species. While significant progress has been made on the identification and function of mouse DC subsets, much less is known about human cells. Here we review the fascinating biology of human skin DCs and describe tolerogenic principles that are critical in maintaining immune homeostasis and for controlling inflammation, as well as mechanisms that are fundamental to confer immunity. We surmise that these principles could be applied to DCs across organs, and could be harnessed for the treatment of various human autoimmune, inflammatory diseases, as well as cancer. Importantly, to leverage the relevance of basic research to the clinical setting, it is first necessary to determine the functional homology between mouse and human DCs. We discuss practical steps towards this aim.

Keywords Dendritic cells (DCs) · Langerhans cells (LCs) · Cytokines · Dermal DCs · $CD8^+$ T cells · Mouse DCs · Human DCs

Introduction

Dendritic cells (DCs) are key antigen presenting cells that control both immunity and tolerance (Banchereau and Steinman 1998). DCs are localized in surface barriers and function as sentinels for the recognition of pathogens in many tissues. DCs specialize in the capture and processing of antigens in vivo, converting proteins to peptides that are presented on major histocompatibility complex (MHC) molecules for recognition by T cells. Stimulation of innate signaling receptors induce DCs to

E. Klechevsky (✉)
Washington University School of Medicine, St. Louis, USA
e-mail: eklechevsky@path.wustl.edu

S. P. Schoenberger et al. (eds.), *Crossroads Between Innate and Adaptive Immunity V,*
Advances in Experimental Medicine and Biology 850, DOI 10.1007/978-3-319-15774-0_4

migrate from the periphery to secondary lymphoid organs. This distinguishes DCs from macrophages, which are relatively sessile and largely remain in tissues after inflammation is initiated. Emerging data indicate that peripheral DCs are divided into distinct subsets, each with unique immunological functions. For example, certain DC subsets promote humoral immunity, while others augment cellular immunity. An important contemporary goal is to identify all of the DC subsets and determine their specific functions in immune regulation in humans. The diverse range of evolving pathogens and environmental stimuli resulted in substantial evolutionary changes in the dendritic cell family, as well as within species. Indeed, differences in the basic biology of DCs are only relevant if they prevent us from translating biomedical research to the clinic. It is therefore important to identify functional DC homologs between mouse and human and develop improved preclinical models. Ultimately, targeting or activating distinct DC subsets, with different specializations, will allow controlling the outcome of the immune response. This will be useful for the design of novel vaccines and immunotherapies (Klechevsky and Banchereau 2013).

The Dendritic Cell Discovery and their Exploration in Man

Currently, the central role of dendritic cells in sensing pathogen infection, priming T cell responses and shaping adaptive immune responses is indisputable. But in 1973, when Ralph Steinman first described this "novel cell type" (Steinman and Cohn 1973), the significance of these findings was questioned. Ziegler and Unanue established that the macrophage performed an essential intracellular Ag processing step in T cell activation (Ziegler and Unanue 1981), and with Allen showing that an antigen peptide was presented in the context of MHC molecule in order to activate T cells (Babbitt et al. 1985). So it was established that myeloid cells function as accessory cells to present antigen and produce instructive signals to T cells leading to immune reactivity or tolerance. Two camps formed debating the significance of the dendritic cell in this process relative to macrophages. While both cell types present antigen in the context of MHC, ultimately the dendritic cells were proven to be the most powerful among the APC family in priming T lymphocyte responses.

Langerhans Cells (LCs), dendritic cells of the epidermis, the upper layer of the skin, were first identified in 1868 by Paul Langerhans and were mistaken for nerve cells (Langerhans 1868). The introduction of LCs into the dendritic cell family (Schuler and Steinman 1985) occurred over 100 years later when Schuler and Steinman demonstrated that LCs express MHC-Class II and can induce the mixed lymphocyte reaction. Importantly, this established the concept of "subsets," i.e that DCs, although sharing basic functions, populate different tissues and appear in different forms. The diversity of the DC family was later also explored in the human dermis, where at least three cell populations have been identified (Artyomov et al. 2015; Haniffa et al. 2012; Klechevsky et al. 2008; Nestle et al. 1998, 1993; Zaba et al. 2007).

Peripheral blood is easily accessible from the human body, and because of limited access to human tissue, much of the early work in humans has focused on

blood DCs. However, given the critical function of DCs in barriers, it is important to dissect the instructive signals that DCs produce to maintain tolerance or induce immunity against a challenge in their unique environment. The painstaking task of purifying DC from tissue has made the study of these cells challenging, thus the biological raison d'être for separate mDC subsets in skin has remained largely unknown. Methods for the in vitro generation of large amounts of human DCs from bone marrow progenitors were critical to advance knowledge of the different DC subsets. The in vitro culture of $CD34^+$ hematopoietic progenitor cells with GM-CSF and a variety of cytokines including TNF-a and FLT3-L generated subpopulations of skin-like DCs that expressed HLA-DR and CD1a or CD14. The former expressed features of LCs including Langerin/CD207, E-cadherin and Birbeck granules. Earlier studies (Caux et al. 1997; Morelli et al. 2005) revealed that human skin DCs have not only different phenotype, but also different immunological functions.

Specific Skin DC Subsets Induce Different Types of Immune Responses

DCs are a complex system of cells with different subsets displaying common, as well as distinct functions. The healthy human skin contains multiple myeloid dendritic cell (mDC) populations. Langerhans cells (LCs) are the major DC in the epidermis and DCs in the dermis are identified based on the expression of CD1a and CD14 (Klechevsky et al. 2008; Lenz et al. 1993; Nestle et al. 1993; Zaba et al. 2007). Our studies revealed that different DCs subsets are each endowed with some unique functions. In particular, the $CD14^+$ interstitial DC subset but not LCs promotes humoral immunity by priming of naïve B cells. It was already apparent at that time (Caux et al. 1996) that the $CD1a^+$ -expressing DCs are more powerful at activating T cells compared to the $CD14^+$ DCs. It took 10 years to officially demonstrate that the preferential differentiation of naïve $CD4^+$ T cells in a specific pathway and successful immunity requires not just a sufficient antigenic stimulant, but is also dependent on the DC subsets that is priming the response (Klechevsky et al. 2008). Experimental depletion of a specific DC subset during infection, or during tumor development can impair the immune response. Such facts emphasize that successful immunity largely depends on the DC for appropriate selection of a specific effector module.

We found that the $CD14^+$ DCs prime the activation of $CD4^+$ T follicular helper cells to induce isotype switching and plasma cell generation. LCs preferentially regulate cellular immunity. They do this by inducing Th2 differentiation of $CD4^+$ T cells and by priming and cross-priming naïve $CD8^+$ T cells (Klechevsky et al. 2008). More recently as we extended our studies, we found, together with others, that human LCs are the main skin DC subset to direct IL-17 and IL-22 specific inflammatory responses (de Jong et al. 2010; Fujita et al. 2009; Penel-Sotirakis et al. 2012). It remains unclear how these accessory cells select the appropriate instructive signal for each pathogen. This issue is critical in designing vaccines capable

of driving cell-mediated immunity protective against intracellular pathogens (e.g., HIV, Mtb, malaria) or cancers when Type 1 immunity and CTL responses are desired. We focus on identifying cytokines and transcriptional circuits presented by a unique DC subset that is dominant in instructive T cell differentiation.

Cytokines and Co-Stimulatory Molecules Drive the Functional Specializations of DC Subsets

An essential step in the initiation of an immune response is the formation of the immunological synapse between different antigen presenting cells and T cells via secreted molecules and cell surface receptors. Along with presenting an antigen in the context of MHC Class I and Class II, DCs also provide additional signals to T cells by expressing an array of co-stimulatory molecules and cytokine expression. All these components will eventually determine the outcome of a naïve T cell polarization. Several of the cytokines produced by $CD14^+$ DCs explain their unique capacity to directly signal B cells. These include IL-6, IL-12, IL-10 and TGF-β (Caux et al. 1997, 1996; Klechevsky et al. 2008). IL-12 secreted by DCs upon CD40 engagement is fundamental in initiating IgM production by naïve B cells and IL-12 synergizes with IL-6 and IL-6Rα chain for this effect. IL-10 induces human B cells to switch isotype and to differentiate into plasma cells. The combination of IL-10 and TGF-β stimulates isotype switching toward IgA (Dullaers et al. 2009). IL-6, IL-10 and TGF-β all promote B cell responses in both humans and mice. But IL-12 is an example of a cytokine that has distinct functions in human. IL-12 in human induces IL-21-secreting $CD4^+$ T cells that can induce isotype switching (Schmitt et al. 2013; Schmitt et al. 2009), but has no role in humoral immunity in mice.

LCs Promote Cellular Immunity over Humoral Immunity In our recent work we found that LCs produce IL-15 which enhances the activation of CTLs (Banchereau et al. 2012a). This is important because the combined effects of IL-15 and IL-6 can initiate Th17 responses (Mathers et al. 2009). The role of IL-15 in LC ability to support memory T cells in the skin or activating NK cell is yet to be established.

Dermal $CD14^+$ DCs Promote Humoral Responses by Inhibiting Cellular Responses In our published work, as well as work from another group, it was found that $CD14^+$ dermal DCs not only promote humoral immunity but they actively inhibit the cellular immune response. First, we found that human dermal $CD14^+$ DCs produce IL-10 (Klechevsky et al. 2008), which together with vitamin D promotes $CD4^+$ Treg development (Chu et al. 2012). The secretion of IL-10 and TGF-β impairs the ability of dermal DCs to prime CTLs (Banchereau et al. 2012a). Furthermore, the expression of the inhibitory receptor, specifically, immunoglobulin-like transcript (ILT) receptors ILT2 and ILT4 functions to attenuate cellular immune responses (Banchereau et al. 2012b). ILTs bind MHC Class I and sterically interfere with CD8-binding. This impairs the interaction of the TCR with peptide-MHC complex, block the priming of CTLs, and instead, promotes the generation of Type 2—cytokine secreting $CD8^+$ T cells (Banchereau et al. 2012b).

Translating Dendritic Cell Biology from Mice to Men

While significant progress has been made in the last 5 years on the identification and function of mouse DC subsets, there is relatively little known about human DC subsets. The difficulty in translating mouse studies to human rises from the fact that phenotypically, DC subsets appear to be distinct in each species. This is most likely due to the incredible array of environmental stimuli we are exposed to. Thus, human DC subsets are likely to be more diverse as compared to mouse. Therefore, unlike the study of T cell subsets, attempts to use specific markers to correlate DC subsets between mouse and human have failed. Classification of DC homology based on function is likely to be the only realistic approach for comparing DC subsets across species. This is especially relevant if studies of DC subsets are to lead to targeted therapy.

The Cross-Presenting DC Subset Enigma

Cross-presentation is a process by which antigens are internalized, processed and presented on MHC class I molecules to $CD8^+$ T cells. In mice, this is a critical function of specific DCs in protecting against certain conditions. In human cancer, there is now clinical evidence suggesting that the induction or activation of $CD8^+$ T cells can contribute to the arrest of tumor growth and increase patient survival. In principle, targeting tumor antigens to DCs may enhance protective $CD8^+$ T cell responses due to the ability of DCs to cross-present exogenous antigens (Segura and Villadangos 2009), resulting in an effective T-cell-based vaccine. Specific DC populations in the mouse, the $CD8\alpha^+$ DCs and a related population found in the mouse dermis expressing CD103 and langerin/CD207 are particularly adept in cross-presentation of antigens compared to others (del Rio et al. 2010; Hildner et al. 2008). This finding in the mouse has fueled the quest to identify the counterpart in humans. In 2010, three groups reported the identification of a blood DC subset characterized by the expression of CD141 (BDCA-3/THBD/thrombomodulin). Compared to another DC subset in the blood (the $CD1c^+$ DC) the $CD141^+$ blood DCs are more efficient at engulfing and cross-presenting soluble antigens to $CD8^+$ T cells. This subset shares several markers with the mouse $CD8a^+$ DCs including TLR-3, CLEC9a, and XCR-1 (Bachem et al. 2010; Jongbloed et al. 2010; Poulin et al. 2010; Romani et al. 2010). Interestingly, the capacity to crosspresent is dependent on TLR-activation and the ability of this cell subset to crossprime naïve $CD8^+$ T cells was not evaluated. A recent study indicates that human CD1c (or BDCA-1) expressing cells (that represent the majority of circulating DCs in human blood) can also cross-present to $CD8^+$ T cells as long as the antigen is delivered through a receptor to early endosome (Cohn et al. 2013).

We looked for the counterpart for the mouse $CD8a^+$ DCs in human tissue. Interestingly, blood derived CD141-expressing cells, identified based on their low CD11c expression relative to other dermal DCs, are found infrequently in the human dermis (Haniffa et al. 2012). A similar scenario occurs in mice, where blood derived cells survey the dermis at steady state (Ginhoux et al. 2007). By examining CD141

expression on dermal resident DCs, we noticed that this marker is not restricted to one subset, and nor is the expression of XCR1 or TLR3. Thus the equivalent for the cross-presenting DC subset in the mouse dermis—the CD103+ langerin + DC cannot be identified solely based on these few markers. Furthermore, in humans, Langerin is only expressed on epidermal LCs and CD103 is not expressed on any of the human skin DC subsets, but is instead expressed on T cells. Overall, defining homology between cell types within species based solely on comparing a limited set of markers is likely to be misleading. We favor the idea that functional similarities reflected by gene expression patterns correlated with biological functions will be the best approach for interspecies comparisons of DC subsets.

Interestingly, in our previous work, we examined the genomic profile of the human skin DC by microarray. We noticed that LCs express genes related to MHC Class I antigen processing and presentation machinery, while the dermal $CD14^+$ DCs displayed enhanced expression of the MHC Class II presentation machinery (Banchereau et al. 2009). A similar gene expression profile was also shown for the mouse $CD8a^+$ DCs and the $CD4^+$ DCs, respectively (Dudziak et al. 2007). This suggested that human LCs share similar functional properties with the mouse $CD8a^+$ DCs. Indeed, this was also ultimately proven in a recent study that we performed to identify functional similarities between human skin and the mouse DC systems. The study which included analyses using genomic systems biology alongside functional approaches was published after this chapter was sent to production (hence details are not included) (Artyomov et al. 2015).

Another important consideration, when cmparing the mouse and human systems, is the ability to induce primary responses (the hallmark of dendritic cells) vs. activating secondary responses. Cross-priming occurs when the antigen is presented to a naïve $CD8^+$ T cell resulting in the initiation of a specific immune response, rather than reactivation of an existing memory T cells. In the mouse, thymic but not splenic $CD8\alpha^+$ DCs can cross-prime antigen to naïve $CD8^+$ T cells (Dresch et al. 2011). Functional differences of that kind need to be worked out in the human system.

T Helper Polarization by Mouse and Human DC Subsets

Identifying functional homologs between the mouse and human DCs with respect to the ability to initiate different modules of $CD4^+$ helper responses has only hardly been examined. In the human dermis the $CD14^+$ DCs can activate T regulatory cells through their IL-10 production, a function shared with the mouse dermal CD11b+ DCs and mouse LCs (that also express CD11b). IRF4-dependent DCs (CD11b + CD103-PDL2+ in the mouse and LCs in the human) specialize in regulating Th2 cell responses (Gao et al. 2013; Kumamoto et al. 2013) (Klechevsky et al. 2008) (Fig. 4.1). Both mouse and human LCs can activate Th17 responses. In the mouse, this type of a polarization is dependent on LC-derived IL-6, a cytokine that is not produced by human LCs. Thus, the means by which Th17 cells are being induced by human LCs is yet to be established. Overall, delineation of accurate functional homologies between human and mouse DC subsets requires broad and unbiased systemic comparisons (Crozat et al. 2010; Robbins et al. 2008).

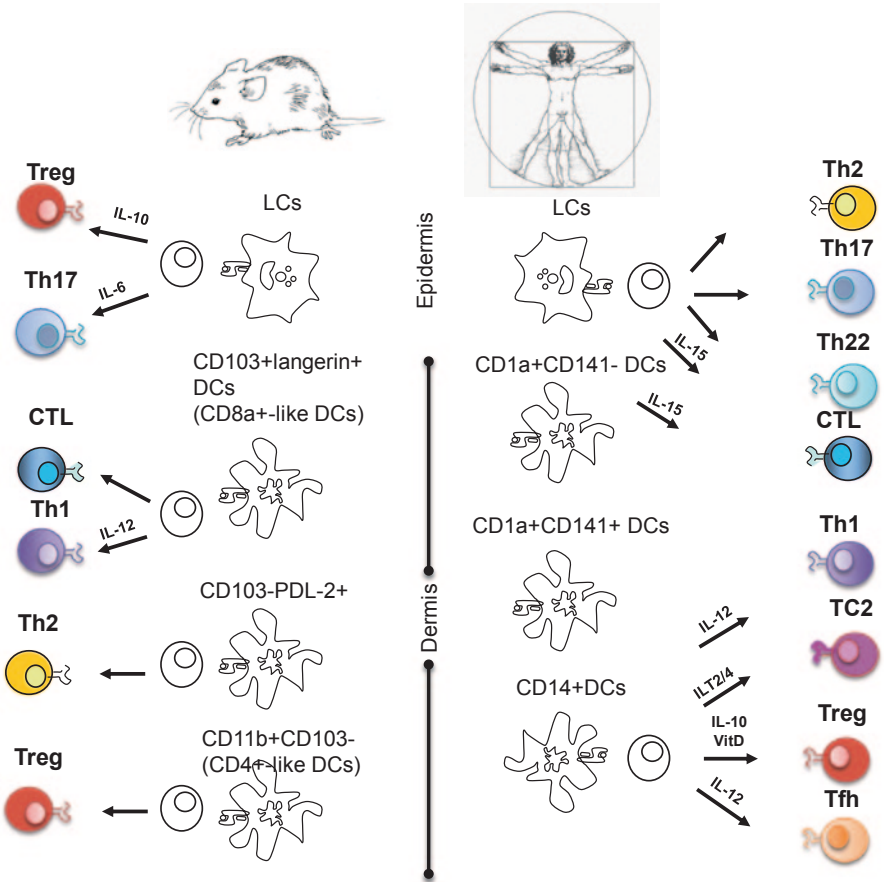

Fig. 4.1 Functional specializations of cutaneous epidermal and dermal dendritic cells in mouse and human

A Controversial Role for Langerhans Cells in Mouse and Human

Human LCs are the most efficient among the dendritic cell subsets in the skin at inducing primary $CD8^+$ T cell responses, by cross-presenting and cross-priming antigens to $CD8^+$ T cells. However, the relevance of LCs to $CD8^+$ T-cell-mediated immunity in mice is still an object of controversy. Particularly since a $CD8a^+$ DC-like population, the $CD103^+$ DCs in the mouse dermis are the most efficient at inducing $CD8^+$ T-cell responses. Although this subset expresses Langerin, it is developmentally distinct from LCs (Nagao et al. 2009). Interestingly, after a 3-day in vitro maturation/differentiation process, murine LCs become very efficient stimulators of T cells suggesting that purified mouse LCs may require an additional signal in comparison to human LCs that can activate T cells immediately following their

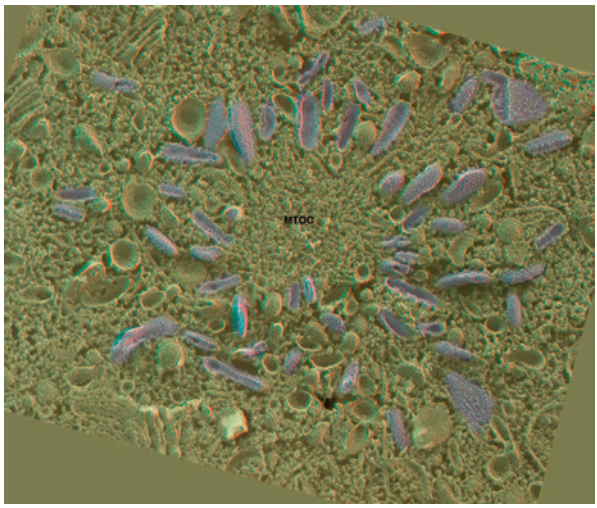

Fig. 4.2 Electron micrograph of Birbeck granules clustering around the cell microtubule-organizing center. (Picture curtsey of John Heuser. Studies were performed in collaboration with Ira Mellman at Yale University)

isolation from tissue (Klechevsky et al. 2008). The identification of two different types of mouse LCs (Sere et al. 2012; Wang et al. 2012), one that is an inflammatory and is present for the short term may be important in the interpretation of studies performed with mouse LCs.

Human LCs do share some similarities with their rodent counterparts. Their physical location as well as the expression of some proteins are similar. For example, Langerin and the associated Birbeck granule (Fig. 4.2), as well as E-cadherin and Epithelial cell adhesion molecule (EpCam) are similarly expressed. However, previously identified differences between mouse and human LCs may help explain the functional discrepancy noted for LCs in the two species. For example, in the mouse, LCs resemble tissue-resident macrophages; they are developmentally dependent on M-CSFR (Ginhoux et al. 2006; Wang et al. 2012), express macrophage-specific markers such as F4/80 and CD11b, migrate poorly to lymph nodes relative to the migration of conventional DCs to lymph nodes, constitutively secrete IL-10 and IL-6 upon activation (Igyarto et al. 2009) and have little to no expression of Zbtb46, which suggests a macrophage identity rather than a DC identity (Meredith et al. 2012; Satpathy et al. 2012). In contrast, human LCs do not express any of the macrophage markers, are negative for CD11b and express IL-15, but not IL-6 or IL-10 (Banchereau et al. 2012a; Klechevsky et al. 2008).

Toll-like receptors are important innate immune sensors. Murine LCs express TLR2, TLR4 and TLR9, but not TLR7 or TLR-3. While human LCs express high levels of transcripts for TLR1, TLR3, TLR5, TLR7 and TLR-8 and weak or absent expression of TLR2, TLR4 or TLR9 (Flacher et al. 2006; Klechevsky et al. 2009). These data suggest that there are interspecies differences in the repertoire of TLR receptors expressed by LCs. Overall human and mouse LCs have evolved differently likely because of distinct microbiota and distinct pathogens.

Concluding Remarks

A transformational change in the treatment of a disease is the ultimate way for demonstrating the value of a basic scientific discovery. Studies in mice have taught us that targeting/depleting a specific DC population can influence the outcome of a disease (Hildner et al. 2008; Hochheiser et al. 2013). The distinct phenotypic and functional divergence among DCs across species limits the direct translation of mouse studies to human. Exploiting the functional diversity of DC subset in humans will be the key to our understanding of human disease processes and viable treatment options. Use of human tissue or the study of patients undergoing efficient therapy is critical and may increase the predictive value of animal models leading to better human clinical trial outcomes. "We count on you," were Ralph's recurrent words to me, urging us scientists in the DC field and other areas of immunology to collaborate and to harness knowledge of this novel cell-type into a novel force in medicine. This is a legacy that we are committed to follow.

References

Artyomov, M. N., et al. (2015). Modular expression analysis reveals functional conservation between human Langerhans cells and mouse cross-priming dendritic cells. *The Journal of Experimental Medicine, 212*(5), 743–757.

Babbitt, B. P., Allen, P. M., Matsueda, G., Haber, E., & Unanue, E. R. (1985). Binding of immunogenic peptides to Ia histocompatibility molecules. *Nature, 317,* 359–361.

Bachem, A., Guttler, S., Hartung, E., Ebstein, F., Schaefer, M., Tannert, A., Salama, A., Movassaghi, K., Opitz, C., Mages, H. W., Henn, V., Kloetzel, P. M., Gurka, S., & Kroczek, R. A. (2010). Superior antigen cross-presentation and XCR1 expression define human CD11c+CD141+ cells as homologues of mouse CD8+ dendritic cells. *The Journal of Experimental Medicine, 207,* 1273–1281.

Banchereau, J., & Steinman, R. M. (1998). Dendritic cells and the control of immunity. *Nature, 392,* 245–252.

Banchereau, J., Klechevsky, E., Schmitt, N., Morita, R., Palucka, K., & Ueno, H. (2009). Harnessing human dendritic cell subsets to design novel vaccines. *Annals of the New York Academy of Sciences, 1174,* 24–32.

Banchereau, J., Thompson-Snipes, L., Zurawski, S., Blanck, J. P., Cao, Y., Clayton, S., Gorvel, J. P., Zurawski, G., & Klechevsky, E. (2012a). The differential production of cytokines by human Langerhans cells and dermal CD14(+) DCs controls CTL priming. *Blood, 119,* 5742–5749.

Banchereau, J., Zurawski, S., Thompson-Snipes, L., Blanck, J. P., Clayton, S., Munk, A., Cao, Y., Wang, Z., Khandelwal, S., Hu, J., McCoy, W. H. t., Palucka, K. A., Reiter, Y., Fremont, D. H., Zurawski, G., Colonna, M., Shaw, A. S., & Klechevsky, E. (2012b). Immunoglobulin-like transcript receptors on human dermal CD14+ dendritic cells act as a CD8-antagonist to control cytotoxic T cell priming. *Proceedings of the National Academy of Sciences of the United States of America, 109,* 18885–18890.

Caux, C., Vanbervliet, B., Massacrier, C., Dezutter-Dambuyant, C., de Saint-Vis, B., Jacquet, C., Yoneda, K., Imamura, S., Schmitt, D., & Banchereau, J. (1996). CD34+ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to GM-CSF+TNF alpha. *The Journal of Experimental Medicine, 184,* 695–706.

Caux, C., Massacrier, C., Vanbervliet, B., Dubois, B., Durand, I., Cella, M., Lanzavecchia, A., & Banchereau, J. (1997). CD34+ hematopoietic progenitors from human cord blood differen-

tiate along two independent dendritic cell pathways in response to granulocyte-macrophage colony-stimulating factor plus tumor necrosis factor alpha: II. Functional analysis. *Blood, 90,* 1458–1470.

Chu, C. C., Ali, N., Karagiannis, P., Di Meglio, P., Skowera, A., Napolitano, L., Barinaga, G., Grys, K., Sharif-Paghaleh, E., Karagiannis, S. N., Peakman, M., Lombardi, G., & Nestle, F. O. (2012). Resident CD141 (BDCA3)+ dendritic cells in human skin produce IL-10 and induce regulatory T cells that suppress skin inflammation. *The Journal of Experimental Medicine, 209,* 935–945.

Cohn, L., Chatterjee, B., Esselborn, F., Smed-Sorensen, A., Nakamura, N., Chalouni, C., Lee, B. C., Vandlen, R., Keler, T., Lauer, P., Brockstedt, D., Mellman, I., & Delamarre, L. (2013). Antigen delivery to early endosomes eliminates the superiority of human blood BDCA3+ dendritic cells at cross presentation. *The Journal of Experimental Medicine, 210,* 1049–1063.

Crozat, K., Guiton, R., Guilliams, M., Henri, S., Baranek, T., Schwartz-Cornil, I., Malissen, B., & Dalod, M. (2010). Comparative genomics as a tool to reveal functional equivalences between human and mouse dendritic cell subsets. *Immunological Reviews, 234,* 177–198.

de Jong, A., Pena-Cruz, V., Cheng, T. Y., Clark, R. A., Van Rhijn, I., & Moody, D. B. (2010). CD1a-autoreactive T cells are a normal component of the human alphabeta T cell repertoire. *Nature Immunology, 11,* 1102–1109.

del Rio, M. L., Bernhardt, G., Rodriguez-Barbosa, J. I., & Forster, R. (2010). Development and functional specialization of CD103+ dendritic cells. *Immunological Reviews, 234,* 268–281.

Dresch, C., Ackermann, M., Vogt, B., de Andrade Pereira, B., Shortman, K., & Fraefel, C. (2011). Thymic but not splenic CD8(+) DCs can efficiently cross-prime T cells in the absence of licensing factors. *European Journal of Immunology, 41,* 2544–2555.

Dudziak, D., Kamphorst, A. O., Heidkamp, G. F., Buchholz, V. R., Trumpfheller, C., Yamazaki, S., Cheong, C., Liu, K., Lee, H. W., Park, C. G., Steinman, R. M., & Nussenzweig, M. C. (2007). Differential antigen processing by dendritic cell subsets in vivo. *Science, 315,* 107–111.

Dullaers, M., Li, D., Xue, Y., Ni, L., Gayet, I., Morita, R., Ueno, H., Palucka, K. A., Banchereau, J., & Oh, S. (2009). A T cell-dependent mechanism for the induction of human mucosal homing immunoglobulin A-secreting plasmablasts. *Immunity, 30,* 120–129.

Flacher, V., Bouschbacher, M., Verronese, E., Massacrier, C., Sisirak, V., Berthier-Vergnes, O., de Saint-Vis, B., Caux, C., Dezutter-Dambuyant, C., Lebecque, S., & Valladeau, J. (2006). Human Langerhans cells express a specific TLR profile and differentially respond to viruses and Gram-positive bacteria. *The Journal of Immunology, 177,* 7959–7967.

Fujita, H., Nograles, K. E., Kikuchi, T., Gonzalez, J., Carucci, J. A., & Krueger, J. G. (2009). Human Langerhans cells induce distinct IL-22-producing CD4+ T cells lacking IL-17 production. *Proceedings of the National Academy of Sciences of the United States of America, 106,* 21795–21800.

Gao, Y., Nish, S. A., Jiang, R., Hou, L., Licona-Limon, P., Weinstein, J. S., Zhao, H., & Medzhitov, R. (2013). Control of T helper 2 responses by transcription factor IRF4-dependent dendritic cells. *Immunity, 39,* 722–732.

Ginhoux, F., Tacke, F., Angeli, V., Bogunovic, M., Loubeau, M., Dai, X. M., Stanley, E. R., Randolph, G. J., & Merad, M. (2006). Langerhans cells arise from monocytes in vivo. *Nature Immunology, 7,* 265–273.

Ginhoux, F., Collin, M. P., Bogunovic, M., Abel, M., Leboeuf, M., Helft, J., Ochando, J., Kissenpfennig, A., Malissen, B., Grisotto, M., Snoeck, H., Randolph, G., & Merad, M. (2007). Blood-derived dermal langerin + dendritic cells survey the skin in the steady state. *The Journal of Experimental Medicine, 204,* 3133–3146.

Haniffa, M., Shin, A., Bigley, V., McGovern, N., Teo, P., See, P., Wasan, P. S., Wang, X. N., Malinarich, F., Malleret, B., Larbi, A., Tan, P., Zhao, H., Poidinger, M., Pagan, S., Cookson, S., Dickinson, R., Dimmick, I., Jarrett, R. F., Renia, L., Tam, J., Song, C., Connolly, J., Chan, J. K., Gehring, A., Bertoletti, A., Collin, M., & Ginhoux, F. (2012). Human tissues contain CD141hi cross-presenting dendritic cells with functional homology to mouse CD103+ nonlymphoid dendritic cells. *Immunity, 37,* 60–73.

Hildner, K., Edelson, B. T., Purtha, W. E., Diamond, M., Matsushita, H., Kohyama, M., Calderon, B., Schraml, B. U., Unanue, E. R., Diamond, M. S., Schreiber, R. D., Murphy, T. L., & Murphy, K. M. (2008). Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. *Science, 322,* 1097–1100.

Hochheiser, K., Heuser, C., Krause, T. A., Teteris, S., Ilias, A., Weisheit, C., Hoss, F., Tittel, A. P., Knolle, P. A., Panzer, U., Engel, D. R., Tharaux, P. L., & Kurts, C. (2013). Exclusive CX3CR1 dependence of kidney DCs impacts glomerulonephritis progression. *Journal of Clinical Investigation, 123,* 4242–4254.

Igyarto, B. Z., Jenison, M. C., Dudda, J. C., Roers, A., Muller, W., Koni, P. A., Campbell, D. J., Shlomchik, M. J., & Kaplan, D. H. (2009). Langerhans cells suppress contact hypersensitivity responses via cognate CD4 interaction and langerhans cell-derived IL-10. *Journal of Immunology, 183,* 5085–5093.

Jongbloed, S. L., Kassianos, A. J., McDonald, K. J., Clark, G. J., Ju, X., Angel, C. E., Chen, C. J., Dunbar, P. R., Wadley, R. B., Jeet, V., Vulink, A. J., Hart, D. N., & Radford, K. J. (2010). Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. *The Journal of Experimental Medicine, 207,* 1247–1260.

Klechevsky, E., & Banchereau, J. (2013). Human dendritic cells subsets as targets and vectors for therapy. *Annals of the New York Academy of Sciences, 1284,* 24–30.

Klechevsky, E., Morita, R., Liu, M., Cao, Y., Coquery, S., Thompson-Snipes, L., Briere, F., Chaussabel, D., Zurawski, G., Palucka, A. K., Reiter, Y., Banchereau, J., & Ueno, H. (2008). Functional specializations of human epidermal Langerhans cells and CD14+ dermal dendritic cells. *Immunity, 29,* 497–510.

Klechevsky, E., Liu, M., Morita, R., Banchereau, R., Thompson-Snipes, L., Palucka, A. K., Ueno, H., & Banchereau, J. (2009). Understanding human myeloid dendritic cell subsets for the rational design of novel vaccines. *Human Immunology, 70,* 281–288.

Kumamoto, Y., Linehan, M., Weinstein, J. S., Laidlaw, B. J., Craft, J. E., & Iwasaki, A. (2013). CD301b(+) Dermal Dendritic Cells Drive T Helper 2 Cell-Mediated Immunity. *Immunity, 39,* 733–743.

Langerhans, P. (1868). Uber die nerven der menschlichen haut. *Archives of Pathological Anatomy, 44,* 325–337.

Lenz, A., Heine, M., Schuler, G., & Romani, N. (1993). Human and murine dermis contain dendritic cells. Isolation by means of a novel method and phenotypical and functional characterization. *Journal of Clinical Investigation, 92,* 2587–2596.

Mathers, A. R., Janelsins, B. M., Rubin, J. P., Tkacheva, O. A., Shufesky, W. J., Watkins, S. C., Morelli, A. E., & Larregina, A. T. (2009). Differential capability of human cutaneous dendritic cell subsets to initiate Th17 responses. *Journal of Immunology, 182,* 921–933.

Meredith, M. M., Liu, K., Darrasse-Jeze, G., Kamphorst, A. O., Schreiber, H. A., Guermonprez, P., Idoyaga, J., Cheong, C., Yao, K. H., Niec, R. E., & Nussenzweig, M.C. (2012). Expression of the zinc finger transcription factor zDC (Zbtb46, Btbd4) defines the classical dendritic cell lineage. *The Journal of Experimental Medicine, 209,* 1153–1165.

Morelli, A. E., Rubin, J. P., Erdos, G., Tkacheva, O. A., Mathers, A. R., Zahorchak, A. F., Thomson, A. W., Falo, L. D. Jr., & Larregina, A. T. (2005). CD4+ T cell responses elicited by different subsets of human skin migratory dendritic cells. *Journal of Immunology, 175,* 7905–7915.

Nagao, K., Ginhoux, F., Leitner, W. W., Motegi, S., Bennett, C. L., Clausen, B. E., Merad, M., & Udey, M. C. (2009). Murine epidermal Langerhans cells and langerin-expressing dermal dendritic cells are unrelated and exhibit distinct functions. *Proceedings of the National Academy of Sciences of the United States of America, 106,* 3312–3317.

Nestle, F. O., Zheng, X. G., Thompson, C. B., Turka, L. A., & Nickoloff, B. J. (1993). Characterization of dermal dendritic cells obtained from normal human skin reveals phenotypic and functionally distinctive subsets. *Journal of Immunology, 151,* 6535–6545.

Nestle, F. O., Filgueira, L., Nickoloff, B. J., & Burg, G. (1998). Human dermal dendritic cells process and present soluble protein antigens. *The Journal of investigative dermatology, 110,* 762–766.

Penel-Sotirakis, K., Simonazzi, E., Peguet-Navarro, J., & Rozieres, A. (2012). Differential capacity of human skin dendritic cells to polarize CD4+ T cells into IL-17, IL-21 and IL-22 producing cells. *PLos One, 7,* e45680.

Poulin, L. F., Salio, M., Griessinger, E., Anjos-Afonso, F., Craciun, L., Chen, J. L., Keller, A. M., Joffre, O., Zelenay, S., Nye, E., Le Moine, A., Faure, F., Donckier, V., Sancho, D., Cerundolo, V., Bonnet, D., & Reis e Sousa, C. (2010). Characterization of human DNGR-1+ BDCA3+ leukocytes as putative equivalents of mouse CD8alpha+ dendritic cells. *The Journal of Experimental Medicine, 207,* 1261–1271.

Robbins, S. H., Walzer, T., Dembele, D., Thibault, C., Defays, A., Bessou, G., Xu, H., Vivier, E., Sellars, M., Pierre, P., Sharp, F. R., Chan, S., Kastner, P., & Dalod, M. (2008). Novel insights into the relationships between dendritic cell subsets in human and mouse revealed by genome-wide expression profiling. *Genome Biology, 9,* R17.

Romani, N., Clausen, B. E., & Stoitzner, P. (2010). Langerhans cells and more: Langerin-expressing dendritic cell subsets in the skin. *Immunological Reviews, 234,* 120–141.

Satpathy, A. T., Kc, W., Albring, J. C., Edelson, B. T., Kretzer, N. M., Bhattacharya, D., Murphy, T. L., & Murphy, K. M. (2012). Zbtb46 expression distinguishes classical dendritic cells and their committed progenitors from other immune lineages. *The Journal of Experimental Medicine, 209,* 1135–1152.

Schmitt, N., Morita, R., Bourdery, L., Bentebibel, S. E., Zurawski, S. M., Banchereau, J., & Ueno, H. (2009). Human dendritic cells induce the differentiation of interleukin-21-producing T follicular helper-like cells through interleukin-12. *Immunity, 31,* 158–169.

Schmitt, N., Bustamante, J., Bourdery, L., Bentebibel, S. E., Boisson-Dupuis, S., Hamlin, F., Tran, M. V., Blankenship, D., Pascual, V., Savino, D. A., Banchereau, J., Casanova, J. L., & Ueno, H. (2013). IL-12 receptor beta1 deficiency alters in vivo T follicular helper cell response in humans. *Blood, 121,* 3375–3385.

Schuler, G., & Steinman, R. M. (1985). Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro. *The Journal of Experimental Medicine, 161,* 526–546.

Segura, E., & Villadangos, J. A. (2009). Antigen presentation by dendritic cells in vivo. *Current Opinion in Immunology, 21,* 105–110.

Sere, K., Baek, J. H., Ober-Blobaum, J., Muller-Newen, G., Tacke, F., Yokota, Y., Zenke, M., & Hieronymus, T. (2012). Two distinct types of Langerhans cells populate the skin during steady state and inflammation. *Immunity, 37,* 905–916.

Steinman, R. M., & Cohn, Z. A. (1973). Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *The Journal of Experimental Medicine, 137,* 1142–1162.

Wang, Y., Szretter, K. J., Vermi, W., Gilfillan, S., Rossini, C., Cella, M., Barrow, A. D., Diamond, M. S., & Colonna, M. (2012). IL-34 is a tissue-restricted ligand of CSF1R required for the development of Langerhans cells and microglia. *Nature Immunology, 13,* 753–760.

Zaba, L. C., Fuentes-Duculan, J., Steinman, R. M., Krueger, J. G., & Lowes, M. A. (2007). Normal human dermis contains distinct populations of CD11c+BDCA-1+ dendritic cells and CD163 + FXIIIA + macrophages. *Journal of Clinical Investigation, 117,* 2517–2525.

Ziegler, K., & Unanue, E. R. (1981). Identification of a macrophage antigen-processing event required for I-region-restricted antigen presentation to T lymphocytes. *Journal of Immunology, 127,* 1869–1875.

Chapter 5
Selective Dependence of Kidney Dendritic Cells on CX_3CR1—Implications for Glomerulonephritis Therapy

Katharina Hochheiser and Christian Kurts

Abstract As central regulators of the adaptive immune response, dendritic cells (DCs) are found in virtually all lymphatic and non-lymphatic organs. A compact network of DCs also spans the kidneys. DCs play a central role in maintenance of organ homeostasis as well as in induction of immune responses against invading pathogens. They can mediate protective or destructive functions in a context-dependent manner.

We recently identified CX_3CR1 as a kidney-specific "homing receptor" for DCs. There was a strong reduction of DCs in the kidneys of CX_3CR1-deficient mice compared to controls. This reduction was not observed in other organs except the small intestine. As a possible underlying reason we found a strong expression of the CX_3CR1 ligand fractalkine in the kidneys. Due to this CX_3CR1-dependent reduction of DCs, especially in the renal cortex, a glomerulonephritis (GN) model was ameliorated in CX_3CR1-deficient mice. In contrast, the immune defense against the most common renal infection, bacterial pyelonephritis (PN), was not significantly influenced by CX_3CR1-deficiency. This was explained by the much smaller CX_3CR1-dependency of medullary DCs, which recruit effector cells into the kidney during PN. Additionally, once neutrophils had been recruited by mechanisms distinct from CX_3CR1, they carried out some of the functions of DCs.

Taken together, we suggest CX_3CR1 as a therapeutic target for GN treatment, as the absence of CX_3CR1 selectively influences DCs in the kidney without rendering mice more susceptible towards bacterial kidney infections.

Keywords Kidney DCs · Kidney physiology · Glumeronephritis (GN) · Interstitial nephritis (tubulointerstitium) · CX_3CR1

K. Hochheiser (✉) · C. Kurts
Institute of Experimental Immunology(IMMEI), Rheinische Friedrich-Wilhelms University, 53105 Bonn, Germany
e-mail: khochhei@uni-bonn.de

C. Kurts
e-mail: ckurts@web.de

S. P. Schoenberger et al. (eds.), *Crossroads Between Innate and Adaptive Immunity V,*
Advances in Experimental Medicine and Biology 850, DOI 10.1007/978-3-319-15774-0_5

Dendritic Cells

Dendritic cells were first described in 1973 by Steinman and Cohn as a new morphologically unique cell type in peripheral lymphoid organs, differing from macrophages (apart from their morphology) by a higher ability to activate naive T cells and a reduced phagocytic activity (Steinman and Cohn 1993). The name of these "dendritic cells" is based on their cytoplasmic extensions reminescent of the branches of a tree (Greek: dendron). Since their first discription it has become clear that there are many distinct types of DCs differing from each other by the expression of various surface molecules, their ontogeny, function and localization (Naik 2008; Shortman and Liu 2002). Today we roughly distinguish between plasmacytoid DCs (pDCs), which are found primarily in the blood and lymphoid organs, and classical DCs (cDCs), which in turn can be divided into lymphoid tissue-resident and migratory DCs (Shortman and Naik 2007). Lymphoid tissue-resident as well as migratory DCs again consist of different subtypes with distinct phenotypes and functions. The two main types of cDCs are $CD8\alpha^+$ $CD11b^-$ DCs (or $CD103^+$ $CD11b^-$ DCs in non-lymphoid organs) on the one hand and $CD8\alpha^-$ $CD103^-$ $CD11b^+$ DCs on the other hand (Vremec et al. 1992; Merad et al. 2013). Simultaneous expression of CD11c and MHC-II is common to almost all DCs and is therefore often used to define murine DCs (Merad et al. 2013).

DCs are found in virtually all lymphoid and non-lymphoid organs, where they monitor their immediate environment (Steinman et al. 2003). For this purpose they take up antigens, process them and transport them to the draining lymph nodes, in which the antigen fragments are presented on the DC surface in a Major Histocompatibility (MHC) Molecule bound form (Randolph et al. 2005). Only in this MHC-bound form the antigens can be detected by T-cells. Depending on the maturation state of the antigen presenting DC, which in turn depends on the nature and origin of the captured antigens and their detection by innate receptors, antigen specific T cells can either be activated or tolerized (Kurts et al. 1997; Banchereau et al. 2000). But not only the surveillance of the periphery and an initial activation or tolerance induction of naive T cells in lymphoid organs fall within the functional area of DCs. In addition, DCs can regulate local immune responses through interaction with the infiltrating effector or memory T cells and the production of various pro-and anti-inflammatory cytokines (Wakim et al. 2008; Heymann et al. 2009; Riedel et al. 2012).

Macrophages

Macrophages are specialized in phagocytosis and subsequent killing of invading microorganisms and infected cells. Additionally, they contribute to the elimination of apoptotic cells and cell debris and thus to the maintenance of tissue homeostasis. Their anti-infectious effector functions are not limited to phagocytosis but include secretion of pro-inflammatory cytokines and cytotoxic mediators, recruitment of other immunocytes to inflamed tissues as well as wound healing and scar formation (Geissmann et al. 2010a; Wynn et al. 2013).

Normally, MPs can kill phagocytosed pathogens but in some cases the microorganisms escape the effector functions of MPs and survive intracellularly (Shaughnessy and Swanson 2007). If this is the case, MPs must be activated by T cells. MPs present fragments of the captured pathogens on their surface in the context of MHC-II molecules. If an activated T cell detects its specific antigen, it activates the antigen presenting MP by cell contact-dependent mechanisms and the production of cytokines such as IFN-γ (Adams and Hamilton 1984). Activated macrophages increase the production of pro-inflammatory cytokines and additionally produce cytotoxic substances such as reactive oxygen species (ROS), which eventually make them capable of killing the pathogens. However, MP activation can also lead to tissue damage, as the produced mediators are toxic not only for the microorganisms but also for the cells of the body.

Anatomy and Physiology of the Kidney

The kidneys excrete substances via the urine. In addition, they regulate maintenance of water and electrolyte balance, acid-base balance and formation and metabolism of some hormones. Macroscopically, the kidney tissue is divided into two compartments: the renal cortex (cortex renalis) and the medulla (medulla renalis), which is located between the renal cortex and the renal pelvis, in which the urine is collected. The smallest functional unit of the kidney is the nephron, which consists of the glomerulus sheathed by the Bowman's capsule, the proximal tubule, the loop of Henle, the distal tubule, and the collecting duct. The mouse kidneys each contain about 8000 nephrons, which run through the kidney cortex and medulla with their different sections (Georgas et al. 2008). The glomeruli and parts of the proximal tubules are located in the cortex. The descending branch of the loop of Henle leaves the cortex and enters the medulla, from where the ascending branch again pulls up into the cortex. Here, the Henle loop merges into the distal tubule. Finally, the distal tubules open into the collecting ducts, which cross the renal cortex and the renal medulla and finally open into the renal pelvis.

