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Bali Pulendran
Peter D. Katsikis
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Crossroads between Innate and Adaptive Immunity III

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Crossroads Between Innate and Adaptive Immunity III

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Preface

This volume presents a collection of reviews derived from work presented at the Aegean Conference, "3rd Crossroads between Innate and Adaptive Immunity," which occured from September 27 to October 2, 2009 at the Minoa Palace Conference Center in Chania, Crete, Greece. This meeting was the third in a series, and assembled a team of scientists working on mechanisms by which the innate immune system of the host senses pathogens, the cellular and signaling networks that orchestrate the innate response and antigen presentation and adaptive immunity. The various facets of the innate response, including dendritic cells, T cells, B cells, NK cells, NK-T cells and the complement cascade during the host response to pathogens and tumors is only now starting to be elucidated. The respective fields that focus on these immune cells and molecules have tended to be relatively compartmentalized, and yet emerging evidence points to the interconnectedness of these facets in coordinating the innate response, and its subsequent impact on the adaptive response. The goal of this conference was to initiate cross-talk between these diverse immunological fields, and promote and facilitate discussion on the interactions between the innate immune response and the adaptive immune response and ultimately facilitate collaboration between these areas of study. Following on the footsteps of the outstanding success of its precursors, the "3rd Crossroads between Innate and Adaptive Immunity" Aegean Conference was highly successful in bringing together and connecting scientists and experts from around the world to address critical areas of Innate and Adaptive Immunity.

Acknowledgements

We are grateful to all the participants of this conference whose enthusiasm and willingness to share new data and discuss new ideas truly represent the lifeblood of the "Crossroads" experience. Those who have contributed chapters to this book deserve an extra measure of our gratitude, as we are keenly aware of the dedication they have shown to this field through their extraordinary efforts in this regard. We are indebted to our colleagues at Springer for giving us the support and opportunity to publish these proceedings in their Advances in Experimental Medicine and Biology Book Series, with special thanks to Samantha Lewis for her great help in assembling and formatting the chapters. As always, we are deeply indebted to the Aegean Conference Organization for the outstanding job they do in making this possible, and particularly to Dimitrios Lambris for his superb organizational, logistical and interpersonal skills, that make this meeting such an effortless pleasure for the three of us. Finally, we wish to express our most sincere gratitude to the various organizations that made this meeting possible, through their very generous support, including the Aegean Conferences, Centocor, Drexel University, Springer, Pfizer, and Kirin.

We look forward to seeing all of you in 2011!

Peter D. Katsikis M.D., Ph.D.
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Chapter 1
Is Transcription the Dominant Force During Dynamic Changes in Gene Expression?

Martin Turner

Abstract Dynamic changes in gene expression punctuate lymphocyte development and are a characteristic of lymphocyte activation. A prevailing view has been that these changes are driven by DNA transcription factors, which are the dominant force in gene expression. Accumulating evidence is challenging this DNA centric view and has highlighted the prevalence and dynamic nature of RNA handling mechanisms. Alternative splicing and differential polyadenylation appear to be more widespread than first thought. Changes in mRNA decay rates also affect the abundance of transcripts and this mechanism may contribute significantly to gene expression. Additional RNA handling mechanisms that control the intracellular localization of mRNA and association with translating ribosomes are also important. Thus, gene expression is regulated through the coordination of transcriptional and post-transcriptional mechanisms. Developing a more "RNA centric" view of gene expression will allow a more systematic understanding of how gene expression and cell function are integrated.

> The greatest tragedy of science – the slaying of a beautiful hypothesis by an ugly fact.
>
> Thomas Henry Huxley 1825–1895

1.1 RNA Decay as a Determinant of Gene Expression

The extent to which the abundance of most mRNAs is a reflection of their rate of synthesis remains unclear. The majority of transcripts are present at low numbers and only a few studies have assessed transcription in intact cells, typically focusing on genes expressed at high copy number in a tissue specific manner such as β-globin

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or immunoglobulin H and L chains. For most genes, the relative contribution of transcription and mRNA decay to mRNA abundance during dynamic changes in gene expression is unknown. In the *C. elegans* germline many genes are regulated principally through their 3′ untranslated regions and not their promoters [1]. In yeast, whole genome measurements of transcription during the shift from glucose to galactose metabolism indicate a role for transcription but highlight mRNA stability as a key regulator of mRNA abundance [2]. Analysis of mRNA half-life in yeast using microarrays also revealed widespread variation in mRNA half-life and that transcripts encoding proteins with functional associations had similar decay rates [3]. Similar principles have been noted in human cells. The decay of mRNAs encoding proteins with linked functions was also similar; metabolic enzymes were encoded by relatively stable mRNAs and transcription factors by labile mRNAs [4].

Microarrays have been used to estimate mRNA half-life following activation of primary T cells with anti-CD3, anti-CD3+anti-CD28, or transformed T cells with phorbol ester and calcium ionophore followed by treatment with actinomycin D which intercalates into DNA, causing transcriptional inhibition [5, 6]. This revealed many transcripts that showed stimulus-dependent changes in half-life and provided evidence for coordinated regulation of transcripts with related functions. Analysis of mRNA half-life in transformed B cells following transcriptional inhibition also indicated that many transcripts were short-lived and genes with related functions were encoded by mRNAs with similar half-lives [7]. More recently, the development of methods to measure both RNA synthesis and decay in intact cells by incorporation of labeled nucleotides, without need for the use of transcriptional inhibitors has added further insight into the importance of mRNA turnover in B cells [8, 9]. These methods allow for the identification of newly synthesized mRNA and the measurement of total, pre-existing, and newly synthesized mRNA following labeling for a specific time and the rate of synthesis and decay of certain transcripts. When applied to the human EBV-transformed BL41 B cell line, short-lived transcripts were enriched among mRNA encoding transcription factors and signal transduction components [10]. However, the previously noted relationship between labile transcripts, apoptosis, and cell cycle regulators [4] was not evident from the analysis performed. Genes regulating energy balance and the proteasomes were found to be encoded by long-lived mRNAs, while those for ribosomal sub-units and translational control had intermediate half-lives. Taken together, these studies indicate gene expression is coordinated at the level of mRNA decay. These observations provide support for the concept advocated by Jack Keene of “post-transcriptional operons” whereby groups of transcripts with related biological function are coordinately regulated at the RNA level [11].

1.2 Translational Control Is a Dynamic and Diverse Regulator of Gene Expression

A second important regulatory process operating at the RNA level is translational control. In yeast, the widespread importance of translational control is evident from the poor correlation between mRNA abundance and protein abundance for many

genes [12–14]. Translational control is also widespread in mammalian cells [15–17]. In some cases, mRNA can exist within cells without giving rise to any detectable protein. Preformed cytokine mRNA has been documented in many independent studies. The mRNA encoding the chemokine RANTES is present in memory CD8 cells, but is not translated into protein until the cells receive a stimulus through their TCR [18]. It has been proposed that this mechanism contributes to the rapid response following a second exposure to antigen. A subset of T cells selected by MHCII expressed by thymocytes contained IL-4 mRNA [19]. This phenomenon has also been observed for Interferon γ in NK and NK T cells and in antigen-primed T cells [20, 21] using knock-in mice where IFNγ mRNA was tagged by the inclusion of an internal ribosome entry site (IRES), upstream of a yellow fluorescent protein reporter. This revealed yellow cells, which had accumulated the bicistronic mRNA but did not contain IFNγ protein. Cap-dependent translation of IFNγ mRNA was thus attenuated while cap-independent IRES-mediated translation was still taking place. This suggests the IFNγ mRNA is not sequestered away from the translating ribosomes, but that cap-dependent translation is specifically affected. Consistent with this, eIF2α was phosphorylated and attenuated in primed T cells and dephosphorylated upon re-stimulation of primed T cells [21]. In another study NK cell activation was accompanied by a stabilization of IFNγ mRNA, which is mediated in part through activation of the p38 MAP-kinase pathway, suggesting that mRNA stability and translation are coordinated to promote rapid output of IFNγ protein [22]. The phosphorylation of eIF2α is linked to the sequestration of mRNA in macromolecular structures called stress granules where un-translated mRNAs accumulate. Many RNA binding proteins are components of stress granules [23]. TIA-1 and TIAR may link signaling pathways to stress granule formation through eIF2α phosphorylation [24].