In the glomeruli the primary urine is generated by filtration of blood depending on the hydrostatic and colloid osmotic pressure in the afferent and efferent vessels. This primary urine has a composition similar to the blood plasma but lacks molecules >45 kDa due to a three barrier filter consisting of capillary endothelium, basement membrane and podocytes (Haraldsson and Sorensson 2004). Smaller molecules are freely filtered and concentrated in the kidney by absorption through the tubular cells. Among them are foreign antigens (e.g. from the diet) which are absorbed by DCs in the tubulointerstitium (Dong et al. 2005). Tolerance must be maintained against these freely filtered antigens in order to prevent constitutive inflammatory reactions against harmless foreign substances in the kidney.

In the following sections of the nephron the primary urine is changed in its composition by secretion and active and passive absorption, so that the organism can excrete waste products of metabolism and regain most of the filtered water, electrolytes and nutrients. This urine concentration requires an osmotic gradient from the

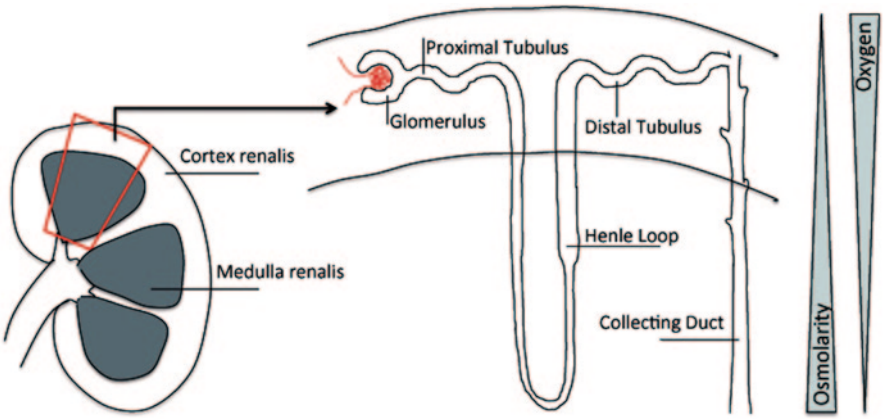

Fig. 5.1 Schematic cartoon of one nephron spanning the compartments of the kidney

outer edge of the renal cortex to the inner edge of the renal medulla, which is formed primarily through the loop of Henle. This osmotic gradient leads to reabsorbtion of water from the collecting ducts. Finally, the urine reaches the renal pelvis and will be transported through the ureters into the bladder.

To maintain the osmotic gradient, cortex and medulla are supplied by different vascular systems with blood and oxygen. While the renal cortex is well supplied with blood and oxygen-rich, the renal medulla is supplied much worse. A better blood flow to the renal medulla would mean that the osmotic gradient would be washed out and a urine concentration could no longer take place. Thus, cells are exposed not only to four-fold higher osmolarities, but also severely hypoxic conditions in the medulla compared to those in the cortex. Whether the different conditions affect the function of cells in the renal cortex and renal medulla, is not known (Fig. 5.1).

Inflammatory Kidney Disease

Acute or chronic inflammatory kidney diseases can both affect the glomeruli (glomerulonephritis (GN)) and the tubulointerstitium (interstitial nephritis). Nephritis can have many causes, including allergic reactions, autoimmune reactions, but also bacterial or viral infections.

Glomerulonephritis and the Model of Nephrotoxic Nephritis

The pathogenesis of GN is still incompletely understood. The pathomechanisms can be diverse and have different triggers, but the immune system seems to be always involved. GN can be triggered by binding of antibodies to kidney-specific

antigens or by immune complexes, which settle in the fine capillaries of the glomeruli. However, T-cell-mediated immune responses may also play a role. GNs are classified according to their etiology, histology and clinical course.

Nephrotoxic nephritis (NTN) is a murine model for human rapidly progressing crescentic GN, which is characterized by a glomerular damage with crescent formation (Tipping and Holdsworth 2006). This form of GN is very aggressive and, if left untreated, usually leads to end-stage renal failure requiring dialysis. In mice, NTN is induced by systemic immunization with a heterologous nephrotoxic serum (NTS) from sheep that were vaccinated with murine cortical tissue (Assmann et al. 1985). Immunization results in the activation of $CD4^+$ T cells in secondary lymphoid organs (Timoshanko et al. 2001; Paust et al. 2009). Due to the specificity of the ovine immunoglobulins for the murine renal cortex, the immune response is directed to the kidney. There, the activated T cells produce pro-inflammatory cytokines and activate tubulointerstitial and glomerular monocytes and macrophages (Kitching et al. 2005; Duffield et al. 2005). Activated mononuclear cells then produce cytokines and reactive oxygen species (ROS), which cause damage to the Bowman's capsule and the glomerular basement membrane with compensatory epithelial proliferation (crescent formation) and the recruitment of further immune cells (Kurts et al. 2007). In contrast to MPs, kidney-resident DCs seem to be protective, at least in the initiation phase of NTN (Riedel et al. 2012; Scholz et al. 2008).

Inflammation, which starts at the glomeruli, is not limited to these structures, but extends over the tubulointerstitium with progression of disease. This is due to the damage of the glomerular barrier, which is associated with a loss of filter function. This loss results in increased protein content in the ultra filtrate and proteinuria. Via partly unknown mechanisms proteinuria leads to further damage of the kidney. One of the reasons is a protein overload in the resorbing tubular epithelial cells, which react with the secretion of chemokines, cytokines, and complement, and thus recruit immune cells to the tubulointerstitium (Abbate et al. 2006; Strutz 2009). This leads to tubulointerstitial inflammation which is often associated with fibrosis, ultimately resulting in a loss of kidney function (Andrews 1977; Bohle 1982; Rees 2009).

Interstitial Nephritis and the Model of Bacterial Pyelonephritis

Bacterial pyelonephritis (PN) is the most common kidney infection and is often caused by uropathogenic Escherichia coli (UPECs), bearing virulence factors such as fimbriae that facilitate attachment to uroepithelial cells and ascension from the bladder to the kidney (Mulvey et al. 1998). PN can be treated by antibiotics but scarring of the kidneys is a common complication and can cause kidney failure (Beauchamp and Bergeron 1999; Hewitt et al. 2008). In mice, PN can be induced by two UPEC instillations into the urinary bladder at an interval of 3 h. The double instillation causes a rise of the UPECs from the bladder into the kidneys in 86 % of cases, compared to only 13 % after a single instillation of UPEC (Tittel et al. 2011).

Among all immunocytes, neutrophils contribute most to clearance of bacteria by phagocytosis. In the healthy kidney, however, they are very rare, so that they must be recruited into the kidney in case of infection in order to exert their effector function. Recruitment from the bloodstream is mediated via the chemokine receptor CXCR2 on neutrophils. CXCR2 binds ligands such as CXCL2, which is produced when bacteria are detected by innate receptors (Hang et al. 1999). Renal DCs are the main producers of the CXCL2 and recruit neutrophils immediately after infection. In the absence of DCs, influx of neutrophils and the antibacterial immune response are severely delayed (Tittel et al. 2011).

DCs in the kidney cortex and medulla show different functions in NTN and PN.

A contiguous network of DCs spans the tubulointerstitium of the kidneys and serves the surveillance of the renal parenchyma (Soos et al. 2006). Within glomeruli DCs are normally not found (Kruger et al. 2004). DCs make up the largest part of the intrarenal leukocytes. In healthy kidneys, there is a small population of $CD103^+$ DCs that accounts for app. 5 % of all renal DCs (Ginhoux et al. 2009). The remaining DCs display a $CD11b^+$ $F4/80^+CX_3CR1^+$ phenotype. Due to the strong F4/80-expression that, in the spleen, is specific for macrophages, it has been a matter of debate whether the $CD11c^+$ $MHC\text{-}II^+$ cells belonged to the DC or MP lineage (Hume and Gordon 1983). However, it has been shown that these cells morphologically resemble DCs and also have functional characteristics of DCs rather than MPs (Kruger et al. 2004). Furthermore, a transcriptome analysis of renal $CD11c^+$ cells has confirmed their affiliation to the DC lineage (Miller et al. 2012).

Renal DCs can innitiate and amplify immune responses within the kidney through the production of cytokines and chemokines (Tittel et al. 2011; Edgtton et al. 2008). Additionally, they can take up glomerular, tubular or filtered antigens and present them to T cells in the kidney or in the renal lymph node (Kurts et al. 1997; Heymann et al. 2009; Edgtton et al. 2008; Roake et al. 1995).

In the healthy kidney, renal DCs and DCs in the renal lymph nodes contribute to the maintenance of homeostasis through the induction of peripheral tolerance (Kurts et al. 1997; Lukacs-Kornek et al. 2008). Resident DCs can exert both protective and pro-inflammatory functions in the inflamed kidney, depending on the disease model (Hochheiser et al. 2011). The role of renal DCs in the GN has recently been clarified. While DCs seem to be protective during initiation of disease by recruiting regulatory NKT cells and inducing IL-10 production in T cells in an ICOS-dependent manner, they mature with progression of disease acquiring the capacity to restimmulate pro-inflammatory T cells thereby switching from a protective to a pro-inflammatory phenotype (Riedel et al. 2012; Scholz et al. 2008; Hochheiser et al. 2011).

Functional in vitro analyzes of DCs in the renal cortex and the renal medulla suggest that mainly the cortical DCs promote NTN (Hochheiser et al. 2013). Only these cells, but not medullary DCs from nephritic mice were able to stimulate OT-II cells, albeit DC maturation was not confined to the cortical DCs, which are located next to inflamed glomeruli. The expression of the costimulatory molecules CD80 and CD86 was equally increased at day 10 after NTN induction in cortical and medullary DCs. In some experiments, cortical DCs showed a slightly increased

expression of MHC-II and CD40 compared to medullary DCs but these marginal differences alone can hardly explain the relatively large discrepancies in the T-cell stimulation. Analysis of endocytosis capabilities of DCs in the two compartments showed a relative superiority of the medullary cells. The differences, however, were much more pronounced in vivo than in vitro, suggesting that the stronger antigen uptake in vivo was mainly due to a greater availability in the renal medulla and only partly due to cell intrinsic differences. Nevertheless, the increased endocytotic activity of medullary DCs (also apparent in vitro) lead us to the assumption that medullary $CD11c^+$ cells have more MP functionality, while cortical $CD11c^+$ cells are more similar to DCs (Hochheiser et al. 2013). The delineation of DCs and MPs is a controversial issue (Geissmann et al. 2010b). Murine DCs were originally defined and distinguished from other cell types such as MPs by the simultaneous expression of CD11c and MHC-II. As antigen presenting cells (APCs) MPs also express MHC-II, but differ from DCs by the lack of expression of CD11c. However, there are exceptions to this rule as some tissue macrophages, such as MPs in the lung, also express high levels of CD11c. Furthermore, there are DC-types, which display only little CD11c expression. In addition, activation of DCs leads to changes in CD11c expression levels. This makes a distinction between DCs and MPs on phenotypic level difficult, so that functional parameters must be considered. But also in terms of the function, there is increasing evidence showing that the originally characterized macrophages (large scavenger cells) and dendritic cells (T-cell activators) defined in lymphoid organs do not exist in these classical forms in peripheral tissues. Rather, they represent two extremes of a more or less continuous phenotype of myeloid APCs (Geissmann et al. 2010a, b). These myeloid cell populations may exercise functions of both classical macrophages as well as classical DCs and sometimes cannot be clearly assigned to one or the other cell type. For the renal $CD11c^+$ cells, it was recently shown in a study that they functionally resemble DCs and not MPs (Kruger et al. 2004). Furthermore, a transcriptome analysis of renal $CD11c^+$ cells has confirmed their affiliation to the DC lineage (Miller et al. 2012). However, these studies analyzed total $CD11c^+$ cells from the kidney, not discriminating between cortex and medulla. Therefore, it is possible that only a portion of these cells (i.e. the cells of the renal cortex) are typical DCs while medullary $CD11c^+$ cells might still resemble MPs. The hypothesis that medullary APCs are more similar to MPs matches the lower CD11c expression, an increased endocytotic activity and little T-cell stimulation compared to cortical APCs (Geissmann et al. 2010a). Classical MPs take up a lot of antigen but degrade it very quickly and cannot present it as efficiently as DCs (Delamarre et al. 2005). Interestingly, we found that medullary APCs showed no rapid antigen degradation, as is typical for MPs (Delamarre et al. 2005). However, they expressed lower H-2M and invariant chain, two proteins that are essential for the loading of MHC class II molecules and thus antigen presentation and T-cell activation (Bryant and Ploegh 2004). These results suggest that medullary APCs are not as capable of loading processed peptides on MHC-II molecules and thus presentation of antigens to T cells compared to cortical APCs. Together with the decreased CD40 expression this could be a reason for poor T-cell activation by medullary APCs. Further possible reasons, which were not considered in our

study, include differences in the expression of inhibitory molecules, such as CTLA-4 or PD-L1 or PD-L2, or of pro-inflammatory and/or regulatory cytokines. Due to the lack of compelling evidence for an affiliation of the medullary APCs to the MP lineage, we here continue to refer to them as medullary DCs. The analysis of further MP- and DC-characteristics, eg NO production might be helpful for classification of the cells in the DC-MP-continuum.

While cortical DCs stimulate T-cells in NTN, medullary DCs are found in an appropriate position for the early detection of bacterial infections, as they are located adjescent to the collecting ducts, through which bacteria can enter the kidneys (Mulvey et al. 1998; Soos et al. 2006). In fact, when we analyzed DC functions in a pyelonephritis model, we found that a high percentage of medullary DCs produced large amounts of neutrophil-recruiting chemokine CXCL2. CXCL2-production by cortical DCs was significantly lower (Geissmann et al. 2010b). The function described for renal DCs in pyelonephritis is therefore executed mainly by the DCs in the renal medulla (Tittel et al. 2011).

So, are cortical DCs specialized in the initiation of adaptive immune responses (adaptive function) and medullary DCs in the induction of innate immune responses (innate function)? This question cannot be conclusively answered from the results of our present study, since only two disease models were considered: a T-cell mediated glomerulonephritis model (NTN) mainly affecting the renal cortex, in which DCs have more adaptive functions, and an infection model (PN) primarily affecting the renal medulla, in which DCs carry out innate functions. Overall, it can be concluded at this point that DCs in the renal cortex drive progression of glomerulonephritis by T-cell stimulation, while medullary DCs are responsible for the initiation of anti-bacterial immune responses in pyelonephritis. Whether the cells are intrinsically different or the functions are determined by their location and the considered models, remains unclear at this point. The question of whether a selective absence of cortical or medullary DCs would affect the progression of NTN or PN, can not be answered, as mice that permit the selective depletion of cortical or medullary DCs, which could bring further insight into the function of DCs in different models, are lacking.

Another question that arises in this context is, what influences the function of the cortical and medullary DCs. Inflammatory stimuli lead to maturation of DCs. However, cortical and medulary DCs also differ under homeostatic conditions with respect to CD11c expression and endocytotic activity (Hochheiser et al. 2013). Whether cortical and medullary DCs have the same precursor and represent different stages of maturation or differentiation of the same cell or whether they represent different types of cells is not clear. It is likely, however, that phenotype and function of the DCs may be influenced by the local environment in the cortex and medulla, respectively. It is known that high salt concentrations are associated with the differentiation of naive T cells in Th17 cells and that also other cell types can be influenced by salt (Wu et al. 2013; Kleinewietfeld et al. 2013; Machnik et al. 2009). In the renal medulla salt concentrations are comparatively high. Additionally, the hypoxic environment could influence DC-differentiation or -function in this compartment. Studies that prove this, do not yet exist.

CX_3CR1 and CX_3CL1

The majority of renal DCs and MPs expresses the chemokine receptor CX_3CR1. However, its function in these cells is not known. The CX_3CR1 ligand CX_3CL1 is the only known representative of the CXXXC chemokine family (Murphy et al. 2000). CX_3CL1 is produced mainly by endothelial cells and exists, unlike any other chemokines except for CXCL16, in both a membrane bound and a soluble form. Membrane bound CX_3CL1 acts as an adhesion molecule, while soluble CX_3CL1 acts as a chemoattractant for CX_3CR1-expressing cells.

With the help of $CX_3CR1^{GFP/+}$ reporter mice and $CX_3CR1^{GFP/GFP}$ CX_3CR1-deficient mice, we recently showed that CX_3CR1 was essential for colonization of the kidneys with DCs (Hochheiser et al. 2013). Under homeostatic conditions, the number of DCs was significantly reduced in the kidneys of CX_3CR1-deficient mice by an average of 75 %. Interestingly, the CX_3CR1-deficiency mainly had an effect on DCs in the renal cortex, and less so on the DCs in the renal medulla. Furthermore, the colonization of the kidneys with MPs, which also express CX_3CR1, was not significantly affected. There was even a slight increase in the number of MPs, although not statistically significant. Other cell types such as mast cells, NK cells and T-cells, subsets of which can express low levels of CX_3CR1, were hardly influenced by the CX_3CR1 deficiency. In other organs, including lung, liver, heart, bladder and lymph nodes, no significant changes in DC and MP numbers were observed. Only in the small intestine, the receptor had an effect on the APC colonization. Here, MPs were slightly affected, as it was also shown in another recent study. The spleens of CX_3CR1-deficient mice had increased numbers of DCs compared to spleens of CX_3CR1-competent mice, which might be explained by a redistribution of DC precursors. The CX_3CR1 ligand CX_3CL1 was highly expressed in the kidneys and intestines, which could be an explanation for the CX_3CR1-dependence of the DC or MP numbers in these tissues. CX_3CR1 can influence the recruitment, the differentiation as well as the survival of leukocytes, as shown in models of atherosclerosis and liver fibrosis (Landsman et al. 2009; Tacke et al. 2007; Karlmark et al. 2010). Whether DC numbers in the kidneys were affected by reduced recruitment of DC precursors, inhibited differentiation, reduced survival or reduced retention by the CX_3CR1 deficiency is unclear. The slow turnover of renal DCs makes it difficult to distinguish between these possibilities (Dong et al. 2005). Nevertheless, these results identify CX_3CR1 as a chemokine receptor, which more or less specifically affects DC numbers in the kidneys (Hochheiser et al. 2013).

The preferential effect of CX_3CR1 on DCs in the renal cortex cannot be explained with the data currently present. At the mRNA level, CX_3CL1 was similarly expressed in cortex and medulla. Possible explanations could be different expression levels at the protein level in renal cortex and medulla or the distribution of CX_3CL1 protein in renal tissue. Furthermore, MPs were not affected by the CX_3CR1 deficiency and also medullary DCs were less dependent on CX_3CR1 than cortical DCs. As described above, medullary DCs display several characteristics of MPs, while cortical DCs seem to be rather typical DCs. Why DCs but not MPs were affected by the receptor-deficiency, even though both types of cells express the receptor, cannot

be answered at this point. Further studies are necessary to elucidate the ontogeny and migration of renal DCs and MPs (Nelson et al. 2012).

CX_3CR1 Recruits DC Precursors to the Kidney in NTN

CX_3CR1 also contributed to DC colonization of the kidneys under inflammatory conditions (Hochheiser et al. 2013). The number of DC in the kidneys of nephritic CX_3CR1-deficient mice was reduced in comparison to nephritic CX_3CR1-competent mice. Also, the number of MPs was slightly, albeit not statistically significantly, reduced in nephritic CX_3CR1-deficient mice, which suggests that the common precursors of inflammatory DCs and MPs, the monocytes, might be hampered to migrate into the kidney. As it is also the case under homeostatic conditions, other CX_3CR1-expressing cells were much less affected. The transfer of bone marrow from CX_3CR1-reporter mice resulted in more GFP^+ cells in the kidneys of nephritic wt mice compared with the transfer of bone marrow from CX_3CR1-deficient mice. Regardless of the bone marrow donor, GFP^+ cells were able to differentiate into DCs inside the kidneys. These results show that the reduced DC numbers in nephritic mice were not only a result of lower colonization under homeostatic conditions, but also resulted directly from reduced migration, survival or retention of DC precursors under inflammatory conditions. In contrast to the kidneys, there were more CX_3CR1-deficient cells in the spleen compared to CX_3CR1-competent cells. The increased colonization of the spleen by CX_3CR1-deficient bone marrow cells excludes the possibility that the decreased cell numbers in the kidneys were due solely to differences in the survival of DC precursors in the blood (Landsman et al. 2009). Nevertheless, the immigration of CX_3CR1-deficient cells in the kidneys was only partially inhibited, which means that other chemokine receptors contribute to the migration of mononuclear cells into the nephritic kidneys. Possible candidates are CCR1 and CCR2, which are expressed by monocytes, the precursors of inflammatory DCs and MPs, and for which a role in the recruitment into the inflamed kidneys has already been shown (Geissmann 2010a; Nelson et al. 2012; Panzer et al. 2006; Li et al. 2008; Turner et al. 2008). One could investigate the respective contribution of a chemokine receptor to the recruitment of DC- and MP-precursors with the help of mice deficient for one or more chemokine receptors, or with various combinations of chemokine receptor inhibitors. However, the large redundancy in the chemokine-chemokine receptor interactions must be considered in such experiments. Unlike CX_3CL1, which is the only ligand for CX_3CR1 and which cannot bind to other chemokine receptors, many other chemokines can bind to several receptors. In addition, chemokine concentrations can be regulated by binding to the respective receptors (Panzer et al. 2006; Turner et al. 2008). The blockade or deficiency of a receptor can thus result in increased chemokine concentrations and recruitment via other receptors. Furthermore, CCR2-deficient mice harbour a relative monocytes deficiency because inflammatory Ly6Chi monocyte need CCR2 to emigrate from the bone marrow into the blood, which makes the analysis of these

mice more complicated (Serbina and Pamer 2006). With the help of transfer experiments in which equal numbers of chemokine receptor-competent and -deficient monocytes are transferred, these limitations can be overcome (Li et al. 2008).

NTN is Reduced in CX_3CR1-Deficient Mice

DC numbers and concomitantly disease severity were reduced in nephritic CX_3CR1-deficient mice (Hochheiser et al. 2013). DC functions themselves were not affected by the CX_3CR1 deficiency, so that the improvement of the disease is probably due to the reduced number of DC, which resulted in decreased T cell restimulation in the kidneys. At a first glance, these results are inconsistent with a study by Haskell et al., in which no significant differences of disease severity were detected when comparing the kidney damage in GN in CD1 mice with and without CX_3CR1 deficiency (Haskell et al. 2001). However, the observed differences might be due to different genetic backgrounds of the mice used in the respective studies, which can influence the course of nephritis (Kurts et al. 2007). Furthermore, two different GN models were used. While Haskell et al. analysed the accelerated NTN model, in which the mice had been immunized before the injection of nephrotoxic serum with irrelevant sheep immunoglobulin, non-accelerated NTN, that depends less on immune complexes and more on the T cell mediated response, was examined in our study.

The observations fit the results showing that depletion of the mature DCs on day 7 of NTN resulted in an improvement of disease. However, in CX_3CR1-deficient mice, also the protective effect of DCs in the early phase of NTN, which is mediated by CXCL16-mediated recruitment of regulatory NKT cells and the induction of IL-10 in T cells, should be reduced (Riedel et al. 2012; Scholz et al. 2008). Disease severity in CX_3CR1-deficient mice at early time points of NTN was not analyzed in this study. Therefore, it is possible that CX_3CR1-deficient mice show an initial worsening of disease, which is reversed by late protective effects of CX_3CR1-deficiency. However, in the study by Scholz et al. showing that early DC depletion aggravated disease, a second DC depletion at late time points could not compensate for the negative effects of early DC depletion. For these different results, there are some possible explanations: (1) The protective properties of DCs in early NTN might be mediated by CX_3CR1-independent DC subsets, eg $CD103^+$ DCs. (2) The efficiency of the DC-depletion (day 5 of NTN: 85 %) in CD11c.DTR mice that were used in the depletion-study is greater than the reduction of DCs in CX_3CR1-deficient mice (homeostasis: 75 %, day 10 of NTN: 50 %) and the remaining DCs in the latter mice are sufficient to mediate the protective effects in early NTN. (3) The (small) reductions of MPs and other CX_3CR1-expressing cells (eg mast cells and T cells) in CX_3CR1-deficient mice provide additional protective effects that are lacking in CD11c.DTR mice (Tipping et al. 1998; Timoshanko et al. 2006). (4) DT injection into CD11c.DTR mice leads to DC-depletion in all tissues, whereas in CX_3CR1-deficient mice DCs are selectively reduced in the kidney (and less so

in the intestine). This could influence the course of NTN by systemic effects. (5) DC depletion in CD11c.DTR mice is associated with prominent neutrophilia (Tittel et al. 2012). The high neutrophil numbers in the blood could affect NTN, because neutrophils appear to migrate into the kidneys early in NTN and may increase tissue damage (Turner et al. 2012; Ryan et al. 2011).

To investigate the effect of CX_3CR1-deficiency on protective DC-mediated functions, earlier time points in NTN must be analyzed. Nevertheless, protective effects of CX_3CR1-deficiency seem to outweigh potential negative effects of early DC-reduction. CX_3CR1 might therefore be a promising therapeutic target for GN.

The Anti-bacterial Immune Response is not Influenced by CX_3CR1 Deficiency

As the anti-bacterial immune response in PN depends on renal DCs, which recruit neutrophils into the kidney by CXCL2-production, CX_3CR1-deficiency or CX_3CR1-directed GN therapy might result in higher susceptibility towards kidney infection (Tittel et al. 2011). To investigate this issue, CX_3CR1-competent and -deficient mice were infected the UPEC and neutrophil infiltration as well as bacterial load of the kidneys were analyzed. Surprisingly, the anti-bacterial immune response was not significantly influenced by CX_3CR1-deficiency. Further studying these effects, we found two reasons for these unexpected results: First of all, medullary DCs, which produce most CXCL2 were only slightly reduced in CX_3CR1-deficient mice and could initiate neutrophil influx. Second, recruited neutrophils produced large amounts of CXCL2, thereby overcoming the slight initial reduction in CXCL2-production and neutrophil-recruitment by renal DCs. These observations go hand in hand with another study, showing that 6h after infection, neutrophils are the major source of CXCL2 inside the kidney (Hang et al. 1999). Nevertheless, renal DCs seem to be indispensable for initiation of neutrophil infiltration, as under steady state conditions, neutrophils are very rare in the kidney. Another recent study by Tittle at al could not detect CXCL2-production by neutrophils up until 3h after infection (Tittel et al. 2011). Neutrophils might therefore need accumulating infectious stimuli to be able to produce chemokines and enhance recruitment. The physiological relevance for this belayedly innitiated enhancement of neutrophil recruitment might be a reduced need of neutrophil influx in minor infections where few neutrophils are sufficient to clear infection. Overwhelming neutrophil influx could then damage tissue rather than clear pathogens, which should be avoided. In contrast to earlier studies, we could not detect CXCL2-production by non-hematopoietic $CD45^-$ cells (Hang et al. 1999; Chassin et al. 2006; Samuelsson et al. 2004; Patole et al. 2005). However, we cannot exclude that some cell types, such as epithelial cells, were lost by processing of the tissues for flow cytometric analysis.

Taken together, the anti-bacterial immune response is not significantly hampered in CX_3CR1-deficient mice and thus might not be an adverse side effect in CX_3CR1-directed GN therapy.

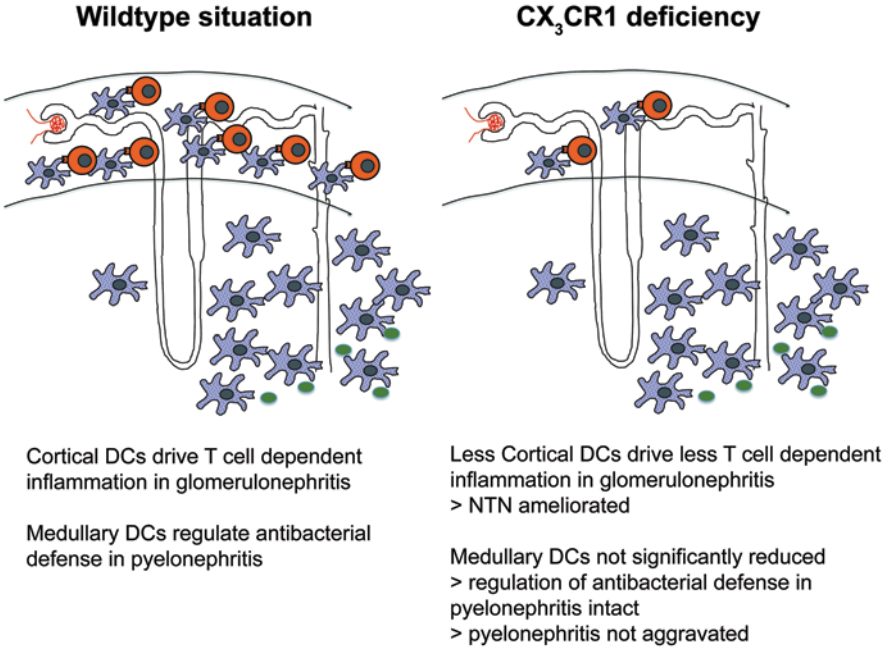

Fig. 5.2 Effect of CX3CR1-deficiency on kidney DCs in cortex and medulla and consequences for GN and PN

Future studies applying CX_3CR1 inhibitors or blocking antibodies need to follow in order to evaluate CX_3CR1 as a therapeutic target for GN and exclude non-specific effects in transgenic mice (Fig. 5.2).

References

Abbate, M., Zoja, C., & Remuzzi, G. (2006). How does proteinuria cause progressive renal damage? *Journal of the American Society of Nephrology, 17,* 2974–2984.

Adams, D. O., & Hamilton, T. A. (1984). The cell biology of macrophage activation. *Annual Review of Immunology, 2,* 283–318.

Andrews, P. M. (1977). A scanning and transmission electron microscopic comparison of puromycin aminonucleoside-induced nephrosis to hyperalbuminemia-induced proteinuria with emphasis on kidney podocyte pedicel loss. *Laboratory Investigation: A Journal of Technical Methods and Pathology, 36,* 183–197.

Assmann, K. J., Tangelder, M. M., Lange, W. P., Schrijver, G., & Koene, R. A. (1985). Anti-GBM nephritis in the mouse: Severe proteinuria in the heterologous phase. *Virchows Arch A Pathological Anatomy and Histopathology, 406,* 285–299.

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y. J., Pulendran, B., & Palucka, K. (2000). Immunobiology of dendritic cells. *Annual Review of Immunology, 18,* 767–811.

Beauchamp, D., & Bergeron, M. G. (1999). Pharmacologic basis for the treatment of pyelonephritis. *Current Infectious Disease Reports, 1,* 371–378.

Bohle, A. (1982). Importance of the renal interstitium for kidney function. *Klinische Wochenschrift, 60,* 1186–1190.

Bryant, P., & Ploegh, H. (2004). Class II MHC peptide loading by the professionals. *Current Opinion in Immunology, 16,* 96–102.

Chassin, C., Goujon, J. M., Darche, S., du Merle, L., Bens, M., Cluzeaud, F., Werts, C., Ogier-Denis, E., Le Bouguenec, C., Buzoni-Gatel, D., et al. (2006). Renal collecting duct epithelial cells react to pyelonephritis-associated Escherichia coli by activating distinct TLR4-dependent and -independent inflammatory pathways. *Journal of Immunology, 177,* 4773–4784.

Delamarre, L., Pack, M., Chang, H., Mellman, I., & Trombetta, E. S. (2005). Differential lysosomal proteolysis in antigen-presenting cells determines antigen fate. *Science, 307,* 1630–1634.

Dong, X., Swaminathan, S., Bachman, L. A., Croatt, A. J., Nath, K. A., & Griffin, M. D. (2005). Antigen presentation by dendritic cells in renal lymph nodes is linked to systemic and local injury to the kidney. *Kidney International, 68,* 1096–1108.

Duffield, J. S., Tipping, P. G., Kipari, T., Cailhier, J. F., Clay, S., Lang, R., Bonventre, J. V., & Hughes, J. (2005). Conditional ablation of macrophages halts progression of crescentic glomerulonephritis. *The American Journal of Pathology, 167,* 1207–1219.

Edgtton, K. L., Kausman, J. Y., Li, M., O'Sullivan, K., Lo, C., Hutchinson, P., Yagita, H., Holdsworth, S. R., & Kitching, A. R. (2008). Intrarenal antigens activate $CD4^+$ cells via co-stimulatory signals from dendritic cells. *Journal of the American Society of Nephrology, 19,* 515–526.

Geissmann, F., Manz, M. G., Jung, S., Sieweke, M. H., Merad, M., & Ley, K. (2010a). Development of monocytes, macrophages, and dendritic cells. *Science, 327,* 656–661.

Geissmann, F., Gordon, S., Hume, D. A., Mowat, A. M., & Randolph, G. J. (2010b). Unravelling mononuclear phagocyte heterogeneity. *Nature Reviews Immunology, 10,* 453–460.

Georgas, K., Rumballe, B., Wilkinson, L., Chiu, H. S., Lesieur, E., Gilbert, T., & Little, M. H. (2008). Use of dual section mRNA in situ hybridisation/immunohistochemistry to clarify gene expression patterns during the early stages of nephron development in the embryo and in the mature nephron of the adult mouse kidney. *HistoChemistry and Cell Biology, 130,* 927–942.

Ginhoux, F., Liu, K., Helft, J., Bogunovic, M., Greter, M., Hashimoto, D., Price, J., Yin, N., Bromberg, J., Lira, S. A., et al. (2009). The origin and development of nonlymphoid tissue CD103+ DCs. *The Journal of Experimental Medicine, 206,* 3115–3130.

Hang, L., Haraoka, M., Agace, W. W., Leffler, H., Burdick, M., Strieter, R., & Svanborg, C. (1999). Macrophage inflammatory protein-2 is required for neutrophil passage across the epithelial barrier of the infected urinary tract. *Journal of Immunology, 162,* 3037–3044.

Haraldsson, B., & Sorensson, J. (2004). Why do we not all have proteinuria? An update of our current understanding of the glomerular barrier. *News in Physiological Sciences, 19,* 7–10.

Haskell, C. A., Hancock, W. W., Salant, D. J., Gao, W., Csizmadia, V., Peters, W., Faia, K., Fituri, O., Rottman, J. B., & Charo, I. F. (2001). Targeted deletion of CX(3)CR1 reveals a role for fractalkine in cardiac allograft rejection. *The Journal of clinical investigation, 108,* 679–688.

Hewitt, I. K., Zucchetta, P., Rigon, L., Maschio, F., Molinari, P. P., Tomasi, L., Toffolo, A., Pavanello, L., Crivellaro, C., Bellato, S., et al. (2008). Early treatment of acute pyelonephritis in children fails to reduce renal scarring: Data from the Italian renal infection study trials. *Pediatrics, 122,* 486–490.

Heymann, F., Meyer-Schwesinger, C., Hamilton-Williams, E. E., Hammerich, L., Panzer, U., Kaden, S., Quaggin, S. E., Floege, J., Grone, H. J., & Kurts, C. (2009). Kidney dendritic cell activation is required for progression of renal disease in a mouse model of glomerular injury. *The Journal of Clinical Investigation, 119,* 1286–1297.

Hochheiser, K., Engel, D. R., Hammerich, L., Heymann, F., Knolle, P. A., Panzer, U., & Kurts, C. (2011). Kidney dendritic cells become pathogenic during crescentic glomerulonephritis with proteinuria. *Journal of the American Society of Nephrology, 22,* 306–316.

Hochheiser, K., Heuser, C., Krause, T. A., Teteris, S., Ilias, A., Weisheit, C., Hoss, F., Tittel, A. P., Knolle, P. A., Panzer, U., et al. (2013). Exclusive CX3CR1 dependence of kidney DCs impacts glomerulonephritis progression. *The Journal of clinical investigation, 123,* 4242–4254.

Hume, D. A., & Gordon, S. (1983). Mononuclear phagocyte system of the mouse defined by immunohistochemical localization of antigen F4/80. Identification of resident macrophages

in renal medullary and cortical interstitium and the juxtaglomerular complex. *The Journal of Experimental Medicine, 157,* 1704–1709.

Karlmark, K. R., Zimmermann, H. W., Roderburg, C., Gassler, N., Wasmuth, H. E., Luedde, T., Trautwein, C., & Tacke, F. (2010). The fractalkine receptor CX(3)CR1 protects against liver fibrosis by controlling differentiation and survival of infiltrating hepatic monocytes. *Hepatology, 52,* 1769–1782.

Kitching, A. R., Turner, A. L., Wilson, G. R., Semple, T., Odobasic, D., Timoshanko, J. R., O'Sullivan, K. M., Tipping, P. G., Takeda, K., Akira, S., et al. (2005). IL-12p40 and IL-18 in crescentic glomerulonephritis: IL-12p40 is the key Th1-defining cytokine chain, whereas IL-18 promotes local inflammation and leukocyte recruitment. *Journal of the American Society of Nephrology, 16,* 2023–2033.

Kleinewietfeld, M., Manzel, A., Titze, J., Kvakan, H., Yosef, N., Linker, R. A., Muller, D. N., & Hafler, D. A. (2013). Sodium chloride drives autoimmune disease by the induction of pathogenic TH17 cells. *Nature, 496,* 518–522.

Kruger, T., Benke, D., Eitner, F., Lang, A., Wirtz, M., Hamilton-Williams, E. E., Engel, D., Giese, B., Muller-Newen, G., Floege, J., et al. (2004). Identification and functional characterization of dendritic cells in the healthy murine kidney and in experimental glomerulonephritis. *Journal of the American Society of Nephrology, 15,* 613–621.

Kurts, C., Kosaka, H., Carbone, F. R., Miller, J. F., & Heath, W. R. (1997). Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive CD8(+) T cells. *The Journal of Experimental Medicine, 186,* 239–245.

Kurts, C., Heymann, F., Lukacs-Kornek, V., Boor, P., & Floege, J. (2007). Role of T cells and dendritic cells in glomerular immunopathology. *Seminars in Immunopathology, 29,* 317–335.

Landsman, L., Bar-On, L., Zernecke, A., Kim, K. W., Krauthgamer, R., Shagdarsuren, E., Lira, S. A., Weissman, I. L., Weber, C., & Jung, S. (2009). CX3CR1 is required for monocyte homeostasis and atherogenesis by promoting cell survival. *Blood, 113,* 963–972.

Li, L., Huang, L., Sung, S. S., Vergis, A. L., Rosin, D. L., Rose, C. E. Jr., Lobo, P. I., & Okusa, M. D. (2008). The chemokine receptors CCR2 and CX3CR1 mediate monocyte/macrophage trafficking in kidney ischemia-reperfusion injury. *Kidney International, 74,* 1526–1537.

Lukacs-Kornek, V., Burgdorf, S., Diehl, L., Specht, S., Kornek, M., & Kurts, C. (2008). The kidney-renal lymph node-system contributes to cross-tolerance against innocuous circulating antigen. *Journal of Immunology, 180,* 706–715.

Machnik, A., Neuhofer, W., Jantsch, J., Dahlmann, A., Tammela, T., Machura, K., Park, J. K., Beck, F. X., Muller, D. N., Derer, W., et al. (2009). Macrophages regulate salt-dependent volume and blood pressure by a vascular endothelial growth factor-C-dependent buffering mechanism. *Natural Medicines, 15,* 545–552.

Merad, M., Sathe, P., Helft, J., Miller, J., & Mortha, A. (2013). The dendritic cell lineage: Ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annual Review of Immunology, 31,* 563–604.

Miller, J. C., Brown, B. D., Shay, T., Gautier, E. L., Jojic, V., Cohain, A., Pandey, G., Leboeuf, M., Elpek, K. G., Helft, J., et al. (2012). Deciphering the transcriptional network of the dendritic cell lineage. *Nature Immunology, 13,* 888–899.

Mulvey, M. A., Lopez-Boado, Y. S., Wilson, C. L., Roth, R., Parks, W. C., Heuser, J., & Hultgren, S. J. (1998). Induction and evasion of host defenses by type 1-piliated uropathogenic *Escherichia coli*. *Science, 282,* 1494–1497.

Murphy, P. M., Baggiolini, M., Charo, I. F., Hebert, C. A., Horuk, R., Matsushima, K., Miller, L. H., Oppenheim, J. J., & Power, C. A. (2000). International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacological Reviews, 52,* 145–176.

Naik, S. H. (2008). Demystifying the development of dendritic cell subtypes, a little. *Immunology and Cell Biology, 86,* 439–452.

Nelson, P. J., Rees, A. J., Griffin, M. D., Hughes, J., Kurts, C., & Duffield, J. (2012). The renal mononuclear phagocytic system. *Journal of the American Society of Nephrology, 23,* 194–203.

Panzer, U., Steinmetz, O. M., Stahl, R. A., & Wolf, G. (2006). Kidney diseases and chemokines. *Current Drug Targets, 7,* 65–80.

Patole, P. S., Schubert, S., Hildinger, K., Khandoga, S., Khandoga, A., Segerer, S., Henger, A., Kretzler, M., Werner, M., Krombach, F., et al. (2005). Toll-like receptor-4: Renal cells and bone marrow cells signal for neutrophil recruitment during pyelonephritis. *Kidney International, 68,* 2582–2587.

Paust, H. J., Turner, J. E., Steinmetz, O. M., Peters, A., Heymann, F., Holscher, C., Wolf, G., Kurts, C., Mittrucker, H. W., Stahl, R. A., et al. (2009). The IL-23/Th17 axis contributes to renal injury in experimental glomerulonephritis. *Journal of the American Society of Nephrology, 20,* 969–979.

Randolph, G. J., Angeli, V., & Swartz, M. A. (2005). Dendritic-cell trafficking to lymph nodes through lymphatic vessels. *Nature Reviews Immunology, 5,* 617–628.

Rees, A. (2009). Cross dendritic cells anger T cells after kidney injury. *Journal of the American Society of Nephrology, 20,* 3–5.

Riedel, J. H., Paust, H. J., Turner, J. E., Tittel, A. P., Krebs, C., Disteldorf, E., Wegscheid, C., Tiegs, G., Velden, J., Mittrucker, H. W., et al. (2012). Immature renal dendritic cells recruit regulatory CXCR6(+) invariant natural killer T cells to attenuate crescentic GN. *Journal of the American Society of Nephrology, 23,* 1987–2000.

Roake, J. A., Rao, A. S., Morris, P. J., Larsen, C. P., Hankins, D. F., & Austyn, J. M. (1995). Dendritic cell loss from nonlymphoid tissues after systemic administration of lipopolysaccharide, tumor necrosis factor, and interleukin 1. *The Journal of Experimental Medicine, 181,* 2237–2247.

Ryan, J., Ma, F. Y., Kanellis, J., Delgado, M., Blease, K., & Nikolic-Paterson, D. J. (2011). Spleen tyrosine kinase promotes acute neutrophil-mediated glomerular injury via activation of JNK and p38 MAPK in rat nephrotoxic serum nephritis. *Laboratory Investigation: A Journal of Technical Methods and Pathology, 91,* 1727–1738.

Samuelsson, P., Hang, L., Wullt, B., Irjala, H., & Svanborg, C. (2004). Toll-like receptor 4 expression and cytokine responses in the human urinary tract mucosa. *Infection and Immunity, 72,* 3179–3186.

Scholz, J., Lukacs-Kornek, V., Engel, D. R., Specht, S., Kiss, E., Eitner, F., Floege, J., Groene, H. J., & Kurts, C. (2008). Renal dendritic cells stimulate IL-10 production and attenuate nephrotoxic nephritis. *Journal of the American Society of Nephrology, 19,* 527–537.

Serbina, N. V., & Pamer, E. G. (2006). Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nature Immunology, 7,* 311–317.

Shaughnessy, L. M., & Swanson, J. A. (2007). The role of the activated macrophage in clearing Listeria monocytogenes infection. *Frontiers in Bioscience: A Journal and Virtual Library, 12,* 2683–2692.

Shortman, K., & Liu, Y. J. (2002). Mouse and human dendritic cell subtypes. *Nature reviews. Immunology, 2,* 151–161.

Shortman, K., & Naik, S. H. (2007). Steady-state and inflammatory dendritic-cell development. *Nature Reviews Immunology, 7,* 19–30.

Soos, T. J., Sims, T. N., Barisoni, L., Lin, K., Littman, D. R., Dustin, M. L., & Nelson, P. J. (2006). CX3CR1+ interstitial dendritic cells form a contiguous network throughout the entire kidney. *Kidney International, 70,* 591–596.

Steinman, R. M., & Cohn, Z. A. (1973). Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *The Journal of Experimental Medicine, 137,* 1142–1162.

Steinman, R. M., Hawiger, D., & Nussenzweig, M. C. (2003). Tolerogenic dendritic cells. *Annual Review of Immunology, 21,* 685–711.

Strutz, F. M. (2009). EMT and proteinuria as progression factors. *Kidney International, 75,* 475–481.

Tacke, F., Alvarez, D., Kaplan, T. J., Jakubzick, C., Spanbroek, R., Llodra, J., Garin, A., Liu, J., Mack, M., van Rooijen, N., et al. (2007). Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques. *The Journal of Clinical Investigation, 117,* 185–194.

Timoshanko, J. R., Kitching, A. R., Holdsworth, S. R., & Tipping, P. G. (2001). Interleukin-12 from intrinsic cells is an effector of renal injury in crescentic glomerulonephritis. *Journal of the American Society of Nephrology, 12,* 464–471.

Timoshanko, J. R., Kitching, A. R., Semple, T. J., Tipping, P. G., & Holdsworth, S. R. (2006). A pathogenetic role for mast cells in experimental crescentic glomerulonephritis. *Journal of the American Society of Nephrology, 17,* 150–159.

Tipping, P. G., & Holdsworth, S. R. (2006). T cells in crescentic glomerulonephritis. *Journal of the American Society of Nephrology, 17,* 1253–1263.

Tipping, P. G., Huang, X. R., Qi, M., Van, G. Y., & Tang, W. W. (1998). Crescentic glomerulonephritis in CD4- and CD8-deficient mice. Requirement for CD4 but not CD8 cells. *The American Journal of Pathology, 152,* 1541–1548.

Tittel, A. P., Heuser, C., Ohliger, C., Knolle, P. A., Engel, D. R., & Kurts, C. (2011). Kidney dendritic cells induce innate immunity against bacterial pyelonephritis. *Journal of the American Society of Nephrology, 22,* 1435–1441.

Tittel, A. P., Heuser, C., Ohliger, C., Llanto, C., Yona, S., Hammerling, G. J., Engel, D. R., Garbi, N., & Kurts, C. (2012). Functionally relevant neutrophilia in CD11c diphtheria toxin receptor transgenic mice. *Nature Methods, 9,* 385–390.

Turner, J. E., Paust, H. J., Steinmetz, O. M., Peters, A., Meyer-Schwesinger, C., Heymann, F., Helmchen, U., Fehr, S., Horuk, R., Wenzel, U., et al. (2008). CCR5 deficiency aggravates crescentic glomerulonephritis in mice. *Journal of Immunology, 181,* 6546–6556.

Turner, J. E., Paust, H. J., Bennstein, S. B., Bramke, P., Krebs, C., Steinmetz, O. M., Velden, J., Haag, F., Stahl, R. A., & Panzer, U. (2012). Protective role for CCR5 in murine lupus nephritis. *American Journal of Physiology: Renal Physiology, 302,* F1503–1515.

Vremec, D., Zorbas, M., Scollay, R., Saunders, D. J., Ardavin, C. F., Wu, L., & Shortman, K. (1992). The surface phenotype of dendritic cells purified from mouse thymus and spleen: Investigation of the CD8 expression by a subpopulation of dendritic cells. *The Journal of Experimental Medicine, 176,* 47–58.

Wakim, L. M., Waithman, J., van Rooijen, N., Heath, W. R., & Carbone, F. R. (2008). Dendritic cell-induced memory T cell activation in nonlymphoid tissues. *Science, 319,* 198–202.

Wu, C., Yosef, N., Thalhamer, T., Zhu, C., Xiao, S., Kishi, Y., Regev, A., & Kuchroo, V. K. (2013). Induction of pathogenic TH17 cells by inducible salt-sensing kinase SGK1. *Nature, 496,* 513–517.

Wynn, T. A., Chawla, A., & Pollard, J. W. (2013). Macrophage biology in development, homeostasis and disease. *Nature, 496,* 445–455.

Chapter 6
Mechanisms of Memory T Cell Activation and Effective Immunity

Grégoire Lauvau and Saïdi M'Homa Soudja

Abstract Effective immunization induces the development of populations of robust effector lymphocytes specific for the immunizing antigens. Amongst them are cytotoxic/CD8$^+$T lymphocytes, which few will further differentiate into long-lived memory cells persisting in the host and exhibiting improved functional characteristics. The current model is that such memory cells can confer rapid host protection upon cognate antigen-mediated activation and direct killing of infected cells. In this chapter, we discuss work from our group and others that highlight the contribution of inflammatory cytokines to memory CD8$^+$ T cell activation and of cytolysis-independent mechanisms of host protection.

Keywords Memory T lymphocytes · Monocytes · Inflammation · Vaccination · Protective immunity

Introduction

Formation of long-lasting antigen-specific memory CD8$^+$ T cells involves complex sets of events that comprise initial activation (priming), proliferation (expansion) and massive apoptosis (contraction), after which the cells that survive give rise to long-lived memory cells (Cui and Kaech 2010; Harty and Badovinac 2008; McKinstry et al. 2010). Through this process, memory cells acquire multiple unique functional features which make them able not only to respond to, but also to actively provide different signals ultimately culminating in host protection. While the current view is that cognate antigen is the only essential trigger to reactivate the

G. Lauvau (✉) · S. M. Soudja
Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY, USA
e-mail: gregoire.lauvau@einstein.yu.edu

S. M. Soudja
e-mail: saidi.soudja@inserm.fr

S. P. Schoenberger et al. (eds.), *Crossroads Between Innate and Adaptive Immunity V,*
Advances in Experimental Medicine and Biology 850, DOI 10.1007/978-3-319-15774-0_6

memory T cells, several evidence in the literature, pointed to an important role of cytokines and chemokines in these processes. Thus, in this chapter, we most specifically present results that our group and others have generated over the past years and that support such evidence.

Non-Cognate Differentiation of Memory CD8$^+$ T Cells Into Robust Effector Cells

Cytokines and Pathways Initial hints came from early work by the Sprent group showing that CD8$^+$ T cells exhibiting features of memory cells (CD44^{hi}) could proliferate in response to the cytokine IL-15 in vitro and in vivo, and independently of T cell receptor (TCR) triggering (Zhang et al. 1998). A further report from the Forman lab using mice immunized with the intracellular bacterium *Listeria monocytogenes (Lm)* established that in response to the cytokines IL-12 and IL-18 only, *Lm*-specific memory but not naive CD8$^+$ T cells were able to differentiate into IFN-γ-secreting effector cells, which could confer some protection to immunized hosts (Berg et al. 2003). Also using *Lm* as a model, we reported that protection of vaccinated host mice during recall infection was occuring within 6–8 h post challenge infection (Narni-Mancinelli et al. 2011). Interestingly, control of bacterial growth was correlated with the rapid clustering and activation (IFN-γ$^+$) of memory CD8$^+$ T cells to the red pulp/marginal zone area of the spleen of challenged mice (Bajenoff et al. 2010), within bacteria-containing area where macrophages actively filter the blood (Aoshi et al. 2008). These 'effector-clusters' were exclusively formed by memory but not naive T cells, and included rapidly recruited blood-derived innate immune cells such as Ly6C$^+$ 'inflammatory' monocytes, the most abundant subset of blood-derived monocytes (Auffray et al. 2009), and neutrophils. Quite unexpectedly, we found that the memory CD8$^+$T cells underwent comparable activation (as measured by expression of IFN-γ, Granzyme B, CD25, NKG2D etc) whether cognate antigen was present or not (Soudja et al. 2012). Investigating further the underlying mechanisms, we could show that the cytokines IL-18 and IL-15 were major drivers of early memory T cell effector differentiation respectively into IFN-γ-secreting and cytolytic (GrB$^+$) cells. This involved the triggering of the inflammasome (IL-18) and the IRF3/type I IFN (IL-15) pathways. While such rapid memory T cell-differentiation did not require cognate antigen recognition by the memory cells, cellular proliferation and robust secondary expansion did not occur without T cell receptor (TCR) triggering. Similar findings were also reported by other groups (Chu et al. 2013; Kupz et al. 2012).

Innate Immune Cells An initial study from the Lefrancois group suggested the unexpected importance of CD11c^{hi} dendritic cells (DCs) for optimal activation and expansion of memory CD8$^+$ T cells in mice immunized with *Lm* and two distinct viruses, the vesicular stomatitis and the influenza viruses (Zammit et al. 2005).

Our study and that from the Bedoui group investigating the mechanisms of antigen-independent activation of memory $CD8^+$ T cells reported an essential role for different subsets of innate myeloid cells in providing key activating cytokines to the memory cells (Kupz et al. 2012; Soudja et al. 2012). While the latter study showed the implication of the NLRC4 inflammasome inside $CD11c^+$ DCs, leading to IL-18 production, we reported that $Ly6C^+$ monocytes could produce both IL-18 and IL-15. Of note, $Ly6C^+$ monocytes appeared as exclusive providers of IL-15 very early on -by 6–8 h post infection-, yet other cell types such as macrophages or DCs could compensate as early as 24 h after the challenge infection. Though substantial differences could account for this discrepancy, including the experimental systems that were used, we favor the idea that both antigen-presenting cell (APC) types are indeed contributing to reactivating the memory cells. As initially proposed by Busch and colleagues (Neuenhahn et al. 2006), and further established by the Murphy lab (Edelson et al. 2011), amongst $CD11c^+$ DCs, the $CD8\alpha^+$ DCs are required to carry live blood bacteria to spleens, thus it is certainly possible that DCs act as initiators while $Ly6C^+$ monocytes, which undergo massive mobilization from the bone-marrow (Serbina and Pamer 2006), quickly amplify and sustain the cytokinic signals.

In addition to providing activating cytokines and along the lines of the early work from Lefrancois and colleagues (Zammit et al. 2005), we also found that $Ly6C^+$ monocytes contribute to antigen-dependent expansion of the memory $CD8^+$ T cells (Soudja et al. 2012). Selective depletion of $Ly6C^+$ monocytes in immunized mice, substantially decreased memory $CD8^+$ T cell expansion during the challenge infection, as also shown following $CD11c^+$ DC elimination. Both of these observations may indeed be accounted for by indirect 'inflammatory' effects, rather than by direct presentation of cognate antigen to the memory cells, as also proposed by Badovinac and Harty (Wirth et al. 2011). The contribution of antigen-presentation by DC and/or $Ly6C^+$ monocytes to memory T cell activation and expansion still remains to be defined.

In summary, all these reports supported the notion that APCs which act as early sentinel cells of the immune system, in particular DCs and $Ly6C^+$ monocytes, play an essential role for optimal cytokine- as well as cognate antigen-mediated activation of memory $CD8^+$ T cells in vivo. This mechanism also contributes to host mechanisms of innate immune protection.

Effective Memory T Cell-Mediated Immunity

$Ly6C^+$ monocytes and $CD11c^+$ DCs are required to promote optimal memory T cell reactivation during a recall infection by acute intracellular pathogens such as *Lm* or viruses like the lymphochorionmeningitis virus (LCMV Armstrong) or the Vaccinia virus. However, how each of these signals translates into effective protection of the hosts is still incompletely understood. The prominent view proposes

that reactivated memory CD8$^+$ T cells rapidly express cytolytic effector functions (such as perforin, Granzyme and Fas) that allow for direct killing of pathogen-infected cells, representing the major mechanism of host protection (Harty et al. 2000). Reactivated memory T cells are also shown to secrete important amounts of proinflammatory cytokines and chemokines such as IFNγ, CCL3, CCL4 and CCL5, which promote immune cell activation and recruitment (Dorner et al. 2002; Sallusto et al. 2000). Ultimately, effective protection of vaccinated hosts likely involves both direct and indirect mechanisms orchestrated by memory T cells, yet we and others have sought to most accurately define the relative contribution of the possible different mechanisms. Of note, which effector mechanisms will be most important will depend on each infection as previously reviewed elsewhere (Harty et al. 2000).

Numerous studies, in particular from the Harty lab, have used both mice lacking essential effector molecules (such as IFN-γ, TNF-α, Perforin, CD95/Fas), as well as adoptive transfer experiments of memory CD8$^+$ T cells purified from mice immunized with *Lm* as model (Badovinac and Harty 2000; Harty and Bevan 1995; White et al. 2000a, b). Wild-type or knockout immunized mice were challenged and their ability to clear the infection compared. Likewise, memory CD8$^+$ T cells from knockout or WT immunized mice were purified and transferred to naïve recipient mice that were subsequently challenged to assess the contribution of distinct effector mechanisms to host protection. Several major conclusions could be drawn from these studies, specifically that (i) not one but multiple mechanisms accounted for host protection, (ii) all immunized knockout mice listed above turned out to be protected against recall infection and (iii) a mechanism that required TNF-α yet was independent of memory T cell-cytolytic activity and IFN-γ was implicated (White et al. 2000a). Further investigations using potent TNF-α neutralizing reagents in vivo, by several groups including ours, established the importance of TNF-α for the protection of immunized hosts during the recall infection (Narni-Mancinelli et al. 2007; Neighbors et al. 2001). While discrepant with the initial studies using mice lacking TNF-α or its receptor TNFRI (p55) (White et al. 2000a), it did underline that knockout mice can develop compensatory mechanisms that substantially differ from that of WT mice. Alternative explanation, though not exclusive, may be that adoptively transferred memory T cells conferred protection to recipient mice through substantially distinct mechanisms than those of vaccinated mice undergoing the challenge infection.

Building on these observations, we further explored the mechanisms of TNF-α-dependent protective immunity. In agreement with prior work (Cook et al. 1999), memory CD8$^+$ T cells could secrete the proinflammatory cytokine CCL3, yet very rapidly following challenge infection, which promoted the differentiation of blood-derived phagocytes -both Ly6C$^+$ monocytes and neutrophils- into TNF-α and reactive oxygen species (ROS) producing cells (Narni-Mancinelli et al. 2007). This oxidative burst enhanced antimicrobial autophagy which was correlated with intracellular pathogen killing (Narni-Mancinelli et al. 2011). Collectively, these results provided solid proof of concept supporting the importance of innate immune effector cells for rapid memory CD8$^+$ T cell-mediated protection in vaccinated hosts. CCL3, however, was released by the memory cells only upon cognate antigen trig-

gering, suggesting that other, possibly earlier, signals were contributing to innate immune cell mobilization.

From prior work by us and others (Berg et al. 2003; Kupz et al. 2012; Soudja et al. 2012), IFN-γ secreted by memory T cells in response to IL-18, IL-12 and IL-15, appeared as a strong candidate. Production of IFN-γ by pathogen-specific memory T cells indeed starts already by 4 h post recall infection and can occur in a cognate-antigen independent manner. Through series of advanced genetic depletion and bone-marrow chimera experiments, results from our lab provided compelling evidence that IFN-γ from memory T cells, but not other lymphocytes such as NK or NK T cells, was most essential in instructing innate myeloid and lymphoid cell activation and differentiation into robust microbicidal effector cells. IFN-γ signaling to myeloid cells such as Ly6C$^+$ monocytes, neutrophils, macrophages and DCs was required for them to secrete proinflammatory cytokines (TNF-α) and chemokines (CXCL9, CXCL10, CCL2, CXCL1), express the inducible nitric oxid synthase (iNOS), and upregulate costimulatory (CD80, CD86, CD40) and antigen-presenting molecules. While the importance of IFN-γ as a key modulator of immune responses has long been investigated and documented in humans and mice models (Hu and Ivashkiv 2009), such links to the mechanisms of memory T cell-mediated protection in a relevant in vivo model of vaccination/infection had not been previously established. Analysis of Ly6C$^+$ monocytes genetic expression program in vaccinated mice undergoing challenge infection revealed an overall 'IFN-γ-skewed' program, notably with genes encoding for the guanylate binding protein (gbp) GTPases involved in microbial killing (Kim et al. 2011; Yamamoto et al. 2012). Importantly, both Ly6C$^+$ monocytes, CD11c$^+$ DCs and subsets of tissue macrophages contributed to effective protection of vaccinated hosts during the recall infection, in an IFN-γ-dependent manner. Another key aspect to outline that directly results from IFN-γ modulation is the secreted levels of chemokines such as CXCL9, CXCL10 and others, which are essential for immune cell recruitment to infected foci. Elegant studies from the Germain and the von Andrian labs have illustrated the importance of such mechanism orchestrated by memory CD8$^+$ T cells for rapid pathogen containment near the peripheral entry portal of lymph-borne bacterial and viral pathogens (Kastenmuller et al. 2013; Sung et al. 2012).

Interestingly too, we could extend some of our findings to a relevant mucosal model of infection, using mice vaccinated intravaginally by attenuated TK$^-$ herpes simplex virus 2 (HSV-2). We found that Ly6C$^+$ monocytes and neutrophils underwent better recruitment and activation during recall infection with WT virulent HSV-2 than that of non-vaccinated counterparts, a finding that is particularly relevant in the context of prior and recent literature on the importance of mucosal resident memory T (T_{RM}) cell-derived IFN-γ for protective immunity against HSV-2 (Ariotti et al. 2014; Gebhardt et al. 2009; Iijima and Iwasaki 2014; Schenkel et al. 2013, 2014). In all of these studies, T_{RM} were reported to carry the very initial immune 'alarming' functions, using IFN-γ as the most essential lymphokine.

In summary, results from several studies largely conducted in our lab support the notion that indirect mechanisms of protection, distinct from the 'usual' cytolytic effector mechanisms expressed by CD8$^+$ T cells, are significant contributors to the

protection of vaccinated hosts during virulent pathogen infection. This mechanism implicates the coordinated orchestration of innate immune cell activation and differentiation by the memory T cells. Altogether, this body of work reveals the importance for such indirect mechanisms of protection, and further emphasizes their need to be accounted for when evaluating vaccine efficacy.

Conclusion

This book chapter summarizes recent developments from our group and others investigating mechanisms of in vivo memory T cell-activation and effective host protection that are taking place in vaccinated hosts during an acute challenge infection (See also summary Fig. 6.1). Current evidence reveal the complexity of these processes and suggest the implication of multiple steps of innate and adaptive cell cross-talks that ultimately lead to microbial pathogen containment and killing, and host protection. Cognate memory T cell antigens, cytokines and chemokines, and the differentiation of robust microbicidal innate and adaptive effector cells are all contributing to an optimal and effective immune response. Further studies, in particular using relevant models of mucosal immunizations, will be necessary to fully dissect and understand these mechanisms, and harness them for potential therapies.

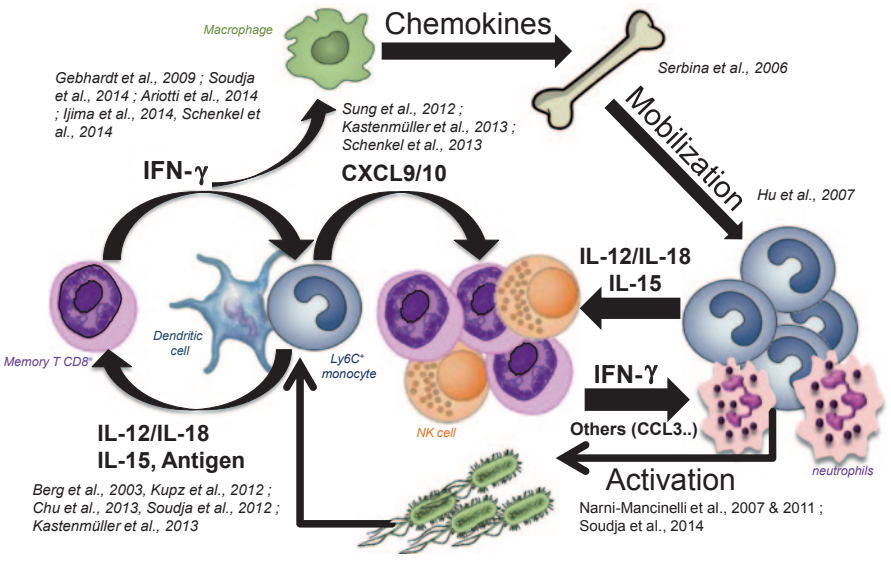

Fig. 6.1 Crosstalk between memory T cells and innate immune cells occurring during recall infection of vaccinated hosts

References

Aoshi, T., Zinselmeyer, B. H., Konjufca, V., Lynch, J. N., Zhang, X., Koide, Y., & Miller, M. J. (2008). Bacterial entry to the splenic white pulp initiates antigen presentation to $CD8^+$ T cells. *Immunity, 29,* 476–486.

Ariotti, S., Hogenbirk, M. A., Dijkgraaf, F. E., Visser, L. L., Hoekstra, M. E., Song, J. Y., Jacobs, H., Haanen, J. B., & Schumacher, T. N. (2014). T cell memory. Skin-resident memory CD8(+) T cells trigger a state of tissue-wide pathogen alert. *Science, 346,* 101–105.

Auffray, C., Sieweke, M. H., & Geissmann, F. (2009). Blood monocytes: Development, heterogeneity, and relationship with dendritic cells. *Annual Review of Immunology, 27,* 669–692.

Badovinac, V. P., & Harty, J. T. (2000). Adaptive immunity and enhanced $CD8^+$ T cell response to Listeria monocytogenes in the absence of perforin and IFN-gamma. *Journal of Immunology, 164,* 6444–6452.

Bajenoff, M., Narni-Mancinelli, E., Brau, F., & Lauvau, G. (2010). Visualizing early splenic memory CD8 + T cells reactivation against intracellular bacteria in the mouse. *PLoS One, 5,* e11524.

Berg, R. E., Crossley, E., Murray, S., & Forman, J. (2003). Memory $CD8^+$ T cells provide innate immune protection against listeria monocytogenes in the absence of cognate antigen. *The Journal of Experimental Medicine, 198,* 1583–1593.

Chu, T., Tyznik, A. J., Roepke, S., Berkley, A. M., Woodward-Davis, A., Pattacini, L., Bevan, M. J., Zehn, D., & Prlic, M. (2013). Bystander-activated memory CD8 T cells control early pathogen load in an innate-like, NKG2D-dependent manner. *Cell Reports, 3,* 701–708.

Cook, D. N., Smithies, O., Strieter, R. M., Frelinger, J. A., & Serody, J. S. (1999). $CD8^+$ T cells are a biologically relevant source of macrophage inflammatory protein-1 alpha in vivo. *Journal of Immunology, 162,* 5423–5428.

Cui, W., & Kaech, S. M. (2010). Generation of effector $CD8^+$ T cells and their conversion to memory T cells. *Immunological Reviews, 236,* 151–166.

Dorner, B. G., Scheffold, A., Rolph, M. S., Huser, M. B., Kaufmann, S. H., Radbruch, A., Flesch, I. E., & Kroczek, R. A. (2002). MIP-1alpha, MIP-1beta, RANTES, and ATAC/lymphotactin function together with IFN-gamma as type 1 cytokines. *Proceedings of the National Academy of Sciences of the United States of America, 99,* 6181–6186.

Edelson, B. T., Bradstreet, T. R., Hildner, K., Carrero, J. A., Frederick, K. E., Kc, W., Belizaire, R., Aoshi, T., Schreiber, R. D., Miller, M. J., et al. (2011). CD8alpha(+) Dendritic Cells Are an Obligate Cellular Entry Point for Productive Infection by Listeria monocytogenes. *Immunity, 35,* 236–248.

Gebhardt, T., Wakim, L. M., Eidsmo, L., Reading, P. C., Heath, W. R., & Carbone, F. R. (2009). Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. *Nature Immunology, 10,* 524–530.

Harty, J. T., & Badovinac, V. P. (2008). Shaping and reshaping CD8 + T-cell memory. *Nature Reviews Immunology, 8,* 107–119.

Harty, J. T., & Bevan, M. J. (1995). Specific immunity to Listeria monocytogenes in the absence of IFN gamma. *Immunity, 3,* 109–117.

Harty, J. T., Tvinnereim, A. R., & White, D. W. (2000). CD8 + T cell effector mechanisms in resistance to infection. *Annual Review of Immunology, 18,* 275–308.

Hu, X., & Ivashkiv, L. B. (2009). Cross-regulation of signaling pathways by interferon-gamma: Implications for immune responses and autoimmune diseases. *Immunity, 31,* 539–550.

Iijima, N., & Iwasaki, A. (2014). T cell memory. A local macrophage chemokine network sustains protective tissue-resident memory CD4 T cells. *Science, 346,* 93–98.

Kastenmuller, W., Brandes, M., Wang, Z., Herz, J., Egen, J. G., & Germain, R. N. (2013). Peripheral prepositioning and local CXCL9 chemokine-mediated guidance orchestrate rapid memory CD8(+) T Cell responses in the lymph node. *Immunity, 38,* 502–513.

Kim, B. H., Shenoy, A. R., Kumar, P., Das, R., Tiwari, S., & MacMicking, J. D. (2011). A family of IFN-gamma-inducible 65-kD GTPases protects against bacterial infection. *Science, 332,* 717–721.

Kupz, A., Guarda, G., Gebhardt, T., Sander, L. E., Short, K. R., Diavatopoulos, D. A., Wijburg, O. L., Cao, H., Waithman, J. C., Chen, W., et al. (2012). NLRC4 inflammasomes in dendritic cells regulate noncognate effector function by memory CD8(+) T cells. *Natural Immunology, 13,* 115-117.

McKinstry, K. K., Strutt, T. M., & Swain, S. L. (2010). The potential of CD4 T-cell memory. *Immunology, 130,* 1–9.

Narni-Mancinelli, E., Campisi, L., Bassand, D., Cazareth, J., Gounon, P., Glaichenhaus, N., & Lauvau, G. (2007). Memory $CD8^+$ T cells mediate antibacterial immunity via CCL3 activation of TNF/ROI + phagocytes. *The Journal of Experimental Medicine, 204,* 2075–2087.

Narni-Mancinelli, E., Soudja, S. M., Crozat, K., Dalod, M., Gounon, P., Geissmann, F., & Lauvau, G. (2011). Inflammatory monocytes and neutrophils are licensed to kill during memory responses in vivo. *PLoS Pathogens, 7*(12):e1002457.

Neighbors, M., Xu, X., Barrat, F. J., Ruuls, S. R., Churakova, T., Debets, R., Bazan, J. F., Kastelein, R. A., Abrams, J. S., & O'Garra, A. (2001). A critical role for interleukin 18 in primary and memory effector responses to listeria monocytogenes that extends beyond its effects on Interferon gamma production. *The Journal of Experimental Medicine, 194,* 343–354.

Neuenhahn, M., Kerksiek, K. M., Nauerth, M., Suhre, M. H., Schiemann, M., Gebhardt, F. E., Stemberger, C., Panthel, K., Schroder, S., Chakraborty, T., et al. (2006). CD8alpha + dendritic cells are required for efficient entry of Listeria monocytogenes into the spleen. *Immunity, 25,* 619–630.

Sallusto, F., Mackay, C. R., & Lanzavecchia, A. (2000). The role of chemokine receptors in primary, effector, and memory immune responses. *Annual Review of Immunology, 18,* 593–620.

Schenkel, J. M., Fraser, K. A., Vezys, V., & Masopust, D. (2013). Sensing and alarm function of resident memory CD8(+) T cells. *Nature Immunology, 14,* 509–513.

Schenkel, J. M., Fraser, K. A., Beura, L. K., Pauken, K. E., Vezys, V., & Masopust, D. (2014). T cell memory. Resident memory CD8 T cells trigger protective innate and adaptive immune responses. *Science, 346,* 98–101.

Serbina, N. V., & Pamer, E. G. (2006). Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nature Immunology, 7,* 311–317.

Soudja, S. M., Ruiz, A. L., Marie, J. C., & Lauvau, G. (2012). Inflammatory Monocytes Activate Memory CD8(+) T and Innate NK Lymphocytes Independent of Cognate Antigen during Microbial Pathogen Invasion. *Immunity, 37,* 549–562.

Sung, J. H., Zhang, H., Moseman, E. A., Alvarez, D., Iannacone, M., Henrickson, S. E., de la Torre, J. C., Groom, J. R., Luster, A. D., & von Andrian, U. H. (2012). Chemokine guidance of central memory T cells is critical for antiviral recall responses in lymph nodes. *Cell, 150,* 1249–1263.

White, D. W., Badovinac, V. P., Fan, X., & Harty, J. T. (2000a). Adaptive immunity against Listeria monocytogenes in the absence of type I tumor necrosis factor receptor p 55. *Infection and Immunity, 68,* 4470–4476.

White, D. W., Badovinac, V. P., Kollias, G., & Harty, J. T. (2000b). Cutting edge: Antilisterial activity of $CD8^+$ T cells derived from TNF-deficient and TNF/perforin double-deficient mice. *Journal of Immunology, 165,* 5–9.

Wirth, T. C., Martin, M. D., Starbeck-Miller, G., Harty, J. T., & Badovinac, V. P. (2011). Secondary $CD8^+$ T-cell responses are controlled by systemic inflammation. *European Journal of Immunology, 41,* 1321–1333.

Yamamoto, M., Okuyama, M., Ma, J. S., Kimura, T., Kamiyama, N., Saiga, H., Ohshima, J., Sasai, M., Kayama, H., Okamoto, T., et al. (2012). A cluster of interferon-gamma-inducible p65 GTPases plays a critical role in host defense against Toxoplasma gondii. *Immunity, 37,* 302–313.

Zammit, D. J., Cauley, L. S., Pham, Q. M., & Lefrancois, L. (2005). Dendritic cells maximize the memory CD8 T cell response to infection. *Immunity, 22,* 561–570.

Zhang, X., Sun, S., Hwang, I., Tough, D. F., & Sprent, J. (1998). Potent and selective stimulation of memory-phenotype $CD8^+$ T cells in vivo by IL-15. *Immunity, 8,* 591–599.

Chapter 7
Molecular Programming of Immunological Memory in Natural Killer Cells

Aimee M. Beaulieu, Sharline Madera and Joseph C. Sun

Abstract Immunological memory is a hallmark of the adaptive immune system. Although natural killer (NK) cells have traditionally been classified as a component of the innate immune system, they have recently been shown in mice and humans to exhibit certain features of immunological memory, including an ability to undergo a clonal-like expansion during virus infection, generate long-lived progeny (i.e. memory cells), and mediate recall responses against previously encountered pathogens—all characteristics previously ascribed only to adaptive immune responses by B and T cells in mammals. To date, the molecular events that govern the generation of NK cell memory are not completely understood. Using a mouse model of cytomegalovirus infection, we demonstrate that individual pro-inflammatory IL-12, IL-18, and type I-IFN signaling pathways are indispensible and play non-redundant roles in the generation of virus-specific NK cell memory. Furthermore, we discovered that antigen-specific proliferation and protection by NK cells is mediated by the transcription factor Zbtb32, which is induced by pro-inflammatory cytokines and promotes a cell cycle program in activated NK cells. A greater understanding of the molecular mechanisms controlling NK cell responses will provide novel strategies for tailoring vaccines to target infectious disease.

Keywords Adaptive immune system · Innate immune system · NK cell memory · Cytokine stimulation

Natural Killer Cells Bridge Innate and Adaptive Immunity

Historical convention has divided the immune system into two compartments—the innate immune system and the adaptive immune system. Until recently, immune cells were strictly assigned to one category or the other, with the primary distinction

Aimee M. Beaulieu and Sharline Madera contributed equally.

J. C. Sun (✉) · A. M. Beaulieu · S. Madera
Memorial Sloan Kettering Cancer Center, 408 East 69th Street,
ZRC-1402, New York, NY 10065, USA
e-mail: sunj@mskcc.org

S. P. Schoenberger et al. (eds.), *Crossroads Between Innate and Adaptive Immunity V,*
Advances in Experimental Medicine and Biology 850, DOI 10.1007/978-3-319-15774-0_7

hinging on a cellular capacity for immunological memory. Memory has been broadly defined as the ability to respond more robustly to a specific antigen or pathogen following subsequent re exposure or reinfection; cells that possessed this feature were classified as adaptive and those that did not were classified as innate. In mammals, B cells and classical $CD4^+$ and $CD8^+$ T cells were historically considered to be the sole members of the adaptive immune system, and all other immune cells fell under the umbrella of the innate immune system. However, new data indicate that the strict adaptive versus innate classification system may not be applicable to all cell types.

NK cells have long been considered to be part the innate immune system. Although derived from a common lymphoid precursor shared with B and T cells, NK cells lack somatically rearranged antigen receptors and instead rely on signals from germ line-encoded surface receptors for activation (Sun and Lanier 2011). Effector responses by NK cells are typified by rapid degranulation of cytolytic granules, which lyse infected or stressed target cells, and by production of a variety of inflammatory cytokines, which serve to alert and activate other cells of the immune system. Because NK cells express a limited number of genetically-fixed activating receptors, and the diversity of pathogenic antigens is inconceivably vast, previous research did not consider that NK cells might be capable of antigen-specific immunological memory.

However, intriguing studies in mice in the early 2000s demonstrated that certain receptors expressed by NK cells could bind, with remarkably high affinity, specific viral proteins expressed on the surface of infected cells. The most notable example was the receptor-ligand pairing of the Ly49H receptor on NK cells in C57BL/6 mice and the mouse cytomegalovirus (MCMV)-encoded glycoprotein, m157 (Arase et al. 2002; Smith et al. 2002). Although not definitive for immunological memory *per se*, the interactions revealed in these studies fulfilled a key prerequisite of adaptive memory responses—that of antigen-specificity—and laid the groundwork for the idea that certain innate lymphocytes, despite their limited receptor repertoire, might be capable of immunological memory. Indeed, in 2009, Sun et al. revealed that NK cells expressing Ly49H could undergo a robust clonal-like expansion—as much as 1000-fold expansion—following MCMV challenge in vivo (Sun et al. 2009). The magnitude and kinetics of the virus-specific NK cell response in these mice, which peaked at around day 7 post-infection and then entered a defined contraction phase, were highly reminiscent of antigen-specific $CD8^+$ T cell responses. Most importantly, a small subset of these effector NK cells could persist for months after the contraction phase and resolution of the primary infection (Fig. 7.1). When these long-lived "memory" cells were re-exposed to MCMV, their response was amplified and more protective than that of their naïve counterparts—fundamental hallmarks of immunological memory.

Subsequent studies in humans have corroborated the mouse experiments. Analogous to the $Ly49H^+$ population in MCMV-infected mice, individuals previously infected with human CMV were found to harbor an expanded and long-lived pool of NK cells expressing the NKG2C receptor (Bjorkstrom et al. 2011; Della Chiesa et al. 2012; Guma et al. 2006; Lopez-Verges et al. 2011). Transfer of these memory-like $NKG2C^+$ NK cells into new patients (e.g. in the course of hematopoietic stem cell transplantation) correlated with enhanced effector responses in the context of

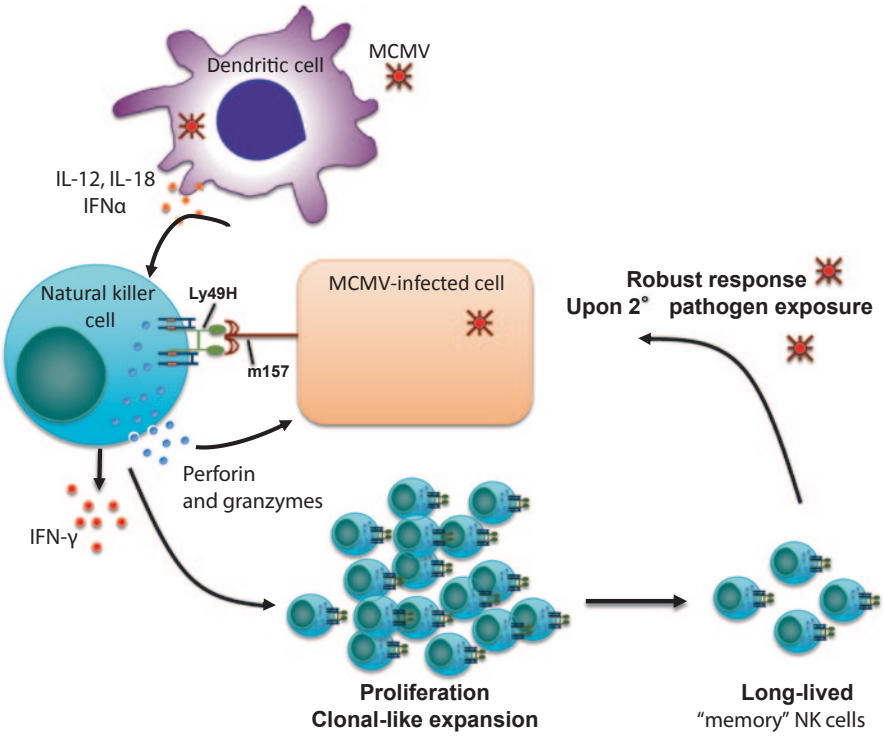

Fig. 7.1 The NK cell response against MCMV. A subset of natural killer cells in the C57BL/6 mouse express the activating receptor Ly49H, which recognizes the MCMV-encoded glycoprotein m157. Antigen receptor engagement synergizes with inflammatory signal to fully activate NK cells and thereby promote cytokine production (IFN-γ), cell-mediated cytotoxicity (perforin and granzymes), and the clonal-like proliferation of Ly49H$^+$ NK cells. Virally expanded antigen-specific NK cells undergo a contraction phase that leaves behind a pool of long-lived "memory" NK cells, which have heightened protective capabilities upon secondary pathogen encounter

HCMV viral reactivation, including more robust proliferation and cytokine production (Foley et al. 2012a, b).

Independent groups have now confirmed that NK cells can mount adaptive immune responses to a wide variety of pathogen and non-pathogen antigens. For example, von Andrian and colleagues have shown that NK cells can mount antigen-specific memory responses against chemical haptens, despite the presumed absence of cognate receptors for specific chemical molecules. Hapten-primed NK cells are capable of mediating contact hypersensitivity, even in the absence of B and T cells, and can confer hypersensitivity to naïve animals when adoptively transferred into new hosts. NK cell-mediated contact hypersensitivity is strictly antigen-specific, as it is never observed when different haptens are used for re-challenge (O'Leary et al. 2006; Paust et al. 2010). Similarly, vaccination against specific viral antigens from influenza, vesicular stomatitis virus (VSV) or human immunodeficiency virus type 1 (HIV-1) generates NK cells capable of protecting naive mice against lethal viral challenge in a virus/antigen-specific manner (Paust et al. 2010).

Pro-Inflammatory Cytokines Control Effector and Memory responses by NK cells

To better understand the molecular events that govern immunological memory in NK cells, genetically altered mouse strains are now being employed to explore the impact of various signaling pathways on NK cell effector and memory responses. Among the pathways that have been explored to date, signaling by pro-inflammatory cytokines is the most well-studied. CMV infection is known to cause a marked upsurge in systemic pro-inflammatory cytokine production, in particular IL-12, IL-18, TNF-α, and Type I Interferons (Orange and Biron 1996a, b; Pien and Biron 2000). All of these factors have been shown to potently activate NK cells through cytokine receptors constitutively expressed on the surface of resting NK cells (Andrews et al. 2003; Nguyen et al. 2002; Orange and Biron 1996b). The role of pro-inflammatory cytokines on antiviral NK cell memory remained largely unexplored until a recent study demonstrated the crucial role of IL-12 and STAT4 in the generation of a protective memory NK cell response during MCMV infection (Sun et al. 2012). NK cells lacking the IL-12 receptor failed to expand and were incapable of forming a long-lasting memory NK cell pool following infection with MCMV. This inability to generate a memory response was independent of IFN-γ as NK cells lacking the IFN-γ receptor behaved similarly to wildtype NK cells. In contrast to IL-12, IL-18, a cytokine known for synergizing with IL-12 for maximal IFN-γ production in NK cells is not necessary for the generation of antiviral NK cell memory (Madera et al., 2015). Similarly, a crucial and non-redundant role for type I interferon (IFN-α) on the Survival of effector NK cells but not memory formation *per se,* was also observed during MCMV infection (Madera and et al., 2015).

NK cell memory has also been described in a non-antigen specific setting, where NK cell activation is driven solely by pro-inflammatory cytokine treatment. NK cells pretreated with a cocktail of IL-12, IL-18 and IL-15 and then adoptively transferred into Rag-deficient hosts, could persist for weeks after transfer. Like memory NK cells induced by viral infection, these pretreated transferred cells produced more IFN-γ following cytokine restimulation than their untreated counterparts (Cooper et al. 2009). Furthermore, cytokine-activated NK cells were more protective than untreated NK cells in a transplantable tumor model (Ni et al. 2012). Similarly, human NK cells pretreated with pro-inflammatory cytokines also exhibit enhanced IFN-γ production upon restimulation (Romee et al. 2012). Although the mechanism of cytokine-induced memory is still unknown, the long-lasting and presumably heritable nature of cytokine-treated NK cells may be “imprinted” by epigenetic modifications induced during pro-inflammatory cytokines exposure. Future studies will determine the specific molecular signals prompted by pro-inflammatory cytokine treatments that influence NK cell function and longevity.

An additional form of non-pathogenic NK cell memory has been described in the setting of homeostatic proliferation. Common gamma family cytokines, such as IL-2, IL-7, and IL-15, are thought to be the main driving force behind the homeostatic proliferation exhibited by mature NK cells and, as expected, mice lacking

the common gamma chain receptor (*Il2rγ*$^{-/-}$) are deficient in NK cells. However, wild-type NK cells will undergo rapid proliferation when adoptively transferred into lymphopenic *Rag2 x Il2rγ*$^{-/-}$ mice (Jamieson et al. 2004; Prlic et al. 2003; Ranson et al. 2003). Like antigen-specific memory NK cells, these homeostatically expanded NK cells demonstrate memory-like properties such as long term persistence in both lymphoid and nonlymphoid organs and enhanced function that is sustained for weeks after transfer (Keppel et al. 2013; Sun et al. 2011).

Zbtb32 and miR155 Link Inflammation to Antigen-Driven Clonal Expansion in NK cells

In addition to the targeted studies focused on cytokine activation, recent genome-wide expression studies have also led to the discovery of several novel pathways involved in regulating antigen-specific effector and memory NK cell responses. For example, microarray analyses revealed the unexpected finding that MCMV infection induces high expression of the transcription factor, Zbtb32, in Ly49H^{+} NK cells (Bezman et al. 2012). Zbtb32 is part of a larger protein family, known as BTB-ZF transcription factors, many of which function as critical regulators of lineage commitment, development, and effector function in other lymphocytes. Among these are ThPOK, which directs CD4^{+} T cell lineage commitment (He et al. 2005; Muroi et al. 2008; Sun et al. 2005); PLZF, which regulates the development and function of NKT and γδ T cells (Alonzo et al. 2010; Kovalovsky et al. 2008; Kreslavsky et al. 2009; Savage et al. 2008); and Bcl-6, which is required for germinal center B-cell and follicular T helper cell formation (Dent et al. 1997; Johnston et al. 2009; Nurieva et al. 2009; Ye et al. 1997; Yu et al. 2009). Zbtb32 itself had previously been shown to regulate Th2 cytokine production (e.g. IL-4, IL-5, and IL-13) by acting as an antagonist of Gata-3 in T cells, both during in vitro activation and in the context of mouse models of allergic hypersensitivity in vivo (Hirahara et al. 2008; Hirasaki et al. 2011; Miaw et al. 2000; Omori et al. 2003).

Using Zbtb32-deficient mice, Beaulieu et al. demonstrated that Zbtb32 was essential for NK cell-mediated antiviral immunity (Beaulieu et al. 2014). Zbtb32-deficient NK cells failed to protect against lethal doses of MCMV and VSV, due to their inability to undergo a proliferative burst and clonally expand in response to infection. Using in vitro cytokine stimulation assays and viral infection of cytokine receptor-deficient mice, Zbtb32 was shown to be induced downstream of pro-inflammatory cytokine signals, including IL-12, IL-18, and type I interferons, where it functioned as the molecular 'hub' through which inflammation was translated into a pro-proliferative cue in activated NK cells (Fig. 7.2). Mechanistically, Zbtb32 was necessary for NK cell proliferation because of its role in antagonizing the tumor suppressor factor, Blimp-1, revealing a previously uncharacterized antagonistic interaction between Zbtb32 and Blimp-1 in lymphocytes (Beaulieu et al. 2014).

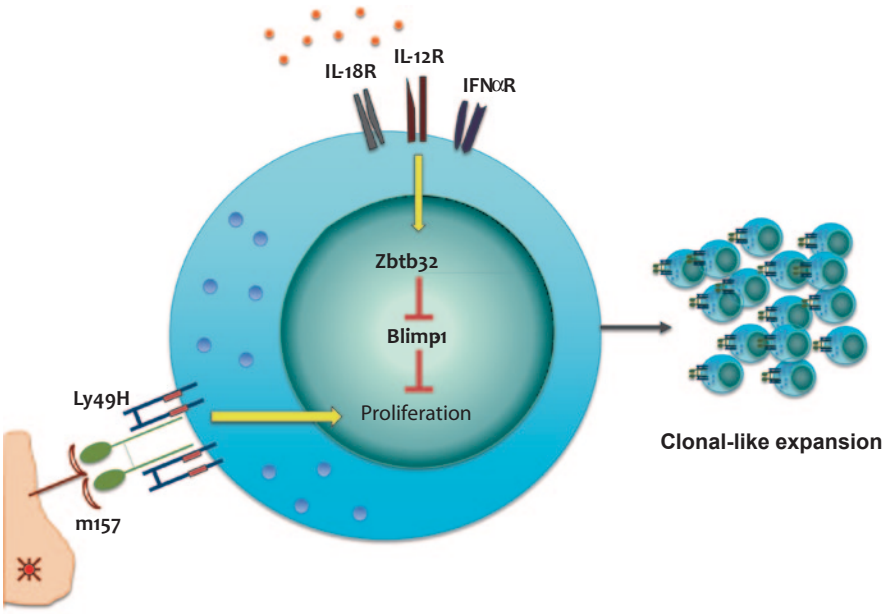

Fig. 7.2 Zbtb32 regulates NK cells proliferation during viral infection. Zbtb32 acts downstream of pro-inflammatory cytokine receptors (e.g. the IL-12R, IFNαR, and IL-18R) to suppress Blimp-1 and thereby promote the "clonal burst" of Ly49H$^+$ NK cells responding to MCMV infection

In addition to Zbtb32, recent studies have revealed other novel molecular pathways involved in regulating memory responses by antigen-specific NK cells. For example, the small regulatory microRNA, miR-155, was shown to be required for memory NK cell responses. Using miR-155-deficient mice, Zawislak et al. showed that NK cells were severely impaired in their ability to expand and establish a memory population during homeostatic proliferation or following MCMV infection (Zawislak et al. 2013). This defect was associated with loss of miR-155-mediated suppression of the pro-apoptotic factor Noxa and the inhibitor of STAT activation, SOCS1, as demonstrated by the finding that NK cells engineered to constitutively express Noxa or SOCS1 also exhibited profound expansion and memory cell defects in response to MCMV infection. Like Zbtb32, miR-155 expression was upregulated in activated NK cells downstream of signals from the pro-inflammatory cytokines IL-12 and IL-18, highlighting yet another pathway linking inflammation to NK cell effector and memory cell respones.