1.3 Post-transcriptional Control Begins in the Nucleus

RNA regulation begins in the nucleus and is coincidental with transcription. Splicing and polyadenylation are key nuclear processes, mediated by RNA binding proteins (RBP) and each transcript is coated with multiple RBP from the moment of its production. The rate at which RNA is turned over is regulated by the exosome [25] and other RNA processing enzymes such as drosha, which in addition to processing microRNA precursors, regulates ribosomal RNA [26]. Microarray analysis of RNA from naive and memory T cells revealed an extensive program of alternative splicing, involving hundreds of genes, with important consequences for cell function. Splicing was regulated by heterogenous nuclear ribonucleoprotein hnRNPLL, which was identified by two groups as a critical regulator of the T lymphocyte splicing program [27, 28]. Deep sequencing studies suggest the majority of primary transcripts are subject to alternative splicing [29–32]. Genome wide studies of nucleosome distribution in CD4+ T cells [33] indicates nucleosomes and H3K36me3 histone marks are enriched within exons relative to introns and sites of polyadenylation. Increased nucleosome occupancy in exons appears to be independent of transcription,

thus one function of chromatin appears to be to direct splicing and polyadenylation [34]. Consistent with this, H3K36me3 histone modifications within the FGFR2 gene direct polypyrimidine tract binding protein (PTB), which suppresses splicing, to specific exons [35]. Many RBPs have been reported to regulate polyadenylation site choices. Genome wide studies of polyadenylation indicate this process is systematically regulated during the activation of B and T lymphocytes [36]. A change in polyadenylation site selection resulting in shortened 3′UTRs is associated with lymphocyte cell division [36]. Shorter 3′UTRs are also more prevalent in transformed cells [37]. One consequence of 3′UTR shortening is that mRNA is less subject to regulation by microRNAs, which inhibit gene expression. Transcripts with truncated 3′UTRs will also be unable to interact with RBPs and this may either increase or decrease the output from any particular transcript.

1.4 mRNA Sequence and Structure Dictate Post-transcriptional Control

Control of mRNA metabolism is regulated by cis-acting sequences within the mRNA. These may be located within the coding region or within 5′UTR or 3′UTRs. The UTRs are an important component of non-coding RNA and become progressively longer as organisms become more complex [38, 39]. In some cases, UTR sequences are more highly conserved than coding regions. This implies that the specialized cell functions of higher organisms are linked to regulatory processes mediated by UTRs. Regulation by UTRs is manifested at many levels, including secondary structure and primary sequence. The binding of trans-acting factors onto specific sequences present within UTRs is one well-recognized mechanism by which RNA processing, localization, turnover and translation are controlled. MicroRNA recognition elements (MRE) are one important class of cis-acting sequence within 3′ UTRs. MREs also appear to be present in coding regions and to a lesser extent in 5′ UTRs. MicroRNAs are best characterized as promoting mRNA decay and translational inhibition. However, there are several examples of microRNAs promoting translation [40, 41] and it remains to be seen how pervasive this aspect of microRNA function is. Another well-studied class of cis-acting sequences present in 3′UTRs are the AU-rich elements (AREs). These have been estimated to be present in 5–8% of protein coding genes [42]. The presence of AREs correlate with instability, but is not an absolute predictor of instability, and may additionally regulate translational control [43]. An important early study defined the ARE as being common in cytokine mRNA and showed that the 3′UTR from GMCSF conferred instability upon the normally stable β-globin mRNA [44]. Although the components of the RNA stability machinery were not identified, this study showed that the instability was conditional in that GMCSF mRNA was stabilized in cells treated with phorbol ester. A subsequent study demonstrated the differential regulation of ARE-containing mRNAs in a single cell, further indicating the system had the feature of selectivity in translation [45].

1.5 RNA Binding Proteins Are Multifunctional Regulators of Gene Expression

The importance of post-transcriptional control is underscored by the large number of RNA-binding proteins encoded in the genome. This number may exceed that of genes encoding transcription factors [46, 47]. However, the function of many RBPs has been difficult to unravel, partly due to the complex pleiotropic and redundant functions of these proteins.

PTB is a widely expressed 57 kDa RNA-recognition motif-containing protein also known as heterogeneous nuclear ribonucleoprotein type I. It is highly related to neural PTB (nPTB) and to regulator of differentiation 1 (ROD1), which is expressed selectively in hematopoietic cells [48]. PTB can regulate splicing and polyadenylation in the nucleus, but also regulates translation and RNA decay in the cytoplasm. This pleiotropy is a recurrent theme in RNA-binding protein biology. In neurons, the alternative splicing of a large group of exons is regulated through controlling the expression of PTB and nPTB. PTB regulates nPTB splicing to generate a non-functional transcript that is rapidly degraded [49]. This antagonism is relieved upon loss of PTB, resulting in a new program of alternative splicing directed by nPTB. PTB also regulates splicing of ROD1 such that inhibition of PTB promotes functional ROD1 expression [50]. The significance of this cross-regulation in the context of hematopoietic system has yet to be examined.

In addition to controlling alternative splicing, PTB can also enhance or suppress translation of selected mRNAs. In the Drosophila oocyte, PTB mediates the repression of oskar mRNA prior to its localization at the posterior pole. PTB may engage oskar mRNA in cytoplasm rather than the in the nucleus, indicating the potential for interaction with transcripts subsequent to their formation and export from the nucleus [51]. PTB can also operate in concert with other RBPs. Hypoxia Inducible Factor1α translation is increased by treatment with Cobalt Chloride (which mimics hypoxia). This effect is mediated, in part, by the binding of HuR and PTB to the 5′UTR and 3′UTR of HIF-1α [52].

PTB can also regulate stability of CD154 mRNA, which encodes the ligand for CD40. CD154 is expressed by activated T cells and is an essential component of the T-cell help signal that drives B cell-mediated antibody responses. The 3′UTR of CD154 contains evolutionarily conserved CU-rich binding sites, which interact with several complexes defined in RNA-electrophoretic mobility shift assays. One of these termed complex I contains PTB and this complex acts to stabilize CD154 mRNA in activated T cells [53]. In vivo activation of mouse CD4+ T cells by anti-CD3 and anti-CD28 or re-stimulation of antigen-primed T cells in vitro leads to an increase in CD154 mRNA stability [54]. Thus, the TCR signaling pathway regulates a non ARE-mediated mechanism of RNA turnover in activated T cells. It remains unclear whether there is an effect on mRNA translation mediated by the PTB complex or other complexes interacting with the CD154 3′UTR.

Cytoplasmic PTB containing RBP complexes have also been identified in B cells following activation of the TLR9 pathway with CpG DNA. Immuno-precipitation

of PTB from B cells and subsequent analysis of the transcripts recovered revealed over 20 transcripts in PTB containing immune complexes [55]. Some of these were stabilized by CpG treatment of B cells, suggesting that PTB can also regulate mRNA stability in B cells. Experiments to reveal the identity of other protein components of the PTB mRNPs will be required to fully understand how this complex is regulated and regulates target transcripts.

1.6 AREs Bind a Diverse Set of Proteins

The proteins that interact with AREs have been termed AU-binding proteins (AUBP). They include T cell intracellular antigen 1 (TIA-1) and the related TIAR, AU-binding factor 1 (AUF1), human antigen R (HuR), K homology splicing regulatory protein (KSRP), and the TPA inducible sequence 11 (TIS11) family (TTP, BRF-1 and BRF-2), which may either stabilize or destabilize the host mRNA or alter its translational efficiency. These proteins, which recognize a similar RNA motif, contain different RNA-interacting domains including RRM, KH, and CCCH zinc fingers. Competition between AUBP for binding sites on mRNA targets suggests an important role in determining the fate of the target mRNA. Furthermore, functional interactions with microRNAs have been documented [56] and given that both classes of regulators control mRNA decay and translation, this is not surprising. However, the interplay between microRNA and RNABP in the control of translation and decay and the details of how this may operate remain scarce [57]. Unlike microRNA, AUBP can be phosphorylated and this can lead to altered function. The Tpl-2/ERK pathways have been implicated in ARE-mediated control of gene expression [58]. The p38 MAPK pathway via MAPKAPK2 (MK2) mediated phosphorylation of RNA binding proteins TTP [59–62] and BRF1 [63] can promote the translation of mRNA containing AREs. BRF-1 and 2 are also phosphorylated by protein kinase B linking AUBP to the PI3K signaling pathway [64, 65].

1.7 TTP and BRF-1 and 2

Studies using gene-targeted mice have highlighted the importance of AUBP in development and function. TTP-deficiency gives rise to an inflammatory syndrome as a consequence of excess circulating TNFα [66]. This increase in TNFα is attributed to an increase in the stability of TNFα mRNA in macrophages [67, 68] and is mediated by the ARE of TNFα [69]. Many other targets of TTP have been identified using microarray of mutant cells or by immuno-precipitating TTP and performing microarray analysis on the associated transcripts. The biological relevance of these

associations remains to be clarified [70, 71]. BRF-1 (Berg36, TIS11b, cMG1, ERF-1) had been identified as an essential regulator of ARE-dependent mRNA decay in a genetic screen for regulators of IL-3 mRNA [72]. BRF-1 was also identified as an early response gene and a potential mediator of ionophore-induced apoptosis in Ramos-transformed B cells [73]. Mice deficient in BRF-1 die around E9.5 with defects in embryonic and extra-embryonic vascularization [74, 75] that correlated with over-expression of vascular endothelial growth factor (VEGF-A). BRF-1 deficient embryonic fibroblasts (MEF) secreted elevated levels of VEGF-A, suggesting BRF-1 somehow regulated VEGF-A expression [75]. This effect was associated with the enhanced association of VEGF mRNA with polysomes in mutant MEFs, but the stability of VEGF mRNA was unchanged. These findings are significant for several reasons. First, they identified VEGF as a BRF-1 regulated gene; the 3′UTR of VEGF mRNA contains an ARE, and a direct physical interaction between VEGF mRNA and BRF-1 may regulate translation [76]. Secondly, it raises the question of whether other members of the TIS11 family regulate gene activity by affecting processes other than mRNA decay. The possibility that translation of TNFα mRNA might be regulated by TTP has not yet been examined in detail, but could conceivably contribute to increased TNFα levels in TTP mutant mice. Lastly, is that strategies used to identify genes regulated by the TTP family of RNA-binding proteins based solely on changes in mRNA level, or mRNA half life, may not reveal the full spectrum of targets. The role of the TTP family in RNA metabolism may be more extensive than RNA decay, as suggested by the finding the yeast homolog of TTP, is the concept that cth2 is a regulator of polyadenylation [77].

Less still is known about BRF-2. A BRF-2 mutant mouse expressing an amino-terminal truncated form of the protein following interruption of the first exon demonstrated female infertility due to arrested development of the early embryo at the two cell stage [78]. However, a nearly full-length protein containing both zinc fingers was derived from a transcript initiating within the intron. The phenotype of a null mutation in the mouse has revealed an essential role for BRF-2 in hematopoiesis as mutant mice die shortly after birth from anemia [79]. The genes regulated by BRF-2 in this context remain to be identified. It is likely that conditional gene targeting approaches will prove informative in this context. To this end, we have generated conditional alleles of BRF-1 and 2. In the context of CD2Cre, which deletes during the earliest stages of thymic development, mice doubly homozygous for flocked alleles of BRF-1 and 2 develop T cell acute lymphoblastic leukemia (T-ALL) [80]. Prior to the onset of T-ALL, thymic development is abnormal with accumulation of cells that have passed through the β-selection checkpoint without first expressing TCR-β. Notch1 expression is increased in non-transformed thymocytes in the absence of BRF-1 and 2. Both RBPs physically interact with evolutionarily conserved AU-rich elements within the 3′ un-translated region of Notch1 and suppress Notch1 expression. The abnormal expansion of immature thymocytes can be inhibited by a neutralizing antibody to Notch-1, suggesting both BRF-1 and 2 regulate the activity of the Notch-1 pathway in a redundant manner during T cell development.

1.8 Intracellular RNA Localization: It Really Matters Where You Are

An additional function of RBP is to regulate the intracellular localization of mRNA. RNA is unevenly distributed throughout many cells and its location has been proven to be important in drosophila and mammalian systems. Evidence that intracellular RNA localization is of broad significance is suggested by the results of a comprehensive high-resolution fluorescent in situ hybridization analysis of mRNA localization in Drosophila embryos. Over 70% of mRNAs were compartmentalized and mRNA distribution and subsequent protein localization were correlated, suggesting that protein localization could be determined prior to translation [81]. RNA localization may be critical for the normal functioning of some polarized cell types such as neurons and activated lymphocytes, where the nucleus may be far from the synapse [82]. It is also possible that RNA localization will be relevant to asymmetric cell division, which is important for stem cell maintenance.

RNA granules appear to be present in most cell types but their function, particularly during mammalian development, has yet to be elucidated. The TTP family of RBPs has previously been shown to be important for directing ARE containing mRNAs into processing bodies, suggesting the potential for qualitative regulation of processing body content [83]. Dynamic changes in mRNP granules studied using new tags and microscopy in real time [84], combined with new approaches to the isolation and characterization of mRNP contents using MS and high throughput sequencing approaches [85] should provide evidence for the importance of these structures in different developmental settings.

1.9 Conclusion

Changes in the tempo of transcription may play a co-dominant role in the regulation of gene expression. Post-transcriptional mechanisms acting at the level of RNA are increasingly being recognized as fundamental to the regulation of most, if not all, biological processes. Regulation of mRNA transport, stability, localization and translation is more than the fine tuning of a transcriptionally controlled reaction, but acts as a critical arbiter of gene activity and biological responses at the system level. Integrating studies at the level of individual genes, primary cells, and tissues to explore the hypothesis that post-transcriptional processes regulate organismal function holds much promise for the future understanding of immunology and other areas of biology.

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Chapter 2
MicroRNAs: Key Components of Immune Regulation

Donald T. Gracias and Peter D. Katsikis

Abstract The regulation of gene expression at the posttranscriptional level has revealed important control levels for genes important to the immune system. MicroRNAs (miRNAs) are small RNAs that regulate gene expression by inhibiting protein translation or by degrading the mRNA transcript. A single miRNA can potentially regulate the expression of multiple genes and the proteins encoded. MiRNA can influence molecular signaling pathways and regulate many biological processes including immune function. Although the role of miRNAs in development and oncogenesis has been well characterized, their role in the immune system has only begun to emerge. During the past few years, many miRNAs have been found to be important in the development, differentiation, survival, and function of B and T lymphocytes, dendritic cells, macrophages, and other immune cell types. We discuss here recent findings revealing important roles for miRNA in immunity and how miRNAs can regulate innate and adaptive immune responses.

2.1 Introduction

MicroRNAs (miRNAs) are an interesting class of small RNAs that can regulate gene expression by inhibiting protein translation or by degrading the mRNA transcript, thus controlling various biological processes. Ever since the discovery of lin-4 in *Caenorhabditis elegans*, which was found to inhibit the protein expression of lin-14 [36], other miRNAs have also been identified in other species. Approximately 940 miRNA have been identified so far in humans with bioinformatic tools hinting towards the possibility of them targeting as much as 30% of

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all genes [3]. A single miRNA may potentially regulate expression of hundreds of genes and subsequently, the proteins encoded by them [1]. Thus, they have the potential to influence molecular signaling pathways in the cell affecting various cellular processes. An important question regarding the function of miRNA is whether they affect the biology of cells by profoundly affecting a few selective targets or by a more subtle effect on a multitude of targets that result in a significant change in biology. While the role of miRNAs in cell development [19, 57] and cancer [12] has been well characterized, its role in the immune system has only begun to emerge. Various miRNAs have been found to be important in the development, differentiation, survival, and function of B and T lymphocytes, dendritic cells, macrophages, and other immune cell types. This review will focus on the role of miRNAs in innate and adaptive immune responses, their regulation of various immune cell types, and implications for immunological diseases.