Beyond proinflammatory cytokine pathways, other signals are likely to regulate memory responses by NK cells. For example, using antibody blockade of the co-stimulatory receptor DNAM-1 and DNAM-1-deficient (*Cd226*$^{-/-}$) mice, Lanier and colleagues show that DNAM-1 is required in a cell-intrinsic manner for Ly49H$^+$ NK cells to expand and differentiate into memory cells following MCMV infection (Nabekura et al. 2014). Mechanistically, activation through DNAM-1 initiated signaling cascades involving both the Src-family tyrosine kinase Fyn and the serine-

threonine protein kinase C isoform eta (PKCη), and each of these pathways played important and distinct roles in the generation of MCMV-specific effector and memory NK cells. More recently, the pro-apoptotic molecule Bim was shown to be a regulator of the contraction of effector NK cells and the generation of memory NK cells following viral infection (Min-Oo et al. 2014). Lastly, we have observed that NK cells lacking the co-stimulatory receptor CD28 are also defective in generating effector and memory NK cells following adoptive transfer and MCMV infection (Karo and Sun, unpublished findings), suggesting multiple and non-redundant co-stimulatory pathways need to be operational for optimal NK cell responses.

NK Cells as Therapeutic Targets

The requirement for proinflammatory cytokine signaling in antigen-driven clonal expansion by NK cells is analogous to "Signal 3" in the priming of effector T cells, which need signals through the antigen receptor (i.e. the TCR; Signal 1), co-stimulatory receptors (Signal 2), and proinflammatory cytokine receptors (Signal 3) to achieve full activation potential (Fig. 7.3). As in T cells, the interdependency

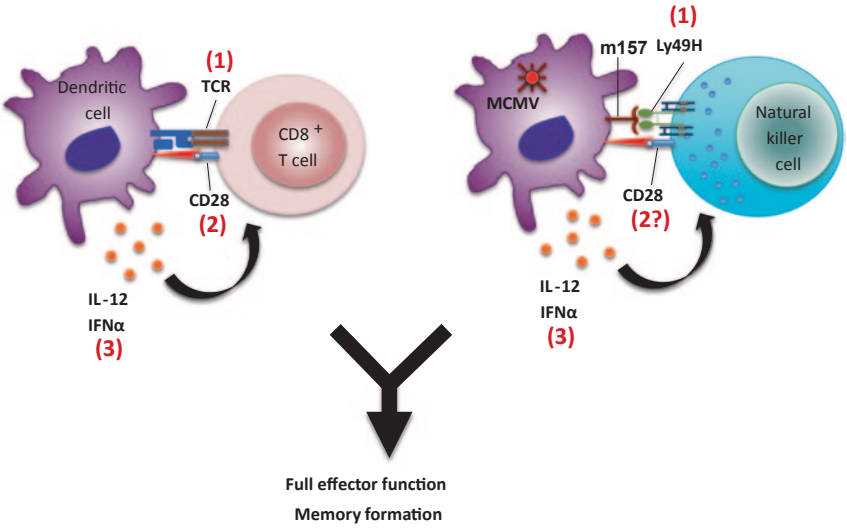

Fig. 7.3 Necessary signals for optimal anti-viral responses in NK and CD8 T cells. Viral infection activates natural killer cells (NK) and $CD8^+$ T cells, which mount a specific response following the triggering of antigen receptors. The full activation of $CD8^+$ T cells depends on three important signals: (1) antigen-mediated TCR engagement, (2) costimulation through costimulatory receptors such as CD28, and (3) exposure to pro-inflammatory cytokine such as IL-12 or type I interferons. Like $CD8^+$ T cells, NK cells also require multiple activation signals to achieve full effector and memory capacity, including antigen-mediated stimulation through the Ly49H receptor ("Signal 1") and stimulation via pro-inflammatory cytokines ("Signal 3"). The role of costimulation has not been explicitly evaluated

of proliferation and inflammation may function as a failsafe against unwanted effector NK cell responses that could contribute to pathological tissue damage and autoimmunity. Conversely, it may be possible to harness the critical and specific role of pro-inflammatory cytokines on NK cell proliferation and memory formation in therapies aimed at treating human disease. Indeed, these findings provide a compelling rationale for the use of pro-inflammatory cytokine pretreatment of NK cells in adoptive immunotherapies against tumors and infection. Cerwenka and colleagues highlighted the potential benefit of cytokine preactivation of NK cells using an established mouse tumor model. The authors found cytokine induced memory-like NK cells displayed sustained effector function and significantly reduced established tumor growth when combined with radiation therapy (Ni et al. 2012). This enhanced function of NK cells was dependent on $CD4^+$ T cell derived IL-2. Further work on the power of pro-inflammatory cytokines to shape the NK cell memory response will add exciting potential for future NK cell immunotherapy strategies.

References

Alonzo, E. S., Gottschalk, R. A., Das, J., Egawa, T., Hobbs, R. M., Pandolfi, P. P., Pereira, P., Nichols, K. E., Koretzky, G. A., Jordan, M. S., et al. (2010). Development of promyelocytic zinc finger and ThPOK-expressing innate gamma delta T cells is controlled by strength of TCR signaling and Id3. *Journal of Immunology, 184,* 1268–1279.

Andrews, D. M., Scalzo, A. A., Yokoyama, W. M., Smyth, M. J., & Degli-Esposti, M. A. (2003). Functional interactions between dendritic cells and NK cells during viral infection. *Nature Immunology, 4,* 175–181.

Arase, H., Mocarski, E. S., Campbell, A. E., Hill, A. B., & Lanier, L. L. (2002). Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. *Science, 296,* 1323–1326.

Beaulieu, A. M., Zawislak, C. L., Nakayama, T., & Sun, J. C. (2014). The transcription factor Zbtb32 controls the proliferative burst of virus-specific natural killer cells responding to infection. *Nature Immunology, 15,* 546–553.

Bezman, N. A., Kim, C. C., Sun, J. C., Min-Oo, G., Hendricks, D. W., Kamimura, Y., Best, J. A., Goldrath, A. W., & Lanier, L. L. (2012). Molecular definition of the identity and activation of natural killer cells. *Nature Immunology, 13,* 1000–1009.

Bjorkstrom, N. K., Lindgren, T., Stoltz, M., Fauriat, C., Braun, M., Evander, M., Michaelsson, J., Malmberg, K. J., Klingstrom, J., Ahlm, C., et al. (2011). Rapid expansion and long-term persistence of elevated NK cell numbers in humans infected with hantavirus. *The Journal of Experimental Medicine, 208,* 13–21.

Cooper, M. A., Elliott, J. M., Keyel, P. A., Yang, L., Carrero, J. A., & Yokoyama, W. M. (2009). Cytokine-induced memory-like natural killer cells. *Proceedings of the National Academy of Sciences of the United States of America, 106,* 1915–1919.

Della Chiesa, M., Falco, M., Podesta, M., Locatelli, F., Moretta, L., Frassoni, F., & Moretta, A. (2012). Phenotypic and functional heterogeneity of human NK cells developing after umbilical cord blood transplantation: A role for human cytomegalovirus? *Blood, 119,* 399–410.

Dent, A. L., Shaffer, A. L., Yu, X., Allman, D., & Staudt, L. M. (1997). Control of inflammation, cytokine expression, and germinal center formation by BCL-6. *Science, 276,* 589–592.

Foley, B., Cooley, S., Verneris, M. R., Curtsinger, J., Luo, X., Waller, E. K., Anasetti, C., Weisdorf, D., & Miller, J. S. (2012a). Human cytomegalovirus (CMV)-induced memory-like NKG2C(+) NK cells are transplantable and expand in vivo in response to recipient CMV antigen. *Journal of Immunology, 189,* 5082–5088.

Foley, B., Cooley, S., Verneris, M. R., Pitt, M., Curtsinger, J., Luo, X., Lopez-Verges, S., Lanier, L. L., Weisdorf, D., & Miller, J. S. (2012b). Cytomegalovirus reactivation after allogeneic transplantation promotes a lasting increase in educated NKG2C + natural killer cells with potent function. *Blood, 119,* 2665–2674.

Guma, M., Budt, M., Saez, A., Brckalo, T., Hengel, H., Angulo, A., & Lopez-Botet, M. (2006). Expansion of CD94/NKG2C^{+} NK cells in response to human cytomegalovirus-infected fibroblasts. *Blood, 107,* 3624–3631.

He, X., He, X., Dave, V. P., Zhang, Y., Hua, X., Nicolas, E., Xu, W., Roe, B. A., & Kappes, D. J. (2005). The zinc finger transcription factor Th-POK regulates CD4 versus CD8 T-cell lineage commitment. *Nature, 433,* 826–833.

Hirahara, K., Yamashita, M., Iwamura, C., Shinoda, K., Hasegawa, A., Yoshizawa, H., Koseki, H., Gejyo, F., & Nakayama, T. (2008). Repressor of GATA regulates TH2-driven allergic airway inflammation and airway hyperresponsiveness. *The Journal of allergy and clinical immunology, 122,* 512–520, e511.

Hirasaki, Y., Iwamura, C., Yamashita, M., Ito, T., Kitajima, M., Shinoda, K., Namiki, T., Terasawa, K., & Nakayama, T. (2011). Repressor of GATA negatively regulates murine contact hypersensitivity through the inhibition of type-2 allergic responses. *Clinical Immunology, 139,* 267–276.

Jamieson, A. M., Isnard, P., Dorfman, J. R., Coles, M. C., & Raulet, D. H. (2004). Turnover and proliferation of NK cells in steady state and lymphopenic conditions. *Journal of Immunology, 172,* 864–870.

Johnston, R. J., Poholek, A. C., DiToro, D., Yusuf, I., Eto, D., Barnett, B., Dent, A. L., Craft, J., & Crotty, S. (2009). Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science, 325,* 1006–1010.

Keppel, M. P., Yang, L., & Cooper, M. A. (2013). Murine NK cell intrinsic cytokine-induced memory-like responses are maintained following homeostatic proliferation. *Journal of Immunology, 190,* 4754–4762.

Kovalovsky, D., Uche, O. U., Eladad, S., Hobbs, R. M., Yi, W., Alonzo, E., Chua, K., Eidson, M., Kim, H. J., Im, J. S., et al. (2008). The BTB-zinc finger transcriptional regulator PLZF controls the development of invariant natural killer T cell effector functions. *Nature Immunology, 9,* 1055–1064.

Kreslavsky, T., Savage, A. K., Hobbs, R., Gounari, F., Bronson, R., Pereira, P., Pandolfi, P. P., Bendelac, A., & von Boehmer, H. (2009). TCR-inducible PLZF transcription factor required for innate phenotype of a subset of gammadelta T cells with restricted TCR diversity. *Proceedings of the National Academy of Sciences of the United States of America, 106,* 12453–12458.

Lopez-Verges, S., Milush, J. M., Schwartz, B. S., Pando, M. J., Jarjoura, J., York, V. A., Houchins, J. P., Miller, S., Kang, S. M., Norris, P. J., et al. (2011). Expansion of a unique CD57(+)NKG-2Chi natural killer cell subset during acute human cytomegalovirus infection. *Proceedings of the National Academy of Sciences of the United States of America, 108,* 14725–14732.

Madera S., & Sun J. C. (2015) Cutting edge: Stage-specific requirement for IL-18 for antiviral NK expansion. *Journal of immunology, 194,* 1408–1412.

Miaw, S. C., Choi, A., Yu, E., Kishikawa, H., & Ho, I. C. (2000). ROG, repressor of GATA, regulates the expression of cytokine genes. *Immunity, 12,* 323–333.

Min-Oo, G., Bezman, N. A., Madera, S., Sun, J. C., & Lanier, L. L. (2014). Proapoptotic Bim regulates antigen-specific NK cell contraction and the generation of the memory NK cell pool after cytomegalovirus infection. *The Journal of Experimental Medicine, 211,* 1289–1296.

Muroi, S., Naoe, Y., Miyamoto, C., Akiyama, K., Ikawa, T., Masuda, K., Kawamoto, H., & Taniuchi, I. (2008). Cascading suppression of transcriptional silencers by ThPOK seals helper T cell fate. *Nature Immunology, 9,* 1113–1121.

Nabekura, T., Kanaya, M., Shibuya, A., Fu, G., Gascoigne, N. R., & Lanier, L. L. (2014). Costimulatory molecule DNAM-1 Is essential for optimal differentiation of memory natural killer cells during mouse cytomegalovirus infection. *Immunity, 40,* 225–234.

Nguyen, K. B., Salazar-Mather, T. P., Dalod, M. Y., Van Deusen, J. B., Wei, X. Q., Liew, F. Y., Caligiuri, M. A., Durbin, J. E., & Biron, C. A. (2002). Coordinated and distinct roles for IFN-

alpha beta, IL-12, and IL-15 regulation of NK cell responses to viral infection. *Journal of immunology, 169,* 4279–4287.

Ni, J., Miller, M., Stojanovic, A., Garbi, N., & Cerwenka, A. (2012). Sustained effector function of IL-12/15/18-preactivated NK cells against established tumors. *The Journal of Experimental Medicine, 209,* 2351–2365.

Nurieva, R. I., Chung, Y., Martinez, G. J., Yang, X. O., Tanaka, S., Matskevitch, T. D., Wang, Y. H., & Dong, C. (2009). Bcl6 mediates the development of T follicular helper cells. *Science, 325,* 1001–1005.

O'Leary, J. G., Goodarzi, M., Drayton, D. L., & von Andrian, U. H. (2006). T cell- and B cell-independent adaptive immunity mediated by natural killer cells. *Nature Immunology, 7,* 507–516.

Omori, M., Yamashita, M., Inami, M., Ukai-Tadenuma, M., Kimura, M., Nigo, Y., Hosokawa, H., Hasegawa, A., Taniguchi, M., & Nakayama, T. (2003). CD8 T cell-specific downregulation of histone hyperacetylation and gene activation of the IL-4 gene locus by ROG, repressor of GATA. *Immunity, 19,* 281–294.

Orange, J. S., & Biron, C. A. (1996a). An absolute and restricted requirement for IL-12 in natural killer cell IFN-gamma production and antiviral defense. Studies of natural killer and T cell responses in contrasting viral infections. *Journal of Immunology, 156,* 1138–1142.

Orange, J. S., & Biron, C. A. (1996b). Characterization of early IL-12, IFN-alphabeta, and TNF effects on antiviral state and NK cell responses during murine cytomegalovirus infection. *Journal of Immunology, 156,* 4746–4756.

Paust, S., Gill, H. S., Wang, B. Z., Flynn, M. P., Moseman, E. A., Senman, B., Szczepanik, M., Telenti, A., Askenase, P. W., Compans, R. W., et al. (2010). Critical role for the chemokine receptor CXCR6 in NK cell-mediated antigen-specific memory of haptens and viruses. *Nature Immunology, 11,* 1127–1135.

Pien, G. C., & Biron, C. A. (2000). Compartmental differences in NK cell responsiveness to IL-12 during lymphocytic choriomeningitis virus infection. *Journal of Immunology, 164,* 994–1001.

Prlic, M., Blazar, B. R., Farrar, M. A., & Jameson, S. C. (2003). In vivo survival and homeostatic proliferation of natural killer cells. *The Journal of Experimental Medicine, 197,* 967–976.

Ranson, T., Vosshenrich, C. A., Corcuff, E., Richard, O., Muller, W., & Di Santo, J. P. (2003). IL-15 is an essential mediator of peripheral NK-cell homeostasis. *Blood, 101,* 4887–4893.

Romee, R., Schneider, S. E., Leong, J. W., Chase, J. M., Keppel, C. R., Sullivan, R. P., Cooper, M. A., & Fehniger, T. A. (2012). Cytokine activation induces human memory-like NK cells. *Blood, 120,* 4751–4760.

Savage, A. K., Constantinides, M. G., Han, J., Picard, D., Martin, E., Li, B., Lantz, O., & Bendelac, A. (2008). The transcription factor PLZF directs the effector program of the NKT cell lineage. *Immunity, 29,* 391–403.

Smith, H. R., Heusel, J. W., Mehta, I. K., Kim, S., Dorner, B. G., Naidenko, O. V., Iizuka, K., Furukawa, H., Beckman, D. L., Pingel, J. T., et al. (2002). Recognition of a virus-encoded ligand by a natural killer cell activation receptor. *Proceedings of the National Academy of Sciences of the United States of America, 99,* 8826–8831.

Sun, J. C., & Lanier, L. L. (2011). NK cell development, homeostasis and function: Parallels with CD8(+) T cells. *Nature Reviews Immunology, 11,* 645–657.

Sun, G., Liu, X., Mercado, P., Jenkinson, S. R., Kypriotou, M., Feigenbaum, L., Galera, P., & Bosselut, R. (2005). The zinc finger protein cKrox directs CD4 lineage differentiation during intrathymic T cell positive selection. *Nature Immunology, 6,* 373–381.

Sun, J. C., Beilke, J. N., & Lanier, L. L. (2009). Adaptive immune features of natural killer cells. *Nature, 457,* 557–561.

Sun, J. C., Beilke, J. N., Bezman, N. A., & Lanier, L. L. (2011). Homeostatic proliferation generates long-lived natural killer cells that respond against viral infection. *The Journal of Experimental Medicine, 208,* 357–368.

Sun, J. C., Madera, S., Bezman, N. A., Beilke, J. N., Kaplan, M. H., & Lanier, L. L. (2012). Proinflammatory cytokine signaling required for the generation of natural killer cell memory. *The Journal of Experimental Medicine, 209,* 947–954.

Ye, B. H., Cattoretti, G., Shen, Q., Zhang, J., Hawe, N., de Waard, R., Leung, C., Nouri-Shirazi, M., Orazi, A., Chaganti, R. S., et al. (1997). The BCL-6 proto-oncogene controls germinal-centre formation and Th2-type inflammation. *Nature Genetics, 16,* 161–170.

Yu, D., Rao, S., Tsai, L. M., Lee, S. K., He, Y., Sutcliffe, E. L., Srivastava, M., Linterman, M., Zheng, L., Simpson, N., et al. (2009). The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity, 31,* 457–468.

Zawislak, C. L., Beaulieu, A. M., Loeb, G. B., Karo, J., Canner, D., Bezman, N. A., Lanier, L. L., Rudensky, A. Y., & Sun, J. C. (2013). Stage-specific regulation of natural killer cell homeostasis and response against viral infection by microRNA-155. *Proceedings of the National Academy of Sciences of the United States of America, 110,* 6967–6972.

Chapter 8
Induction of Immune Tolerance to Dietary Antigens

Kwang Soon Kim and Charles D. Surh

Abstract The intestinal immune system is continuously exposed to massive amounts of diverse antigens derived from both food and intestinal microbes. Immunological tolerance to these enteric antigens is critical for ensuring intestinal and systemic immune homeostasis. Oral tolerance is a specific type of peripheral tolerance induced by the exposure of antigen via the oral route, emphasizing the role of intestinal immune system for preventing unnecessary hypersensitivity reactions to innocuous dietary and microbial antigens. Here, we discuss how dietary antigens are recognized by intestinal immune systems and highlight the role of $Foxp3^+$ regulatory $CD4^+$ T cells (Tregs) in establishment of oral tolerance, the tolerogenic features of intestinal dendritic cells that induce development of $Foxp3^+$ Tregs, and the factors that promote development of the intestinal dendritic cells.

Keywords Intestinal immune system · Gut-associated lymphoid tissues (GALT) · Dietary antigens · $Foxp3^+$ regulatory $CD4^+$ T cells · Intestinal dendritic cells

Introduction

The main physiological task of the immune system is to protect the host by distinguishing self from non-self. The generation of an enormous diversity of antigen-reactive receptors during lymphocyte development enables mature T and B

C. D. Surh (✉) · K. S. Kim
Academy of Immunology and Microbiology (AIM), Institute for Basic Science (IBS), San 31, Hyoja-Dong, Nam-Gu, Pohang 790-784, Republic of Korea
e-mail: csurh@ibs.re.kr

Department of Integrative Biosciences and Biotechnology, Pohang University of Science and Technology (POSTECH), Pohang, Republic of Korea

C. D. Surh
Division of Developmental Immunology, La Jolla institute for Allergy and Immunology, La Jolla, CA, USA

S. P. Schoenberger et al. (eds.), *Crossroads Between Innate and Adaptive Immunity V,* Advances in Experimental Medicine and Biology 850, DOI 10.1007/978-3-319-15774-0_8

cells to recognize a vast array of self and non-self antigens. The adaptive immune system needs to respond to harmful pathogenic microbes by eradicating the invading pathogens and establishing immunological memory to prevent re-infection by the same pathogens. Nonetheless, the immune system also needs to be regulated to ensure immunological tolerance against self-antigens and innocuous environmental antigens. While the process of negative selection during lymphocyte development can eliminate most self-reactive lymphocytes (defined as central tolerance), peripheral tolerance mechanisms are also required to prevent activation of autoreactive lymphocytes that have eluded negative selection (Schwartz 1989; Starr et al. 2003). In addition, a fraction of T cells that strongly recognizes self-antigens, including tissue-specific antigens, can circumvent death during development by differentiating into one of several populations of T cells with a specialized function, including regulatory $CD4^+$ T cells expressing the forkhead box P3 transcription factor (Foxp3) (Sakaguchi 2005). These thymus-derived $Foxp3^+$ $CD4^+$ T cells have the ability to regulate the activity of other T cells and play an essential role in maintaining peripheral tolerance by suppressing mature autoreactive T cells from responding to self-antigens (Sakaguchi 2005). However, such a mechanism may not be sufficient for establishing tolerance to innocuous foreign antigens exposed through the mucosal sites, such as gastrointestinal tract, skin or respiratory tract, since these foreign antigens are not encountered during lymphocyte development (Pabst and Mowat 2012). Hence, a parallel mechanism may have evolved for this purpose.

Oral tolerance is defined as a specific type of antigen-specific hypo-responsiveness that is induced after the immune system is stimulated by an antigen delivered orally, i.e., thru the gut mucosa and gut-associated lymphoid tissues (GALT). Hence, pre-exposure through the gastrointestinal tract often causes hypo-responsiveness to subsequent local or systemic challenge with the same antigen. Similar phenomenon applies for antigens delivered through the respiratory tract indicating that the immune system at mucosal surfaces is biased to display tolerance rather than immunity (Akbari et al. 2001; Tsitoura 1999). In this regard, mucosal, rather than oral, tolerance is a better terminology as it more appropriately reflects the features of mucosal immune system. However, oral tolerance was the first phenomenon to be described in this type of immune response (Chase 1946) and we will continue to use this term in this chapter to reiterate our focus on immune tolerance to dietary antigens. In this chapter, the recognition of dietary antigens by the intestinal immune system and the underlying mechanisms involved in establishing oral tolerance will be discussed with particular emphases on (1) the role of $Foxp3^+$ Tregs in establishment of oral tolerance, (2) the features of intestinal DCs promoting the generation of $Foxp3^+$ Tregs, and (3) the local environmental factors that condition intestinal DCs and $Foxp3^+$ Tregs to induce oral tolerance.

Recognition of Dietary Antigens by the Intestinal Immune System

Migration of CD103$^+$ DCs from the Small Intestine to Mesenteric Lymph Node is Crucial for Establishing Oral Tolerance

Oral tolerance is of crucial importance to the host because the GALT are chronically stimulated by considerable amounts of foreign antigens from the gut, which is colonized with vast numbers of commensal microbes, collectively referred to as intestinal microbiota, and continuously filled with large amounts of food. The human intestinal microbiota outnumbers host cells by ~10-folds, and more importantly, possesses ~100-fold higher genetic diversity than the host (Backhed et al. 2005). In addition, we typically ingest more than 100 g of dietary proteins every day (Weiner et al. 2011). Unlike the skin where most exogenous substances are efficiently prevented from entering the body, the gastrointestinal tract allows absorption of essential nutrients through a monolayer of columnar epithelium (Schulz and Pabst 2013). Massive and chronic antigenic challenges at the surface and through the intestinal epithelium are managed and attenuated by sophisticated systems that maintain the integrity of intestine and protect the host. These include physical and biochemical barriers inherent of the epithelium, such as tight junctions between intestinal epithelial cells and the layer of mucus that is charged with anti-microcidal peptides, which are secreted by some of the key cell types of the intestinal epithelium, the goblet and paneth cells, respectively.

More importantly, enormous amount of innate and adaptive immune cells (10^{12} lymphoid cells in a meter of human small intestine (Weiner et al. 2011)) are dispersed throughout the GALT, including intraepithelial lymphocytes (IELs) situated within the intestinal epithelium, lymphoid and myeloid lineage cells in the lamina propria (LP), isolated lymphoid follicles (ILFs), Peyer's patches (PPs) and the mesenteric lymph nodes (mLNs). The intestinal epithelium and the lamina propria are thought to be effector sites, possessing activated/memory phenotype lymphocytes and antibody producing plasma cells. ILFs, PPs and mLNs are organized lymphoid structures and inductive sites of immune responses, containing B cell follicles and T cell zones; naïve B and T cells can gain entry into these GALT through the high endothelial venules (HEV), similar to the lymphoid tissues in periphery, except that ILFs and PPs are devoid of afferent lymph vessels (Pabst and Mowat 2012).

The important task of intestinal immune system is to mount protective immune responses against opportunistic intestinal pathogenic microbes and toxins while maintaining tolerance to commensal bacteria and food antigens. Enteric antigens derived from food and commensal microbiota are not simply ignored. Rather, some of these antigens appear to be actively recognized by the intestinal immune systems, as evidenced by the fact that most of CD4$^+$ T lymphocytes, especially those in the LP, display an activated or memory phenotype, even in the absence of enteric pathogens (Peters et al. 1989; Schieferdecker et al. 1992; Josefowicz et al. 2012). Even in

the germ-free mice, most $CD4^+$ T cells in the small intestine LP display a memory phenotype, although less in the colonic LP compared with that from conventional mice (Josefowicz et al. 2012), suggesting that food is the primary source of antigens for activation of LP $CD4^+$ T cells in the small intestine.

As in other non-lymphoid tissues, antigen-presenting cells (APCs), especially macrophages (MØs) and dendritic cells (DCs), populate throughout the gastrointestinal tract to perceive, process and integrate environmental cues to regulate the intestinal immunological responses. Various subpopulations of MØs and CD11c expressing DCs of different origins and functions are distributed in both inductive and effector sites of the small intestine. Common DC precursors (CDPs) differentiate from MØ-DC progenitors (MDPs) to give rise to circulating DC precursors (pre-DCs). After migration into the intestine, pre-DCs further differentiate into $CD11c^+$ DCs expressing CD103, a receptor for epithelial E-cadherin, in a fms-like tyrosine kinase 3 (Flt3) ligand-driven pathway (Varol et al. 2009; Hashimoto et al. 2011). $CD11c^+$ $CD103^+$ DCs are the most abundant subsets of APCs residing in the small intestinal LP and can be further divided by CD11b expression. $CD103^+$ LP DCs can uptake luminal antigens and migrate into mLNs, where they stimulate naïve T and B cells to undergo activation and acquire gut-tropism (Schulz et al. 2009). In addition to DC, $Ly6c^{hi}$ monocytes originating directly from MDPs to give rise to $CD11c^+$ $CX3CR1^{hi}$ MØs and $CD11c^+$ $CX3CR1^{int}$ inflammatory DCs in a GM-CSF-dependent pathway (Varol et al. 2009; Rivollier et al. 2012). $CX3CR1^{hi}$ MØs are abundant in LP of the small intestine; these cells are not migratory to mLNs under normal physiological conditions, but can carry commensal bacteria to mLNs in the absence of Myd88 or under conditions of dysbiosis (Diehl et al. 2013). $CX3CR1^{int}$ inflammatory DCs are also known to develop in colon during colonic inflammation (Rivollier et al. 2012).

The importance of intestinal DCs for mediating oral tolerance was initially recognized from the finding that expansion of DCs induced by treatment with Flt3-ligand enhanced efficiency of oral tolerance (Viney et al. 1998). Subsequent studies ascribed $CD103^+$ DCs to the role of processing and presenting dietary antigens to T cells (Johansson-Lindbom et al. 2005; Schulz et al. 2009). Intestinal DCs constantly traffic into mLNs in a CCR7-dependent manner. Hence, in CCR7-deficient mice, $CD103^+$ LP DCs have an impaired ability to migrate into mLNs, leading to a reduction of $CD103^+$ DCs in mLNs and preventing induction of oral tolerance (Johansson-Lindbom et al. 2005; Jang et al. 2006; Worbs et al. 2006). Furthermore, the induction of oral tolerance is hampered by the surgical removal of mLNs (mesenteric lymphadenoctomy) (Worbs et al. 2006) or when development of mLNs is prevented by a genetic mutation (Spahn et al. 2001, 2002), suggesting that migration of $CD11c^+$ $CD103^+$ LP DCs into mLNs is critical for establishing oral tolerance. Intriguingly, DCs in the other inductive sites of GALT, such as PPs and ILFs, seem to play only a subordinate role in establishing oral tolerance. This is despite the presence of Microfold (M) cells, the enterocytes lacking microvilli in follicle-associated epithelium (FAE) of PPs, which transcytose luminal antigens to the DCs in PPs for sampling. Hence, oral tolerance can be induced in PP-null mice (Spahn et al. 2001, 2002) or by the injection of soluble proteins into ligated small

intestine loops made within the bowel segments free of PPs (Kraus et al. 2005). In addition, adoptive transfer of LP DCs, but not PP DCs, from ovalbumin (OVA) fed mice suppressed delayed type hypersensitivity (DTH) responses, indicative of their tolerogenic function (Chirdo et al. 2005). Interestingly, in mice intragastrically challenged with *Enterobacter cloacae*, *E. Cloacae* were detected in DCs of PPs as well as mLNs, but not in LP DCs (Macpherson and Uhr 2004). Thus, it is generally considered that PP DCs are important for the recognition of particulate antigens, such as intestinal bacteria and viruses delivered by M cells, rather than soluble dietary antigens.

Sampling of Dietary Antigens by Migratory CD103$^+$ DCs is Facilitated by CX3CR1^{hi} MØs, Intestinal Epithelial Cells and Goblet Cells

As mentioned above, migration of CD103$^+$ LP DCs to mLNs is critical for establishing oral tolerance. Although it was reported that some CD103$^+$CD11b$^+$ DCs could migrate near the basal membrane and capture luminal *Salmonella* by extending projections between the epithelial cells, this type of sampling by CD103$^+$ DCs is inefficient (Farache et al. 2013a; Chang et al. 2013). Hence, CD103$^+$ DCs require help provided by CX3CR1^{hi} MØs, intestinal epithelial cells (IECs) and goblet cells in order to efficiently induce oral tolerance (Fig. 8.1) (Farache et al. 2013b). For CX3CR1^{hi} MØs, these cells can extend protrusions into the intestinal lumen much more efficiently than CD103$^+$ DCs, as such extensions are largely dependent on CX3CR1 expression (Niess et al. 2005). Accordingly, CX3CR1^{hi} MØs can efficiently sample soluble antigens introduced into the intestine much better than CD103$^+$ DCs. This kind of division of labor between CD103$^+$ DCs and CX3CR1^{hi} MØs suggests that there might be an interaction between the two types of cells in order for non-migratory CX3CR1^{hi} MØs to hand off antigens to migratory CD103$^+$ DCs. Indeed, it was recently reported that CX3CR1^{hi} MØs can transfer peptide-MHC class II complex on their cell surface to CD103$^+$ DCs via a gap junction-mediated mechanism and the genetic deletion of a gap junction component, connexin-43, impairs induction of oral tolerance (Mazzini et al. 2014). Transfer of surface membrane proteins such as peptide-MHC class II complex in a contact dependent manner is called trogocytosis (Joly and Hudrisier 2003). Precedence for DCs "cross-dressed" with peptide-MHC I complex obtained from other cells via trogocytosis that can be presented to T cells has been previously reported (Wakim and Bevan 2011). This mode of gap junction-mediated transfer of antigens from CX3CR1^{hi} MØs to CD103$^+$ DCs is in part responsible for the impairment of oral tolerance in CX3CR1-deficient mice (Hadis et al. 2011).

Besides CX3CR1^{hi} MØs, IECs can also take up luminal soluble antigens (Farache et al. 2013a; Chang et al. 2013). Although more than 90 % of absorbed proteins are degraded, antigenic molecules can be released from the basolateral membrane of IECs as partially degraded antigenic peptides or exosomes with peptide-MHC class II complexes, which are subsequently taken up by CD103$^+$ DCs (Menard et al.

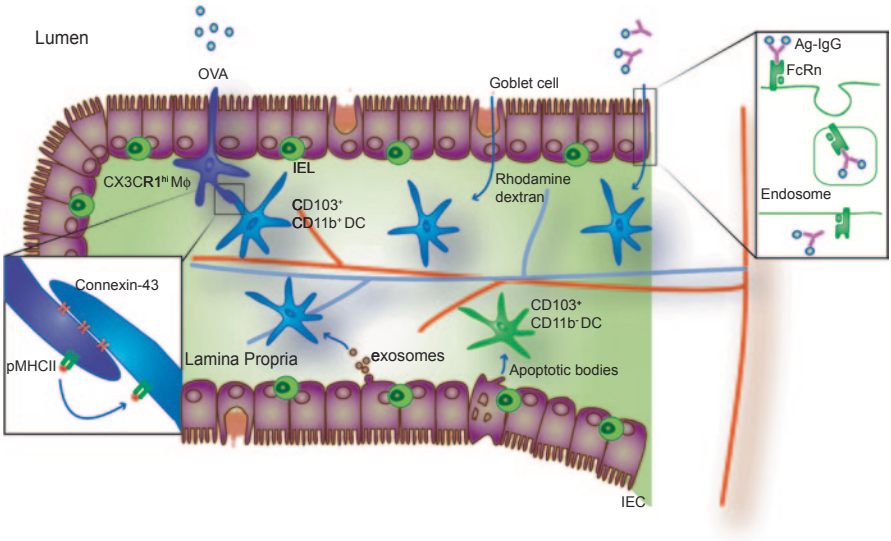

Fig. 8.1 Sampling of dietary antigens by migratory CD103$^+$ DCs. In order to efficiently induce oral tolerance, enteric antigen acquisition by the CD103$^+$ DCs in the lamina propria (*LP*), not in Peyer's patches, and their migration into the mesenteric lymph nodes (*mLNs*) are required. Sampling of the luminal soluble enteric antigens in the LP is efficiently acquired by CX3CR1^{hi} macrophages (*MØs*), intestinal epithelial cells (*IECs*) and goblet cells, but less efficiently by CD103$^+$ DCs. In this regard, several mechanisms orchestrated by CX3CR1^{hi} MØs, IEC and goblet cells are involved in delivering antigens to migratory CD103$^+$ LP DCs. I. CX3CR1^{hi} MØs, non-migratory under steady state conditions, can extend dendrites into the lumen, acquire antigens and then pass them in the form of peptide MHC class II complexes to migratory CD103$^+$ DCs via a gap junction-mediated mechanism. II. IECs can acquire luminal soluble antigens and release partially degraded antigenic peptides or exosomes containing peptide-MHC class II complexes into the basolateral membrane of IECs. III. Apoptotic bodies from IECs, containing dietary antigens, can be taken up by CD103$^+$CD11b$^-$ DCs expressing DNGR-1, which is required for uptake of apoptotic bodies. IV. Luminal antigens such as Rhodamine dextran can accumulate in goblet cells and can be subsequently taken up by CD103$^+$ DCs. V. Antigen-IgG complexes from breast milk in the lumen of neonatal mice can be efficiently taken up by IECs via neonatal Fc receptor (*FcRn*)

2010). Furthermore, apoptotic bodies from IECs can be endocytosed by migratory DCs, although exclusively by CD103$^+$ CD11b$^-$ DCs due to their expression of DNGR-1, which is required for uptake of apoptotic cells (Huang et al. 2000; Poulin et al. 2012; Cerovic et al. 2014). CD103$^+$ DCs can also probe goblet cells actively with their dendrites and capture soluble antigens from goblet cells (McDole et al. 2012). However, the precise role of IEC- and goblet cell-mediated sampling of soluble antigens by CD103$^+$ DCs in the context of oral tolerance remains to be elucidated. Interestingly, breast milk-mediated transfer of antigen-IgG complexes can potently induce oral tolerance in neonatal mice through the participation of neonatal Fc receptor (FcRn) (Mosconi et al. 2010). FcRn is expressed on the IECs and mediates the uptake of antigen-IgG complexes. Antigen-IgG complexes also protect antigens from catabolism within IECs, thus enhancing their presentation by CD103$^+$ DCs (Menard et al. 2010; Verhasselt 2010).

Mechanisms for Establishing Oral Tolerance

The initial proposed mechanisms of oral tolerance mirrored that suggested for peripheral tolerance, namely, clonal deletion or anergy of Ag-specific T cells, which can be induced by suboptimal antigenic stimulation, typically in the absence of costimulatory signals (Chen et al. 1995a; Gutgemann et al. 1998). However, these mechanisms could not explain the fact that oral tolerance can be transferred into naïve recipients by the adoptive transfer of immune cells or the T lymphocyte fraction from the tolerized mice (Mattingly and Waksman 1978; Richman et al. 1978). Experimental approaches aimed at studying the tolerogenic responses actively mediated by "suppressor cells" had eventually delineated another mechanism of oral tolerance. It was generally accepted that $CD4^+$ T cells play a central role in establishing oral tolerance. Hence, oral tolerance is abrogated when $CD4^+$ T cells are depleted in vivo (Garside et al. 1995; Barone et al. 1995), and more importantly, $CD4^+$ T cells from tolerized mice can transfer oral tolerance (Chen et al. 1994; Chen et al. 1995b). Coincidently, a subset of $CD4^+$ T cells constitutively expressing CD25 under steady state was found to confer regulatory functions (Sakaguchi et al. 1995). These regulatory T cells (Tregs) turned out to express X-linked transcription factor Foxp3, which is required for their development and to mediate their suppressive functions (Hori et al. 2003; Fontenot et al. 2003; Khattri et al. 2003). Null-mutation of Foxp3 gene causes the lymphoproliferative disease known as immune polyendocrinopathy X-linked (IPEX) syndrome in human and similar lymphoproliferative immune disorders in mice. Affected individuals develop various immune disorders, including type I diabetes, allergic skin disease and intestinal inflammation, suggesting that $Foxp3^+$ Tregs are critical for the maintenance of peripheral tolerance, including tolerance in the gastrointestinal tract (Bennett et al. 2001; Wildin et al. 2001; Brunkow et al. 2001). Other $CD4^+$ T cell subsets, such as Th3 and Tr1 secreting TGF-β and IL-10, respectively, were also demonstrated to have regulatory functions implicated in oral tolerance (Chen et al. 1994; Zhou et al. 2010). However, Th3 and Tr1 are not well characterized due to the absence of markers specifically associated with these cells. Their effector functions, such as the secretion of TGF-β and IL-10, respectively, also overlap with those of $Foxp3^+$ Tregs. Thus, we will primarily focus on the role of $Foxp3^+$ Tregs in establishing oral tolerance.

$Foxp3^+$ Regulatory $CD4^+$ T Cells Play a Decisive Role in Establishing Tolerance to Dietary Antigens

$Foxp3^+$ Tregs can develop from hematopoietic progenitors in the thymus through high-avidity interactions with the self peptide-MHC class II complexes (Sakaguchi 2005; Jordan et al. 2001). However, $Foxp3^+$ Tregs can also develop extrathymically in the peripheral lymphoid organs through the differentiation of conventional mature $CD4^+$ T cells under specific conditions (Curotto de Lafaille and Lafaille 2009). In this regard, $Foxp3^+$ Tregs can be divided into two types, thymically-derived Treg

(tTreg) and peripherally-derived Treg (pTreg) (Abbas et al. 2013), with pTregs being more important for oral tolerance than tTregs. The initial suggestion on the role of Foxp3$^+$ Tregs in oral tolerance was the finding that feeding ovalbumin (ova) to ova-specific TCR transgenic mice (DO11.10) led to expansion of CD4$^+$ CD25$^+$ T cells, which can suppress immune responses to ova upon adoptive transfer into naïve secondary hosts (Zhang et al. 2001). More specifically, Lafaille group showed that oral administration of ova to DO11.10 mice in a RAG knockout background, which unlike in a RAG$^+$ background are deficient in Foxp3$^+$ Tregs, induces de novo generation of Foxp3$^+$ DO11.10 pTregs and adoptive transfer of these cells suppressed IgE responses in naïve recipient mice (Mucida et al. 2005; Curotto de Lafaille et al. 2008). Further support came from the finding that depletion of Foxp3$^+$ CD4$^+$ T cells by injections of diphtheria toxin into DEREG mice, which express human diphtheria toxin receptor under control of Foxp3 promoter, abrogates the ability of oral tolerance to be induced in these mice (Hadis et al. 2011). Moreover, pTregs were found to have nonredundant functions and are important for the local tolerance at mucosal tissues. Relevantly, Haribhai and colleagues showed that both tTregs and pTregs were shown to be necessary to prevent mortality and morbidity in Foxp3-null mice. Hence, therapeutic adoptive transfer of tTregs could protect Foxp3-null mice from lethality, but not from excessive inflammation (Haribhai et al. 2011). However, adoptive transfer of mixed populations of tTregs and pTregs (or conventional CD4$^+$ T cells with a capacity to generate pTregs in vivo) could rescue Foxp3-null mice completely and reduced the inflammation at mucosal surfaces. Similar findings also applied to the model of colitis induced by adoptive transfer of naïve CD4$^+$ T cells into T cell-depleted immunodeficient mice (Haribhai et al. 2009).

The underlying mechanisms behind the unique function for pTregs remain largely elusive. While Foxp3$^+$ Tregs display unique transcriptional signatures in comparison to conventional CD4$^+$ T cells (Sugimoto et al. 2006; Hill et al. 2007), the gene expression profiling of in vivo generated tTregs and pTregs (and/or total Foxp3$^+$ Tregs) showed a high level of similarity (Haribhai et al. 2011). These efforts did not lead to discovery of genes exclusively expressed by pTregs that confer their unique functions, but nonetheless revealed a way to distinguish pTregs from tTregs, especially at the mucosal site under steady state conditions. Initially, Helios, a member of Ikaros transcription factor family, was proposed as a marker of tTregs (Thornton et al. 2010). However, subsequent studies showed that pTregs could express Helios upon repeated T cell stimulation (Gottschalk et al. 2012). More recently, Lafaille and Bluestone groups independently showed that tTregs, but not pTregs, express Neuropilin-1 (Nrp1) on their cell surface, especially for Foxp3$^+$ Tregs found in secondary lymphoid tissues under normal physiological conditions (Weiss et al. 2012; Yadav et al. 2012). Accordingly, Nrp1^{lo} pTregs are more abundantly present in the LP of both small intestine and colon than in secondary lymphoid organs. Interestingly, colonic Nrp1^{lo} pTregs are dramatically depleted in germ-free mice, indicating that generation of colonic pTregs is induced by intestinal microbiota (Weiss et al. 2012). Rudensky group also showed that Foxp3$^+$ Tregs at mucosal sites are reduced more significantly in mice with a deficiency in CNS1 of Foxp3 locus, which precludes binding to the Foxp3 gene locus the transcription

factors (NFAT, Smad3 and RAR/RXR) necessary for pTreg generation (Zheng et al. 2010; Josefowicz et al. 2012). Accordingly, $Nrp1^{lo}$ pTregs are reduced in various tissues of $CNS1^{-/-}$ mice with a prominent decrease at the mucosal sites (Weiss et al. 2012). However, a considerable percentage of $Nrp1^{lo}$ pTregs was still detectable at mucosal sites of $CNS1^{-/-}$ mice (Weiss et al. 2012) and the percentage of colonic $Foxp3^+$ Tregs in $CNS1^{-/-}$ mice were also decreased upon the treatment of antibiotics which can mimic the conditions of GF mice (Josefowicz et al. 2012). Further studies are necessary to resolve the issue of why $CNS1^{-/-}$ mice still possess $Nrp1^{lo}$ cells. Another issue raised from these studies is that most transcription profiling was done by using $Foxp3^+$ Tregs in secondary lymphoid tissues but not those in mucosal sites. It is possible that gene expression patterns of pTregs in secondary lymphoid tissues might not be identical to that of intestinal pTregs due to different state of activation or differential influence from local tissue microenvironments.

Studies have shown that the TCR repertoire of pTregs is largely non-overlapping to that of tTregs (Haribhai et al. 2011). This is expected since pTregs are derived from conventional T cells and their TCR repertoire bears little resemblance to that of tTregs (Hsieh et al. 2006; Pacholczyk et al. 2006). In this respect, despite their remarkable similarity in gene expression profiles to tTregs, pTregs are still different from tTregs by showing an ability to occupy unique niches at the mucosal tissues. For instance, although it is expected that the small intestinal pTregs are generated in response to dietary antigens, this notion is yet to be directly demonstrated. One way to accomplish this will be by examining for the expression of Nrp1 on Tregs from mice raised only with protein-free or chemically defined elemental diet deficient of antigenic macromolecules. Indeed, we have recently observed that $Nrp1^{lo}$ pTregs are severely depleted when conventional and germ-free mice that were raised solely on an elemental diet (Kim and Surh, unpublished observation).