2.2 Biogenesis and Mode of Function

miRNAs have been identified within the introns of protein encoding genes as well as within the exons and introns of noncoding RNAs [52]. These genes can be transcribed by either RNA polymerase II [37] or RNA polymerase III [7] to form the primary miRNA transcript (pri–miRNA). These transcripts are then further processed by a microprocessor complex, which consists of the enzymes Drosha (RNAse III) and DGCR8 (DiGeorge syndrome critical region gene 8 or Pasha). This processing leads to the approximately 70-nucleotide (nt) long precursor miRNA (pre-miRNA) in the nucleus [15, 20, 24]. This hairpin structure is then exported to the cytoplasm by exportin-5, which is associated with ran guanosine triphosphate (Ran GTP) [6]. The absence of exportin−5 leads to a decrease in mature miRNAs, but not the accumulation of pre-miRNA in the nucleus, which potentially implicates a role for exportin−5 in protecting pre-miRNA against nuclear digestion [42, 66]. Following the export of pre-miRNA into the cytoplasm, the RNAse III enzyme or dicer complex with its dsRBP cofactor, TRBP, cleaves the pre-miRNAs into ~22 nt long miRNA duplex strands [31]. Deletion of dicer leads to an abrogation of mature miRNA production [22, 38], indicating its crucial role in miRNA biogenesis.

One strand of the mature miRNA is packaged into the RNA- induced silencing complex (RISC), which consists of argonaute (Ago2) proteins, dicer and TRBP [21]. This mature miRNA then guides the RISC to its target mRNA and interacts with its 3′ untranslated region (3′UTR) [35]. This interaction is dependent on partial sequence complementary between the 3′UTR elements and the 5′ region of the miRNA centered on nucleotides 2–7, which is referred to as the miRNA “seed” region [4]. This results in regulation of gene expression through translational repression or mRNA degradation [9].While miRNAs were initially thought to repress its target protein output with little or no impact on mRNA [51], a recent study has indicated that mammalian miRNAs may

predominantly act by decreasing target mRNA levels [23]. Even though most studies have focused on its ability to silence genes, some recent data has also suggested that some miRNAs may also increase protein translation under certain conditions [61].

2.3 Role of MicroRNAs in Innate Immunity

The innate immune system provides the first line of defense against various pathogenic infections and is mediated by innate immune cells such as monocytes, granulocytes and natural killer cells. These cells detect pathogen-associated molecular patterns (PAMPs) through several conserved pattern recognition receptors, of which the Toll-like receptors are best characterized. Recent studies have indicated a role for several miRNAs such as miR-146a, miR-125b, miR-155, miR-9, etc. in regulating inflammatory responses downstream of TLR activation after recognition of pathogens [5,30,49,60]. Other data suggests an absolute requirement for miRNAs in the development and functioning of these cell types. As we shall discuss below, a number of innate immune cells are regulated by miRNA.

2.3.1 Monocytes and Macrophages

Monocytes and macrophages play a critical role during innate immune responses to infection through the induction of inflammatory cytokine production and phagocytosis of microbial pathogens. In human monocytes, analysis of the expression profile of 200 miRNAs in response to LPS stimulation revealed an increased expression of miR-146a/b, miR-155, and miR-132 [58]. miR-146 was found to be regulated in a NF-κB-dependent manner, controlling expression of IRAK1 (IL-1 receptor-associated kinase 1) and TRAF6 (TNF receptor associated factor 6) [58]. IRAK1 and TRAF6 are adaptor molecules that act downstream of the TLR signaling pathway. TLR stimulation leads to the activation of AP-1 and NF-kB transcription factors that upregulate various immune responsive genes. miR-146a feedback also negatively inhibited type I IFN production triggered by vesicular stomatitis virus through modulation of the RIG-I pathway, targeting IRAK1, IRAK2, and TRAF6 in macrophages [26]. Thus, miR-146a may play a role in downregulating TLR and cytokine signaling during innate responses.

The induction of miR-9 in monocytes after TLR activation and by pro-inflammatory cytokines (e.g., TNF-α and IL-1β) leads to regulation of NFKB1, causing a feedback control of NF-kB dependent responses [5]. Similarly, miR-147, a TLR4-induced miRNA, was shown to negatively regulate inflammatory responses in murine macro-phages [40].

miR-125b, which directly represses TNF-α mRNA transcripts during steady state expression, is downregulated in the presence of LPS [60]. In contrast, miR-155 is known to play an important role in mediating inflammatory responses, and is

substantially upregulated in murine macrophages, with exposure to Poly(I:C) and IFNβ [49]. Additionally, miR-155 was found to enhance TNF-α translation in response to LPS and directly target the expression of Ripk I [Receptor (TNFR superfamily)-interacting serine-threonine kinase I], IκB kinase ε (IKKε), and Fas-associated death domain (FADD) [60]. miR-155 was also found to directly target inositol phosphatase SHIP1, a negative regulator of the phosphoinositide 3-kinase pathway, increasing activation of the kinase Akt, which drives the inflammatory response [50].

2.3.2 Dendritic Cells

Dendritic cells are essential for initiating and controlling immune responses and can be derived from circulating blood monocytes, as well as myeloid progenitors in the bone marrow. miRNAs may play important roles in the differentiation of human monocyte derived dendritic cells (MDDC), as microarray profiling revealed differential expression of 20 miRNAs in MDDC during a course of 5 days [25]. In that study, miR-21 and miR-34 were found to be critical in MDDC differentiation by regulating their target genes, WNT1 and JAG1. The addition of exogenous Wnt-1 and Jagged-1 or inhibiting these miRNAs dysregulated the DC-SIGN/CD14 ratio and reduced the endocytic function of differentiating MDDCs. miR-146a is differentially expressed by myeloid-derived DC subsets, with higher constitutive expression levels in Langerhans cells (LC), relative to interstitial DCs (intDCs) [30]. This miRNA is induced by the transcription factor PU.1 in response to TGF-β1, regulating DC activation by decreasing TLR2-dependent NF-kB signaling. The high expression of miR-146a in LCs may be induced by the epithelial microenvironment to decrease their sensitivity to inappropriate activation by commensal bacterial ligands.

miR-155 was also found to be requisite for the optimal function of dendritic cells [53]. While it did not affect maturation as indicated by expression of major histo-compatability complex-II (MHC-II) and co-stimulatory molecules, miR-155-deficient DCs could not efficiently activate T cells, suggesting defective antigen presentation. miR-155 can also regulate the pathogen uptake of DCs by decreasing expression of DC-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN) by directly repressing PU.1 expression [44]. Interestingly, it may also modulate the IL-1 signaling pathway by controlling levels of TAB2 in activated human MDDCs, which decreases inflammatory cytokine production as part of a negative feedback loop [8]. Thus, miRNAs may play an important role in modulating DC function during innate and adaptive immune responses.

2.3.3 Granulocytes

miRNAs may also regulate key processes in granulocytes, including proliferation and function [29]. In the absence of miR-223, there is increased myeloid progenitor cell proliferation and neutrophil hypersensitivity to pathogenic stimuli, leading to

exaggerated inflammatory responses. It directly targets Mef2c, a transcription factor that is critical for myeloid progenitor proliferation but not function, suggesting that other targets may also be involved. Therefore, miR-223 may act to fine tune granulocyte responses during activation to prevent aberrant or inappropriate responses that lead to pathology. miR-9 is induced by LPS in human neutrophils in a MYD88/NF-κB dependent manner to control cell function through an inhibitory feedback loop [5].

Other groups have also identified a role for miR-155 in granulocyte/macrophage (GM) expansion during inflammation and during certain cases of acute myeloid leukemia (AML), with SHIP1 being an important target [50]. The overexpression of miR-155 in mice results in myelo-proliferative disorders, indicative of the tight balance maintained in regulating miRNA expression during innate immunity [50].

2.3.4 Other Innate Immune Cells

Invariant NK T (iNKT) cells are a distinct class of innate-like T cells expressing an invariant T cell receptor (TCR) specific for lipids presented by CD1d, a MHC class I-like molecule. It displays a distinct miRNA expression profile relative to other T cells, with miR-21 being over-expressed and 13 other miRNAs being under-expressed in iNKT cells [18]. Deletion of dicer, which is critical for functional miRNA generation, results in significantly reduced numbers of iNKT cells in both the thymus and the periphery due to increased cell death [69].

Other innate cells such as mast cells reside in most tissues, particularly near exposed surfaces such as the skin, gastrointestinal tract, and lung airways. They may mediate various functions during infections and allergic responses, which could also be regulated by miRNAs. miR-221-222 is highly upregulated in mast cells upon activation, reducing cell proliferation and regulating the cell cycle through partial inhibition of cell cycle inhibitor $p27^{kip}$ [45]. miRNAs may play a decisive role in regulating innate immune responses by striking a fine balance between allowing pathogen clearance and preventing excessive inflammatory responses that could result in immune pathology.

2.4 Role of MicroRNAs in Adaptive Immunity

The adaptive immune response is characterized by activation and subsequent clonal expansion of antigen-specific T and B lymphocytes leading to cytotoxic effector responses, and cytokine and antibody production in response to infections. In addition to regulating responses in innate immunity, miRNAs also play an important role in controlling adaptive immune responses. A number of different miRNAs are critical for B and T cell development, activation, survival, and proliferation.

2.4.1 B Cells

The importance of miRNAs in B cell development can be gauged from a study done on dicer-deficient B cells where development was blocked, probably as a consequence of increased Bim expression, a target of miR-17-92 [34]. In that study, miR-17-92 was also shown to dysregulate V(D)J gene rearrangements in mature B cells. Genome wide expression profiling of purified B cell subsets such as naive memory B cells and germinal center (GC) lymphocytes (centroblasts) revealed temporal changes in miRNA levels during B cell differentiation [43]. It also showed a highly unique miRNA expression signature in centroblasts, having an upregulated expression of miR-125b that targets the expression of IRF4 and PRDM1 (BLIMP1). These factors can repress BCL6 expression, which is necessary for differentiation to plasma and memory B cells. This means that miR-125b could play a role in regulating B cell differentiation. Memory B cells however, express miR-223 that downregulates the expression of LMO2, which is preferentially expressed in GC lymphocytes (that have lower levels of miR-223). While the function for LMO2 in GC cells has not yet been elucidated, its expression level in diffuse large B-cell lymphoma (DLBCL) patients has been correlated with increased survival [47].

Other miRNAs may also regulate various aspects of B cell differentiation and function. miR-181a is preferentially expressed by cells of the B lymphoid lineage in the mouse bone marrow and its ectopic expression in hematopoietic progenitors results in a doubling of B lymphoid cells with no increase in T lymphoid cells [10]. Another related member, miR-181b has been found to impair class switch recombination (CSR) in activated B cells by directly regulating activation-induced cytidine deaminase (AID), thereby restricting B cell malignancies [14]. Similarly, the DLE2/miR-15a/16-1 cluster may control B cell proliferation by regulating G_0/G_1-S phase related target genes [32]. Increased expression of miR-17-92 may result in lymphoproliferative disease and auto-immunity as a consequence of increased proliferation and less activation-induced cell death due to direct targeting of the tumor suppressor PTEN and Bim [65].

During lymphocyte development, miR-150 is highly expressed in mature and resting lymphocytes and targets the transcription factor c-Myb, the deletion of which blocks pro to pre-B cell transition and prevents B1 cell formation [64]. With miR-150 ablation, there is increased B1 cell expansion and enhanced humoral immune responses.

The best-characterized miRNA during a B cell response is miR-155, the deletion of which results in defective B cell immunity [53, 59]. miR-155 deficiency leads to a reduced number of germinal center B cells and extra-follicular B cells, with B cells failing to produce high affinity IgG1 antibodies. Here, miR-155 can regulate the formation of plasma cells by directly repressing its target gene PU.1 [62]. It can also suppress AID-mediated Myc-Igh translocation, thus acting as a tumor suppressor to reduce potentially oncogenic translocations [16].

2.4.2 T Cells

The development of T cells requires different signaling cascades, which are in regulated by various miRNAs. There are distinct patterns of miRNA expression in several stages of T cell development, with the miRNAs upregulated controlling functions such as differentiation and activation [48]. Additionally, the expression profile of naïve, effector and memory CD8+T cells has revealed dynamic changes in the levels of miRNA within each subset [63]. Global downregulation of miRNAs occurs in effector CD8+T cells relative to naïve CD8+T cells, with their level again increasing in memory CD8+T cells. Also, with activation, proliferating CD4+T cells tend to express mRNAs with shorter 3′UTRs, making them less prone to regulation by miRNAs [55]. These results therefore suggest that miRNAs may be critical for modulating gene expression during antigen-induced activation and differentiation of T cells.

T-cell specific deletion of dicer results in impaired T cell development, decreasing survival of αβ-expressing thymocytes, as well as dysregulating T helper cell differentiation and function [11]. Peripheral T cells are reduced in numbers, poorly proliferate with activation and undergo increased apoptosis [46]. An example of a miRNA that is important for T cell development is miR-181a. It can regulate the strength of TCR signaling by suppressing multiple phosphatases, thus influencing negative and positive selection [39]. Its expression also allows for maintenance of central tolerance through deletion of moderate-affinity interacting T cell clones.

Other studies have also indicated a role for various miRNAs, such as miR-155 in T cell differentiation, activation, and function. In the absence of miR-155, CD4+T cells are more prone towards T_H2 differentiation than T_H1 leading to increased production of cytokines like IL-4, IL-5 and IL-10 [53]. This may be due to increased c-maf levels, a potent trans-activator of the IL-4 promoter. miR-155 also represses the expression of IFNγRα on CD4+T cells [2], making them less sensitive to the anti-proliferative effects of IFNγ . Elevated miR-155 levels may also confer resistance on CD4+T cells to regulatory T cell-mediated suppression [56].

miR-326 on the other hand, was found to promote T_H17 differentiation by targeting Ets-1, potentially playing a critical role in the pathogenesis of multiple sclerosis [17]. Similarly, miR-17-92 was determined to regulate the differentiation of T follicular helper (T_{FH}) cells, which are important for maintaining germinal center B cells and antibody responses [67]. In addition to its role in B cells, T cells over-expressing this miRNA also showed an increased ability to proliferate and survive [65]. T cell activation leads to an induction of miR-146a, which may impair activator protein 1 (AP-1) and IL-2 production brought about by TCR triggering [13], thus regulating adaptive immune responses. It can directly repress expression of Fas-associated death domain (FADD), thereby modulating activation-induced cell death. Signaling through both, the TCR and co-stimulatory molecules are required for appropriate T cell activation, possibly through regulating expression of miRNAs, in addition to other functions. CD28 co-stimulation of T cells increases miR-214 levels, which in turn allows for enhanced proliferation through targeting of Pten [28].

miRNAs have also been found to be important for the development and function of regulatory T cells. In the absence of dicer in Forkhead box P3 (Foxp3) + Treg cells; there is down-regulation of transcription factor Foxp3 as well as loss of suppressor functions in vivo [68]. Foxp3 upregulates expression of miR-155 in Treg cells, which may be critical for Treg development [33] or for maintaining Treg proliferative activity and homeostasis by regulating SOCS1 and γc chain cytokine signaling [41]. Foxp3 also downregulates miR-142-3p in CD4 + CD25 + Treg cells, which targets adenylyl cyclase (AC) 9 mRNA, allowing for increased levels of cAMP, which is required for suppressor function [27]. On the other hand, miRNAs such as miR-31 and miR-21 may regulate expression of Foxp3 in T cells [54].

From the above, it is apparent that there is a body of evidence emerging which indicates that miRNAs play a critical role in regulating the development, differentiation, and function of T cell subsets and controlling aspects of immune responses to infections and auto-immunity.

2.5 Conclusion

miRNA are increasingly being identified as key players in the immune system, regulating processes such as the development, differentiation and function of immune cells. Several miRNAs have been identified in the different immune cell types regulating a number of responses. An interesting pattern has also emerged, where a single miRNA, such as miR-155, may influence global immune responses through its effect on macrophages, dendritic cells, and B and T lymphocytes through the direct regulation of distinct target genes. From the various studies done, it is now increasingly apparent that dysregulated expression of miRNA in the immune system could contribute to disease pathogenesis, especially in the case of inflammatory, cancer-related and auto-immune disorders. Additionally, miRNAs may also be critical for immune responses to pathogens such as viruses and bacteria, contributing to host immunity. More studies are needed to dissect the role of miRNA in vivo and confirm their function, but also the mechanism by which they mediate their effect. It remains to be established using in vivo models whether miRNA target and strongly modulate individual genes to alter immunity or if they have a more subtle mode of action affecting sets of genes moderately, but with profound biological outcome. Clearly more studies will be required to elucidate and help improve our understanding of the complex role played by miRNAs in immunity, possibly allowing for the eventual development of novel therapeutic approaches to treat diseases.