$Foxp3^+$ Tregs utilize a diverse set of mechanisms for maintaining peripheral tolerance (Vignali et al. 2008). $Foxp3^+$ Tregs can secrete inhibitory cytokines such as TGF-β, IL-10 and IL-35, express granzymes to induce direct cytolysis of effector T cells, deplete local IL-2 to inhibit effector T cell proliferation, and/or modulate DC maturation or function. Multiple suppressive mechanisms enable $Foxp3^+$ Tregs to restrain diverse types of immune responses mediated by both innate and adaptive immune cells. However, it is doubtful that all of these suppressive mechanisms are necessary for $Foxp3^+$ Tregs to mediate their suppressive functions at different conditions. Rather, it is more likely that depending on the environmental context, $Foxp3^+$ Tregs tailor their suppressive mechanisms (Chaudhry and Rudensky 2013). For example, $Foxp3^+$ Treg-specific ablation of IL-10 gene results in increased inflammation at environmental surfaces of mucosal tissues, but not systemically (Rubtsov et al. 2008). Consistent with the idea that $Foxp3^+$ Tregs customize their responses according to the inflammatory conditions, it has been shown that the capacity of $Foxp3^+$ Tregs to control distinct types of T helper cell responses requires the acquisition of transcription factors expressed by the effector T helper cells (Chaudhry and Rudensky 2013; Cretney et al. 2013). Hence, T-bet sufficient $Foxp3^+$ Tregs is superior to T-bet deficient ones in suppressing Th1 responses in vitro and in vivo (Koch et al. 2009). IRF4 or GATA3 deficiency specifically in $Foxp3^+$ Tregs causes Th2

mediated inflammation (Zheng et al. 2009; Wohlfert et al. 2011; Rudra et al. 2012) and Foxp3$^+$ Tregs control Th17 responses in a STAT3 dependent manner (Chaudhry et al. 2009). In addition, Foxp3 can interact with various transcription factors as envisaged by the Foxp3 interactome (Rudra et al. 2012). The expression of effector CD4$^+$ helper T cell-specific transcription factors not only contribute directly to suppressive functions of Foxp3$^+$ Tregs, but also influences their functions in vivo by affecting their homing to inflammatory sites (Cretney et al. 2013). However, it is not yet clear whether pTregs at mucosal sites need to specifically express a specific transcription factor or a gene to establish tolerance to dietary antigens.

As an integral part of maintaining intestinal immune homeostasis, pTregs in gastrointestinal tract establish and maintain oral tolerance. As aforementioned, the migration of CD103$^+$ DCs harboring enteric antigens into mLNs is a critical step for establishing oral tolerance. It was generally believed that pTregs are generated in mLNs and then they migrate into the intestine or disperse systemically to suppress immune responses the gut or systemically, respectively. However, the idea that pTregs can migrate systemically from the mLNs was challenged by the finding that both the oral and the ensuing systemic tolerance could not be established in mice deficient of CCR9 and α4β7 integrin, which are necessary for gut homing of effector T cells and Foxp3$^+$ Tregs (Hadis et al. 2011; Cassani et al. 2011). These results suggest that establishment of oral tolerance is a step-wise process: induction of pTregs in mLNs by migratory CD103$^+$ DCs, migration of pTregs into the small intestine for their conditioning and expansion, and dispersion of pTregs systemically throughout the body (Fig. 8.2).

Induction of pTregs with their Gut Tropism in mLNs by CD103$^+$ DCs Requires Transforming Growth Factor-β and Retinoic Acid

Two critical steps for establishing oral tolerance, the induction of pTregs in mLNs and imprinting these T cells for gut tropism, are both mediated by CD103$^+$ DCs that migrate from LP of the small intestine to mLNs. The ability of CD103$^+$ mLN DCs as well as CD103$^+$ LP DCs to induce pTreg generation is dependent on TGF-β, an important cytokine for the conversion of conventional naïve CD4$^+$ T cells into Foxp3$^+$ Tregs (Chen et al. 2003; Fantini et al. 2004). It was shown that TGF-β produced by CD103$^+$ DCs is sufficient to promote conversion of naïve CD4$^+$ T cells into Foxp3$^+$ Tregs in vitro. CD103$^+$ mLN DCs also express a higher level of latent associated binding protein 3 (LTBP3) and tissue plasminogen activator (tPA), important for secretion and activation of latent TGF-β, respectively, than CD103$^-$ DCs in mLN (Coombes et al. 2007). Furthermore, intestine-derived CD103$^+$ DCs, but not CD103$^-$ mLN DCs or splenic DCs, also specifically express integrin αvβ8 (Paidassi et al. 2011; Worthington et al. 2011), which have been shown to activate latent TGF-β. While other agents that can activate latent TGF-β exist, such as tPA, protease plasmin, thrombosponin-1, MMP2 and MMP9, αvβ8 appears essential for

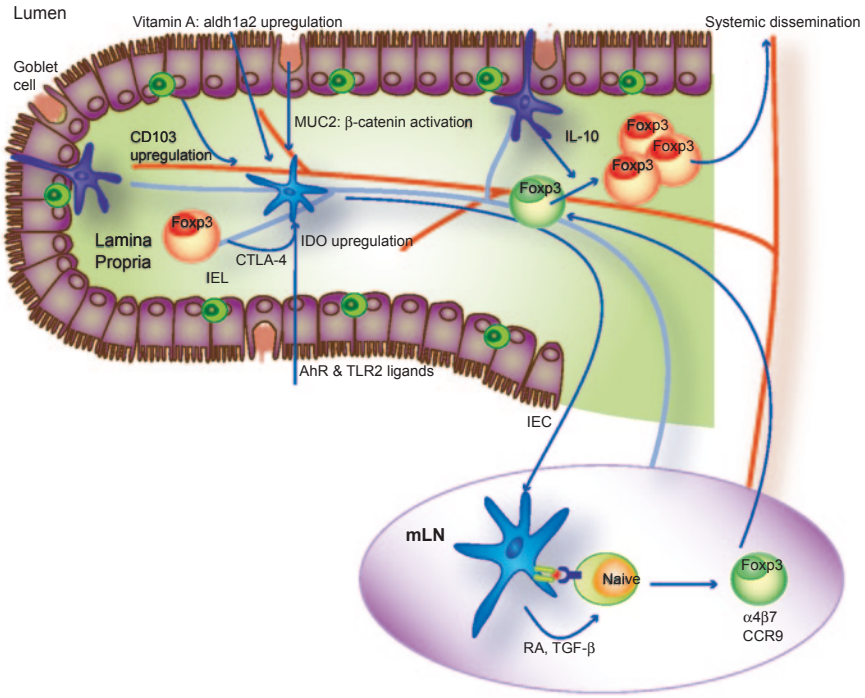

Fig. 8.2 Mechanisms for establishing oral tolerance Oral tolerance is a step-wise process initiated by the induction of peripheral Foxp3$^+$ Tregs (pTregs) in the mLNs by CD103$^+$ DCs that have migrated from the small intestine. Homing of the pTregs specific to dietary antigens from mLNs to the small intestine LP followed by their further conditioning and/or expansion at this site is also critical to establish intestinal and systemic tolerance to the dietary antigens. Intestinal CD103$^+$ DCs constitutively traffic into mLNs with luminal soluble enteric antigens and produce TGF-β and retinoic acids (*RA*) to preferentially induce the generation of pTregs. RA acts as a co-factor for TGF-β induced Foxp3$^+$ Treg conversion and also induces the expression of chemokine receptors such as α4β7 and CCR9 on effector CD4$^+$ T cells. Expansion and acquisition of suppressive effector function of gut-tropic pTregs in the small intestine is induced by IL-10 and other mediators produced by CX3CR1^{hi} MØs and DCs. The intestinal environment induces DCs to become more tolerogenic than inflammatory through several mechanisms involving signals from the diet, microbial components, and other immune and non-immune cells

generation of pTregs (Annes et al. 2003). Hence, CD11$^+$ cell-specific ablation of β8 integrin (Travis et al. 2007) or the deficiency of αv integrin specifically in epithelial cells and hematopoietic cells (αv-tie2 mice) (Lacy-Hulbert et al. 2007) causes autoimmunity and colitis. In contrast, mice lacking other agents did not show inflammation in the gut (Carmeliet et al. 1994; Crawford et al. 1998; Parks and Shapiro 2001). Moreover, CD103$^+$ mLN DCs from αv-tie2 mice, with a deletion of αv gene in endothelial and hematopoietic cells, are not able to induce Foxp3$^+$ Treg generation under known in vitro conditions in the presence of latent TGF-β or even under known in vivo conditions for ova-specific CD4$^+$ T cells upon oral administration of ova (Paidassi et al. 2011).

Although the addition of active form of TGF-β in culture containing CD013$^+$ DCs can further augment Foxp3$^+$ Treg conversion in vitro, this does not apply for CD103$^-$ DCs in mLNs, even when treated with a higher concentration of TGF-β(Coombes et al. 2007). CD103$^+$ DCs, but not CD103$^-$ DCs, in mLNs can metabolize vitamin A (retinol) from diet into retinoic acid (RA) by expressing retinaldehyde dehydrogenase 2 (RALDH2) encoded by *aldh1a2*. Indeed, CD103$^-$ DCs in mLNs when exposed to RA in the presence of exogenous TGF-β could promote the Foxp3$^+$ Treg conversion as efficiently as CD103$^+$ DCs, indicating that RA can act as a cofactor for TGF-β induced Foxp3$^+$ Treg conversion (Coombes et al. 2007; Sun et al. 2007; Benson et al. 2007). Since TGF-β can regulate both pro- and anti-inflammatory immune responses, in the presence of pro-inflammatory cytokines such as IL-6, TGF-β can lead to the differentiation of Th17 cells instead of pTregs (Bettelli et al. 2006; Mangan et al. 2006; Veldhoen et al. 2006). Interestingly, splenic DCs are more efficient at inducing generation of Th17 cells in vitro than mLN DCs in the presence of exogenous TGF-β plus IL-6; moreover, the addition of RA antagonist LE135 enhanced mLN DCs' ability to promote Th17 cell differentiation, indicating that RA reciprocally regulate TGF-β dependent T cell differentiation (Mucida et al. 2007). RA also allows the Foxp3$^+$ Treg conversion in the presence of high levels of co-stimulation (Benson et al. 2007).

RA also plays an important role in the induction of CCR9 and α4β7 integrin on activated CD4$^+$ T cells in vitro as well as in vivo (Iwata et al. 2004). Hence, in mice fed with vitamin A deficient diet (VAD mice), the gut homing of activated T cells is severely impaired and results in the reduction of CD4$^+$ T cells in the LP of small intestine (Iwata et al. 2004; Cha et al. 2010). In this regard, oral tolerance induction in VAD mice is impaired and rescued by the adoptive transfer of gut tropic CD4$^+$ T cells (Cassani et al. 2011). Although RA produced by CD103$^+$ DCs can enhance Foxp3$^+$ Treg differentiation in vitro or ex vivo (Coombes et al. 2007; Sun et al. 2007; Benson et al. 2007), its in vivo role in pTreg generation is not clear. In RARα-deficient mice in which RA signaling is defective, or in VAD mice, the percentage of Foxp3$^+$ Tregs in the LP of small intestine is comparable with that of wild type mice or mice fed with vitamin A sufficient diet, respectively (Hill et al. 2008; Cha et al. 2010). However, it is quite possible that the LP Tregs in these mice are mostly tTregs and that abrogation of oral tolerance in VAD mice might be caused by selective impairment of gut homing of pTregs to the LP of small intestine. This validity of this possibility is yet to be examined.

Indoleamine 2,3 Dioxygenase (IDO) is Constitutively Expressed in CD103$^+$ DCs and is Involved in Foxp3$^+$ Treg Induction

Another important mediator for tolerogenic functions of intestinal DCs is indoleamine 2,3 dioxygenase (IDO), an enzyme involved in the oxidative catabolism of the essential amino acid tryptophan (TRP). CD103$^+$ mLN and LP DCs, but not

CD103$^-$ counterparts, express IDO (Onodera et al. 2009; Matteoli et al. 2010) and treatment of mice with 1-methyl-DL-Tryptophan, an IDO competitive inhibitor, suppresses Foxp3$^+$ Treg differentiation and the establishment of oral tolerance (Matteoli et al. 2010). IDO contributes to metabolic immune regulation by TRP deprivation and by the production of kynurenin, the first breakdown product in the IDO dependent degradation pathway (Munn and Mellor 2013). Deprivation of amino acids, including TRP, activates GCN2 kinase in T cells leading to the phosphorylation of eukaryotic initiation factor (eIF) 2α; subsequent phosphorylation of eIF2α blocks ribosomal translation of most mRNA resulting in proliferative arrest and anergy in effector T cells (Munn et al. 2005). It has been shown that GCN2 activation blocks Th17 cell differentiation (Sundrud et al. 2009) and enhances the suppressive activity of mature Foxp3$^+$ Tregs (Sharma et al. 2007), although the underlying mechanisms for the differential effects of TRP deprivation remains unclear. Kynurenin induces the differentiation of Foxp3$^+$ Tregs from naïve CD4$^+$ T cells in vitro synergistically with tryptophan deprivation (Fallarino et al. 2006). This effect of kynurenin on Foxp3$^+$ Treg differentiation was shown to be dependent on aryl hydrocarbon receptor (AhR) expressed in T cells (Mezrich et al. 2010).

AhR was first discovered as a transcription factor responsible for the immunotoxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), one of the impurities in the industrial organic synthesis of herbicide (Stockinger et al. 2014). It has turned out that the activation of AhR has been implicated in numerous aspects of immunological functions including the development and functions of RORγt$^+$ innate lymphoid cells type 3 (ILC3) (Kiss et al. 2011; Qiu et al. 2012), effector CD4$^+$ T cell differentiation, namely, Foxp3$^+$ Tregs and Th17 cells (Quintana et al. 2008; Quintana et al. 2010), and the induction of tolerogenic DCs (Quintana et al. 2010), which will be further discussed later. Several exogenous and endogenous compounds have been shown to possess AhR-activating properties. The important exogenous sources of AhR ligands are indoles, such as indole-3-carbinol (I3C), which can be derived from cruciferous vegetables in the diet (Kiss et al. 2011), or indole-3-acetic acid (IAA) and indole-3-acetaldehyde (IAId), which are produced by bacterial metabolism of tryptophan (Zelante et al. 2013). Endogenous AhR ligands include kynurenin, 6-formylindolo[3,2-b]carbazole (FICZ) produced by UVB radiation of tryptophan (Rannug et al. 1987) and 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) isolated from lung tissues (Song et al. 2002). It has been shown that AhR activation regulates Foxp3$^+$ Treg and Th17 cell differentiation in a ligand-specific manner (Quintana et al. 2008; Quintana et al. 2010). Hence, AhR activation by kynurenin, TCDD or ITE can promote the differentiation of Foxp3$^+$ Tregs, whereas FICZ mediated activation of AhR promote Th17 cell, but not Foxp3$^+$ Treg differentiation (Mezrich et al. 2010; Quintana et al. 2008; Quintana et al. 2010). These finding suggest that the outcome of AhR activation-induced modulation of T cell differentiation depends on the specific signaling pathways induced by the ligands.

The ability to Induce pTregs by Intestinal CD103$^+$ DC Subsets is Shared by DCs at Other Mucosal Sites

As aforementioned, CD103$^+$ DCs can be further divided based on the expression of CD11b. The development of CD103$^+$ CD11b$^+$ DCs is dependent on Notch2 and IRF4. Thus, in Notch2 deficient mice, CD103$^+$ CD11b$^+$ DCs in both the mLNs and LP of small intestine are completely depleted but CD103$^+$ CD11b$^-$ DCs are normal (Satpathy et al. 2013). IRF4 seems to play a role in the survival of CD103$^+$ CD11b$^+$ DCs but not in their differentiation from the precursors, as IRF4 deficient DCs are more susceptible to the apoptosis (Persson et al. 2013). In this regard, although CD103$^+$ CD11b$^+$ mLN DCs are almost absent in the mLN of IRF4-deficient mice, they still possess CD103$^+$ CD11b$^+$ DCs in the LP of small intestine at an approximately 50% reduced level. A fraction of CD103$^+$ CD11b$^-$ DCs express CD8α and resembles CD8α$^+$ DCs in the secondary lymphoid tissues due to their dependency on Batf3, IRF8 and Id2 for differentiation (Hashimoto et al. 2011). CD103$^+$ CD11b$^-$ LP DCs were shown to be poor inducers of Foxp3$^+$ Tregs in the presence of TGF-β in vitro and do not express *aldh1a2*, suggesting that they are dispensable for establishing the oral tolerance (Fujimoto et al. 2011). However, conflicting results have also been reported (Shan et al. 2013) and, despite of the functional similarity of CD103$^+$ DCs between mLNs and the LP of small intestine, CD103$^+$ CD11b$^-$ mLN DCs was shown to express *aldh1a2* (Guilliams et al. 2010). Furthermore, mice deficient of IRF4 in DCs have normal Foxp3$^+$ Tregs in the LP of small intestine (Persson et al. 2013) and develop oral tolerance (Persson et al., unpublished results and reviewed in (Bekiaris et al. 2014)). Similarly, Batf3$^{-/-}$ mice are deficient of CD103$^+$ CD11b$^-$ LP DCs but have normal numbers of Foxp3$^+$ Tregs in the LP of small intestine (Edelson et al. 2010). Mice lacking both subsets of CD103$^+$ LP DCs cause the reduction of Foxp3$^+$ Tregs in both mLNs and the LP of small intestine (Welty et al. 2013). However, the effect of the depletion of both CD11b$^+$ and CD11b$^-$ subsets of CD103$^+$ LP DCs on the establishment of oral tolerance remains to be elucidated. Collectively, these results suggested that both subsets of CD103$^+$ DCs play redundant roles in the generation and/or maintenance of Foxp3$^+$ Tregs in the LP of small intestine.

It seems that depending on the anatomical location of DCs, DCs having different origins can share functional specialization for establishing tolerance at mucosal sites. Most lung CD103$^+$ DCs originate from the same recirculating pre-DC that give arise to CD103$^+$ CD11b$^-$ LP DCs and depend on Batf3 for their development (Hashimoto et al. 2011;Edelson et al. 2010). Lung CD103$^+$ DCs play an essential role in establishing airway tolerance through the TGF-β- and RA-dependent pathways as observed for CD103$^+$ LP DCs (Khare et al. 2013). In addition, dermis-derived CD103$^-$ dendritic cells can produce RA and induce Foxp3$^+$ Tregs (Guilliams et al. 2010). These results suggest that the ability to produce RA is not restricted to CD103$^+$ DCs in the LP of small intestine. Although RA produced by DCs at different mucosal tissues participates in the induction of Foxp3$^+$ Tregs, RA produced by extra-intestinal DCs does not seem to imprint T cells to gut homing (Guilliams et al.

2010). Whether RA imprints tropism towards the DC-originating tissue remains to be established.

Intestinal Environment Conditions DCs and Foxp3$^+$ Tregs to Establish Oral Tolerance Through Multifaceted Mechanisms

CD103$^+$ mLN and LP DCs mediate oral tolerance by generating pTregs and imprinting their gut tropism in a manner dependent on TGF-β and RA. However, CD103$^+$ DCs from colitic mice have impaired ability to induce Foxp3$^+$ Tregs and promote increased generation of IFN-γ producing CD4$^+$ T cells (Laffont et al. 2010). Furthermore, even under steady-state conditions, CD103$^+$ CD11b$^+$ DCs, but not CD103$^+$ CD11b$^-$ DCs, are also essential for Th17 development (Persson et al. 2013; Schlitzer et al. 2013), indicating that the tolerogenic features of CD103$^+$ CD11b$^+$ DCs are not permanent but rather malleable. Accumulating evidence supports the notion that intestinal DCs are conditioned by local environment involving multiple stimuli from dietary, microbial components and interactions with other immune and non-immune cells, such as IEC (Fig. 8.2) (Scott et al. 2011; Manicassamy and Pulendran 2011). The functional plasticity of intestinal DCs relies on the expression of a diverse array of cell surface or intracellular receptors of which ligands are abundant at mucosal sites.

Although RA produced by CD103$^+$ DCs are critical for inducing expression of gut homing receptor on responding T cells, RA itself can also condition CD103$^+$ DCs to be tolerogenic through the RAR/RXR mediated induction of *aldh1a2* (Jaensson-Gyllenback et al. 2011). In this regard, CD103$^+$ DCs in VAD mice display reduced *aldh1a2* expression. Although the diet is the only source of RA for animal species (Blomhoff and Blomhoff 2006), bile retinol secreted from the liver, the major reservoir of retinol in the body, into the small intestine lumen was shown to be sufficient to induce expression of *aldh1a2* in CD103$^+$ DCs, enabling them to induce CCR9 expression on T cells even in a condition of restricted uptake in dietary retinol (Jaensson-Gyllenback et al. 2011). Several factors also influence *aldh1a2* expression, including TLR-2 ligands such as zymosan (Manicassamy et al. 2009), peroxisome proliferator-activated receptor-g agonists (Szatmari et al. 2006), GM-CSF and IL-4 (Yokota et al. 2009). However, these factors play redundant roles as mLN DCs from Myd88$^{-/-}$Trif$^{-/-}$ and IL-4Rα$^{-/-}$ mice display normal or only a modestly decreased level of *aldh1a2* (Guilliams et al. 2010). IECs have also been shown to produce RA as well as TGF-β (Iliev et al. 2009). Hence, bone marrow derived or splenic DCs conditioned in vitro by the pre-incubation with supernatants from epithelial cell lines or by the contact with monolayer forming epithelial cell lines can promote the differentiation of gut tropic Foxp3$^+$ Tregs in TGF-β and RA-dependent manner.

Accompanying their unique ability to promote the generation of Foxp3$^+$ Tregs is the inability of CD103$^+$ mLN or LP DCs to efficiently produce pro-inflammatory cytokines, such as IL-6, TNF-α, IL-12p70 or IL-23, in response to TLR ligand

stimulation, in a sharp contrast to $CD103^-$ mLN DCs or splenic DCs. Instead of inflammatory cytokines, $CD103^+$ LP DCs constitutively produce anti-inflammatory cytokines IL-10 and TGF-β (Coombes et al. 2007; Monteleone et al. 2008). It has been shown that intestinal DCs require intact Wnt-β-catenin signaling in order to display these optimal tolerogenic characteristics (Manicassamy et al. 2010). Thus, DCs in the LP of small intestine constitutively express several Wnt-ligands compared with splenic DCs and β-catenin signaling pathway is constitutively active in the intestinal DCs. In mice with β-catenin deficiency specifically in DCs, LP DCs express less anti-inflammatory mediators such as TGF-β, IL-10 and *aldh1a2*, and display increased levels of pro-inflammatory cytokines. Accordingly, $Foxp3^+$ Tregs in both small intestine and colon, but not in spleen, are dramatically reduced and these mice are highly susceptible to dextran sulfate sodium (DSS)-induced intestinal inflammation. Furthermore, subsequent study showed that MUC2, the building block of the gut mucus, can be recognized by the intestinal APCs as well as the IECs and enables them to produce anti-inflammatory mediators and suppress the production of pro-inflammatory cytokines (Shan et al. 2013). MUC2 binds receptor complex consisting of galectin-3, Dectin-1, FCγ RIIB on DCs and promotes their tolerizing features by inducing β-catenin activation. As in mice deficient of β-catenin in DCs, $MUC2^{-/-}$ mice display reduced level of $Fox3^+$ Tregs in the LP of small intestine and failed to establish oral tolerance.

As mentioned previously, $CD103^+$ mLN and LP DCs constitutively express IDO. Although the mechanisms inducing IDO expression in intestinal DCs have yet to be elucidated directly, several factors regulating IDO gene expression in DCs have been identified, including interferons (IFNs), TGF-β, prostaglandin E2, TLR9 ligation, AhR activation, and reverse signaling induced by CTLA4 expressed on $Foxp3^+$ Tregs (Munn and Mellor 2013). Some of these mechanisms are likely to be involved in inducing IDO in the intestinal DCs, which are chronically exposed to intestinal microbes and TGF-β abundantly present at this mucosal site. In addition to its effect on $CD4^+$ T cell differentiation, AhR activation can also regulate tolerogenic function of DCs. Hence, AhR ligand ITE can induce tolerogenic DCs to increased expression of IL-10, TGF-β and *aldh1a1*, another member of aldehyde dehydrogenase family related to *adlh1a2*, and promote the generation of $Foxp3^+$ Tregs in RA-dependent manner (Quintana et al. 2010). Furthermore, TCDD-mediated AhR activation can induce IDO expression in DCs (Mezrich et al. 2010). However, it remains to be determined whether endogenous AhR ligands, such as kynurenin, ITE or indole compounds, produced by intestinal microbes-mediated catabolism of dietary tryptophan, are involved in inducing IDO expression of intestinal DCs. Interestingly, reverse signals from T cells to DCs through the interaction of CD80/CD86 with CTLA4 expressed on $Foxp3^+$ Tregs can also induce IDO expression in DCs (Fallarino et al. 2003). Intestinal $Foxp3^+$ Tregs express CTLA4 at a markedly higher level than those in other secondary lymphoid tissues (Guo et al. 2008). In this regard, $Foxp3^+$ Treg-specific ablation of CTLA4 causes a reduction of IDO^+ DCs in mLN as well as IDO activity (Onodera et al. 2009). It seems that mutual interaction between DCs and $Foxp3^+$ Tregs might augment the tolerogenic functions of intestinal DCs and act as feed-forward mechanisms to maintain oral tolerance.

Besides of the role of CX3CR1^{hi} MØs in sampling intestinal luminal antigens, CX3CR1^{hi} MØs in the LP of small intestine also spontaneously produce IL-10 (Denning et al. 2007), a pleiotrophic cytokine regulating a variety of functions of hematopoietic cells, including suppressing immunostimulatory functions of APCs and inhibiting Th1 differentiation (Moore et al. 2001). In this regard, IL-10 produced by CX3CR1^{hi} MØs and DCs can contribute to the formation of the tolerogenic intestinal environment. It has been shown that LP CX3CR1^{hi} MØs can induce Foxp3^{+} Treg differentiation in the presence of TGF-β in vitro (Denning et al. 2007), even at a high APC:T cell ratio, which is detrimental for the ability of CD103^{+} LP DCs to induce Foxp3^{+} Treg conversion (Denning et al. 2011). A role for IL-10 is also indicated by the finding that blockade of IL-10 signaling suppresses Foxp3^{+} Treg differentiation induced by CX3CR1^{hi} MØs (Denning et al. 2007). However, given that CX3CR1^{hi} MØs are sessile under steady state conditions (Schulz et al. 2009), the role of CX3CR1^{hi} MØs in the generation of small intestinal pTregs and the establishment of oral tolerance is unclear. Hence, although the expansion of Foxp3^{+} Tregs locally in the LP of the small intestine as well as oral tolerance are impaired in CX3CR1$^{-/-}$ mice (Hadis et al. 2011), whether these problems are due to the absence of CX3CR1-produced IL-10 is not clear. Nonetheless, it has been proposed that IL-10 signaling on Foxp3^{+} Tregs is necessary to mediate their suppressive functions in order to inhibit Th1 and Th17 effector cells and to prevent T cell transfer-induced colitis (Murai et al. 2009; Chaudhry et al. 2011). Indeed, IL-10 has been shown to promote phosphorylation of STAT3, which is required in Foxp3^{+} Tregs to suppress Th17-mediated intestinal inflammation in the colon (Chaudhry et al. 2009). Moreover, Tregs deficient in the expression of IL-10R produce a reduced amount of IL-10 (Chaudhry et al. 2011), suggesting that IL-10 produced by Foxp3^{+} Tregs and other cellular sources in the intestine, such as DCs and CX3CR1^{hi} MØs, augments tolerogenic functions of Foxp3^{+} Tregs. In support of this idea, the adoptive transfer of wild type naïve CD4^{+} T cells, but not T cells from both IL-10 and IL-10R deficient mice, rescued oral tolerance defect in CCR9$^{-/-}$ mice (Cassani et al. 2011).

Conclusion

Oral tolerance is a stepwise process mediated by multifaceted mechanisms. Intestinal immune system is of critical importance not only for the generation of tolerogenic DCs, enabling them to induce development of Foxp3^{+} Tregs in response to dietary antigens, but also for the conditioning and maintaining these cells to ensure tolerance in both mucosal and systemic immune system. Although several key factors for establishing oral tolerance have been elucidated, many other issues remain poorly understood. Foxp3^{+} Tregs are heterogeneous populations based on their origin and their effector function, as indicated by their differential expression of effector transcriptional factors and homing receptors in response to the environment cues. Although pTregs generated in response to orally administrated antigens are

important mediators of oral tolerance, many aspects of these cells are still poorly understood. Hence, it is still unknown whether diet-induced pTregs can be distinguished from pTregs generated in response to intestinal microbes, whether these two populations use different mechanisms to establish oral tolerance and, if so, which effector transcriptional factors are associated with the unique functions of diet- and microbe-specific pTregs. Another important issue to be addressed is the question of how locally induced tolerance mechanisms mediated by intestinal DCs result in systemic suppression to orally administrated antigens. Although gut tropism preferentially sequesters effector T cells to the intestine where pTregs expanded preferentially, pTregs may migrate out from the intestine to the periphery to mediate the systemic suppression. However, the underlying mechanisms for systemic spreading of tolerance remain largely unknown. Better understandings of these processes may lead to identification of new targets for the therapeutic enhancement of oral tolerance to treat severe food allergic responses.

Acknowledgements We thank Drs. Sung Wook Hong and Yun Ji Park for commenting on the manuscript and creating illustrative figures. This work was supported by Institute for Basic Science (IBS).

References

Abbas, A. K., Benoist, C., Bluestone, J. A., Campbell, D. J., Ghosh, S., Hori, S., et al. (2013). Regulatory T cells: Recommendations to simplify the nomenclature. *Nature Immunology, 14*(4), 307–308. doi:10.1038/ni.2554.

Akbari, O., DeKruyff, R. H., & Umetsu, D. T. (2001). Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nature Immunology, 2*(8), 725–731. doi:10.1038/90667.

Annes, J. P., Munger, J. S., & Rifkin, D. B. (2003). Making sense of latent TGFbeta activation. *Journal of Cell Science, 116*(Pt 2), 217–224.

Backhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A., & Gordon, J. I. (2005). Host-bacterial mutualism in the human intestine. *Science, 307*(5717), 1915–1920. doi:10.1126/science.1104816.

Barone, K. S., Jain, S. L., & Michael, J. G. (1995). Effect of in vivo depletion of CD4+ and CD8+ cells on the induction and maintenance of oral tolerance. *Cellular Immunology, 163*(1), 19–29. doi:10.1006/cimm.1995.1094.

Bekiaris, V., Persson, E. K., & Agace, W. W. (2014). Intestinal dendritic cells in the regulation of mucosal immunity. *Immunological Reviews, 260*(1), 86–101. doi:10.1111/imr.12194.

Bennett, C. L., Christie, J., Ramsdell, F., Brunkow, M. E., Ferguson, P. J., Whitesell, L., et al. (2001). The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nature Genetics, 27*(1), 20–21. doi:10.1038/83713.

Benson, M. J., Pino-Lagos, K., Rosemblatt, M., & Noelle, R. J. (2007). All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. *The Journal of Experimental Medicine, 204*(8), 1765–1774. doi:10.1084/jem.20070719.

Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T. B., Oukka, M., et al. (2006). Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature, 441*(7090), 235–238. doi:10.1038/nature04753.

Blomhoff, R., & Blomhoff, H. K. (2006). Overview of retinoid metabolism and function. *Journal of Neurobiology, 66*(7), 606–630. doi:10.1002/neu.20242.

Brunkow, M. E., Jeffery, E. W., Hjerrild, K. A., Paeper, B., Clark, L. B., Yasayko, S. A., et al. (2001). Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nature Genetics, 27*(1), 68–73. doi:10.1038/83784.

Carmeliet, P., Schoonjans, L., Kieckens, L., Ream, B., Degen, J., Bronson, R., et al. (1994). Physiological consequences of loss of plasminogen activator gene function in mice. *Nature, 368*(6470), 419–424. doi:10.1038/368419a0.

Cassani, B., Villablanca, E. J., Quintana, F. J., Love, P. E., Lacy-Hulbert, A., Blaner, W. S., et al. (2011). Gut-tropic T cells that express integrin alpha4beta7 and CCR9 are required for induction of oral immune tolerance in mice. *Gastroenterology, 141*(6), 2109–2118. doi:10.1053/j.gastro.2011.09.015.

Cerovic, V., Houston, S. A., Westlund, J., Utriainen, L., Davison, E. S., Scott, C. L., et al. (2014). Lymph-borne CD8alpha dendritic cells are uniquely able to cross-prime CD8 T cells with antigen acquired from intestinal epithelial cells. *Mucosal Immunology*. doi:10.1038/mi.2014.40.

Cha, H. R., Chang, S. Y., Chang, J. H., Kim, J. O., Yang, J. Y., Kim, C. H., et al. (2010). Downregulation of Th17 cells in the small intestine by disruption of gut flora in the absence of retinoic acid. *Journal of Immunology, 184*(12), 6799–6806. doi:10.4049/jimmunol.0902944.

Chang, S. Y., Song, J. H., Guleng, B., Cotoner, C. A., Arihiro, S., Zhao, Y., et al. (2013). Circulatory antigen processing by mucosal dendritic cells controls CD8(+) T cell activation. *Immunity, 38*(1), 153–165. doi:10.1016/j.immuni.2012.09.018.

Chase, M. W. (1946). Inhibition of experimental drug allergy by prior feeding of the sensitizing agent. *Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine (New York. NY), 61,* 257–259.

Chaudhry, A., & Rudensky, A. Y. (2013). Control of inflammation by integration of environmental cues by regulatory T cells. *The Journal of Clinical Investigation, 123*(3), 939–944. doi:10.1172/JCI57175.

Chaudhry, A., Rudra, D., Treuting, P., Samstein, R. M., Liang, Y., Kas, A., et al. (2009). CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. *Science, 326*(5955), 986–991. doi:10.1126/science.1172702.

Chaudhry, A., Samstein, R. M., Treuting, P., Liang, Y., Pils, M. C., Heinrich, J. M., et al. (2011). Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. *Immunity, 34*(4), 566–578. doi:10.1016/j.immuni.2011.03.018.

Chen, Y., Kuchroo, V. K., Inobe, J., Hafler, D. A., & Weiner, H. L. (1994). Regulatory T cell clones induced by oral tolerance: Suppression of autoimmune encephalomyelitis. *Science, 265*(5176), 1237–1240.

Chen, Y., Inobe, J., & Weiner, H. L. (1995a). Induction of oral tolerance to myelin basic protein in CD8-depleted mice: Both CD4+ and CD8+ cells mediate active suppression. *Journal of Immunology, 155*(2), 910–916.

Chen, Y., Inobe, J., Marks, R., Gonnella, P., Kuchroo, V. K., & Weiner, H. L. (1995b). Peripheral deletion of antigen-reactive T cells in oral tolerance. *Nature, 376*(6536), 177–180. doi:10.1038/376177a0.

Chen, W., Jin, W., Hardegen, N., Lei, K. J., Li, L., Marinos, N., et al. (2003). Conversion of peripheral CD4+ CD25- naive T cells to CD4+ CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *The Journal of Experimental Medicine, 198*(12), 1875–1886. doi:10.1084/jem.20030152.

Chirdo, F. G., Millington, O. R., Beacock-Sharp, H., & Mowat, A. M. (2005). Immunomodulatory dendritic cells in intestinal lamina propria. *European Journal of Immunology, 35*(6), 1831–1840.

Coombes, J. L., Siddiqui, K. R., Arancibia-Carcamo, C. V., Hall, J., Sun, C. M., Belkaid, Y., et al. (2007). A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *The Journal of Experimental Medicine, 204*(8), 1757–1764. doi:10.1084/jem.20070590.

Crawford, S. E., Stellmach, V., Murphy-Ullrich, J. E., Ribeiro, S. M., Lawler, J., Hynes, R. O., et al. (1998). Thrombospondin-1 is a major activator of TGF-beta1 in vivo. *Cell, 93*(7), 1159–1170.

Cretney, E., Kallies, A., & Nutt, S. L. (2013). Differentiation and function of Foxp3(+) effector regulatory T cells. *Trends in Immunology, 34*(2), 74–80. doi:10.1016/j.it.2012.11.002.

Curotto de Lafaille, M. A., & Lafaille, J. J. (2009). Natural and adaptive foxp3+ regulatory T cells: More of the same or a division of labor? *Immunity, 30*(5), 626–635. doi:10.1016/j.immuni.2009.05.002.

Curotto de Lafaille, M. A., Kutchukhidze, N., Shen, S., Ding, Y., Yee, H., & Lafaille, J. J. (2008). Adaptive Foxp3+ regulatory T cell-dependent and -independent control of allergic inflammation. *Immunity, 29*(1), 114–126. doi:10.1016/j.immuni.2008.05.010.

Denning, T. L., Wang, Y. C., Patel, S. R., Williams, I. R., & Pulendran, B. (2007). Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses. *Nature Immunology, 8*(10), 1086–1094. doi:10.1038/ni1511.

Denning, T. L., Norris, B. A., Medina-Contreras, O., Manicassamy, S., Geem, D., Madan, R., et al. (2011). Functional specializations of intestinal dendritic cell and macrophage subsets that control Th17 and regulatory T cell responses are dependent on the T cell/APC ratio, source of mouse strain, and regional localization. *Journal of Immunology, 187*(2), 733–747. doi:10.4049/jimmunol.1002701.

Diehl, G. E., Longman, R. S., Zhang, J. X., Breart, B., Galan, C., Cuesta, A., et al. (2013). Microbiota restricts trafficking of bacteria to mesenteric lymph nodes by CX(3)CR1(hi) cells. *Nature, 494*(7435), 116–120. doi:10.1038/nature11809.

Edelson, B. T., Kc, W., Juang, R., Kohyama, M., Benoit, L. A., Klekotka, P. A., et al. (2010). Peripheral CD103+ dendritic cells form a unified subset developmentally related to CD8alpha+ conventional dendritic cells. *The Journal of Experimental Medicine, 207*(4), 823–836. doi:10.1084/jem.20091627.

Fallarino, F., Grohmann, U., Hwang, K. W., Orabona, C., Vacca, C., Bianchi, R., et al. (2003). Modulation of tryptophan catabolism by regulatory T cells. *Nature Immunology, 4*(12), 1206–1212. doi:10.1038/ni1003.

Fallarino, F., Grohmann, U., You, S., McGrath, B. C., Cavener, D. R., Vacca, C., et al. (2006). The combined effects of tryptophan starvation and tryptophan catabolites down-regulate T cell receptor zeta-chain and induce a regulatory phenotype in naive T cells. *Journal of Immunology, 176*(11), 6752–6761.

Fantini, M. C., Becker, C., Monteleone, G., Pallone, F., Galle, P. R., & Neurath, M. F. (2004). Cutting edge: TGF-beta induces a regulatory phenotype in CD4+ CD25- T cells through Foxp3 induction and down-regulation of Smad7. *Journal of Immunology, 172*(9), 5149–5153.

Farache, J., Koren, I., Milo, I., Gurevich, I., Kim, K. W., Zigmond, E., et al. (2013a). Luminal bacteria recruit CD103+ dendritic cells into the intestinal epithelium to sample bacterial antigens for presentation. *Immunity, 38*(3), 581–595. doi:10.1016/j.immuni.2013.01.009.

Farache, J., Zigmond, E., Shakhar, G., & Jung, S. (2013b). Contributions of dendritic cells and macrophages to intestinal homeostasis and immune defense. *Immunology and Cell Biology, 91*(3), 232–239. doi:10.1038/icb.2012.79.

Fontenot, J. D., Gavin, M. A., & Rudensky, A. Y. (2003). Foxp3 programs the development and function of CD4+ CD25+ regulatory T cells. *Nature Immunology, 4*(4), 330–336. doi:10.1038/ni904.

Fujimoto, K., Karuppuchamy, T., Takemura, N., Shimohigoshi, M., Machida, T., Haseda, Y., et al. (2011). A new subset of CD103+ CD8alpha+ dendritic cells in the small intestine expresses TLR3, TLR7, and TLR9 and induces Th1 response and CTL activity. *Journal of Immunology, 186*(11), 6287–6295. doi:10.4049/jimmunol.1004036.

Garside, P., Steel, M., Liew, F. Y., & Mowat, A. M. (1995). CD4+ but not CD8+ T cells are required for the induction of oral tolerance. *International Immunology, 7*(3), 501–504.

Gottschalk R. A., Corse E., & Allison J. P. (2012). Expression of helios in peripherally induced Foxp3+ regulatory T cells. *Journal of immunology 188*(3): 976–980. doi:10.4049/jimmunol.1102964.

Guilliams, M., Crozat, K., Henri, S., Tamoutounour, S., Grenot, P., Devilard, E., et al. (2010). Skin-draining lymph nodes contain dermis-derived CD103(−) dendritic cells that constitutively produce retinoic acid and induce Foxp3(+) regulatory T cells. *Blood, 115*(10), 1958–1968. doi:10.1182/blood-2009-09-245274.

Guo, Z., Jang, M. H., Otani, K., Bai, Z., Umemoto, E., Matsumoto, M., et al. (2008). CD4+ CD25+ regulatory T cells in the small intestinal lamina propria show an effector/memory phenotype. *International Immunology, 20*(3), 307–315. doi:10.1093/intimm/dxm143.

Gutgemann, I., Fahrer, A. M., Altman, J. D., Davis, M. M., & Chien, Y. H. (1998). Induction of rapid T cell activation and tolerance by systemic presentation of an orally administered antigen. *Immunity, 8*(6), 667–673.

Hadis, U., Wahl, B., Schulz, O., Hardtke-Wolenski, M., Schippers, A., Wagner, N., et al. (2011). Intestinal tolerance requires gut homing and expansion of FoxP3+ regulatory T cells in the lamina propria. *Immunity, 34*(2), 237–246. doi:10.1016/j.immuni.2011.01.016.

Haribhai, D., Lin, W., Edwards, B., Ziegelbauer, J., Salzman, N. H., Carlson, M. R., et al. (2009). A central role for induced regulatory T cells in tolerance induction in experimental colitis. *Journal of Immunology, 182*(6), 3461–3468. doi:10.4049/jimmunol.0802535.

Haribhai, D., Williams, J. B., Jia, S., Nickerson, D., Schmitt, E. G., Edwards, B., et al. (2011). A requisite role for induced regulatory T cells in tolerance based on expanding antigen receptor diversity. *Immunity, 35*(1), 109–122. doi:10.1016/j.immuni.2011.03.029.

Hashimoto, D., Miller, J., & Merad, M. (2011). Dendritic cell and macrophage heterogeneity in vivo. *Immunity, 35*(3), 323–335.

Hill, J. A., Feuerer, M., Tash, K., Haxhinasto, S., Perez, J., Melamed, R., et al. (2007). Foxp3 transcription-factor-dependent and -independent regulation of the regulatory T cell transcriptional signature. *Immunity, 27*(5), 786–800. doi:10.1016/j.immuni.2007.09.010.

Hill, J. A., Hall, J. A., Sun, C. M., Cai, Q., Ghyselinck, N., Chambon, P., et al. (2008). Retinoic acid enhances Foxp3 induction indirectly by relieving inhibition from CD4+CD44hi cells. *Immunity, 29*(5), 758–770. doi:10.1016/j.immuni.2008.09.018.

Hori, S., Nomura, T., & Sakaguchi, S. (2003). Control of regulatory T cell development by the transcription factor Foxp3. *Science, 299*(5609), 1057–1061. doi:10.1126/science.1079490.

Hsieh, C. S., Zheng, Y., Liang, Y., Fontenot, J. D., & Rudensky, A. Y. (2006). An intersection between the self-reactive regulatory and nonregulatory T cell receptor repertoires. *Nature Immunology, 7*(4), 401–410. doi:10.1038/ni1318.

Huang, F. P., Platt, N., Wykes, M., Major, J. R., Powell, T. J., Jenkins, C. D., et al. (2000). A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes. *The Journal of Experimental Medicine, 191*(3), 435–444.

Iliev, I. D., Mileti, E., Matteoli, G., Chieppa, M., & Rescigno, M. (2009). Intestinal epithelial cells promote colitis-protective regulatory T-cell differentiation through dendritic cell conditioning. *Mucosal Immunology, 2*(4), 340–350. doi:10.1038/mi.2009.13.

Iwata, M., Hirakiyama, A., Eshima, Y., Kagechika, H., Kato, C., & Song, S. Y. (2004). Retinoic acid imprints gut-homing specificity on T cells. *Immunity, 21*(4), 527–538. doi:10.1016/j.immuni.2004.08.011.

Jaensson-Gyllenback, E., Kotarsky, K., Zapata, F., Persson, E. K., Gundersen, T. E., Blomhoff, R., et al. (2011). Bile retinoids imprint intestinal CD103+ dendritic cells with the ability to generate gut-tropic T cells. *Mucosal Immunology, 4*(4), 438–447. doi:10.1038/mi.2010.91.

Jang, M. H., Sougawa, N., Tanaka, T., Hirata, T., Hiroi, T., Tohya, K., et al. (2006). CCR7 is critically important for migration of dendritic cells in intestinal lamina propria to mesenteric lymph nodes. *Journal of Immunology, 176*(2), 803–810.

Johansson-Lindbom, B., Svensson, M., Pabst, O., Palmqvist, C., Marquez, G., Forster, R., et al. (2005). Functional specialization of gut CD103+ dendritic cells in the regulation of tissue-selective T cell homing. *The Journal of Experimental Medicine, 202*(8), 1063–1073. doi:10.1084/jem.20051100.

Joly, E., & Hudrisier, D. (2003). What is trogocytosis and what is its purpose? *Nature Immunology, 4*(9), 815. doi:10.1038/ni0903-815.

Jordan, M. S., Boesteanu, A., Reed, A. J., Petrone, A. L., Holenbeck, A. E., Lerman, M. A., et al. (2001). Thymic selection of CD4+ CD25+ regulatory T cells induced by an agonist self-peptide. *Nature Immunology, 2*(4), 301–306. doi:10.1038/86302.

Josefowicz, S. Z., Niec, R. E., Kim, H. Y., Treuting, P., Chinen, T., Zheng, Y., et al. (2012). Extrathymically generated regulatory T cells control mucosal TH2 inflammation. *Nature, 482*(7385), 395–399. doi:10.1038/nature10772.

Khare, A., Krishnamoorthy, N., Oriss, T. B., Fei, M., Ray, P., & Ray, A. (2013). Cutting edge: Inhaled antigen upregulates retinaldehyde dehydrogenase in lung CD103+ but not plasmacytoid dendritic cells to induce Foxp3 de novo in CD4+ T cells and promote airway tolerance. *Journal of Immunology, 191*(1), 25–29. doi:10.4049/jimmunol.1300193.