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Chapter 3
KIR/HLA: Genetic Clues for a Role of NK Cells in the Control of HIV

Lena Fadda and Galit Alter

Abstract Early events following HIV infections determine the course of disease progression. Mounting evidence suggests that antiviral immune responses induced soon after infection, prior to the induction of adaptive immune responses, are key to early control of viral infection. Among the early innate immune effector cells, natural killer (NK) cells represent a unique subset of lymphoctyes that do not express an antigen specific receptor. Rather, these cells integrate signals from an arsenal of non-specific inhibitory and activating receptors that are expressed on their cell surface. As such, these cells are classified as cells of the innate immune system, and they are able to lyse certain tumor targets and infected cells without the need for prior antigen sensitization. Over the past decade, accumulating evidence suggests that these innate lymphocytes may not be as innate as once believed, but that individual NK cell clones may show some target cell specificity, and play a critical early role following infection with HIV.

3.1 Introduction

NK cells are large, bone marrow-derived, granular lymphocytes [1–3] that are thought to play a critical role in the innate immune response to a number of infections [4], as they are able to respond to the incoming pathogen prior to the induction of an adaptive immune response. In this capacity, NK cells are able to rapidly lyse infected cells without the need for antigen sensitization. In addition to their direct cytotoxic role, these cells are also able to modulate the acute immune response through two separate mechanisms. First, NK cells have the propensity to release large quantities

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of pro-inflammatory cytokines that are critical for the early induction of adaptive immune responses [1, 5]. Secondly, NK cells have been shown to play a critical role in editing dendritic cell (DC) populations [5–7], thereby indirectly modulating the quality and frequency of DC subsets that are able to gain access to inductive sites where they may induce T and B cell responses. Thus, our appreciation for the role of NK cells in the immune response has expanded dramatically, beyond the notion that these cells are simply a subset of non-specific killers.

Human NK cells are classified as lymphocytes lacking B (CD19/20) and T (CD3) cell markers, but that do express CD56 and/or the Fcγ-receptor 3a (CD16) [1]. These cells can be divided into three functionally distinct subsets based on their surface density of CD56. Roughly 10% of NK cells in the peripheral circulation express high densities of CD56 ($CD56^{bright}$) and are thought to be critical immunoregulators because they lack the capacity to kill, but are able to secrete large quantities of pro-inflammatory cytokines. The $CD56^{bright}$ NK cells express abundant levels of the NCRs and c-type lectins, but lack the expression of most killer immunglobulin-like receptors (KIRs) [1], a family of inhibitory or activating NK cell receptors with a specificity for MHC class I molecules. The major population of NK cells in the peripheral blood express an intermediate level of CD56 ($CD56^{dim}$), contain large quantities of cytolytic granules, express intermediate levels of NCRs and c-type lectins, express abundant levels of KIR, and are typically referred to as the cytolytic subset of cells as they are able to spontaneously lyse certain tumor targets without the need for antigen sensitization [1]. As $CD56^{bright}$ NK cells do not express KIR, much debate exists as to whether these cells represent an immature state of NK cells, with $CD56^{dim}$ NK cells representing a more mature phenotype, or whether they represent an independent lineage of NK cells that are involved in non KIR-related NK-mediated immune responses [8, 9]. Finally, a third population of NK cells that express no CD56 have been recently defined that accumulate in the setting of chronic viral infection, $CD56^{neg}$ [10, 11]. These cells express similar patterns of other NK cell receptors as the $CD56^{dim}$ subset of NK cells, but contain little perforin, and are therefore poorly cytolytic and do not secrete cytokines. This subset is consequently referred to as anergic.

3.2 The "Missing Self" Hypothesis and NK Cell Self-tolerance

Control of NK cell activation is now understood to be a highly complex system of diverse inhibitory and activating receptor-ligand interactions. NK cells achieve inhibition mainly through interaction with MHC class I molecules via NK cell receptors that suppress rather than activate NK cell function. These receptors mediate a regulatory mechanism that is thought to protect normal cells from autologous NK cell attack. Lack of engagement of inhibitory receptors, along with the engagement of activating receptors by activating ligands on potential target cells results in target cell killing [12–14]. This central concept in NK cell recognition, termed the "missing self" hypothesis, postulates that engagement of inhibitory receptors by self MHC

class I molecules is necessary to inhibit NK cell activation. NK cells are therefore programmed to kill target cells that have lost or express low levels of MHC class I, which can occur during viral infection or tumor transformation. Furthermore, NK cells can sense abnormal cells that over-express ligands for activating NK cell receptors. NK cells are able to eliminate cells when a sufficiently potent activating receptor is stimulated despite expression of self-MHC class I on these cells [15].

NK cell inhibitory receptors for target cell MHC class I molecules play major roles in controlling the NK cell response to potential target cells. How these receptors are related to self-tolerance, however, is still not completely understood. Studies in humans and mice have shown that during NK cell development, NK cells undergo a self-MHC class I-dependent maturation process. Engagement of NK cell inhibitory receptors with self MHC class I provides a positive signal that leads to licensing of fully competent peripheral NK cells that can sense and lyse autologous target cells that lose MHC class I [16, 17]. In the absence of an inhibitory receptor-self MHC class I ligand interaction, NK cells are unlicensed and represent a hyporesponsive subset that fail to respond to a number of stimuli [17]. Recent evidence has suggested that NK cell licensing is a quantitative process. A hierarchy of responses to class I-negative cells by NK subsets has been observed that is directly proportional to the number of different inhibitory KIRs for self-HLA [18]. Furthermore, NK cells receiving stronger inhibitory signals during development through engagement with self MHC class I have been shown to respond more strongly and with increased frequency to MHC class I-deficient target cells than NK cells licensed on weaker inhibitory receptor-ligand combinations [19]. Certain KIR and HLA alleles are associated with more responsive NK cells [20, 21], suggesting that certain KIR and HLA combinations contribute more to NK cell potency than other combinations.

3.3 NK Cell Receptors

Among the arsenal of receptors that modulate NK cell function [3], some are unique to NK cells, such as the natural cytotoxicity receptors (NCRs) [22], while others are found on NK cells as well as other lymphocyte subsets. Among these additional NK cell receptors, two families of receptors, the c-type lectins (NKG2) and the killer immunoglobulin like receptors (KIR), have received the most attention.

The c-type lectins are conserved across mice and men, and consist of a family of both activating and inhibitory receptors that are abundantly expressed on NK cells, as well as particular subsets of T cells [23]. These receptors are expressed on NK cells early during development, suggesting that members of this family of receptors may not only play a role in the effector response of NK cells in the peripheral circulation, but may also be linked to early NK cell development [24]. Although these receptors are highly conserved, they interact with groups of MHC-class I homologues including HLA-E, G, and the stress inducible MHC class I chain homologue-A and -B that are more heterogeneous. Particular alleles of these ligands have been associated with differential outcomes in some auto-immune diseases [25]. Interestingly, the ligands

for these NK cell receptors are induced on the surface of infected or malignant cells following stress, and many viruses including HIV, have devised evasion mechanisms to avoid detection through these receptors [26]. In particular, HIV infection of CD4+ T cells can result in the upregulation of HLA-E, the dominant ligand for the inhibitory NKG2A [27]. HIV can also mediate the retention of MIC-A/B, the primary ligands for the activating NKG2D in the endoplasmic reticulum [28] as well as the release of this molecule from the cell surface following enzymatic surface cleavage [29]. Furthermore, recent data strongly implicates the activating NKG2D receptor in aberrant NK cell-mediated editing of DCs during chronic HIV infection [30], suggesting that members of this family of NK cell receptors may be involved in HIV associated immunopathogenesis.

The second family of receptors that has been investigated in great detail are members of the KIR family of receptors [31]. Unlike the highly conserved c-type lectins, KIRs are encoded on chromosome 19 within the human genome's second most polymorphic region, following the major histocompatibility locus. Similar to the c-type lectins, KIRs can be both inhibitory and activating and are classified by the number of loops in their extracellular domain (2D – two loops or 3D – three loops) and the length of their intracellular tails (L-long or S- short). The long intracellular tails contain two consecutive inhibitory ITIMs that permit the recruitment of the phosphatases SHP-1 and -2 upon ligation of inhibitory KIRs by their HLA ligands, they protect the cell from NK cell-mediated lysis [32]. Activating KIR with short intracellular tails lack these inhibitory motifs but contain a charged lysine residue in their transmembrane domain that allows interaction with ITAM-containing adaptor proteins that transmit the activating signal to NK cells [32] following ligation [33]. The known ligands of the KIR receptors consist of MHC-class I receptors and homologues. Several groups have speculated that rapid evolution at the MHC locus may have driven the co-evolution of their binding KIRs in response to infections in higher mammals [31]. Along these lines, particular KIR/HLA combinations have been associated with protection against several viral infections including HPV, HCV, and HIV, suggesting that particular populations of NK cells, bearing specific KIR receptors, may mediate control of viral infections in the context of their MHC-class I ligands [31].

KIR expression is defined during NK cell development in the bone marrow. During development, the pattern of KIR expression is determined through a stochastic process that randomly shuts off the expression of some receptors, but not others, resulting in the generation of some NK cells expressing anywhere from one to all encoded KIRs [33]. This random process leads to the variegated expression of KIRs, and gives rise to a diverse repertoire of NK cell clones with unique capacities to recognize target cells. However, KIR expression is locked during NK cell development, and once shut off in a given clone, KIR expression is thought to be fixed on a clonal level [34].

As mentioned above, KIRs are primarily expressed on CD56^{dim} cytolytic NK cells and are therefore likely central to modulating the functional responses mediated by this cell subset. In the peripheral circulation, the inhibitory KIRs are thought to play a critical role in monitoring for missing self, and therefore maintain a tonic inhibitory signal upon interactions with MHC-class I expressed on all self-cells. A loss of MHC class I, a common event in viral infection or tumor transformation, would

result in a lack of interaction with the inhibitory KIR, and would alert NK cells of the presence of an aberrant target cell. Less is known about target cell recognition by activating KIRs, as ligands for these receptors are largely unknown. Thus far, only direct binding of KIR2DS1 to HLA-C2 has been demonstrated – the same ligand for its inhibitory counterpart KIR2DL1 [35]. The role of activating KIR is not clear. Given the high sequence homology in the extracellular domain to their inhibitory counterparts, it may be that these receptors either recognize MHC class I molecules with a specific peptide related to viral or pathological conditions, or that they recognize MHC class I-like molecules. Even less is known about the functional interactions that result in activation of NK cells that encode non-expressed KIRs, such as KIR3DL1*004.

Crystal structures of KIR/MHC class I complexes show that KIR interacts with the α1 and α2 helix of MHC class I and makes direct contact with the C-terminal portion of the bound peptide [36, 37]. The critical nature of the peptide in the groove for the interaction of KIR with MHC has been further documented in a number of studies that clearly demonstrated that amino acid changes in peptide motifs, particularly at positions 7 or 8 within the peptide, resulted in the abrogation of inhibitory KIR recognition of target cells and the lysis of these cells [38–43]. Thus, several groups speculate that while self-peptides bound to MHC class I provide a strong inhibitory signal to the inhibitory KIR, it is possible that a stress or viral peptide produced during infection or following transformation may alter the capacity of the inhibitory KIR to bind (thereby lowering the inhibitory threshold) and increase the affinity of the activating KIR for MHC class I.

3.4 Role of KIR and HLA Polymorphisms in HIV-1 Infection

Genome wide association studies (GWAS) have shown that slower HIV disease progression is strongly associated with the expression of particular MHC class I alleles [44]. Two particular protective SNPs identified in the first reported GWAS were directly linked to MHC class I alleles, one that marked HLA-B57 (previously known to provide protection against HIV disease progression), and a second novel SNP 35 kb upstream from HLA-C thought to be related to increased HLA-C expression on the cell surface. The protective effect of this latter SNP was not attributable to a specific HLA-C allele [45], suggesting a potential non-CD8-dependent protective mechanism. Given that HLA-C is a ligand for KIR2D receptors [35, 46] present in the genomes of nearly all individuals, it is tempting to speculate that this protective effect in HIV infection is NK cell-dependent through the interaction of KIR2D with HLA-C. As mentioned earlier, NK cells licensed on stronger inhibitory signals during development are thought to respond more strongly and with increased frequency to loss of MHC class I than NK cells licensed on weaker inhibitory signals [19, 20]. Therefore, it may be possible that increased HLA-C expression may provide stronger licensing signals during KIR2D^{+} NK cells during development, contributing to protection when encountering an appropriate target cell.

Interestingly, epidemiological studies have shown that a number of additional HLA-Class I alleles, in addition to HLA-B57, are also associated with slower HIV disease progression [47]. These protective alleles fall within the MHC class I – B subgroup, and belong to a subset of alleles that express a serological epitope on their surface, Bw4. Furthermore, HLA-Bw4 homozygosity has been shown to be protective against HIV disease progression [48]. Additionally, a specific subset of HLA-Bw4 alleles that encode an isoleucine at position 80 within the antigen-binding groove, also referred to as HLA-Bw480I, have been shown to be largely dominate this protective effect. This subgroup of HLA class I alleles includes HLA-B57 in addition to a number of additional relevant HLA class I alleles that have been associated with slower HIV disease progression [47]. Given that the major role of HLA-class I molecules is to present intracellular peptides to patrolling CD8+ T cells, the popular hypothesis over the past two decades has been that these HLA class I alleles mediate their protective effect via the recruitment of HIV-1-specific cytotoxic CD8+ T cells.

More recently, epidemiologic studies have shown that the protective effect of some of these HLA class I alleles is dramatically enhanced by the co-expression of particular KIR alleles [49, 50]. While KIRs with two extracellular domains are thought to interact mainly with MHC class I-C alleles, the three domain KIRs interact with MHC class I-B alleles (and some MHC class I-A alleles that have HLA-Bw4 motifs) [33]. Among the three domain KIRs, many KIR3DL1 allotypes exist, including two activating variants (KIR3DS1) and at least three sub classes of inhibitory variants. These include a group that is expressed at high levels on the surface of NK cells (KIR3DL1^{hi}), one that is expressed at lower levels (KIR3DL1^{lo}), and a single variant that is not expressed on the cell surface (KIR3DL1*004) [21, 51]. Among these different allotypes, KIR3DS1, KIR3DL1*004, and the KIR3DL1^{hi} allotypes have all been shown to further enhance the protective effect of HLA-Bw4 alleles, including HLA-B57 [49, 52]. These associations strongly suggest that at least part of the protective effect of HLA-Bw4 alleles may be mediated through the recognition of HIV-1-infected cells by NK cells through particular KIR, potentially early in infection prior to the induction of adaptive immune responses, rather than through CD8+ T cells alone. However, the underlying functional mechanism by which an activating group of inhibitors and a non-expressive variant of KIR3DL1/S1 gene can all mediate protection against HIV-disease progression remains unclear, and has drawn a great deal of attention to the field of NK cell biology in response to this viral infection.

3.5 New Insights into NK Cell-Mediated Control of HIV

Progressive HIV infection is associated with striking changes in the phenotype of NK cell markers on NK cells during HIV infection, including a loss of NCRs (NKp46 and NKp30) expression [53], reduced NKG2D expression, and an inverted ratio of NKG2A:NKG2C expression [54]. Most notably, KIR expression has shown increase on both NK cells as well as CD8+ T cells with progressive infection [55]. Given that KIR expression is fixed during NK cell development, this data suggests

that these changes are likely related to the preferential expansion of KIR+NK cells with disease progression HIV infection. Furthermore, KIR3DS1+ NK cells exhibit enhanced function during early infection [56] and have been shown to expand preferentially following acute HIV infection in subjects that co-express its putative ligand, HLA-Bw480I [57]. Furthermore, while the physical interaction between the KIR3DS1 molecule and HLA-Bw480I molecules has yet to be shown, KIR3DS1+ NK cells respond potently to HIV-infected autologous CD4+ T cells in an HLA-Bw480I dependent manner [50]. These findings strongly suggest that early signals delivered by HIV-infected HLA-Bw480I+CD4+ T cells drive the rapid proliferation of KIR3DS1+ NK cells that may provide more robust antiviral control during acute infection, at a time when the adaptive immune response is just developing. However, the precise mechanism by which KIR3DS1 recognizes virally infected cells through HLA-Bw480I requires further investigation.