Khattri, R., Cox, T., Yasayko, S. A., & Ramsdell, F. (2003). An essential role for scurfin in CD4+ CD25+ T regulatory cells. *Nature Immunology, 4*(4), 337–342. doi:10.1038/ni909.

Kiss, E. A., Vonarbourg, C., Kopfmann, S., Hobeika, E., Finke, D., Esser, C., et al. (2011). Natural aryl hydrocarbon receptor ligands control organogenesis of intestinal lymphoid follicles. *Science, 334*(6062), 1561–1565. doi:10.1126/science.1214914.

Koch, M. A., Tucker-Heard, G., Perdue, N. R., Killebrew, J. R., Urdahl, K. B., & Campbell, D. J. (2009). The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nature Immunology, 10*(6), 595–602. doi:10.1038/ni.1731.

Kraus, T. A., Brimnes, J., Muong, C., Liu, J. H., Moran, T. M., Tappenden, K. A., et al. (2005). Induction of mucosal tolerance in Peyer's patch-deficient, ligated small bowel loops. *The Journal of Clinical Investigation, 115*(8), 2234–2243.

Lacy-Hulbert, A., Smith, A. M., Tissire, H., Barry, M., Crowley, D., Bronson, R. T., et al. (2007). Ulcerative colitis and autoimmunity induced by loss of myeloid alphav integrins. *Proceedings of the National Academy of Sciences of the United States of America, 104*(40), 15823–15828. doi:10.1073/pnas.0707421104.

Laffont, S., Siddiqui, K. R., & Powrie, F. (2010). Intestinal inflammation abrogates the tolerogenic properties of MLN CD103+ dendritic cells. *European Journal of Immunology, 40*(7), 1877–1883. doi:10.1002/eji.200939957.

Macpherson, A. J., & Uhr, T. (2004). Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. *Science, 303*(5664), 1662–1665.

Mangan, P. R., Harrington, L. E., O'Quinn, D. B., Helms, W. S., Bullard, D. C., Elson, C. O., et al. (2006). Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature, 441*(7090), 231–234. doi:10.1038/nature04754.

Manicassamy, S., & Pulendran, B. (2011). Dendritic cell control of tolerogenic responses. *Immunological Reviews, 241*(1), 206–227. doi:10.1111/j.1600-065X.2011.01015.x.

Manicassamy, S., Ravindran, R., Deng, J., Oluoch, H., Denning, T. L., Kasturi, S. P., et al. (2009). Toll-like receptor 2-dependent induction of vitamin A-metabolizing enzymes in dendritic cells promotes T regulatory responses and inhibits autoimmunity. *Nature Medicine, 15*(4), 401–409. doi:10.1038/nm.1925.

Manicassamy, S., Reizis, B., Ravindran, R., Nakaya, H., Salazar-Gonzalez, R. M., Wang, Y. C., et al. (2010). Activation of beta-catenin in dendritic cells regulates immunity versus tolerance in the intestine. *Science, 329*(5993), 849–853. doi:10.1126/science.1188510.

Matteoli, G., Mazzini, E., Iliev, I. D., Mileti, E., Fallarino, F., Puccetti, P., et al. (2010). Gut CD103+ dendritic cells express indoleamine 2,3-dioxygenase which influences T regulatory/T effector cell balance and oral tolerance induction. *Gut, 59*(5), 595–604. doi:10.1136/gut.2009.185108.

Mattingly, J. A., & Waksman, B. H. (1978). Immunologic suppression after oral administration of antigen. I. Specific suppressor cells formed in rat Peyer's patches after oral administration of sheep erythrocytes and their systemic migration. *Journal of Immunology, 121*(5), 1878–1883.

Mazzini, E., Massimiliano, L., Penna, G., & Rescigno, M. (2014). Oral tolerance can be established via gap junction transfer of fed antigens from CX3CR1(+) macrophages to CD103(+) dendritic cells. *Immunity, 40*(2), 248–261. doi:10.1016/j.immuni.2013.12.012.

McDole, J. R., Wheeler, L. W., McDonald, K. G., Wang, B., Konjufca, V., Knoop, K. A., et al. (2012). Goblet cells deliver luminal antigen to CD103+ dendritic cells in the small intestine. *Nature, 483*(7389), 345–349. doi:10.1038/nature10863.

Menard, S., Cerf-Bensussan, N., & Heyman, M. (2010). Multiple facets of intestinal permeability and epithelial handling of dietary antigens. *Mucosal Immunology, 3*(3), 247–259. doi:10.1038/mi.2010.5.

Mezrich, J. D., Fechner, J. H., Zhang, X., Johnson, B. P., Burlingham, W. J., & Bradfield, C. A. (2010). An interaction between kynurenine and the aryl hydrocarbon receptor can generate regulatory T cells. *Journal of Immunology, 185*(6), 3190–3198. doi:10.4049/jimmunol.0903670.

Monteleone, I., Platt, A. M., Jaensson, E., Agace, W. W., & Mowat, A. M. (2008). IL-10-dependent partial refractoriness to Toll-like receptor stimulation modulates gut mucosal dendritic cell function. *European Journal of Immunology, 38*(6), 1533–1547. doi:10.1002/eji.200737909.

Moore, K. W., de Waal, M. R., Coffman, R. L., & O'Garra, A. (2001). Interleukin-10 and the interleukin-10 receptor. *Annual Review of Immunology, 19,* 683–765. doi:10.1146/annurev.immunol.19.1.683.

Mosconi, E., Rekima, A., Seitz-Polski, B., Kanda, A., Fleury, S., Tissandie, E., et al. (2010). Breast milk immune complexes are potent inducers of oral tolerance in neonates and prevent asthma development. *Mucosal Immunology, 3*(5), 461–474. doi:10.1038/mi.2010.23.

Mucida, D., Kutchukhidze, N., Erazo, A., Russo, M., Lafaille, J. J., & Curotto de Lafaille, M. A. (2005). Oral tolerance in the absence of naturally occurring Tregs. *The Journal of Clinical Investigation, 115*(7), 1923–1933. doi:10.1172/JCI24487.

Mucida, D., Park, Y., Kim, G., Turovskaya, O., Scott, I., Kronenberg, M., et al. (2007). Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science, 317*(5835), 256–260. doi:10.1126/science.1145697.

Munn, D. H., & Mellor, A. L. (2013). Indoleamine 2,3 dioxygenase and metabolic control of immune responses. *Trends in Immunology, 34*(3), 137–143. doi:10.1016/j.it.2012.10.001.

Munn, D. H., Sharma, M. D., Baban, B., Harding, H. P., Zhang, Y., Ron, D., et al. (2005). GCN2 kinase in T cells mediates proliferative arrest and anergy induction in response to indoleamine 2,3-dioxygenase. *Immunity, 22*(5), 633–642. doi:10.1016/j.immuni.2005.03.013.

Murai, M., Turovskaya, O., Kim, G., Madan, R., Karp, C. L., Cheroutre, H., et al. (2009). Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. *Nature Immunology, 10*(11), 1178–1184. doi:10.1038/ni.1791.

Niess, J. H., Brand, S., Gu, X., Landsman, L., Jung, S., McCormick, B. A., et al. (2005). CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science, 307*(5707), 254–258. doi:10.1126/science.1102901.

Onodera, T., Jang, M. H., Guo, Z., Yamasaki, M., Hirata, T., Bai, Z., et al. (2009). Constitutive expression of IDO by dendritic cells of mesenteric lymph nodes: Functional involvement of the CTLA-4/B7 and CCL22/CCR4 interactions. *Journal of Immunology, 183*(9), 5608–5614. doi:10.4049/jimmunol.0804116.

Pabst, O., & Mowat, A. M. (2012).Oral tolerance to food protein. *Mucosal Immunology, 5*(3), 232–239. doi:10.1038/mi.2012.4.

Pacholczyk, R., Ignatowicz, H., Kraj, P., & Ignatowicz, L. (2006). Origin and T cell receptor diversity of Foxp3+ CD4+ CD25+ T cells. *Immunity, 25*(2), 249–259. doi:10.1016/j.immuni.2006.05.016.

Paidassi, H., Acharya, M., Zhang, A., Mukhopadhyay, S., Kwon, M., Chow, C., et al. (2011). Preferential expression of integrin alphavbeta8 promotes generation of regulatory T cells by mouse CD103+ dendritic cells. *Gastroenterology, 141*(5), 1813–1820. doi:10.1053/j.gastro.2011.06.076.

Parks, W. C., & Shapiro, S. D. (2001). Matrix metalloproteinases in lung biology. *Respiratory Research, 2*(1), 10–19. doi:10.1186/rr33.

Persson, E. K., Uronen-Hansson, H., Semmrich, M., Rivollier, A., Hagerbrand, K., Marsal, J., et al. (2013). IRF4 transcription-factor-dependent CD103(+)CD11b(+) dendritic cells drive mucosal T helper 17 cell differentiation. *Immunity, 38*(5), 958–969. doi:10.1016/j.immuni.2013.03.009.

Peters, M. G., Secrist, H., Anders, K. R., Nash, G. S., Rich, S. R., & MacDermott, R. P. (1989). Normal human intestinal B lymphocytes. Increased activation compared with peripheral blood. *The Journal of Clinical Investigation, 83*(6), 1827–1833. doi:10.1172/JCI114088.

Poulin, L. F., Reyal, Y., Uronen-Hansson, H., Schraml, B. U., Sancho, D., Murphy, K. M., et al. (2012). DNGR-1 is a specific and universal marker of mouse and human Batf3-dependent dendritic cells in lymphoid and nonlymphoid tissues. *Blood, 119*(25), 6052–6062. doi:10.1182/blood-2012-01-406967.

Qiu, J., Heller, J. J., Guo, X., Chen, Z. M., Fish, K., Fu, Y. X., et al. (2012). The aryl hydrocarbon receptor regulates gut immunity through modulation of innate lymphoid cells. *Immunity, 36*(1), 92–104. doi:10.1016/j.immuni.2011.11.011.

Quintana, F. J., Basso, A. S., Iglesias, A. H., Korn, T., Farez, M. F., Bettelli, E., et al. (2008). Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. *Nature, 453*(7191), 65–71. doi:10.1038/nature06880.

Quintana, F. J., Murugaiyan, G., Farez, M. F., Mitsdoerffer, M., Tukpah, A. M., Burns, E. J., et al. (2010). An endogenous aryl hydrocarbon receptor ligand acts on dendritic cells and T cells to suppress experimental autoimmune encephalomyelitis. *Proceedings of the National Academy of Sciences of the United States of America, 107*(48), 20768–20773. doi:10.1073/pnas.1009201107.

Rannug, A., Rannug, U., Rosenkranz, H. S., Winqvist, L., Westerholm, R., Agurell, E., et al. (1987). Certain photooxidized derivatives of tryptophan bind with very high affinity to the Ah receptor and are likely to be endogenous signal substances. *The Journal of Biological Chemistry, 262*(32), 15422–15427.

Richman, L. K., Chiller, J. M., Brown, W. R., Hanson, D. G., & Vaz, N. M. (1978). Enterically induced immunologic tolerance. I. Induction of suppressor T lymphoyctes by intragastric administration of soluble proteins. *Journal of Immunology, 121*(6), 2429–2434.

Rivollier, A., He, J., Kole, A., Valatas, V., & Kelsall, B. L. (2012). Inflammation switches the differentiation program of Ly6Chi monocytes from antiinflammatory macrophages to inflammatory dendritic cells in the colon. *The Journal of Experimental Medicine, 209*(1), 139–155. doi:10.1084/jem.20101387.

Rubtsov, Y. P., Rasmussen, J. P., Chi, E. Y., Fontenot, J., Castelli, L., Ye, X., et al. (2008). Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity, 28*(4), 546–558. doi:10.1016/j.immuni.2008.02.017.

Rudra, D., deRoos, P., Chaudhry, A., Niec, R. E., Arvey, A., Samstein, R. M., et al. (2012). Transcription factor Foxp3 and its protein partners form a complex regulatory network. *Nature Immunology, 13*(10), 1010–1019. doi:10.1038/ni.2402.

Sakaguchi, S. (2005). Naturally arising Foxp3-expressing CD25+ CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nature Immunology, 6*(4), 345–352. doi:10.1038/ni1178.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., & Toda, M. (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *Journal of Immunology, 155*(3), 1151–1164.

Satpathy, A. T., Briseno, C. G., Lee, J. S., Ng, D., Manieri, N. A., Kc, W., et al. (2013). Notch2-dependent classical dendritic cells orchestrate intestinal immunity to attaching-and-effacing bacterial pathogens. *Nature Immunology, 14*(9), 937–948. doi:10.1038/ni.2679.

Schieferdecker, H. L., Ullrich, R., Hirseland, H., & Zeitz, M. (1992). T cell differentiation antigens on lymphocytes in the human intestinal lamina propria. *Journal of Immunology, 149*(8), 2816–2822.

Schlitzer, A., McGovern, N., Teo, P., Zelante, T., Atarashi, K., Low, D., et al. (2013). IRF4 transcription factor-dependent CD11b+ dendritic cells in human and mouse control mucosal IL-17 cytokine responses. *Immunity, 38*(5), 970–983. doi:10.1016/j.immuni.2013.04.011.

Schulz, O., & Pabst, O. (2013). Antigen sampling in the small intestine. *Trends in Immunology, 34*(4), 155–161. doi:10.1016/j.it.2012.09.006.

Schulz, O., Jaensson, E., Persson, E. K., Liu, X., Worbs, T., Agace, W. W., et al. (2009). Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. *The Journal of Experimental Medicine, 206*(13), 3101–3114. doi:10.1084/jem.20091925.

Schwartz, R. H. (1989). Acquisition of immunologic self-tolerance. *Cell, 57*(7), 1073–1081.

Scott, C. L., Aumeunier, A. M., & Mowat, A. M. (2011). Intestinal CD103+ dendritic cells: Master regulators of tolerance? *Trends in Immunology, 32*(9), 412–419. doi:10.1016/j.it.2011.06.003.

Shan, M., Gentile, M., Yeiser, J. R., Walland, A. C., Bornstein, V. U., Chen, K., et al. (2013). Mucus enhances gut homeostasis and oral tolerance by delivering immunoregulatory signals. *Science, 342*(6157), 447–453. doi:10.1126/science.1237910.

Sharma, M. D., Baban, B., Chandler, P., Hou, D. Y., Singh, N., Yagita, H., et al. (2007). Plasmacytoid dendritic cells from mouse tumor-draining lymph nodes directly activate mature Tregs via indoleamine 2,3-dioxygenase. *The Journal of Clinical Investigation, 117*(9), 2570–2582. doi:10.1172/JCI31911.

Song, J., Clagett-Dame, M., Peterson, R. E., Hahn, M. E., Westler, W. M., Sicinski, R. R., et al. (2002). A ligand for the aryl hydrocarbon receptor isolated from lung. *Proceedings of the National Academy of Sciences of the United States of America, 99*(23), 14694–14699. doi:10.1073/pnas.232562899.

Spahn, T. W., Fontana, A., Faria, A. M., Slavin, A. J., Eugster, H. P., Zhang, X., et al. (2001). Induction of oral tolerance to cellular immune responses in the absence of Peyer's patches. *European Journal of Immunology, 31*(4), 1278–1287.

Spahn, T. W., Weiner, H. L., Rennert, P. D., Lugering, N., Fontana, A., Domschke, W., et al. (2002). Mesenteric lymph nodes are critical for the induction of high-dose oral tolerance in the absence of Peyer's patches. *European Journal of Immunology, 32*(4), 1109–1113.

Starr, T. K., Jameson, S. C., & Hogquist, K. A. (2003). Positive and negative selection of T cells. *Annual Review of Immunology, 21,* 139–176. doi:10.1146/annurev.immunol.21.120601.141107.

Stockinger, B., Di Meglio, P., Gialitakis, M., & Duarte, J. H. (2014). The aryl hydrocarbon receptor: Multitasking in the immune system. *Annual Review of Immunology, 32,* 403–432. doi:10.1146/annurev-immunol-032713-120245.

Sugimoto, N., Oida, T., Hirota, K., Nakamura, K., Nomura, T., Uchiyama, T., et al. (2006). Foxp3-dependent and -independent molecules specific for CD25+ CD4+ natural regulatory T cells revealed by DNA microarray analysis. *International Immunology, 18*(8), 1197–1209. doi:10.1093/intimm/dxl060.

Sun, C. M., Hall, J. A., Blank, R. B., Bouladoux, N., Oukka, M., Mora, J. R., et al. (2007). Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *The Journal of Experimental Medicine, 204*(8), 1775–1785. doi:10.1084/jem.20070602.

Sundrud, M. S., Koralov, S. B., Feuerer, M., Calado, D. P., Kozhaya, A. E., Rhule-Smith, A., et al. (2009). Halofuginone inhibits TH17 cell differentiation by activating the amino acid starvation response. *Science, 324*(5932), 1334–1338. doi:10.1126/science.1172638.

Szatmari, I., Pap, A., Ruhl, R., Ma, J. X., Illarionov, P. A., Besra, G. S., et al. (2006). PPARgamma controls CD1d expression by turning on retinoic acid synthesis in developing human dendritic cells. *The Journal of Experimental Medicine, 203*(10), 2351–2362. doi:10.1084/jem.20060141.

Thornton, A. M., Korty, P. E., Tran, D. Q., Wohlfert, E. A., Murray, P. E., Belkaid, Y., et al. (2010). Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *Journal of Immunology, 184*(7), 3433–3441. doi:10.4049/jimmunol.0904028.

Travis, M. A., Reizis, B., Melton, A. C., Masteller, E., Tang, Q., Proctor, J. M., et al. (2007). Loss of integrin alpha(v)beta8 on dendritic cells causes autoimmunity and colitis in mice. *Nature, 449*(7160), 361–365. doi:10.1038/nature06110.

Tsitoura, D. C., DeKruyff, R. H., Lamb, J. R., & Umetsu, D. T. (1999). Intranasal exposure to protein antigen induces immunological tolerance mediated by functionally disabled CD4+ T cells. *Journal of Immunology, 163*(5), 2592–2600.

Varol, C., Vallon-Eberhard, A., Elinav, E., Aychek, T., Shapira, Y., Luche, H., et al. (2009). Intestinal lamina propria dendritic cell subsets have different origin and functions. *Immunity, 31*(3), 502–512.

Veldhoen, M., Hocking, R. J., Atkins, C. J., Locksley, R. M., & Stockinger, B. (2006). TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity, 24*(2), 179–189. doi:10.1016/j.immuni.2006.01.001.

Verhasselt, V. (2010). Oral tolerance in neonates: From basics to potential prevention of allergic disease. *Mucosal Immunology, 3*(4), 326–333. doi:10.1038/mi.2010.25.

Vignali, D. A., Collison, L. W., & Workman, C. J. (2008). How regulatory T cells work. *Nature Reviews Immunology, 8*(7), 523–532. doi:10.1038/nri2343.

Viney, J. L., Mowat, A. M., O'Malley, J. M., Williamson, E., & Fanger, N. A. (1998). Expanding dendritic cells in vivo enhances the induction of oral tolerance. *Journal of Immunology, 160*(12), 5815–5825.

Wakim, L. M., & Bevan, M. J. (2011). Cross-dressed dendritic cells drive memory CD8+ T-cell activation after viral infection. *Nature, 471*(7340), 629–632. doi:10.1038/nature09863.

Weiner, H. L., da Cunha, A. P., Quintana, F., & Wu, H. (2011). Oral tolerance. *Immunological Reviews, 241*(1), 241–259. doi:10.1111/j.1600-065X.2011.01017.x.

Weiss, J. M., Bilate, A. M., Gobert, M., Ding, Y., Curotto de Lafaille, M. A., Parkhurst, C. N., et al. (2012). Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not mucosa-generated induced Foxp3+ T reg cells. *The Journal of Experimental Medicine, 209*(10):1723–1742, S1721. doi:10.1084/jem.20120914.

Welty, N. E., Staley, C., Ghilardi, N., Sadowsky, M. J., Igyarto, B. Z., & Kaplan, D. H. (2013). Intestinal lamina propria dendritic cells maintain T cell homeostasis but do not affect commensalism. *The Journal of Experimental Medicine, 210*(10), 2011–2024. doi:10.1084/jem.20130728.

Wildin, R. S., Ramsdell, F., Peake, J., Faravelli, F., Casanova, J. L., Buist, N., et al. (2001). X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nature Genetics, 27*(1), 18–20. doi:10.1038/83707.

Wohlfert, E. A., Grainger, J. R., Bouladoux, N., Konkel, J. E., Oldenhove, G., Ribeiro, C. H., et al. (2011). GATA3 controls Foxp3(+) regulatory T cell fate during inflammation in mice. *The Journal of Clinical Investigation, 121*(11), 4503–4515. doi:10.1172/JCI57456.

Worbs, T., Bode, U., Yan, S., Hoffmann, M. W., Hintzen, G., Bernhardt, G., et al. (2006). Oral tolerance originates in the intestinal immune system and relies on antigen carriage by dendritic cells. *The Journal of Experimental Medicine, 203*(3), 519–527.

Worthington, J. J., Czajkowska, B. I., Melton, A. C., & Travis, M. A. (2011). Intestinal dendritic cells specialize to activate transforming growth factor-beta and induce Foxp3+ regulatory T cells via integrin alphavbeta8. *Gastroenterology, 141*(5), 1802–1812. doi:10.1053/j.gastro.2011.06.057.

Yadav, M., Louvet, C., Davini, D., Gardner, J. M., Martinez-Llordella, M., Bailey-Bucktrout, S., et al. (2012). Neuropilin-1 distinguishes natural and inducible regulatory T cells among regulatory T cell subsets in vivo. *The Journal of Experimental Medicine, 209*(10):1713–1722, S1711–1719. doi:10.1084/jem.20120822.

Yokota, A., Takeuchi, H., Maeda, N., Ohoka, Y., Kato, C., Song, S. Y., et al. (2009). GM-CSF and IL-4 synergistically trigger dendritic cells to acquire retinoic acid-producing capacity. *International Immunology, 21*(4), 361–377. doi:10.1093/intimm/dxp003.

Zelante, T., Iannitti, R. G., Cunha, C., De Luca, A., Giovannini, G., Pieraccini, G., et al. (2013). Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22. *Immunity, 39*(2), 372–385. doi:10.1016/j.immuni.2013.08.003.

Zhang, X., Izikson, L., Liu, L., & Weiner, H. L. (2001). Activation of CD25(+)CD4(+) regulatory T cells by oral antigen administration. *Journal of Immunology, 167*(8), 4245–4253.

Zheng, Y., Chaudhry, A., Kas, A., deRoos, P., Kim, J. M., Chu, T. T., et al. (2009). Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. *Nature, 458*(7236), 351–356. doi:10.1038/nature07674.

Zheng, Y., Josefowicz, S., Chaudhry, A., Peng, X. P., Forbush, K., & Rudensky, A. Y. (2010). Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature, 463*(7282), 808–812. doi:10.1038/nature08750.

Zhou, Y., Kawasaki, H., Hsu, S. C., Lee, R. T., Yao, X., Plunkett, B., et al. (2010). Oral tolerance to food-induced systemic anaphylaxis mediated by the C-type lectin SIGNR1. *Nature Medicine, 16*(10), 1128–1133. doi:10.1038/nm.2201.

Chapter 9
The Role of p110δ in the Development and Activation of B Lymphocytes

Rebecca Newman and Martin Turner

Abstract The phosphatidylinositol-3-kinase (PI3K) pathway has an essential role in signal transduction, where it is required for a number of different cellular processes including proliferation, differentiation, development, migration and growth. In the immune system, PI3K regulates inflammation by controlling the activation and recruitment of leukocytes. The generation of conditional knockout mice has allowed the study of PI3K isoforms specifically in B and T lymphocytes, and demonstrates the importance of intact signalling in their development and function. PI3K signalling must be tightly regulated in lymphocytes as excessive PI3K can lead to autoimmunity, immunodeficiency or cancer, whilst diminished signalling can result in developmental defects and immunodeficiency. Recent advances in the understanding of PI3K signalling have hastened the application of isoform-specific PI3K inhibitors, which are currently undergoing clinical trials. This review will focus on the p110δ catalytic subunit of the class 1A family of PI3K, and its role in the development and activation of B lymphocytes through various downstream effectors.

Keywords PI3K · p110δ · B cells · AKT

PI3K in Lymphocytes

There is now considerable evidence to show that the PI3K pathway is essential for the development, activation and function of B lymphocytes. Indeed inactivation of the PI3K pathway results in the absence of a number of mature B cell subsets, indicating the requirement for this pathway for the differentiation and survival of B cells (Clayton et al. 2002; Okkenhaug et al. 2002; Henley et al. 2008; Kovesdi

R. Newman (✉) · M. Turner
Laboratory of Lymphocyte Signalling and Development, The Babraham Institute, Babraham Research Campus, Babraham CB22 3AT, UK
e-mail: rebecca.newman@babraham.ac.uk

M. Turner
e-mail: martin.turner@babraham.ac.uk

S. P. Schoenberger et al. (eds.), *Crossroads Between Innate and Adaptive Immunity V,*
Advances in Experimental Medicine and Biology 850, DOI 10.1007/978-3-319-15774-0_9

et al. 2010; Ramadani et al. 2010; Suzuki et al. 1999; Fruman 1999). Mature B cell survival relies on tonic signalling through the B cell antigen receptor (BCR), which is dependent on the PI3K pathway (Okkenhaug and Fruman 2010; Lam et al. 1997; Tze et al. 2005; Srinivasan et al. 2009). Forced overexpression of PI3K promotes mature B cell survival in the absence of BCR expression (Srinivasan et al. 2009). A number of studies have also indicated that functional PI3K is required for the responses of mature B cells to thymus-independent (TI) and thymus-dependent (TD) antigens (Clayton et al. 2002; Durand et al. 2009; Okkenhaug et al. 2002; Okkenhaug and Vanhaesebroeck 2003). Whilst PI3K is essential for B cells, signalling through this pathway must be tightly regulated. Several B cell malignancies show evidence of PI3K deregulation, and may be dependent upon this pathway for survival and retention within lymphoid tissues (Ringshausen et al. 2002). A key example of this is chronic lymphocytic leukaemia (CLL), where PI3K activity is significantly increased compared to normal B cells (Herman et al. 2010; Ringshausen et al. 2002). The recently described activated PI3K-δ syndrome (APDS) results from a dominant gain-of-function mutation (E1021K) in the p110δ protein (Angulo et al. 2013). These patients have increased recirculating transitional cells, which may reflect a developmental block prior to the mature B cell stage, or mature B cell survival defects, and impaired antibody responses (Angulo et al. 2013). T cell development in the thymus appears relatively normal in the absence of both p85α regulatory subunit of PI3K and when p110δ is deleted or inactive (Suzuki et al. 1999; Fruman 1999; Okkenhaug and Fruman 2010; Okkenhaug et al. 2002). However, in mice with a catalytic inactivation of p110δ, peripheral T cells are reduced in number, and maintain a 'naïve' phenotype (Okkenhaug et al. 2002). As such, PI3K signalling is required for the maturation and survival of peripheral T cells (Okkenhaug and Fruman 2010). Like B cells, the levels of PI3K signalling must be tightly controlled in T cells (Lucas et al. 2014). T cells from APDS patients are prone to activation-induced cell death, prior to any substantial cytokine responses (Angulo et al. 2013). This cell death could be attributable to an increase in TCR signal strength due to upregulation of PI3K signalling. Another recent paper details a separate group of patients with gain-of-function-mutations in *PIK3CD*, the gene encoding p110δ, who have acute impairments in their development of functional memory B and T cells (Lucas et al. 2014). Overall this demonstrates a dose-dependent necessity for PI3K signalling in B and T lymphocytes.

Introduction to PI3K

Class 1 phosphatidylinositol-3-kinases (PI3K) comprise an 110kDa catalytic subunit which forms a heterodimer with a family of tightly associated adaptor proteins that regulate subcellular localisation and enzymatic activity. PI3Ks are lipid kinases which phosphorylate phosphoinositides at the D-3 position of the inositol ring, leading to the production of numerous products including the second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 can then act as a

docking platform for various pleckstrin-homology-domain-containing proteins including AKT, Bruton's tyrosine kinase (Btk), Phospholipase C (PLC)γ2, Tec kinases, integrin-linked kinase, Rac guanine nucleotide exchange factors (P-REX) and phosphoinositide-dependent kinase (PDK). This initiates various downstream signalling events important for proliferation, differentiation and survival (Rommel et al. 2007; Fruman and Bismuth 2009). This pathway is negatively regulated by PTEN which dephosphorylates PIP3 at the 3' position and SH2 containing inositol phosphatase (SHIP) which dephosphorylates at the 5' position generating phosphatidylinositol-3,4-bisphosphate, which is also a second messenger. Deletion of PTEN at the pro-B cell stage blocks B cell development, demonstrating that levels of PI3K need to be tightly regulated for B cell development (Alkhatib et al. 2012). In addition to its role as a lipid phosphatase, PTEN has also been shown to exhibit protein phosphatase activity (Tibarewal et al. 2012; Leslie et al. 2009). PTEN's auto-dephosphorylation at threonine 366 is thought to be important for regulating its lipid phosphatase activity (Tibarewal et al. 2012). Further evidence suggests a PI3K-independent role for PTEN as a tumour suppressor, which relies on its nuclear compartmentalisation (Song et al. 2011; Shen et al. 2007). This function for PTEN is independent of its phosphatase activity, and instead relies on the role of PTEN in maintaining genome stability, and its interactions with the APC-CDH1 tumour suppressive complex (Shen et al. 2007; Song et al. 2011).

The class 1 PI3K are further subdivided into class 1A which consists of the p110α, p110β and p110δ catalytic subunits, whilst p110γ constitutes class 1B. Whilst p110δ is highly expressed in haematopoietic cells, its expression is not limited to the cells of the immune system. Indeed expression of p110δ has been found in transformed epithelial cells, and in endothelial cells, p110δ has been shown to be important for the expression of cell adhesion molecules during airway inflammation, (Lee et al. 2006) and recruitment of neutrophils into inflamed tissues (Puri et al. 2004). Moreover, using a lacZ reporter knocked into the mouse *pik3cd* locus expression of p110δ was found to be highly enriched in the nervous system (Eickholt et al. 2007). This is important to consider when studying $p110\delta^{-/-}$ mice, or the kinase-dead D910A p110δ mutants (Okkenhaug et al. 2002), in which PI3K deficiencies are not restricted to immune cell subsets. Despite this, the majority of p110δ research has been focused on its role in immune cell biology (Fruman and Bismuth 2009). The generation of conditional knockout mice is therefore important to allow the role of p110δ in immune cells to be studied specifically.

P110δ in B Cells

P110δ has been shown to be an essential, non-redundant regulator of B cell development (Clayton et al. 2002; Henley et al. 2008; Kovesdi et al. 2010; Okkenhaug et al. 2002; Ramadani et al. 2010). In p110δ null or catalytically inactive transgenic mice, there are defects in B cell receptor (BCR) signal transduction, basal immunoglobulin production and the loss of marginal zone (MZ) and B1 B cells (Okkenhaug

et al. 2002; Clayton et al. 2002; Kovesdi et al. 2010; Jou et al. 2002; Ramadani et al. 2010). This has been shown to be a defect intrinsic to B cells. Conversely, deficiency of phosphatase and tensin homologue (PTEN), which negatively regulates the PI3K pathway by dephosphorylating PIP3, results in the expansion of the MZ B cell subset (Anzelon et al. 2003; Janas et al. 2008). These B cell specific defects have captured the attention of clinicians, with the hope that therapeutic targeting of the p110δ catalytic subunit be cytotoxic for self-reactive or malignant B cells, with minimal toxicity for other immune cell types (Herman et al. 2010; Lannutti et al. 2011). P110δ specific inhibitors are of particular interest in the treatment of the B cell malignancy chronic lymphocytic leukaemia (CLL). The promising p110δ-specific inhibitor GS1101 (formerly CAL-101) sold by Gilead Sciences, which is currently undergoing phase 3 clinical trials, has been found to be toxic for CLL cells, whilst NK and T cells remain relatively unaffected (Herman et al. 2010; Lannutti et al. 2011; Vanhaesebroeck and Khwaja 2014).

BCR cross-linking results in the phosphorylation of tyrosine residues located within immunoreceptor tyrosine-based activation motifs (ITAMs) of the BCR subunits Igα and Igβ (CD79α and CD79β respectively) (Kurosaki 1997). The BCR has no intrinsic protein tyrosine kinase (PTK) activity, and it utilises cytoplasmic PTKs, including Syk and the Src family kinases such as Lyn and Btk for signal transduction. These promote PI3K activation by phosphorylation of tyrosines that act as docking sites for the PI3K regulatory subunits (Reth and Wienands 1997; Kurosaki 1997; Turner et al. 1997a; Moon et al. 2005). In this way, PI3K acts as a point of convergence for signalling by distinct PTKs. Activated PTKs phosphorylate co-receptors and adaptor proteins, resulting in the recruitment of effector molecules including PI3K (Aiba et al. 2008). Btk is phosphorylated on tyrosine 551 by Src and Syk PTKs following BCR cross-linking (Rawlings et al. 1996; Baba et al. 2001; Clayton et al. 2002). Upon activation, Btk subsequently becomes autophosphorylated on tyrosine 223. In p110δ deficient mice, there was no detectable phosphorylation at either tyrosine residue, indicating that p110δ is required for Btk activation (Clayton et al. 2002).

In addition to its activation downstream of BCR signalling, the PI3K pathway plays a role in signal transduction by a number of other receptors. CD19 is a BCR co-receptor, which is important for regulating BCR signalling thresholds (Inaoki et al. 1997; Tedder et al. 1997). Ligation of membrane IgM results in the phosphorylation of CD19 on multiple tyrosine residues. Of particular importance are two YXXM motifs in the CD19 cytoplasmic tail, which act as binding sites for the p85α regulatory subunit of PI3K (Tuveson et al. 1993; Aiba et al. 2008). A second PI3K adapter molecule BCAP undergoes tyrosine phosphorylation by Syk and Btk upon BCR-crosslinking, enabling it to bind p85α through its four YXXM motifs (Okada et al. 2000; Aiba et al. 2008). BCAP and CD19 have been shown to have overlapping functions in the activation of PI3K mediated by BCR signalling (Aiba et al. 2008). BCAP has also been implicated in the activation of the PI3K/AKT pathway downstream of Toll-like receptor (TLR) signalling, following the discovery that it contains a functional Toll-IL-1 receptor (TIR) domain (Troutman et al.

2012). P110δ has been shown to have a role in determining the differential cytokine responses of B cells induced by TLR ligands, as IL-10 and IL-6 production are partially dependent on p110δ signalling (Dil and Marshall 2009). Furthermore, B cell proliferation is impaired in response to LPS stimulation in mice lacking p110δ (Okkenhaug et al. 2002; Dil and Marshall 2009).

PI3K is activated downstream of most chemokine receptors on T cells (Ward 2004; Patrussi and Baldari 2008). Unlike T lymphocytes, the molecules involved in signal transduction of homing receptors in B cells remain relatively unknown, however PI3K signalling has been shown to be important for CXCR4-mediated activation of Rho-GTPases, and is thought to play a similar role downstream of CXCR5 (de Gorter et al. 2007; Patrussi et al. 2014). Indeed, p110δ has been shown to be required for optimal B cell chemotaxis to CXCL13, a ligand for CXCR5, and for B cell homing to peyers patches, mesenteric lymph nodes and splenic white pulp cords (Reif et al. 2004). PI3K can also function downstream of integrins, and as such have an important role in the migration and retention of B cells in tissues (Pillai and Cariappa 2009). In addition to chemokine signals, B cell positioning in the marginal zone of the spleen is dependent upon sphingosine-1-phosphate (S1P) receptor $S1P_1$ (Cinamon et al. 2004). Marginal zone B cells that lack $S1P_1$ relocate to the splenic follicles (Cinamon et al. 2004, 2008). In T cells, $S1P_1$ gene expression is regulated by Krüppel-like factor 2 (KLF2), which is inhibited by PI3K/AKT signalling (Bai et al. 2007; Skon et al. 2013). Winkelmann et al. suggest that this mechanism does not exist in B cells, as $S1P_1$ expression on B cells is only slightly affected in KLF2 deficient mice (Winkelmann et al. 2011). Instead KLF2 is responsible for controlling the expression of CD62L and $\alpha_4\beta_7$ integrin, which are required for proper B cell homing (Winkelmann et al. 2011).

P110δ may play a role in cytokine-dependent survival of B cells (Bilancio et al. 2006; Henley et al. 2008). B cell-activating factor of the TNF family (BAFF) is required for the maintenance of mature B cell subsets (Schiemann et al. 2001; Batten et al. 2000; Mackay et al. 1999). BAFF or BAFF-R deficient mice exhibit a developmental block at the T1 stage, and have highly reduced marginal zone and follicular B cell compartments due to failure to survive (Schiemann et al. 2001; Miller and Hayes 1991; Thompson et al. 2001). Incubation of mature B cells with BAFF induces phosphorylation of AKT and upregulation of genes required for glycolysis and cell cycle progression (Patke et al. 2006; Woodland et al. 2008). It was later shown that PI3K p110δ signalling is required to mediate cell survival, growth and proliferation downstream of BAFF signalling, and that it may be recruited to the BAFF-R by CD19 (Patke et al. 2006; Henley et al. 2008; Woodland et al. 2008; Jellusova et al. 2013). Constitutive activation of the PI3K pathway by B cell-specific deletion of PTEN restores surface CD21/35 expression, but not CD23, despite this, overall splenic architecture is rescued by PTEN inactivation (Jellusova et al. 2013). However, while BAFF is not required for the B1 lineage, p110δ is critical for the development or survival of B1 B cells providing further evidence for a broader role of this kinase in B cell function.

AKT Dependent Effectors

Activation of the serine/threonine kinase AKT downstream of PI3K signalling is well characterised, however the roles of many AKT substrates have not been well defined in B cell biology (Chen et al. 2010). The evolutionarily conserved Foxo subfamily of forkhead box transcription factors, comprising Foxo1, Foxo3 and Foxo4, are negatively regulated by PI3K-AKT signalling (Chen et al. 2010; Dengler et al. 2008; Calnan and Brunet 2008). Phosphorylation of Foxo proteins at three conserved sites by AKT results in cytoplasmic sequestration, preventing their activity as transcription factors (Calnan and Brunet 2008). Foxo1 is of particular interest in B cell biology, as it has been shown to be essential at many stages of B cell differentiation (Dengler et al. 2008). Early deletion of Foxo1, using mb1-cre to delete at the early pro-B cell stage, results in a developmental block at the pro-B cell stage (Dengler et al. 2008), whilst deletion at a later stage results in mature B cell defects including decreased numbers of follicular B cells and a proportionally expanded marginal zone B cell compartment (Chen et al. 2010). These effects can be partially explained by considering the transcriptional targets of Foxo1 which include *Il7ra, Rag1* and *Rag2*, consistent with the phenotype of p110δ deficient mice (Llorian et al. 2007; Dengler et al. 2008; Amin and Schlissel 2008). More recently it has been shown that Foxo1 is crucial for the proper mRNA splicing of the Krüppel-like zinc finger transcription factor Ikaros, which is required for Rag expression and *IgH* gene recombination (Alkhatib et al. 2012). This suggests a mechanism whereby tonic pre-BCR signalling activates PI3K; suppressing Foxo mediated activation of Rag genes (Verkoczy et al. 2007). Absence of another member of the forkhead family of transcription factors, Foxp1 has similar developmental defects to the Foxo1 deficient mice, suggesting they share transcriptional targets (Hu et al. 2006). Less well characterised is the role of Foxo3a in B cells. Overexpression of Foxo3a in primary B cells causes cell cycle arrest, or delay in cell cycle progression (Yusuf et al. 2004; Dijkers et al. 2002). Furthermore, mutation of serine and threonine residues in the Foxo3a protein, which renders it AKT independent, increases its capacity to block cell cycle progression and induces cell death (Yusuf et al. 2004). This demonstrates the role of PI3K effectors for normal B cell development proliferation and survival.

In addition to transcription factor regulation, the PI3K pathway may also control gene expression through downstream effectors such as RNA binding proteins (RBP). ZFP36l1 (Tis11b, BRF1) are phosphorylated by AKT leading to its sequestration by the 14-3-3 proteins, and subsequent degradation, preventing its ability to destabilise target mRNAs (Benjamin et al. 2006; Schmidlin et al. 2004). ZFP36l1 belongs to a family of conserved zinc-finger containing RBP consisting of ZFP36, ZFP36l1 and ZFP36l2, for which important roles in the immune system are emerging (Hodson et al. 2010; Turner and Hodson 2012; Brooks and Blackshear 2013; Taylor et al. 1996) (R. N. unpublished data).

Notch proteins are essential in a number of cell fate decisions, differentiation and developmental processes. Whilst Notch-1 is critical for the development and

differentiation of T cells, Notch-2 is indispensable for B cell development (Saito et al. 2003; Gibb et al. 2010; Osborne and Minter 2007). The PI3K pathway has been shown to be important for Notch-1 signal transduction, via AKT dependent signalling (McKenzie et al. 2006). PI3K potentiates Notch signals, in part, through AKT mediated phosphorylation of Glycogen Synthase Kinase-3β (GSK3β), which prevents inhibitory phosphorylation of intracellular Notch by GSK3β (McKenzie et al. 2006; Cross et al. 1995). GSK3β has been shown to bind Notch-2 both in vivo and in vitro (Espinosa et al. 2003).It is therefore likely that p110δ is involved in the regulation of Notch-2 signalling in mature B cells.

PI3K/AKT signalling is important for both cellular proliferation, through its regulation of cell cycle progression and for survival (Choudhury et al. 1997; Muise-Helmericks et al. 1998; Chang et al. 2003). P110δ is required for the transcriptional activation of cyclin D_2, which is essential for mediating cell cycle entry in B cells activated by BCR cross-linking (Glassford et al. 2005). Thus, p110δ deficient B cells can enter the early G_1 phase of the cell cycle, but fail to progress to S phase (Glassford et al. 2005). Btk and the p85α subunit of PI3K, which further constitute the BCR signalosome, are also required for cyclin D_2 expression and S phase entry downstream of BCR stimulation (Glassford et al. 2003). Concurrent with this, p110δ $^{D910A/D910A}$ mice which have a p110δ homozygous inactivation fail to proliferate in vitro following BCR stimulation (Okkenhaug et al. 2002). The requirement for p110δ appears to be dose dependent, as p110δ $^{D910A/+}$ mice have an intermediate defect in proliferation, which is not compensated for by other p110 subunits (Bilancio et al. 2006). GSK3 is constitutively active and phosphorylates a number of proteins important for cell cycle progression including cyclin D_1 and c-Myc promoting their degradation (Cantley 2002; Chang et al. 2003). Cyclin D_1 is required to initiate phosphorylation of the retinoblastoma protein (Rb), which facilitates subsequent phosphorylation by cyclin E and Cdk2 (Hatakeyama et al. 1994; Liang and Slingerland 2003). When p110δ is genetically or pharmacologically inactivated, BCR-mediated upregulation of cyclin D_2, A and E does not occur (Bilancio et al. 2006). This correlated with a lack of Rb phosphorylation by CDK-4 and -6 during early G_1, indicating the mechanistic basis for a lack of cell cycle progression in the absence of functional p110δ (Bilancio et al. 2006). C-Myc is important for inhibiting the expression of a number of negative cell cycle regulators such as p27 and p21 (Liang and Slingerland 2003; Vlach et al. 1996; Gartel and Shchors 2003). P27 degradation may also be regulated through a component of the SCF^{SKP2} ubiquitin ligase complex, SKP2, which is positively regulated by the PI3K pathway (Mamillapalli et al. 2001; Liang and Slingerland 2003). Mice with inactive p110δ fail to downregulate p27 following BCR cross-linking (Bilancio et al. 2006). Evidence suggests that p21 may be directly phosphorylated by AKT, resulting in its cytoplasmic accumulation, preventing interaction with cyclin-Cdk targets which reside in the nucleus (Zhou et al. 2001). Thus PI3K signalling is essential for the G_1/S transition of the cell cycle (Brennan et al. 1997). In addition to positive regulation of cell cycle progression, the PI3K pathway is important for negatively regulating apoptosis. AKT phosphorylation of the proapoptotic protein Bad, leads to its sequestration and inhibition by the 14-3-3 proteins (Brunet et al. 2001; Datta et al. 1997). This prevents the interac-

tion of Bad with the anti-apoptotic proteins Bcl-2 and Bcl-X_L thus promoting cell survival (Brunet et al. 2001; Cantley 2002; Chang et al. 2003).

AKT Independent Effectors

Whilst the role of AKT signalling has been extensively characterised, the role of PI3K signalling in the regulation of other signalling molecules is sometimes overlooked. Btk is a TEC family kinase expressed in B cells, which contributes to the activation of phospholipase C-γ (PLC-γ) through tyrosine phosphorylation (Scharenberg and Kinet 1998). PLC-γ catalyses the conversion of phosphatidylinositol-4,5-bisphosphate (PIP2) to form the second messengers diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3). IP3 then plays an important role in the mobilisation of intracellular calcium stores followed by extracellular calcium influx into the cytosol to produce sustained calcium signalling (Scharenberg and Kinet 1998). P110δ is required for efficient Btk phosphorylation thus indicating an important role for PI3K in maintaining calcium signalling pathways (Clayton et al. 2002). Importantly, Btk contains a PH domain which enables it to selectively bind PIP3 (Salim et al. 1996). Disruption of PIP3 binding, leads to loss of Btk function, and inhibition of the PI3K pathway prevents membrane recruitment of Btk (Rawlings et al. 1993; Krahn et al. 2004). Intact NFκB signalling is essential for BCR-mediated survival of peripheral B cell subsets (Bendall et al. 1999). NFκB survival signals are linked to BCR signalling through activated Btk (Petro et al. 2000; Petro and Khan 2001; Bajpai et al. 2000). In addition to providing maintenance signals downstream of the BCR, Btk is required for the maturation of immature peripheral B cells (Rawlings et al. 1993; Su et al. 2002). X-linked immunodeficiency (XID) mice, which have a mutated form of Btk have reduced numbers of follicular B cells, and peritoneal B1 cells (Rawlings et al. 1993). As such, Btk has an essential role both in differentiation and maintenance of mature B cell subsets (Su et al. 2004).

The Vav family of proteins comprises Vav-1, Vav-2 and Vav-3, which act as guanine nucleotide exchange factors (GEFs) for the Rho family of guanosine triphosphatases (GTPases) (Turner et al. 1997b; Turner and Billadeau 2002). All three proteins are phosphorylated following BCR crosslinking, and have been shown to be important for normal B cell development (Turner 2002; Doody et al. 2001; Turner and Billadeau 2002; Vigorito et al. 2005). Vav GEF activity has been shown to be both directly and indirectly regulated by PI3K signalling (Han et al. 1998). While Vav1 is required for normal B1 cell development, B cell subsets appear normal in mice lacking either Vav2 or Vav3 (Fujikawa et al. 2003; Doody et al. 2001). Mice lacking Vav1 and Vav2 have highly reduced numbers of mature B cells in the spleen, LN and bone marrow (Doody et al. 2001). Vav 1 and 2 double knock-out mice also have impaired BCR signalling, and T-independent and T-dependent antigen responses (Doody et al. 2001).Disruption of all three Vav family proteins results in a XID-like phenotype, where transitional B cell subsets are intact, but there is a developmental arrest at the T2 to mature B cell transition (Fujikawa et al. 2003;

Vigorito et al. 2005). Additionally, Vav deficient mature B cells have a survival defect, attributed to a requirement for Vav in maintaining NFκB survival signals following BCR cross-linking (Vigorito et al. 2005). Vav also plays a role in the activation of the PI3K pathway in B cells (Vigorito et al. 2004). This study also found that calcium flux elicited by high avidity crosslinking of membrane immunoglobulin had a PI3K-independent component, while membrane Ig/CD19 coligation was totally PI3K dependent.

Ras enzymes have intrinsic GTPase activity which enables them to act as molecular switches, dependent on whether they are bound to GDP or GTP. Inactive, GDP-bound Ras are activated following interaction with GEF proteins, which facilitate the exchange of GDP for the more abundant GTP (Lenzen et al. 1998). Ras is negatively regulated by GTPase activating proteins (GAPs) which promote the GTPase activity of Ras, resulting in GTP hydrolysis (Ehrhardt et al. 2002). PI3K directly interacts with Ras, indicating that it plays a role in the organisation of the actin cytoskeleton (Rodriguez-Viciana et al. 1997; Downward 1997). Ras proteins have also been shown to have important roles in haematopoietic cell development and function (Ehrhardt et al. 2002). Indeed, a B cell developmental block occurs prior to formation of the pre-BCR in mice expressing a dominant-negative form of H-Ras (Iritani et al. 1997). Additionally, TC21 deficient mice have reduced numbers of B and T cells, with fewer MZ and follicular B cells (Delgado et al. 2009). The p110 subunits differ in their capacity to bind Ras, due to structural differences in their Ras-binding domains (RBD) (Amzel et al. 2008; Rodriguez-Viciana et al. 2004). While H-Ras, R-Ras, N-Ras, K-Ras, TC21 and R-Ras3 can potently activate p110α, only R-Ras and TC21 are capable of activating p110δ (Rodriguez-Viciana et al. 2004). Interestingly TC21 has recently been shown to mediate interactions between both p110δ and p85α and the BCR, thus recruiting PI3K to the cell membrane (Delgado et al. 2009).Selectivity in Ras binding may account for some of the differences between p110δ and p110α in their recruitment, and/or activation downstream of BCR signalling (Ramadani et al. 2010).

Concluding Remarks

PI3K has been shown to have an important role in the development, function and activation of lymphocytes. However, overexpression and/or hyperactivation, as well as a lack of PI3K, can have adverse effects on the cells of the immune system. PI3K p110δ plays a number of distinct roles in B and T cells, and these are effected through a range of interactor proteins. In addition to activation of the well characterised serine/threonine kinase AKT, PI3K signalling also leads to the activation of a number of other enzymes independently of AKT. As such it is required for a multitude of responses downstream of the B cell antigen receptor, chemokine receptors, cytokine receptors as well as BCR co-receptors, and many of these downstream pathways converge on one another. Thus research into the control of B cell responses by the PI3K pathway will be essential for therapeutic targeting studies.

Recently there have been huge clinical advances following on from the study of p110δ in the immune system. The success of p110δ inhibitors in treating the B cell malignancies CLL and indolent B cell non-Hodgkin's lymphomas are evident from the clinical trial data. Although PI3K is not mutationally activated in these cancers, p110δ inhibition prevents malignant B cells receiving survival signals from the cancer microenvironment and the BCR (Vanhaesebroeck and Khwaja 2014). It will, however, be important to consider the expression of p110δ outside of haematopoietic cells when continuing with clinical trials of these inhibitors, as this may be consequential for off-target side-effects. Additionally, p110δ expression outside of the immune system may indicate new avenues for exploiting p110δ inhibitor treatment. For example, p110δ has been shown to be abundantly expressed in primary and cancer cells of breast and melanocyte origin, and it is important for the chemotaxis of breast cancer cells in response to EGF (Sawyer et al. 2003; Ho et al. 1997). Thus p110δ inhibitors may be a useful as anti-metastatic drugs in breast cancer and malignant melanoma (Sawyer et al. 2003).

Acknowledgements Work in the authors' laboratory is funded by the Biotechnology and Biological Sciences Research Council. R. N is supported by a CASE studentship in partnership with GSK.

References

Aiba, Y., Kameyama, M., Yamazaki, T., Tedder, T. F., & Kurosaki, T. (2008). Regulation of B-cell development by BCAP and CD19 through their binding to phosphoinositide 3-kinase. *Blood, 111*(3), 1497–1503. doi:10.1182/blood-2007-08-109769.

Alkhatib, A., Werner, M., Hug, E., Herzog, S., Eschbach, C., Faraidun, H., et al. (2012). FoxO1 induces Ikaros splicing to promote immunoglobulin gene recombination. *The Journal of Experimental Medicine, 209*(2), 395–406. doi:10.1084/jem.20110216.

Amin, R. H., & Schlissel, M. S. (2008). Foxo1 directly regulates the transcription of recombination-activating genes during B cell development. *Nature Immunology, 9*(6), 613–622. doi:10.1038/ni.1612.

Amzel, L. M., Huang, C. H., Mandelker, D., Lengauer, C., Gabelli, S. B., & Vogelstein, B. (2008). Structural comparisons of class I phosphoinositide 3-kinases. *Nature Reviews Cancer, 8*(9), 665–669. doi:10.1038/nrc2443.

Angulo, I., Vadas, O., Garcon, F., Banham-Hall, E., Plagnol, V., Leahy, T. R., et al. (2013). Phosphoinositide 3-kinase delta gene mutation predisposes to respiratory infection and airway damage. *Science, 342*(6160), 866–871. doi:10.1126/science.1243292.

Anzelon, A. N., Wu, H., & Rickert, R. C. (2003). Pten inactivation alters peripheral B lymphocyte fate and reconstitutes CD19 function. *Nature Immunology, 4*(3), 287–294. doi:10.1038/ni892.

Baba, Y., Hashimoto, S., Matsushita, M., Watanabe, D., Kishimoto, T., Kurosaki, T., et al. (2001). BLNK mediates Syk-dependent Btk activation. *Proceedings of the National Academy of Sciences of the United States of America, 98*(5), 2582–2586. doi:10.1073/pnas.051626198.

Bai, A., Hu, H., Yeung, M., & Chen, J. (2007). Kruppel-like factor 2 controls T cell trafficking by activating L-selectin (CD62L) and sphingosine-1-phosphate receptor 1 transcription. *Journal of Immunology, 178*(12), 7632–7639.

Bajpai, U. D., Zhang, K., Teutsch, M., Sen, R., & Wortis, H. H. (2000). Bruton's tyrosine kinase links the B cell receptor to nuclear factor kappaB activation. *The Journal of Experimental Medicine, 191*(10), 1735–1744.

Batten, M., Groom, J., Cachero, T. G., Qian, F., Schneider, P., Tschopp, J., et al. (2000). BAFF mediates survival of peripheral immature B lymphocytes. *The Journal of Experimental Medicine, 192*(10), 1453–1466.

Bendall, H. H., Sikes, M. L., Ballard, D. W., & Oltz, E. M. (1999). An intact NF-kappa B signaling pathway is required for maintenance of mature B cell subsets. *Molecular Immunology, 36*(3), 187–195.

Benjamin, D., Schmidlin, M., Min, L., Gross, B., & Moroni, C. (2006). BRF1 protein turnover and mRNA decay activity are regulated by protein kinase B at the same phosphorylation sites. *Molecular and Cellular Biology, 26*(24), 9497–9507. doi:10.1128/MCB.01099-06.

Bilancio, A., Okkenhaug, K., Camps, M., Emery, J. L., Ruckle, T., Rommel, C., et al. (2006). Key role of the p110delta isoform of PI3K in B-cell antigen and IL-4 receptor signaling: Comparative analysis of genetic and pharmacologic interference with p110delta function in B cells. *Blood, 107*(2), 642–650. doi:10.1182/blood-2005-07-3041.

Brennan, P., Babbage, J. W., Burgering, B. M., Groner, B., Reif, K., & Cantrell, D. A. (1997). Phosphatidylinositol 3-kinase couples the interleukin-2 receptor to the cell cycle regulator E2F. *Immunity, 7*(5), 679–689.

Brunet, A., Datta, S. R., & Greenberg, M. E. (2001). Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. *Current opinion in Neurobiology, 11*(3), 297–305.

Calnan, D. R., & Brunet, A. (2008). The FoxO code. *Oncogene, 27*(16), 2276–2288. doi:10.1038/onc.2008.21.

Cantley, L. C. (2002) The phosphoinositide 3-kinase pathway. *Science, 296*(5573), 1655–1657. doi:10.1126/science.296.5573.1655.

Chang, F., Lee, J. T., Navolanic, P. M., Steelman, L. S., Shelton, J. G., Blalock, W. L., et al. (2003). Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: A target for cancer chemotherapy. *Leukemia, 17*(3), 590–603. doi:10.1038/sj.leu.2402824.

Chen, J., Limon, J. J., Blanc, C., Peng, S. L., & Fruman, D. A. (2010). Foxo1 regulates marginal zone B-cell development. *European Journal of Immunology, 40*(7), 1890–1896. doi:10.1002/eji.200939817.

Choudhury, G. G., Karamitsos, C., Hernandez, J., Gentilini, A., Bardgette, J., & Abboud, H. E. (1997) PI-3-kinase and MAPK regulate mesangial cell proliferation and migration in response to PDGF. *The American Journal Of Physiology, 273*(6 Pt 2), F931–938.

Cinamon, G., Matloubian, M., Lesneski, M. J., Xu, Y., Low, C., Lu, T., et al. (2004). Sphingosine 1-phosphate receptor 1 promotes B cell localization in the splenic marginal zone. *Nature Immunology, 5*(7), 713–720. doi:10.1038/ni1083.

Cinamon, G., Zachariah, M. A., Lam, O. M., Foss, F. W. Jr., & Cyster, J. G. (2008). Follicular shuttling of marginal zone B cells facilitates antigen transport. *Nature Immunology, 9*(1), 54–62. doi:10.1038/ni1542.

Clayton, E., Bardi, G., Bell, S. E., Chantry, D., Downes, C. P., Gray, A., et al. (2002). A Crucial Role for the p110 Subunit of Phosphatidylinositol 3-Kinase in B Cell Development and Activation. *The Journal of Experimental Medicine, 196*(6), 753–763. doi:10.1084/jem.20020805.

Cross, D. A. E., Alessi, D. R., Cohen, P., Andjelkovich, M., & Hemmings, B. A. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature, 378*(6559), 785–789.

Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., et al. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell, 91*(2), 231–241.

de Gorter D. J., Beuling, E. A., Kersseboom, R., Middendorp, S., van Gils J. M., Hendriks, R. W., et al. (2007). Bruton's tyrosine kinase and phospholipase Cgamma2 mediate chemokine-controlled B cell migration and homing. *Immunity, 26*(1), 93–104. doi:10.1016/j.immuni.2006.11.012.

Delgado, P., Cubelos, B., Calleja, E., Martinez-Martin, N., Cipres, A., Merida, I., et al. (2009). Essential function for the GTPase TC21 in homeostatic antigen receptor signaling. *Nature Immunology, 10*(8), 880–888. doi:10.1038/ni.1749.

Dengler, H. S., Baracho, G. V., Omori, S. A., Bruckner, S., Arden, K. C., Castrillon, D. H., et al. (2008). Distinct functions for the transcription factor Foxo1 at various stages of B cell differentiation. *Nature Immunology, 9*(12), 1388–1398. doi:10.1038/ni.1667.

Dijkers, P. F., Birkenkamp, K. U., Lam, E. W., Thomas, N. S., Lammers, J. W., Koenderman, L., et al. (2002). FKHR-L1 can act as a critical effector of cell death induced by cytokine withdrawal: Protein kinase B-enhanced cell survival through maintenance of mitochondrial integrity. *The Journal of Cell Biology, 156*(3), 531–542. doi:10.1083/jcb.200108084.

Dil, N., & Marshall, A. J. (2009). Role of phosphoinositide 3-kinase p110 delta in TLR4- and TLR9-mediated B cell cytokine production and differentiation. *Molecular Immunology, 46*(10), 1970–1978. doi:10.1016/j.molimm.2009.03.010.

Doody, G. M., Bell, S. E., Vigorito, E., Clayton, E., McAdam, S., Tooze, R., et al. (2001). Signal transduction through Vav-2 participates in humoral immune responses and B cell maturation. *Nature Immunology, 2*(6), 542–547. doi:10.1038/88748.

Downward, J. (1997). Role of phosphoinositide-3-OH kinase in Ras signaling. *Advances in Second Messenger and Phosphoprotein Research, 31,* 1–10.

Durand, C. A., Hartvigsen, K., Fogelstrand, L., Kim, S., Iritani, S., Vanhaesebroeck, B., et al. (2009). Phosphoinositide 3-kinase p110 delta regulates natural antibody production, marginal zone and B-1 B cell function, and autoantibody responses. *Journal of Immunology, 183*(9), 5673–5684. doi:10.4049/jimmunol.0900432.

Ehrhardt, A., Ehrhardt, G. R., Guo, X., & Schrader, J. W. (2002). Ras and relatives–job sharing and networking keep an old family together. *Experimental Hematology, 30*(10), 1089–1106.

Eickholt, B. J., Ahmed, A. I., Davies, M., Papakonstanti, E. A., Pearce, W., Starkey, M. L., et al. (2007). Control of axonal growth and regeneration of sensory neurons by the p110delta PI 3-kinase. *PloS ONE, 2*(9), e869. doi:10.1371/journal.pone.0000869.

Espinosa, L., Ingles-Esteve, J., Aguilera, C., & Bigas, A. (2003). Phosphorylation by glycogen synthase kinase-3 beta down-regulates Notch activity, a link for Notch and Wnt pathways. *The Journal of Biological Chemistry, 278*(34), 32227–32235. doi:10.1074/jbc.M304001200.

Fruman, D. A. (1999) Impaired B cell development and proliferation in absence of phosphoinositide 3-kinase p85. *Science, 283*(5400), 393–397. doi:10.1126/science.283.5400.393.

Fruman, D. A., & Bismuth, G. (2009) Fine tuning the immune response with PI3K. *Immunological Reviews, 228*(1), 253–272. doi:10.1111/j.1600-065X.2008.00750.x.

Fujikawa, K., Miletic, A. V., Alt, F. W., Faccio, R., Brown, T., Hoog, J., et al. (2003). Vav1/2/3-null mice define an essential role for Vav family proteins in lymphocyte development and activation but a differential requirement in MAPK signaling in T and B cells. *The Journal of Experimental Medicine, 198*(10), 1595–1608. doi:10.1084/jem.20030874.

Gartel, A. L., & Shchors, K. (2003). Mechanisms of c-myc-mediated transcriptional repression of growth arrest genes. *Experimental Cell Research, 283*(1), 17–21. Doi 10.1016/S0014-4827(02)00020-4.

Gibb, D. R., Shikh M, E., Kang, D. J., Rowe, W. J., Sayed R, E., Cichy, J., et al. (2010). ADAM10 is essential for Notch2-dependent marginal zone B cell development and CD23 cleavage in vivo. *The Journal of Experimental Medicine, 207*(3), 623–635. doi:10.1084/jem.20091990.

Glassford, J., Soeiro, I., Skarell, S. M., Banerji, L., Holman, M., Klaus, G. G., et al. (2003). BCR targets cyclin D2 via Btk and the p85alpha subunit of PI3-K to induce cell cycle progression in primary mouse B cells. *Oncogene, 22*(15), 2248–2259. doi:10.1038/sj.onc.1206425.

Glassford, J., Vigorito, E., Soeiro, I., Madureira, P. A., Zoumpoulidou, G., Brosens, J. J., et al. (2005). Phosphatidylinositol 3-kinase is required for the transcriptional activation of cyclin D2 in BCR activated primary mouse B lymphocytes. *European Journal of Immunology, 35*(9), 2748–2761. doi:10.1002/eji.200425812.

Han, J., Luby-Phelps, K., Das, B., Shu, X., Xia, Y., Mosteller, R. D., et al. (1998). Role of substrates and products of PI 3-kinase in regulating activation of Rac-related guanosine triphosphatases by Vav. *Science, 279*(5350), 558–560.

Hatakeyama, M., Brill, J. A., Fink, G. R., & Weinberg, R. A. (1994). Collaboration of G1 cyclins in the functional inactivation of the retinoblastoma protein. *Genes and Development, 8*(15), 1759–1771.

Henley, T., Kovesdi, D., & Turner, M. (2008). B-cell responses to B-cell activation factor of the TNF family (BAFF) are impaired in the absence of PI3K delta. *European Journal of Immunology, 38*(12), 3543–3548. doi:10.1002/eji.200838618.

Herman, S. E., Gordon, A. L., Wagner, A. J., Heerema, N. A., Zhao, W., Flynn, J. M., et al. (2010). Phosphatidylinositol 3-kinase-delta inhibitor CAL-101 shows promising preclinical activity in chronic lymphocytic leukemia by antagonizing intrinsic and extrinsic cellular survival signals. *Blood, 116*(12), 2078–2088. doi:10.1182/blood-2010-02-271171.

Ho, L. K., Liu, D., Rozycka, M., Brown, R. A., & Fry, M. J. (1997). Identification of four novel human phosphoinositide 3-kinases defines a multi-isoform subfamily. *Biochemical and Biophysical Research Communications, 235*(1), 130–137. doi:10.1006/bbrc.1997.6747.

Hodson, D. J., Janas, M. L., Galloway, A., Bell, S. E., Andrews, S., Li, C. M., et al. (2010). Deletion of the RNA-binding proteins ZFP36L1 and ZFP36L2 leads to perturbed thymic development and T lymphoblastic leukemia. *Nature Immunology, 11*(8), 717–724. doi:10.1038/ni.1901.

Hu, H., Wang, B., Borde, M., Nardone, J., Maika, S., Allred, L., et al. (2006). Foxp1 is an essential transcriptional regulator of B cell development. *Nature Immunology, 7*(8), 819–826. doi:10.1038/ni1358.

Inaoki, M., Sato, S., Weintraub, B. C., Goodnow, C. C., & Tedder, T. F. (1997). CD19-regulated signaling thresholds control peripheral tolerance and autoantibody production in B lymphocytes. *The Journal of Experimental Medicine, 186*(11), 1923–1931.

Iritani, B. M., Forbush, K. A., Farrar, M. A., & Perlmutter, R. M. (1997). Control of B cell development by Ras-mediated activation of Raf. *The EMBO Journal, 16*(23), 7019–7031. doi:10.1093/emboj/16.23.7019.

Janas, M. L., Hodson, D., Stamataki, Z., Hill, S., Welch, K., Gambardella, L., et al. (2008). The effect of deleting p110delta on the phenotype and function of PTEN-deficient B cells. *Journal of Immunology, 180*(2), 739–746.

Jellusova, J., Miletic, A. V., Cato, M. H., Lin, W. W., Hu, Y., Bishop, G. A., et al. (2013). Context-specific BAFF-R signaling by the NF-kappaB and PI3K pathways. *Cell Reports, 5*(4), 1022–1035. doi:10.1016/j.celrep.2013.10.022.

Jou, S. T., Carpino, N., Takahashi, Y., Piekorz, R., Chao, J. R., Wang, D., et al. (2002). Essential, nonredundant role for the phosphoinositide 3-kinase p110delta in signaling by the B-cell receptor complex. *Molecular and Cellular Biology, 22*(24), 8580–8591.

Kovesdi, D., Bell, S. E., & Turner, M. (2010). The development of mature B lymphocytes requires the combined function of CD19 and the p110delta subunit of PI3K. *Self Nonself, 1*(2):144–153. doi:10.4161/self.1.2.11796.

Krahn, A. K., Ma, K. W., Hou, S., Duronio, V., & Marshall, A. J. (2004). Two distinct waves of membrane-proximal B cell antigen receptor signaling differentially regulated by Src homology 2-containing inositol polyphosphate 5-phosphatase. *Journal of Immunology, 172*(1), 331–339.

Kurosaki, T. (1997). Molecular mechanisms in B cell antigen receptor signaling. *Current Opinion Immunology, 9,* 309–318.

Lam, K. P., Kuhn, R., & Rajewsky, K. (1997). In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. *Cell, 90*(6), 1073–1083.

Lannutti, B. J., Meadows, S. A., Herman, S. E., Kashishian, A., Steiner, B., Johnson, A. J., et al. (2011). CAL-101, a p110delta selective phosphatidylinositol-3-kinase inhibitor for the treatment of B-cell malignancies, inhibits PI3K signaling and cellular viability. *Blood, 117*(2), 591–594. doi:10.1182/blood-2010-03-275305.

Lee, K. S., Lee, H. K., Hayflick, J. S., Lee, Y. C., & Puri, K. D. (2006) Inhibition of phosphoinositide 3-kinase delta attenuates allergic airway inflammation and hyperresponsiveness in murine asthma model. *The FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology, 20*(3), 455–465. doi:10.1096/fj.05-5045com.

Lenzen, C., Cool, R. H., Prinz, H., Kuhlmann, J., & Wittinghofer, A. (1998). Kinetic analysis by fluorescence of the interaction between Ras and the catalytic domain of the guanine nucleotide exchange factor Cdc25Mm. *Biochemistry, 37*(20), 7420–7430. doi:10.1021/bi972621j.

Leslie, N. R., Maccario, H., Spinelli, L., & Davidson, L. (2009) The significance of PTEN's protein phosphatase activity. *Advances in Enzyme Regulation, 49*(1):190–196. doi:http://dx.doi.org/10.1016/j.advenzreg.2008.12.002.

Liang, J., & Slingerland, J. M. (2003). Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression. *Cell Cycle, 2*(4), 339–345.

Llorian, M., Stamataki, Z., Hill, S., Turner, M., & Martensson, I. L. (2007). The PI3K p110delta is required for down-regulation of RAG expression in immature B cells. *Journal of Immunology, 178*(4), 1981–1985.

Lucas, C. L., Kuehn, H. S., Zhao, F., Niemela, J. E., Deenick, E. K., Palendira, U., et al. (2014). Dominant-activating germline mutations in the gene encoding the PI(3)K catalytic subunit p110delta result in T cell senescence and human immunodeficiency. *Nature Immunology, 15*(1), 88–97. doi:10.1038/ni.2771.

Mackay, F., Woodcock, S. A., Lawton, P., Ambrose, C., Baetscher, M., Schneider, P., et al. (1999). Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations. *The Journal of Experimental Medicine, 190*(11), 1697–1710.

Mamillapalli, R., Gavrilova, N., Mihaylova, V. T., Tsvetkov, L. M., Wu, H., Zhang, H., et al. (2001) PTEN regulates the ubiquitin-dependent degradation of the CDK inhibitor p27(KIP1) through the ubiquitin E3 ligase SCF(SKP2). *Current Biology: CB, 11*(4), 263–267.

McKenzie, G., Ward, G., Stallwood, Y., Briend, E., Papadia, S., Lennard, A., et al. (2006). Cellular Notch responsiveness is defined by phosphoinositide 3-kinase-dependent signals. *BMC Cell Biology, 7,* 10. doi:10.1186/1471-2121-7-10.

Miller, D. J., & Hayes, C. E. (1991). Phenotypic and genetic characterization of a unique B lymphocyte deficiency in strain A/WySnJ mice. *European Journal of Immunology, 21*(5), 1123–1130. doi:10.1002/eji.1830210506.

Moon, K. D., Post, C. B., Durden, D. L., Zhou, Q., De P, Harrison, M. L., et al. (2005). Molecular basis for a direct interaction between the Syk protein-tyrosine kinase and phosphoinositide 3-kinase. *The Journal of Biological Chemistry, 280*(2), 1543–1551. doi:10.1074/jbc.M407805200.

Muise-Helmericks, R. C., Grimes, H. L., Bellacosa, A., Malstrom, S. E., Tsichlis, P. N., & Rosen, N. (1998). Cyclin D expression is controlled post-transcriptionally via a phosphatidylinositol 3-kinase/Akt-dependent pathway. *The Journal of Biological Chemistry, 273*(45), 29864–29872.

Okada, T., Maeda, A., Iwamatsu, A., Gotoh, K., & Kurosaki, T. (2000). BCAP: The tyrosine kinase substrate that connects B cell receptor to phosphoinositide 3-kinase activation. *Immunity, 13*(6), 817–827.

Okkenhaug, K., & Fruman, D. A. (2010). PI3Ks in lymphocyte signaling and development. *Current Topics in Microbiology and Immunology, 346,* 57–85. doi:10.1007/82_2010_45.

Okkenhaug, K., & Vanhaesebroeck, B. (2003). PI3K in lymphocyte development, differentiation and activation. *Nature Reviews Immunology, 3*(4), 317–330. doi:10.1038/nri1056.

Okkenhaug, K., Bilancio, A., Farjot, G., Priddle, H., Sancho, S., Peskett, E., et al. (2002). Impaired B and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice. *Science, 297*(5583), 1031–1034. doi:10.1126/science.1073560.

Osborne, B. A., & Minter, L. M. (2007). Notch signalling during peripheral T-cell activation and differentiation. *Nature Reviews Immunology, 7*(1), 64–75. doi:10.1038/nri1998.

Patke, A., Mecklenbrauker, I., Erdjument-Bromage, H., Tempst, P., & Tarakhovsky, A. (2006). BAFF controls B cell metabolic fitness through a PKC beta- and Akt-dependent mechanism. *The Journal of Experimental Medicine, 203*(11), 2551–2562. doi:10.1084/jem.20060990.

Patrussi, L., & Baldari, C. T. (2008). Intracellular mediators of CXCR4-dependent signaling in T cells. *Immunology Letters, 115*(2), 75–82. doi:10.1016/j.imlet.2007.10.012.

Patrussi, L., Capitani, N., Cannizzaro, E., Finetti, F., Lucherini, O. M., Pelicci, P. G., et al. (2014). Negative regulation of chemokine receptor signaling and B-cell chemotaxis by p66Shc. *Cell Death & Disease, 5,* e1068. doi:10.1038/cddis.2014.44.

Petro, J. B., & Khan, W. N. (2001). Phospholipase C-gamma 2 couples Bruton's tyrosine kinase to the NF-kappaB signaling pathway in B lymphocytes. *The Journal of Biological Chemistry, 276*(3), 1715–1719. doi:10.1074/jbc.M009137200.

Petro, J. B., Rahman, S. M., Ballard, D. W., & Khan, W. N. (2000). Bruton's tyrosine kinase is required for activation of IkappaB kinase and nuclear factor kappaB in response to B cell receptor engagement. *The Journal of Experimental Medicine, 191*(10), 1745–1754.

Pillai, S., & Cariappa, A. (2009). The follicular versus marginal zone B lymphocyte cell fate decision. *Nature Reviews Immunology, 9*(11), 767–777. doi:10.1038/nri2656.

Puri, K. D., Doggett, T. A., Douangpanya, J., Hou, Y., Tino, W. T., Wilson, T., et al. (2004). Mechanisms and implications of phosphoinositide 3-kinase delta in promoting neutrophil trafficking into inflamed tissue. *Blood, 103*(9), 3448–3456. doi:10.1182/blood-2003-05-1667.

Ramadani, F., Bolland, D. J., Garcon, F., Emery, J. L., Vanhaesebroeck, B., Corcoran, A. E., et al. (2010). The PI3K isoforms p110alpha and p110delta are essential for pre-B cell receptor signaling and B cell development. *Science Signaling, 3*(134), ra60. doi:10.1126/scisignal.2001104.

Rawlings, D. J., Saffran, D. C., Tsukada, S., Largaespada, D. A., Grimaldi, J. C., Cohen, L., Mohr, R. N., Bazan, J. F., Howard, M., Copeland, N. G., et al. (1993). Mutation of unique region of Bruton's tyrosine kinase in immunodeficient XID mice. *Science, 261*(5119), 358–361.

Rawlings, D. J., Scharenberg, A. M., Park, H., Wahl, M. I., Lin, S., Kato, R. M., et al. (1996). Activation of BTK by a phosphorylation mechanism initiated by SRC family kinases. *Science, 271*(5250), 822–825.

Reif, K., Okkenhaug, K., Sasaki, T., Penninger, J. M., Vanhaesebroeck, B., & Cyster, J. G. (2004). Cutting edge: Differential roles for phosphoinositide 3-kinases, p110gamma and p110delta, in lymphocyte chemotaxis and homing. *Journal of Immunology, 173*(4), 2236–2240.

Reth, M., & Wienands, J. (1997). Initiation and processing of signals from the B cell antigen receptor. *Annual Review of Immunology, 15,* 453–479.

Ringshausen, I., Schneller, F., Bogner, C., Hipp, S., Duyster, J., Peschel, C., et al. (2002). Constitutively activated phosphatidylinositol-3 kinase (PI-3K) is involved in the defect of apoptosis in B-CLL: Association with protein kinase Cdelta. *Blood, 100*(10), 3741–3748. doi:10.1182/blood-2002-02-0539.

Rodriguez-Viciana, P., Warne, P. H., Khwaja, A., Marte, B. M., Pappin, D., Das, P., et al. (1997). Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. *Cell, 89*(3), 457–467.

Rodriguez-Viciana, P., Sabatier, C., & McCormick, F. (2004). Signaling specificity by Ras family GTPases is determined by the full spectrum of effectors they regulate. *Molecular and Cellular Biology, 24*(11), 4943–4954. doi:10.1128/MCB.24.11.4943-4954.2004.

Rommel, C., Camps, M., & Ji, H. (2007). PI3K delta and PI3K gamma: Partners in crime in inflammation in rheumatoid arthritis and beyond? *Nature reviews Immunology, 7*(3), 191–201. doi:10.1038/nri2036.

Brooks, S. A., & Blackshear, P. J. (2013). Tristetraprolin (TTP): Interactions with mRNA and proteins, and current thoughts on mechanisms of action. *Biochimica et biophysica acta, 1829*(6–7), 666–679. doi:10.1016/j.bbagrm.2013.02.003.

Saito, T., Chiba, S., Ichikawa, M., Kunisato, A., Asai, T., Shimizu, K., et al. (2003). Notch2 Is preferentially expressed in mature B cells and indispensible for marginal zone B lineage development. *Immunity, 18,* 675–685.

Salim, K., Bottomley, M. J., Querfurth, E., Zvelebil, M. J., Gout, I., Scaife, R., et al. (1996). Distinct specificity in the recognition of phosphoinositides by the pleckstrin homology domains of dynamin and Bruton's tyrosine kinase. *The EMBO Journal, 15*(22), 6241–6250.

Sawyer, C., Sturge, J., Bennett, D. C., O'Hare, M. J., Allen, W. E., Bain, J., et al. (2003). Regulation of breast cancer cell chemotaxis by the phosphoinositide 3-kinase p110delta. *Cancer Research, 63*(7), 1667–1675.

Scharenberg, A. M., & Kinet, J. P. (1998). PtdIns-3,4,5-P3: A regulatory nexus between tyrosine kinases and sustained calcium signals. *Cell, 94*(1), 5–8.

Schiemann, B., Gommerman, J. L., Vora, K., Cachero, T. G., Shulga-Morskaya, S., Dobles, M., et al. (2001). An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. *Science, 293*(5537), 2111–2114. doi:10.1126/science.1061964.

Schmidlin, M., Lu, M., Leuenberger, S. A., Stoecklin, G., Mallaun, M., Gross, B., et al. (2004). The ARE-dependent mRNA-destabilizing activity of BRF1 is regulated by protein kinase B. *The EMBO Journal, 23*(24), 4760–4769. doi:10.1038/sj.emboj.7600477.

Shen, W. H., Balajee, A. S., Wang, J., Wu, H., Eng, C., Pandolfi, P. P., et al. (2007). Essential role for nuclear PTEN in maintaining chromosomal integrity. *Cell, 128*(1), 157–170. doi:10.1016/j.cell.2006.11.042.

Skon, C. N., Lee, J. Y., Anderson, K. G., Masopust, D., Hogquist, K. A., & Jameson, S. C. (2013). Transcriptional downregulation of S1pr1 is required for the establishment of resident memory CD8+ T cells. *Nature Immunology, 14*(12), 1285–1293. doi:10.1038/ni.2745.

Song, M. S., Carracedo, A., Salmena, L., Song, S. J., Egia, A., Malumbres, M., et al. (2011). Nuclear PTEN regulates the APC-CDH1 tumor-suppressive complex in a phosphatase-independent manner. *Cell, 144*(2), 187–199. doi:10.1016/j.cell.2010.12.020.

Srinivasan, L., Sasaki, Y., Calado, D. P., Zhang, B., Paik, J. H., DePinho, R. A., et al. (2009). PI3 kinase signals BCR-dependent mature B cell survival. *Cell, 139*(3), 573–586. doi:10.1016/j.cell.2009.08.041.

Su, T. T., Guo, B., Kawakami, Y., Sommer, K., Chae, K., Humphries, L. A., et al. (2002). PKC-beta controls I kappa B kinase lipid raft recruitment and activation in response to BCR signaling. *Nature Immunology, 3*(8), 780–786. doi:10.1038/ni823.

Su, T. T., Guo, B., Wei, B., Braun, J., & Rawlings, D. J. (2004). Signaling in transitional type 2 B cells is critical for peripheral B-cell development. *Immunological Reviews, 197,* 161–178.

Suzuki, H., Terauchi, Y., Fujiwara, M., Aizawa, S., Yazaki, Y., Kadowaki, T., et al. (1999). Xid-like immunodeficiency in mice with disruption of the p85alpha subunit of phosphoinositide 3-kinase. *Science, 283*(5400), 390–392.

Taylor, G. A., Carballo, E., Lee, D. M., Lai, W. S., Thompson, M. J., Patel, D. D., et al. (1996). A pathogenic role for TNFα in the syndrome of cachexia, arthritis, and autoimmunity resulting from Tristetraprolin (TTP) deficiency. *Immunity, 4,* 445–454.

Tedder, T. F., Inaoki, M., & Sato, S. (1997). The CD19-CD21 complex regulates signal transduction thresholds governing humoral immunity and autoimmunity. *Immunity, 6*(2), 107–118.

Thompson, J. S., Bixler, S. A., Qian, F., Vora, K., Scott, M. L., Cachero, T. G., et al. (2001). BAFF-R, a newly identified TNF receptor that specifically interacts with BAFF. *Science, 293*(5537), 2108–2111. doi:10.1126/science.1061965.

Tibarewal, P., Zilidis, G., Spinelli, L., Schurch, N., Maccario, H., Gray, A., et al. (2012). PTEN protein phosphatase activity correlates with control of gene expression and invasion, a tumor-suppressing phenotype, but not with AKT activity. *Science Signaling, 5*(213), ra18. doi:10.1126/scisignal.2002138.

Troutman, T. D., Hu, W., Fulenchek, S., Yamazaki, T., Kurosaki, T., Bazan, J. F., et al. (2012). Role for B-cell adapter for PI3K (BCAP) as a signaling adapter linking Toll-like receptors (TLRs) to serine/threonine kinases PI3K/Akt. *Proceedings of the National Academy of Sciences of the United States of America, 109*(1), 273–278. doi:10.1073/pnas.1118579109.

Turner, M. (2002). The role of Vav proteins in B cell responses. *Advances in Experimental Medicine and Biology, 512,* 29–34.

Turner, M., & Billadeau, D. D. (2002). VAV proteins as signal integrators for multi-subunit immune-recognition receptors. *Nature Reviews Immunology, 2*(7), 476–486. doi:10.1038/nri840.

Turner, M., & Hodson, D. (2012). Regulation of lymphocyte development and function by RNA-binding proteins. *Current Opinion in Immunology, 24*(2), 160–165. doi:10.1016/j.coi.2012.01.011.

Turner, M., Gulbranson-Judge, A., Quinn, M. E., Walters, A. E., MacLennan, I. C. M., & Tybulewicz, V. L. (1997a). Syk Tyrosine kinase is required for the positive selection of immature B cells into the recirculating B Cell pool. *The Journal of Experimental Medicine, 186*(12), 2013–2021.

Turner, M., Mee, P. J., Walters, A. E., Quinn, M. E., Mellor, A. L., Zamoyska, R., et al. (1997b). A requirement for the Rho-family GTP exchange factor Vav in positive and negative selection of thymocytes. *Immunity, 7*(4), 451–460.

Tuveson, D. A., Carter, R. H., Soltoff, S. P., & Fearon, D. T. (1993). CD19 of B cells as a surrogate kinase insert region to bind phosphatidylinositol 3-kinase. *Science, 260*(5110), 986–989.

Tze, L. E., Schram, B. R., Lam, K. P., Hogquist, K. A., Hippen, K. L., Liu, J., et al. (2005). Basal immunoglobulin signaling actively maintains developmental stage in immature B cells. *PLoS Biology, 3*(3), e82. doi:10.1371/journal.pbio.0030082.

Vanhaesebroeck, B., & Khwaja, A. (2014). PI3Kdelta inhibition hits a sensitive spot in B cell malignancies. *Cancer Cell, 25*(3), 269–271. doi:10.1016/j.ccr.2014.02.012.

Verkoczy, L., Duong, B., Skog, P., Ait-Azzouzene, D., Puri, K., Vela, J. L., et al. (2007). Basal B cell receptor-directed phosphatidylinositol 3-kinase signaling turns off RAGs and promotes B cell-positive selection. *Journal of Immunology, 178*(10), 6332–6341.

Vigorito, E., Bardi, G., Glassford, J., Lam, E. W., Clayton, E., & Turner, M. (2004). Vav-dependent and vav-independent phosphatidylinositol 3-kinase activation in murine B cells determined by the nature of the stimulus. *Journal of Immunology, 173*(5), 3209–3214.

Vigorito, E., Gambardella, L., Colucci, F., McAdam, S., & Turner, M. (2005). Vav proteins regulate peripheral B-cell survival. *Blood, 106*(7), 2391–2398. doi:10.1182/blood-2004-12-4894.

Vlach, J., Hennecke, S., Alevizopoulos, K., Conti, D., & Amati, B. (1996). Growth arrest by the cyclin-dependent kinase inhibitor p27Kip1 is abrogated by c-Myc. *The EMBO Journal, 15*(23), 6595–6604.

Ward, S. G. (2004). Do phosphoinositide 3-kinases direct lymphocyte navigation? *Trends in Immunology, 25*(2), 67–74. doi:10.1016/j.it.2003.12.003.

Winkelmann, R., Sandrock, L., Porstner, M., Roth, E., Mathews, M., Hobeika, E., et al. (2011). B cell homeostasis and plasma cell homing controlled by Kruppel-like factor 2. *Proceedings of the National Academy of Sciences of the United States of America, 108*(2), 710–715. doi:10.1073/pnas.1012858108.

Woodland, R. T., Fox, C. J., Schmidt, M. R., Hammerman, P. S., Opferman, J. T., Korsmeyer, S. J., et al. (2008). Multiple signaling pathways promote B lymphocyte stimulator dependent B-cell growth and survival. *Blood, 111*(2), 750–760. doi:10.1182/blood-2007-03-077222.

Yusuf, I., Zhu, X., Kharas, M. G., Chen, J., & Fruman, D. A. (2004). Optimal B-cell proliferation requires phosphoinositide 3-kinase-dependent inactivation of FOXO transcription factors. *Blood, 104*(3), 784–787. doi:10.1182/blood-2003-09-3071.

Zhou, B. P., Liao, Y., Xia, W., Spohn, B., Lee, M. H., & Hung, M. C. (2001). Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. *Nature Cell Biology, 3*(3), 245–252. doi:10.1038/35060032.

Chapter 10
Immune Memory and Exhaustion: Clinically Relevant Lessons from the LCMV Model

D. Zehn and E. J. Wherry

Abstract The development of dysfunctional or exhausted T cells is characteristic of immune responses to chronic viral infections and cancer. Exhausted T cells are defined by reduced effector function, sustained upregulation of multiple inhibitory receptors, an altered transcriptional program and perturbations of normal memory development and homeostasis. This review focuses on (a) illustrating milestone discoveries that led to our present understanding of T cell exhaustion, (b) summarizing recent developments in the field, and (c) identifying new challenges for translational research. Exhausted T cells are now recognized as key therapeutic targets in human infections and cancer. Much of our knowledge of the clinically relevant process of exhaustion derives from studies in the mouse model of *Lymphocytic choriomeningitis virus* (LCMV) infection. Studies using this model have formed the foundation for our understanding of human T cell memory and exhaustion. We will use this example to discuss recent advances in our understanding of T cell exhaustion and illustrate the value of integrated mouse and human studies and will emphasize the benefits of bi-directional mouse-to-human and human-to-mouse research approaches.

Keywords Lymphocytic choriomeningitis virus (LCMV) · T cell exhaustion · Memory CD8 T cells · Infections · Translational research

Introduction: T Cell Responses in Acute and Chronic Infections

If the host survives, viral infections result in one of two outcomes. Either, the virus is cleared acutely (usually within 1–2 weeks) or the virus persists. In the former case, robust immune responses are often generated including the characteristic development of cytotoxic effector CD8 T cell populations capable of eliciting effector

E. J. Wherry (✉)
Department of Microbiology, Institute for Immunology, Philadelphia, PA, USA
e-mail: wherry@mail.med.upenn.edu

D. Zehn
Division of Immunology and Allergy, Lausanne University Hospital, Lausanne, Switzerland
e-mail: dietmar.zehn@chuv.ch

S. P. Schoenberger et al. (eds.), *Crossroads Between Innate and Adaptive Immunity V,*
Advances in Experimental Medicine and Biology 850, DOI 10.1007/978-3-319-15774-0_10

functions such as antiviral cytokine production, beta chemokine production and cytotoxicity. These effector T cells disperse throughout the body and eventually become resident in many non-lymphoid as well as lymphoid tissues (Kaech and Cui 2012; Zhang and Bevan 2011). Following the peak of this effector response, most effector CD8 T cells die, but a small pool of memory precursors further differentiates and seeds the memory pool. These memory CD8 T cells become quiescent, but also acquire the ability to self-renew in the absence of antigen via efficient responsiveness to both IL-7 and IL-15 (Jameson and Masopust 2009). A population of tissue resident memory (T_{RM}) also develops outside the spleen, lymph nodes and bone marrow and establishes permanent residence in the gut, skin, brain, lungs and elsewhere (Mueller et al. 2013). While these T_{RM} appear not to undergo traditional self-renewal like central memory CD8 T cells in lymphoid tissues (Masopust 2006), their maintenance properties remain to be completely defined. Systemic memory CD8 T cell are highly responsive upon antigen re-encounter. Upon re-infection, functional memory CD8 T cells rapidly perform effector functions (within minutes to hours), undergo substantial proliferative re-expansion and re-differentiate into a secondary effector pool. Efficient long-term maintenance coupled to highly efficient effector function and proliferative potential are the basis for protective immunity conferred by memory T cells.

In contrast to this scenario following acutely cleared infections, persisting infections with viruses that sustain high levels of replication often result in T cell dysfunction or exhaustion (Wherry 2011). CD8 T cell exhaustion is defined by the hierarchical loss of effector functions, sustained high expression of multiple inhibitory receptors such as PD-1 (Barber et al. 2006), an altered transcriptional program including distinct expression and use of key transcription factors, and a skewed memory development program that results in an inefficient development of antigen-independence and poor use of IL-7 and IL-15 signals (Wherry et al. 2004; Shin 2007). Severe exhaustion is often observed during infections with high levels of ongoing viral replication such as chronic strains of LCMV (i.e. clone 13, high dose WE, docile) (Oldstone 2002; Ahmed 1984), during untreated human infections with HIV, HCV and HBV (Klenerman and Hill 2005).

CD8 T cells with an exhausted phenotype also appear prominently among tumor-infiltrating lymphocytes (TIL) in human cancer and have a transcriptional signature that resembles in large part the transcriptional landscape of exhausted CD8 T cells from chronic LCMV infection in mice (Kim and Ahmed 2010; Baitsch et al. 2011). Less “severe” chronic infections where viral replication is restrained due to anatomical limitations (i.e. by infecting inaccessible or particularly shielded tissues such as the CNS) or gene programs that support a latent virus persistence also causes alterations in T cell differentiation, but whether these changes reflect exhaustion, senescence, or other forms of altered memory T cell differentiation remains a subject of considerable research (Klenerman and Hill 2005; Hertoghs et al. 2010). For this review, we will focus mainly on aspects of T cell exhaustion observed in severe chronic with high virus replication rates and cancer.

Milestone Achievements Obtained by Using Persisting LCMV Infections

The LCMV infection model in mice has been a highly tractable and productive experimental system with which to make major discoveries about T cell biology, memory and exhaustion. Many of these key discoveries have had major impact on our understanding of human immunology. Of course, the discovery of MHC restriction by Doherty and Zinkernagel using the LCMV system (Zinkernagel and Doherty 1974) represents one of the seminal findings in immunology. However, other important studies using LCMV to define the magnitude, kinetics and long-term durability of virus-specific T cell responses (Butz and Bevan 1998; Murali-Krishna et al. 1998; Lau et al. 1994) accurately predicted many features of human antiviral T cell responses (Miller et al. 2008; Hammarlund et al. 2003; Amanna et al. 2006). The concept that the T cell response becomes exhausted over time during chronic infections was also first defined using strains of LCMV that cause a persisting infection, and was soon followed by experiments demonstrating the presence of virus-specific CD8 T cells that lacked the ability to efficiently elicit effector functions (Gallimore et al. 1998; Moskophidis 1993; Zajac et al. 1998). These observations using, at the time, the novel technology of MHC class I/peptide tetramer staining allowed visualization of virus-specific T cells that could not be identified using previous techniques that relied on elaboration of effector functions such as IFN-γ or IL-2 production or limiting dilution analysis that relied on T cell proliferation *in vitro*. Subsequent studies defined the nature of this dysfunction by demonstrating hierarchical loss of some effector functions before others using the LCMV system (Wherry et al. 2007; Wherry et al. 2003; Fuller and Zajac 2003), and these observations were extended to HIV where the idea of loss of "polyfunctionality" (Harari et al. 2006; Makedonas and Betts 2006) has become ingrained in our understanding of poor T cell quality during persisting infections. Many additional studies in humans also defined similar populations of "exhausted" T cells during HIV, HCV, HBV, HTLV-I (Wherry 2011), persisting bacterial and parasitic infections and during human cancer (Kim and Ahmed 2010; Virgin et al. 2009). Thus, the LCMV model of chronic viral infection was instrumental in defining the field of T cell exhaustion and providing benchmarks to extend to humans. As with any cross-species analysis, not every individual detail aligns perfectly. Nevertheless, the usefulness of the LCMV system for understanding human T cell exhaustion should not be understated.

One of the most important sets of observations in the field has been the discovery that exhausted T cells could be rejuvenated and that their functional defects (at least partly) reversed. These observations were made by first discovering that exhausted T cell during chronic LCMV infection express high levels of the inhibitory receptor PD-1 (Barber et al. 2006; Wherry et al. 2007). Blocking this inhibitory receptor restored function of exhausted CD8 T cells and enhanced viral control (Barber et al. 2006). These findings from the LCMV model were very rapidly extended directly to HIV and SIV infection (Day et al. 2006; Trautmann et al. 2006; Petrovas et al.

2006; Velu 2009). These observations clearly demonstrated that exhausted T cell populations contained at least some cells that were not terminally differentiated and could be reinvigorated. What remained however unclear at the time, was whether all or only a subset of the exhausted T cell population was capable of this re-invigoration. This issue will be addressed below. Moreover, targeting the PD-1 pathway in human cancer is proving to be one of the most effective clinical immune interventions to treat cancer to date with response rates of up to 30–50% even in patients who have failed other immunotherapies (Hamid et al. 2013; Topalian et al. 2012; Brahmer et al. 2012). PD-1 is not, however, the only immunoregulatory pathway operating in exhausted T cells. Exhausted CD8 T cells co-express multiple other inhibitory receptors simultaneously with PD-1 and these other receptors co-regulate exhaustion (Blackburn et al. 2009). Again, these observations of inhibitory receptor co-expression and co-regulation of exhaustion have been rapidly extended to human chronic infections and cancer and have paved the way for combination immunotherapy clinical trials (Wolchok et al. 2013).

An interesting question that has arisen from the studies above and observations in clinical trials is whether all exhausted T cells are the same? In other words, does the heterogeneity that clearly exists phenotypically (Blackburn et al. 2009) affect functionality and reinvigoration during immunotherapies of other interventions? Transcriptional profiling studies have been quite informative at identifying pathways such as PD-1, revealing transcriptional circuits involved in T cell exhaustion and for providing signatures that can be used to identify patterns of exhaustion in human infections such as HIV (Quigley et al. 2010) and cancer (Baitsch et al. 2011). However, such studies are population based and fail to reveal information about heterogeneity within the population. Indeed, subpopulations of exhausted CD8 T cells were identified, first on the basis of PD-1 co-expression with other surface markers (Blackburn et al. 2008), but then using expression of the transcription factors T-bet and Eomes (Paley et al. 2012). When these subsets were separated, nearly all of the remaining proliferative potential was contained in the PD-1^{Int} T-bet^{Hi} subset of exhausted CD8 T cells (Blackburn et al. 2008; Paley et al. 2012). The PD-1^{Hi} $Eomes^{Hi}$ subset was terminally exhausted and failed to proliferate with or without PD-1 blockade (Blackburn et al. 2008). These findings illustrate that the benefit of PD-1 pathway blockade likely occurs by re-invigoration of this PD-1^{Int} subset. Head to head comparisons indicate that, despite this re-invigoration by PD-1 pathway blockade, the proliferative capacity of the PD-1^{int} subset is still substantially worse than that of functional memory CD8 T cells. It remains unclear whether further relevant heterogeneity exists within this T-bet^{Hi} PD-1^{Int} subset. For example, it is possible that an even smaller subset within this T-bet^{Hi} PD-1^{Int} population of exhausted T cells retains high proliferative capacity, but that this small subset is normally not appreciated numerically. Alternatively, the proliferative capacity of this entire T-bet^{Hi} subset may be lower than that of functional memory CD8 T cells. Future studies are necessary to distinguish these possibilities.

These subsets of exhausted CD8 T cells also form a proliferative hierarchy during steady state chronic infection that is essential to maintain the overall population of exhausted T cells (see figure). Many tissues are organized in a proliferative hi-

erarchy where a pool of progenitor cells continually gives rise to a more terminally differentiated population of cells that performs the functions of the tissue. A similar scenario appears to occur for exhausted CD8 T cells and be defined by T-bet^{Hi} and Eomes^{Hi} subpopulations (Paley et al. 2012). The PD-1^{Int} T-bet^{Hi} subset continually gives rise to an extensively divided population of PD-1^{Hi} Eomes^{Hi} terminally differentiated cells in the presence of persisting antigen. While the T-bet^{Hi} subset is slightly better in terms of cytokine production, it is only the Eomes^{Hi} subset that maintains modest cytotoxic capacity indicating that this terminally exhausted subset may have an important role in containing (but not fully eliminating) persisting infections. The Eomes^{Hi} subset is also present at a ~20-fold numerical excess compared to the T-bet^{Hi} subset and more efficiently populates peripheral tissues where virus persists. Importantly, a similar scenario appears to exist in HCV (Paley et al. 2012) and likely HIV infection (Buggert et al. 2014). The signal that drives this continual proliferation and differentiation of the T-bet^{Hi} subset into Eomes^{Hi} terminal progeny is persisting antigen (Paley et al. 2012), consistent with previous observations about the antigen-dependence of exhausted CD8 T cells (Wherry et al. 2004; Shin et al. 2007). Moreover, when antigen is removed in the context of chronic viral infection using an epitope mutant virus, both subsets of exhausted CD8 T cells fail to persist efficiently (Paley et al. 2012). Unlike memory CD8 T cells, at least a subset of exhausted CD8 T cells undergoes continual rapid proliferation in response to antigen rather than IL-7 and IL-15 (Jameson and Masopust 2009). This continual proliferation may have negative consequences in that it results in accumulation of terminally differentiated exhausted CD8 T cell populations.

One important implication from this work is the concept that exhausted T cells, despite their dysfunction, are critical for establishing and maintaining a host-pathogen equilibrium (Virgin et al. 2009; Paley et al. 2012). Indeed, when either the progenitor (T-bet^{Hi}) or terminally differentiated (Eomes^{Hi}) subset of exhausted CD8 T cells was genetically removed, mice lost all ability to contain LCMV clone 13 replication and became viremic for life (Paley et al. 2012). This observation is also consistent with data from CD8 T cell depletion in chronic SIV infection which results in a dramatic increase in viral replication and rapid progression to AIDS (Jin et al. 1999; Schmitz et al. 1999). Moreover, while escape mutation in SIV, HIV and HCV infection can occur during the acute phase of infection, during chronic infection escape mutation is likely driven by exhausted T cells (Leslie et al. 2004; Petrovic et al. 2012). Thus, the idea that exhausted CD8 T cells are inert is a misconception. Rather, exhausted T cells apply pressure on the pathogen and establish a relative host-pathogen standoff allowing the host to survive by balancing pathogen containment and limiting immunopathology. While this situation is obviously not ideal for infections like HIV, HCV, HBV and for cancer, it is unlikely that these scenarios were the evolutionary driver for T cell exhaustion since these infections have entered mammals too recently in evolutionary terms and cancer usually occurs post reproductive age exerting little evolutionary pressure. However, whether the evolutionary impetus for T cell exhaustion was some aspect of self-tolerance or perhaps infections like herpes viruses remains to be determined.

Important Unanswered Questions

Despite these previous studies, a number of important questions remain unanswered. First, it is unclear how exhausted T cells change or differentiate if antigen is eliminated—a situation arising in chronic infections when escape mutants are selected. This scenario is now also of central clinical importance for HCV infection where the introduction of highly effective direct acting antiviral (DAA) drugs will cure many chronically infected HCV subjects (Schinazi et al. 2014). It remains unclear, however, whether these patients will be left with HCV-specific T cell memory and, if so, whether these memory cells will be capable of protection upon re-infection. While the studies mentioned above demonstrate that the vast majority of exhausted CD8 T cells do not possess normal self-renewal, persistence or re-expansion potential, previous evidence from HIV infection suggests that, while the majority of the HIV-specific CD8 T cell population may disappear upon epitope escape or HAART (Alter et al. 2003; Jamieson et al. 2003; Casazza et al. 2001), consistent with antigen-dependent T cell exhaustion, upon treatment interruption, HIV-specific CD8 T cell populations re-emerge (Ortiz et al. 2001). This topic is discussed further below in the context of the notion that small sub-populations with memory (re-)differentiation potential may exist within the exhausted pool.

Second, the role of inhibitory signals such as PD-1 in impacting the function of normal and exhausted T cells remains poorly understood. We typically associate sustained expression of PD-1 with the transition from functional to exhausted T cells and PD-1 has been implicated in causing exhaustion. However, it is unclear whether PD-1 signals are instructive for CD8 T cell exhaustion and cause this altered differentiation pattern. Because of a role for PD-1 in suppressing immunopathology during the early phase of LCMV clone 13 infection, it has been difficult to answer the question of whether T cell exhaustion can or cannot occur without PD-1. This question is also addressed below.

Third, T cell populations in chronic infections are heterogeneous and display different degrees of exhaustion (Blackburn et al. 2009). Presently, it remains unclear what determines this heterogeneity, but different lines of evidence suggest that the strength of TCR stimulation, epitope abundance and viral load are critical elements in determining the development of the exhausted phenotype (Wherry et al. 2003; Blackburn et al. 2009; Lichterfeld et al. 2007; Vigano et al. 2012). Defining the relationship(s) between the quality and quantity of TCR stimulation and cellular differentiation (not only for T cell tolerance (Enouz et al. 2012) or acute infections (Zehn et al. 2009)) but also for chronic infections will be important to further our understanding of the molecular program of T cell differentiation in chronic infections. In particular, transcriptional and molecular profiling studies may provide deeper insights if increasingly homogenous subsets of exhausted T cells could be profiled. Ultimately, single-cell profiling approaches may be necessary to fully dissect this issue. However, it would be highly desirable to develop approaches to selectively interrogate highly homogenous populations of functional effector T cells, exhausted T cells, and T cells at discreet intermediate stages of exhaustion defined phenotypically or functionally.

Finally, the precise role of key transcription factors in T cell exhaustion also remains incompletely understood. The importance of T-bet and Eomes is discussed briefly above. However, one of the more interesting, yet still poorly understood observations is that these two transcription factors (and many others) regulate distinct sets of genes in exhausted versus functional memory T cells (Doering et al. 2012). How this context-specific transcription factor function is regulated remains to be determined, but may have important implications for reversibility and reprogramming of exhausted T cells.

New and emerging data begin to address these questions. Below, we focus on how the findings presented at the 2013 Aegean meeting addressing some of these and related questions.

Insights into the Mechanisms that Maintain T Cell Responses in Chronic Infections

Chronic infections such as HIV and HCV often persist for years during which virus-specific T cells exert partial control over virus replication. Central questions are (i) how are these long-term T cell responses maintained, (ii) what are the dynamics, lifespan and turnover of individual T cells in exhausted populations, and (iii) to what extent population renewal occurs from different sources of T cells.

One long standing question is if recent thymic emigrants and thus *de novo* recruitment of naïve T cells could play a role in maintaining the exhausted T cell populations, but the significance and magnitude of this contribution during chronic infections still remains unclear. In some settings, recent thymic emigrants can be primed on persisting antigen during chronic infection (Vezys et al. 2006; Kemball et al. 2005). However, long-term CD8 T cell responses during LCMV clone 13 infection can be maintained in thymectomized mice (Shin et al. 2007; Miller et al. 2005). Moreover, during HIV infection, there is clear evidence for an impaired thymic output decreasing the likelihood of a substantial pool of recent thymic emigrants contributing to maintenance of HIV-specific T cell populations (Douek et al. 1998).

These considerations strongly suggest that there must be other mechanisms to maintain T cell responses in chronic infections. While the lifespan of individual T cells in chronic infections remains unknown, observations that T cells continue to proliferate suggest a substantial turn-over of T cells in these infections (Shin et al. 2007). For long-term durability of T cell responses, one would therefore predict the persistence of at least some cells in the population with proliferative capacity. Thus, a specialized T cell subpopulation might exist which retains over longer periods proliferative potential. So far, both the turn-over process and the precise characteristics of the T cells which retain the highest proliferative potential is still poorly understood, but several critical insights into these questions have recently been obtained.

Two different lines of evidence strongly supported the existence of a memory-like sub-population in chronic infections. The group of D. Zehn has demonstrated that when antigen-specific T cell populations from chronic LCMV clone 13 infections are transferred into antigen-free host mice, then these transferred populations underwent strong re-expansion after subjecting the host mice to an acute LCMV infection (Utzschneider et al. 2013). Most importantly, this re-expansion occurred even after a 4 week delay between the T cell transfer and applying the acute LCMV infection. Following the transfer, the host mouse T cell compartment was carefully monitored for signs of LCMV-specific T cells responses over the 4 week delay interval. The lack of a response excluded any virus transfer along with the highly pure T cell populations that were injected into the host mice (Utzschneider et al. 2013). These observations signal that a fraction of the transferred cells survived in the absence of further antigen-recognition and inflammation which together with the ability to undergo secondary expansion are hallmark features of memory T cells.

The observation that T cell populations with re-expansion potential could be found in persisting clone 13 infections inevitably raise the concern whether or not the particular kinetics of an LCMV clone 13 infection with a rather short phase of viremia (Zajac et al. 1998; Matloubian et al. 1994) might have supported the formation of the memory-like T cells. In series of unpublished experiments that were presented at the meeting, we could also exclude this possibility. We took advantage of Vβ5 mice which express only the TCRbeta chain from an ovalbumin specific T cell clone (Kelly et al. 1993)—which biases the repertoire towards T cell specificities that are irrelevant for LCMV infections. Since Vβ5 mice express endogenously rearranged TCRalpha chains (Dillon et al. 1994; Zehn and Bevan 2006), the Vβ5 mice bear polyclonal T cells but contain very few LCMV specific T cells—a circumstance that results in very high virus titers following chronic LCMV infections. In fact, LCMV clone 13 virus titers increase up to day 8 and remain stable thereafter even when the mice were supplemented with P14 TCR transgenic T cells which are specific to the LCMV-derived gp33 epitope (Utzschneider and Zehn, unpublished observations). In these hosts, the P14 populations show full signs of T cell exhaustion. Yet a fraction of P14 T cells retains the potential to undergo re-expansion despite the presence of very high virus titers and high antigen load.

As described above, the group of E.J. Wherry has identified subsets of exhausted CD8 T cells that, indeed, differ in their re-expansion and ongoing proliferative potential. Work from the Wherry lab was presented that not only defined the proliferative capacity of these subsets, but also identified the underlying transcriptional control of these events. The $Eomes^{Hi}$ $PD\text{-}1^{Hi}$ subset of exhausted CD8 T cells is terminally differentiated and has minimal residual proliferative capacity. Moreover, this subset of exhausted CD8 T cells cannot be re-invigorated by PD-1 pathway blockade. In contrast, the numerically smaller subset of $T\text{-}bet^{Hi}PD\text{-}1^{Int}$ exhausted CD8 T cells not only undergoes continual proliferation in response to persisting antigen, but can undergo proliferative expansion when re-exposed to antigen (Blackburn et al. 2008; Paley et al. 2012). This subset also responds vigorously to PD-1 pathway blockade. Interestingly, the assay used to interrogate ongoing proliferation can dramatically impact the interpretation. BRDU labeling or Ki67 staining reveals ongoing proliferation in *both* exhausted CD8 T cell subsets. However, lineage trac-

ing experiments using CFSE labeling revealed that the proliferation of Eomes^{Hi} PD-1^{Hi} cells observed by BRDU and Ki67 reflects cells that have recently emerged from the T-bet^{Hi}PD-1^{Int} subset, undergone extensive division and converted into the more terminal subset (Paley et al. 2012). Importantly, both subsets were antigen-dependent and removal of antigen not only eliminated ongoing proliferation but also reduced the persistence of each subset *in vivo* (Paley et al. 2012).

More recent studies have also identified the lineage origin of these exhausted T cells. Interestingly, both exhausted CD8 T cell subsets emerge from the memory precursor (i.e. CD127^{Hi}KLRG1^{Lo}) subset of effector CD8 T cells, while the KLRG1^{Hi} terminally differentiated effector pool was incapable of giving rise to exhausted CD8 T cells (Angelosanto et al. 2012). As memory precursors become exhausted they diverge phenotypically, functionally and transcriptionally from their effector origin and fail to develop into memory T cells (Wherry et al. 2007; Paley et al. 2012; Doering et al. 2012; Angelosanto et al. 2012).

Stable and Flexible Features of T Cell Exhaustion in Chronic Infections

As mentioned above, the Wherry laboratory demonstrated that ongoing proliferation of exhausted CD8 T cells is accompanied by a shift from Tbet^{high} Eomes^{low} to Tbet^{low} Eomes^{high} cells. At this meeting, data was also presented from the Wherry lab that a similar conversion occurs during PD-1 blockade mediated re-invigoration of exhausted T cells. In line with these observations, the group of D. Zehn observed that when T cells derived from acute or chronic infections are transferred into naïve hosts followed by acute LCMV Armstrong induced re-expansion, then the cells originating from the chronic infection showed much higher Eomes expression levels than T cells expanded two times by an acute infection (Utzschneider et al. 2013). Given the switch of infection from chronic to acute, this observation is particularly interesting as it indicates that the capacity to express high levels of Eomes was imprinted during the chronic infection. Thus, a cell intrinsic differentiation program ensures high Eomes expression levels independently from the type of infection that induces T cell re-expansion. The Zehn lab also observed that when cells from chronic infections re-expand in an acute infection, their exhausted cytokine profiles and PD-1 expression pattern persisted (Utzschneider et al. 2013). This unexpected observation contrasts the view that expandable populations might be confined to less exhausted cells within the population. Instead, it highlights that key features of exhausted phenotype can be stably passed on to progeny after re-expansion. It is unclear, however, how this observation relates to the clear improvement in functionality following PD-1 pathway blockade (Barber et al. 2006; Blackburn et al. 2009; Blackburn et al. 2008) and the observation that T cells found later in LCMV clone 13 infection after control of viremia become more functional and PD-1^{Lo} in tissues with low amounts of virus (Wherry et al. 2004; Blackburn et al. 2010). Nonetheless, the aforementioned observations are in line with a report showing that markers of T cell exhaustion may in some cases persist regardless of the outcome of HCV

infections (Kasprowicz et al. 2008). However, new DAA drugs available to treat, and completely cure ongoing HCV infections (Schinazi et al. 2014) in the absence of immunomodulatory drugs such as IFN-α will provide further critical insights into the stability issue of T cell phenotypes in chronic infections.

Together the observations discussed above imply that certain features such as cytokine profiles and elevated PD-1 expression can be passed on to cellular progeny in chronic infections while other features while other features change following proliferation including that cells transition from a Tbet high to an Eomes high phenotype. Of note, this Eomes high stage is also reached when T cells from chronic infections are transferred and re-expanded in acute infections (Utzschneider et al. 2013). How these different observations of stable and changing properties can be aligned remains unclear at present. Similarly, how the observed stability in cytokine expression can be implemented into the established model of a hierarchically occurring loss of certain T cell functions in chronic infections is also unclear at present. A key aspect to consider is that phenotypic changes are typically described at the level of the entire population without tracing the behavior of individual cells and their progeny. Thus, it needs to be taken into account that we often underestimate the dynamics within complex and heterogeneous cell populations. We tend to consider that changes in the overall appearance of a T cell population are linked to a conversion of cells from one phenotype to another. While this is possible in chronic infections, a non-mutually exclusive scenario involves the outgrowth of cells with a particular phenotype over cells showing another characteristic phenotype. Thus, it remains unclear if the functional recovery seen in the population of antigen-specific T cells in chronic infections following PD-1 blockade results from a cellular conversion from a more to a less exhausted T cell or does it result from a selective outgrowth of more functional T cells over more exhausted T cell subsets. Previous studies demonstrated strong evidence in favor of the latter - that PD-1 pathway blockade selectively expands the T-bet^{High} subset of exhausted CD8 T cells leading to a population outgrowth of these cells over the pre-existing pool of Eomes^{High} terminally exhausted CD8 T cells (Blackburn et al. 2008). Nonetheless, many questions about this process remain unanswered and further fate mapping these events will likely provide important insights. To track the progeny of individual cells within a T cell population remains challenging and has so far been rarely investigated though an elegant approach has been demonstrated also at this meeting by the group of D. Busch (Buchholz et al. 2013; Gerlach et al. 2013) (see related article). Altogether, the observations discussed above underline the importance of dedicating more efforts to investigating the dynamics of individual cells within T cell populations in chronic infections.

Are PD-1 Signals Instructive for Exhaustion

Another set of data presented by the Wherry lab at this meeting addressed whether PD-1 signals were necessary to induce CD8 T cell exhaustion. Previous studies were unable to address this issue because genetic PD-1 (or PD-L1) deficiency or

early blockade of this pathway results in lethal immunopathology during the effector phase (Barber et al. 2006; Frebel et al. 2012). However, data were presented using an approach where small numbers of WT and PD-1 KO LCMV specific P14 cells were co-adoptively transferred followed by LCMV clone 13 infection. In this setting the fate of PD-1 KO P14 could be followed during chronic viral infection. Surprisingly, PD-1 KO LCMV-specific CD8 T cells still became exhausted during chronic infection (Odorrizi et al. 2015). In fact, while initial T cell expansion was enhanced in the absence of PD-1, T cell dysfunction in the chronic phase of infection was more severe and PD-1 KO exhausted CD8 T cells expressed higher levels of other inhibitory receptors. Moreover, in the absence of PD-1 the exhausted CD8 T cell population was skewed toward the $Eomes^{Hi}$ subset. Together, these results suggest that, while PD-1 restrains full function and expansion of exhausted CD8 T cells, in the permanent absence of PD-1 signals, exhausted CD8 T cells become further overstimulated, lose function more readily and terminally differentiate. These observations may have implications for long-term treatment with PD-1 pathway inhibitors in the clinic.

General Conclusions/New Challenges

Exhausted T cells are now appreciated to be common in many chronic infections and in cancer (Speiser et al. 2014; Pauken and Wherry 2015). Targeting these cells clinically represents one of the biggest breakthroughs in cancer therapy in years. Indeed, PD-1 pathway blockade drugs have recently been licensed for the treatment of melanoma and FDA approval for similar drugs for other cancers are imminent. Despite the clinical opportunities to target T cell exhaustion, we still understand very little about these cells, their maintenance properties and the details of their population heterogeneity. The precise molecular mechanisms by which PD-1 regulates T cell exhaustion also remain to be defined and the pathways engaged upon re-invigoration of exhausted CD8 T cells by PD-1 pathway blockade have not been identified. Transcriptional control mechanism appear at least partly context specific (Doering et al. 2012) urging caution in extrapolating information about the role of transcription factors in exhaustion based on their functions in other cell types such as functional effector or memory T cells. While studies presented at the meeting highlighted some lineage tracing experiments and the insights they can reveal about T cell exhaustion, much more work is needed in this regard. Population based studies have the inherent limitation that they may underestimate the importance of small subsets of cells. Taking advantage of the LCMV model in this regard may be particularly important since such lineage tracing experiments will be challenging in humans. Finally, it is important to note that much of our understanding of T cell exhaustion derives from studies of CD8 T cells. CD4 T cell exhaustion remains less well understood. Indeed, other studies presented by the Wherry lab at this meeting highlighted the differences between CD4 and CD8 T cell exhaustion (Crawford et al. 2014) and hopefully will provide a foundation for future studies in this area.

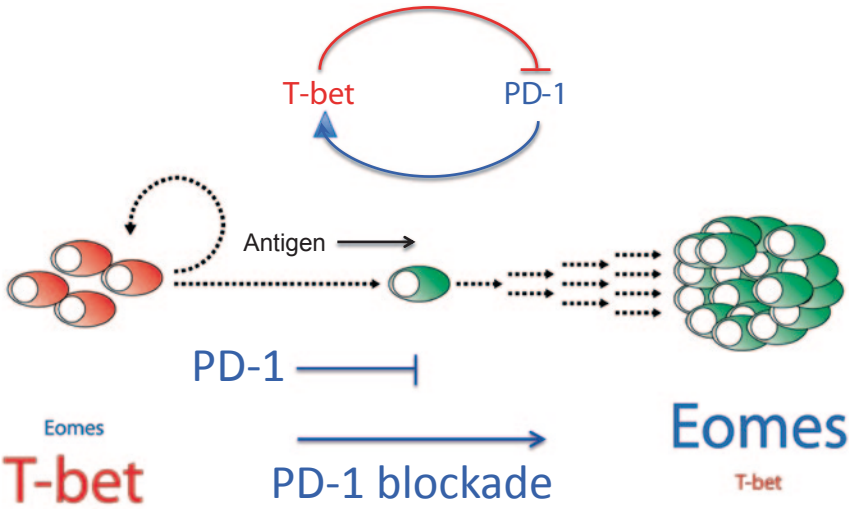

Fig. 10.1 Subsets of exhausted CD8 T cells exist in a proliferative hierarchy controlled by T-bet, Eomes, PD-1 and interactions with persisting antigen. T-bet^{Hi} progenitor exhausted CD8 T cells continually divide, undergo proliferative expansion and give rise to terminal Eomes^{Hi} cells in response to persisting antigen. PD-1 antagonizes this lineage differentiation and preserves the T-bet^{Hi} subset while blockade of this pathway enhances expansion and conversion of T-bet^{Hi} cells into Eomes^{Hi} cells. T-bet directly represses PD-1 expression while high PD-1 appears to foster high Eomes and low T-bet. (Adapted from Blackburn et al. 2008; Paley et al. 2012; Kao et al. 2011)

In summary, a more detailed understanding of the molecular mechanisms of T cell exhaustion and the ability to enhance the stability, persistence and recovery of function from these populations will enable improved clinical therapies for persisting infections and cancer (Fig. 10.1).

References

Ahmed, R., Salmi, A., Butler, L. D., Chiller, J. M., & Oldstone, M. B. (1984). Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice. Role in suppression of cytotoxic T lymphocyte response and viral persistence. *The Journal of Experimental Medicine, 160*(2), 521–540.

Alter, G., Hatzakis, G., Tsoukas, C. M., Pelley, K., Rouleau, D., LeBlanc, R., et al. (2003). Longitudinal assessment of changes in HIV-specific effector activity in HIV-infected patients starting highly active antiretroviral therapy in primary infection. *Journal of Immunology, 171*(1), 477–488.

Amanna, I. J., Slifka, M. K., & Crotty, S. (2006). Immunity and immunological memory following smallpox vaccination. *Immunological Reviews, 211,* 320–337.

Angelosanto, J. M., Blackburn, S. D., Crawford, A., & Wherry, E. J. (2012). Progressive loss of memory T cell potential and commitment to exhaustion during chronic viral infection. *Journal of Virology, 86*(15), 8161–8170.

Baitsch, L., Baumgaertner, P., Devevre, E., Raghav, S. K., Legat, A., Barba, L., et al. (2011). Exhaustion of tumor-specific CD8(+) T cells in metastases from melanoma patients. *The Journal of Clinical Investigation, 121*(6), 2350–2360.

Barber, D. L., Wherry, E. J., Masopust, D., Zhu, B., Allison, J. P., Sharpe, A. H., et al. (2006). Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature, 439*(7077), 682–687.

Blackburn, S. D., Shin, H., Freeman, G. J., & Wherry, E. J. (2008). Selective expansion of a subset of exhausted CD8 T cells by alphaPD-L1 blockade. *Proceedings of the National Academy of Sciences of the United States of America, 105*(39), 15016–15021.

Blackburn, S. D., Shin, H., Haining, W. N., Zou, T., Workman, C. J., Polley, A., et al. (2009). Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nature Immunology, 10*(1), 29–37.

Blackburn, S. D., Crawford, A., Shin, H., Polley, A., Freeman, G. J., & Wherry, E. J. (2010). Tissue-specific differences in PD-1 and PD-L1 expression during chronic viral infection: Implications for CD8 T-cell exhaustion. *Journal of Virology, 84*(4), 2078–2089.

Brahmer, J. R., Tykodi, S. S., Chow, L. Q., Hwu, W. J., Topalian, S. L., Hwu, P., et al. (2012). Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *The New England Journal of Medicine, 366*(26), 2455–2465.

Buchholz, V. R., Flossdorf, M., Hensel, I., Kretschmer, L., Weissbrich, B., Graf, P., et al. (2013). Disparate individual fates compose robust CD8+ T cell immunity. *Science, 340*(6132), 630–635.

Buggert, M., Tauriainen, J., Yamamoto, T., Frederiksen, J., Ivarsson, M. A., Michaelsson, J., et al. (2014). T-bet and Eomes are differentially linked to the exhausted phenotype of CD8+ T cells in HIV infection. *PLoS Pathogens, 10*(7), e1004251.

Butz, E. A., & Bevan, M. J. (1998). Massive expansion of antigen-specific CD8+ T cells during an acute virus infection. *Immunity, 8*(2), 167–175.

Casazza, J. P., Betts, M. R., Picker, L. J., & Koup, R. A. (2001). Decay kinetics of human immunodeficiency virus-specific CD8+ T cells in peripheral blood after initiation of highly active antiretroviral therapy. *Journal of Virology, 75*(14), 6508–6516.

Crawford, A., Angelosanto, J. M., Kao, C., Doering, T. A., Odorizzi, P. M., Barnett, B. E., et al. (2014). Molecular and transcriptional basis of CD4(+) T cell dysfunction during chronic infection. *Immunity, 40*(2), 289–302.

Day, C. L., Kaufmann, D. E., Kiepiela, P., Brown, J. A., Moodley, E. S., Reddy, S., et al. (2006). PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature, 443*(7109), 350–354.

Dillon, S. R., Jameson, S. C., & Fink, P. J. (1994). V beta 5+ T cell receptors skew toward OVA + H-2Kb recognition. *Journal of Immunology, 152*(4), 1790–1801.

Doering, T. A., Crawford, A., Angelosanto, J. M., Paley, M. A., Ziegler, C. G., & Wherry, E. J. (2012). Network analysis reveals centrally connected genes and pathways involved in CD8+ T cell exhaustion versus memory. *Immunity, 37*(6), 1130–1144.

Douek, D. C., McFarland, R. D., Keiser, P. H., Gage, E. A., Massey, J. M., Haynes, B. F., et al. (1998). Changes in thymic function with age and during the treatment of HIV infection. *Nature, 396*(6712), 690–695.

Enouz, S., Carrie, L., Merkler, D., Bevan, M. J., & Zehn, D. (2012). Autoreactive T cells bypass negative selection and respond to self-antigen stimulation during infection. *The Journal of Experimental Medicine, 209*(10), 1769–1779.

Frebel, H., Nindl, V., Schuepbach, R. A., Braunschweiler, T., Richter, K., Vogel, J., et al. (2012). Programmed death 1 protects from fatal circulatory failure during systemic virus infection of mice. *The Journal of Experimental Medicine, 209*(13), 2485–2499.

Fuller, M. J., & Zajac, A. J. (2003). Ablation of CD8 and CD4 T cell responses by high viral loads. *Journal of Immunology, 170*(1), 477–486.

Gallimore, A., Glithero, A., Godkin, A., Tissot, A. C., Pluckthun, A., Elliott, T., et al. (1998). Induction and exhaustion of lymphocytic choriomeningitis virus-specific cytotoxic T lym-

phocytes visualized using soluble tetrameric major histocompatibility complex class I-peptide complexes. *The Journal of Experimental Medicine, 187*(9), 1383–1393.

Gerlach, C., Rohr, J. C., Perie, L., van Rooij N., van Heijst J. W., Velds, A., et al. (2013). Heterogeneous differentiation patterns of individual CD8+ T cells. *Science, 340*(6132), 635–639.

Hamid, O., Robert, C., Daud, A., Hodi, F. S., Hwu, W. J., Kefford, R., et al. (2013). Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. *The New England Journal of Medicine, 369*(2), 134–144.

Hammarlund, E., Lewis, M. W., Hansen, S. G., Strelow, L. I., Nelson, J. A., Sexton, G. J., et al. (2003). Duration of antiviral immunity after smallpox vaccination. *Nature Medicine, 9*(9), 1131–1137.

Harari, A., Dutoit, V., Cellerai, C., Bart, P. A., Du Pasquier R. A., & Pantaleo, G. (2006). Functional signatures of protective antiviral T-cell immunity in human virus infections. *Immunological Reviews, 211,* 236–254.

Hertoghs, K. M., Moerland, P. D., van Stijn A., Remmerswaal, E. B., Yong, S. L., van de Berg P. J., et al. (2010). Molecular profiling of cytomegalovirus-induced human CD8+ T cell differentiation. *The Journal of Clinical Investigation, 120*(11), 4077–4090.

Jameson, S. C., & Masopust, D. (2009). Diversity in T cell memory: An embarrassment of riches. *Immunity, 31*(6), 859–871.

Jamieson, B. D., Yang, O. O., Hultin, L., Hausner, M. A., Hultin, P., Matud, J., et al. (2003). Epitope escape mutation and decay of human immunodeficiency virus type 1-specific CTL responses. *Journal of Immunology, 171*(10), 5372–5379.

Jin, X., Bauer, D. E., Tuttleton, S. E., Lewin, S., Gettie, A., Blanchard, J., et al. (1999). Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. *The Journal of Experimental Medicine, 189*(6), 991–998.

Kaech, S. M., & Cui, W. (2012). Transcriptional control of effector and memory CD8+ T cell differentiation. *Nature Reviews Immunology, 12*(11), 749–761.

Kao, C., Oestreich, K. J., Paley, M. A., Crawford, A., Angelosanto, J. M., Ali, M. A., et al. (2011). Transcription factor T-bet represses expression of the inhibitory receptor PD-1 and sustains virus-specific CD8+ T cell responses during chronic infection. *Nature Immunology, 12*(7), 663–671.

Kasprowicz, V., Schulze Zur Wiesch, J., Kuntzen, T., Nolan, B. E., Longworth, S., Berical, A., et al. (2008). High level of PD-1 expression on hepatitis C virus (HCV)-specific CD8+ and CD4+ T cells during acute HCV infection, irrespective of clinical outcome. *Journal of Virology, 82*(6), 3154–3160.

Kelly, J. M., Sterry, S. J., Cose, S., Turner, S. J., Fecondo, J., Rodda, S., et al. (1993). Identification of conserved T cell receptor CDR3 residues contacting known exposed peptide side chains from a major histocompatibility complex class I-bound determinant. *European Journal of Immunology, 23*(12), 3318–3326.

Kemball, C. C., Lee, E. D., Vezys, V., Pearson, T. C., Larsen, C. P., & Lukacher, A. E. (2005). Late priming and variability of epitope-specific CD8+ T cell responses during a persistent virus infection. *Journal of Immunology, 174*(12), 7950–7960.

Kim, P. S., & Ahmed, R. (2010). Features of responding T cells in cancer and chronic infection. *Current Opinion in Immunology, 22*(2), 223–230.

Klenerman, P., & Hill, A. (2005). T cells and viral persistence: Lessons from diverse infections. *Nature Immunology, 6*(9), 873–879.

Lau, L. L., Jamieson, B. D., Somasundaram, T., & Ahmed, R. (1994). Cytotoxic T-cell memory without antigen. *Nature, 369*(6482), 648–652.

Leslie, A. J., Pfafferott, K. J., Chetty, P., Draenert, R., Addo, M. M., Feeney, M., et al. (2004). HIV evolution: CTL escape mutation and reversion after transmission. *Nature Medicine, 10*(3), 282–289.

Lichterfeld, M., Yu, X. G., Mui, S. K., Williams, K. L., Trocha, A., Brockman, M. A., et al. (2007). Selective depletion of high-avidity human immunodeficiency virus type 1 (HIV-1)-specific CD8+ T cells after early HIV-1 infection. *Journal of Virology, 81*(8), 4199–4214.

Makedonas, G., & Betts, M. R. (2006). Polyfunctional analysis of human T cell responses: Importance in vaccine immunogenicity and natural infection. *Springer Seminars in Immunopathology, 28*(3), 209–219.

Masopust, D., Vezys, V., Wherry, E. J., Barber, D. L., & Ahmed, R. (2006). Cutting edge: Gut microenvironment promotes differentiation of a unique memory CD8 T cell population. *Journal of Immunology, 176*(4), 2079–2083.

Matloubian, M., Concepcion, R. J., & Ahmed, R. (1994). CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. *Journal of Virology, 68*(12), 8056–8063.

Miller, N. E., Bonczyk, J. R., Nakayama, Y., & Suresh, M. (2005). Role of thymic output in regulating CD8 T-cell homeostasis during acute and chronic viral infection. *Journal of Virology, 79*(15), 9419–9429.

Miller, J. D., van der Most R. G., Akondy, R. S., Glidewell, J. T., Albott, S., Masopust, D., et al. (2008). Human effector and memory CD8+ T cell responses to smallpox and yellow fever vaccines. *Immunity, 28*(5), 710–722.

Moskophidis, D., Lechner, F., Pircher, H., & Zinkernagel, R. M. (1993). Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. *Nature, 362*(6422), 758–761.

Mueller, S. N., Gebhardt, T., Carbone, F. R., & Heath, W. R. (2013). Memory T cell subsets, migration patterns, and tissue residence. *Annual Review of Immunology, 31,* 137–161.

Murali-Krishna, K., Altman, J. D., Suresh, M., Sourdive, D. J., Zajac, A. J., Miller, J. D., et al. (1998). Counting antigen-specific CD8 T cells: A reevaluation of bystander activation during viral infection. *Immunity, 8*(2), 177–187.

Odorizzi, P. M., Pauken, K. E., Paley, M. A., Sharpe, A., & Wherry, E. J. (2015). Genetic absence of PD-1 promotes accumulation of terminally differentiated exhausted CD 8 + T cells. *J Exp Med.*

Oldstone, M. B. (2002). Biology and pathogenesis of lymphocytic choriomeningitis virus infection. *Current Topics in Microbiology and Immunology, 263,* 83–117.

Ortiz, G. M., Wellons, M., Brancato, J., Vo, H. T., Zinn, R. L., Clarkson, D. E., et al. (2001). Structured antiretroviral treatment interruptions in chronically HIV-1-infected subjects. *Proceedings of the National Academy of Sciences of the United States of America, 98*(23), 13288–13293.

Paley, M. A., Kroy, D. C., Odorizzi, P. M., Johnnidis, J. B., Dolfi, D. V., Barnett, B. E., et al. (2012). Progenitor and terminal subsets of CD8+ T cells cooperate to contain chronic viral infection. *Science, 338*(6111), 1220–1225.

Pauken, K. E., & Wherry, E. J. (2015). Overcoming T cell exhaustion in infection and cancer. *Trends Immunol*, *36*, 265–276.

Petrovas, C., Casazza, J. P., Brenchley, J. M., Price, D. A., Gostick, E., Adams, W. C., et al. (2006). PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection. *The Journal of Experimental Medicine, 203*(10), 2281–2292.

Petrovic, D., Dempsey, E., Doherty, D. G., Kelleher, D., & Long, A. (2012). Hepatitis C virus-T-cell responses and viral escape mutations. *European Journal of Immunology, 42*(1), 17–26.

Quigley, M., Pereyra, F., Nilsson, B., Porichis, F., Fonseca, C., Eichbaum, Q., et al. (2010). Transcriptional analysis of HIV-specific CD8+ T cells shows that PD-1 inhibits T cell function by upregulating BATF. *Nature Medicine, 16*(10), 1147–1151.

Shin, H., Blackburn, S. D., Blattman, J. N., & Wherry, E. J. (2007). Viral antigen and extensive division maintain virus-specific CD8 T cells during chronic infection. *The Journal of Experimental Medicine, 204*(4), 941–949.

Schinazi, R., Halfon, P., Marcellin, P., & Asselah, T. (2014). HCV direct-acting antiviral agents: The best interferon-free combinations. *Liver International: Official Journal of the International Association for the Study of the Liver, 34*(Suppl 1), 69–78.

Schmitz, J. E., Kuroda, M. J., Santra, S., Sasseville, V. G., Simon, M. A., Lifton, M. A., et al. (1999). Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science, 283*(5403), 857–860.

Speiser, D. E., Utzschneider, D. T., Oberle, S. G., Munz, C., Romero, P., & Zehn, D. (2014). T cell differentiation in chronic infection and cancer: functional adaptation or exhaustion? *Nat Rev Immunol, 14*, 768–774.

Topalian, S. L., Hodi, F. S., Brahmer, J. R., Gettinger, S. N., Smith, D. C., McDermott, D. F., et al. (2012). Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *The New England Journal of Medicine, 366*(26), 2443–2454.

Trautmann, L., Janbazian, L., Chomont, N., Said, E. A., Gimmig, S., Bessette, B., et al. (2006). Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. *Nature Medicine, 12*(10), 1198–1202.

Utzschneider, D. T., Legat, A., Fuertes Marraco, S. A., Carrie, L., Luescher, I., Speiser, D. E., et al. (2013). T cells maintain an exhausted phenotype after antigen withdrawal and population reexpansion. *Nature Immunology, 14*(6), 603–610.

Velu, V., Titanji, K., Zhu, B., Husain, S., Pladevega, A., Lai, L., et al. (2009). Enhancing SIV-specific immunity in vivo by PD-1 blockade. *Nature, 458*(7235), 206–210.

Vezys, V., Masopust, D., Kemball, C. C., Barber, D. L., O'Mara, L. A., Larsen, C. P., et al. (2006). Continuous recruitment of naive T cells contributes to heterogeneity of antiviral CD8 T cells during persistent infection. *The Journal of Experimental Medicine, 203*(10), 2263–2269.

Vigano, S., Utzschneider, D. T., Perreau, M., Pantaleo, G., Zehn, D., & Harari, A. (2012). Functional avidity: A measure to predict the efficacy of effector T cells? *Clinical & Developmental Immunology, 2012,* 153863.

Virgin, H. W., Wherry, E. J., & Ahmed, R. (2009). Redefining chronic viral infection. *Cell, 138*(1), 30–50.

Wherry, E. J. (2011). T cell exhaustion. *Nature Immunology, 12*(6), 492–499.

Wherry, E. J., Blattman, J. N., Murali-Krishna, K., van der Most R., & Ahmed, R. (2003). Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *Journal of Virology, 77*(8), 4911–4927.

Wherry, E. J., Barber, D. L., Kaech, S. M., Blattman, J. N., & Ahmed, R. (2004). Antigen-independent memory CD8 T cells do not develop during chronic viral infection. *Proceedings of the National Academy of Sciences of the United States of America, 101*(45), 16004–16009.

Wherry, E. J., Ha, S. J., Kaech, S. M., Haining, W. N., Sarkar, S., Kalia, V., et al. (2007). Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity, 27*(4), 670–684.

Wolchok, J. D., Kluger, H., Callahan, M. K., Postow, M. A., Rizvi, N. A., Lesokhin, A. M., et al. (2013). Nivolumab plus ipilimumab in advanced melanoma. *The New England Journal of Medicine, 369*(2), 122–133.

Zajac, A. J., Blattman, J. N., Murali-Krishna, K., Sourdive, D. J., Suresh, M., Altman, J. D., et al. (1998). Viral immune evasion due to persistence of activated T cells without effector function. *The Journal of Experimental Medicine, 188*(12), 2205–2213.

Zehn, D., & Bevan, M. J. (2006). T cells with low avidity for a tissue-restricted antigen routinely evade central and peripheral tolerance and cause autoimmunity. *Immunity, 25*(2), 261–270.

Zehn, D., Lee, S. Y., & Bevan, M. J. (2009). Complete but curtailed T-cell response to very low-affinity antigen. *Nature, 458*(7235), 211–214.

Zhang, N., & Bevan, M. J. (2011). CD8(+) T cells: Foot soldiers of the immune system. *Immunity, 35*(2), 161–168.

Zinkernagel, R. M., & Doherty, P. C. (1974). Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature, 248*(5450), 701–702.

Index

S. P. Schoenberger et al. (eds.), *Crossroads Between Innate and Adaptive Immunity V,*
Advances in Experimental Medicine and Biology 850, DOI 10.1007/978-3-319-15774-0