The antiviral role of the combined KIR/HLA genotype KIR3DL1^{hi}/HLA-Bw4 has been more elusive. However, both expression and functional data strongly suggest that NK cells that develop in this genetic background may be more functionally competent due to enhanced licensing signals during NK cell development [20, 58]. Thus, it is plausible that KIR3DL1^{hi}/HLA-Bw4 may deliver a stronger self-inhibitory result during NK cell development, prompting a pool of NK cells with an enhanced antiviral capacity [16, 59] able to respond more aggressively upon HIV infection to target cells with altered MHC class I expression. Along these lines, KIR3DL1+ NK cells from individuals that co-express increasing doses of its ligand, HLA-Bw4 [20] respond more potently to MHC class I negative target cells [20] and KIR3DL1+ NK cells expand preferentially following acute HIV infection in the presence of its ligand [57]. These statistics propose that KIR3DL1^{hi}/HLA-Bw4 may be associated with more functionally competent NK cells that play a critical role in antiviral control of HIV.

3.6 Conclusions

Epidemiologic data and genome wide studies on large units of subjects with differential control of HIV disease progression and potential resistance to infection are starting to shed light on the immunologic pathways that may play a vital role in the antiviral response to HIV infection. These studies have clearly highlighted the critical nature of particular MHC Class I alleles in slowing HIV disease progression, and more recent studies now suggest that particular KIR, preferentially expressed on NK cells, may further enhance the protective activity of these genes. Interestingly, both inhibitory and activating genes of the same locus have been implicated in this protection, and mounting mechanistic data suggests that these receptors mediate their protection in distinct manners that are critically dependent on interaction with protective MHC class I-B alleles. Defining the mechanism by which KIR/HLA are able to mediate antiviral control may offer new avenues by which to manipulate the immune system to gain more effective control of HIV infection.

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Chapter 4
Natural Killer Cell Licensing During Viral Infection

Mark T. Orr and Lewis L. Lanier

Abstract Natural Killer (NK) cell functionality is controlled by inhibitory receptors that recognize self-MHC class I. NK cells that do not interact with self-MHC class I are hypo-responsive to many stimuli and fail to reject MHC class I-deficient cells. Thus, although the mechanisms are unknown, interactions with MHC class I "licensed" NK cells respond efficiently. Surprisingly, these licensed NK cells fail to control viral infection. During mouse cytomegalovirus (MCMV) infection, SHP-1 signaling downstream of inhibitory receptors for MHC class I limits NK cell proliferation. Interactions with MHC class I prevent licensed NK cells from controlling of MCMV replication and pathogenesis; rather, it is the unlicensed NK cells that are not inhibited by self-MHC class I that efficiently control MCMV infection. Therefore, the licensing hypothesis is not sufficient to explain NK cell functionality during viral infection.

4.1 Introduction

Natural Killer (NK) cells play a crucial role in controlling certain viral infections and eliminating tumor cells. Depletion of NK cells renders animals susceptible to infections such as mouse cytomegalovirus (MCMV), ectromelia virus, and Ebola virus [1–3]. Humans preferentially lacking NK cell functions are unable to control Epstein-Barr virus, human cytomegalovirus, and varicella zoster virus, among others [4–6]. NK cells kill target cells by secretion of lytic granules containing perforin and granzymes. Upon activation, NK cells also produce a number of effector molecules including IFN-γ, TNF-α, and MIP-1β that shape the innate and adaptive immune responses to infection and transformation [7]. NK cells express a plethora of inhibitory

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and activating receptors. The integration of activating and inhibitory receptor signaling determines whether an NK cell attacks a potential target cell or not [8].

NK cell activating receptors include: NKR-P1C (NK1.1), the Fc receptor CD16, the activating Ly49 family members Ly49D and Ly49H, the activating members of the Killer Cell Immunoglobulin-like Receptor (KIR) family, NKG2D, and the natural cytotoxicity receptors NKp30, NKp44, and NKp46 [8]. Ligands for these receptors may be broadly divided into self-ligands such as the RAE-1 and ULBP families of stress-induced NKG2D ligands and IgG antibodies recognized by CD16 and non-self ligands including the m157 glycoprotein encoded by MCMV recognized by Ly49H and influenza virus hemagglutinin recognized by NKp46 [8]. These activating receptors typically lack intracellular signaling domains but instead non-covalently associate with signaling adaptors via charged amino acids in the transmembrane domains of the receptors and adaptors. These signaling adaptors include the DAP10 adaptor and the immuno-receptor tyrosine-based activating motif (ITAM)-containing molecules CD3ζ, FcεRIγ, and DAP12. The ITAM adaptors signal via Syk and ZAP70, whereas DAP10 signals via PI-3 kinase and a Vav1 complex [9].

NK cell inhibitory receptors recognize a wide variety of self-molecules. The most well studied ligand families for NK cell inhibitory receptors are the MHC class I molecules recognized by inhibitory Ly49 family members in rodents and inhibitory KIR family members in humans [10]. In mice and humans, the heterodimeric receptor CD94-NKG2A recognizes non-classic MHC class I molecules, mouse Qa-1 or human HLA-E, presenting leader peptides from classical MHC class I molecules. Inhibitory NK cell receptors that bind to ligands other than MHC class I include: KLRG1 (which binds cadherins), 2B4 (which recognizes CD48), CD305 (LAIR-1) that binds collagen, the gp49B receptor for integrin ($\alpha_v\beta_3$), and others [11]. Most of these inhibitory receptors contain an immune-receptor tyrosine-based inhibitory motif (ITIM) in their intracellular domain. Upon receptor engagement and phosphorylation of the ITIM, the protein phosphatases SHP-1 and SHP-2 and the inositol phosphatase SHIP-1 are recruited to the ITIM domain [12]. Although inhibitory receptor triggering results in the dephosphorylation of many proteins, Vav1 seems to be a critical target of SHP-1 [13]. Dephosphorylation of Vav1 abrogates NK cell activation via activating receptors.

4.2 NK Cell Licensing

NK cells in wild-type mice reject normal, syngeneic MHC class I-deficient cells [14]. This suggests that normal cells express one or more unidentified activating ligands, which if unopposed by MHC class I reactive inhibitory receptors, can activate NK cells. NK cells from MHC class I-deficient animals (arising from deletion of *B2m, TAP 1,* or the MHC class I heavy chain genes) are not auto-reactive and do not reject MHC class I-deficient cells, despite the presence of activating ligands [14–16]. Despite appearing phenotypically mature, NK cells from MHC class I-deficient animals are hypo-responsive to activation induced by crosslinking their activating receptors by using plate-bound antibodies against these receptors [17, 18]. Thus, the presence of MHC class I is paradoxically required to engender responsiveness in NK cells.

Many NK cell receptors are expressed in an overlapping, stochastic fashion resulting in many subsets of NK cells defined by their receptor-expression pattern. Although it has been previously postulated that NK cells are selected for the expression of at least one self-MHC class I reactive inhibitory receptor [19], recent studies have found a sizable number of phenotypically mature NK cells that lack inhibitory receptors for self-MHC class I [17, 20, 21]. Similar to the NK cells from MHC class I deficient animals, the NK cells that do not recognize self-MHC class I are hypo-responsive [17, 18, 22]. Responsiveness can be restored to hypo-responsive NK cells by transgenic expression of a MHC class I allele that is able to interact with an inhibitory Ly49 receptor on these NK cells [18]. Responsiveness also requires an intact ITIM domain, suggesting that inhibitory receptor signaling is required. However, SHP-1 and SHIP-1 are dispensable for NK cell responsiveness [18].

Two hypotheses have been proposed to explain the requirement for recognition of self-MHC class I via inhibitory receptors for NK cell responsiveness. The licensing hypothesis suggests that NK cells are initially hypo-responsive and that engagement of MHC class I "licenses" NK cells to become functional [18]. The alternate "disarming" hypothesis proposes that NK cells are initially functional, but constant engagement of activating receptors on NK cells with self ligands, if unopposed by MHC class I-specific inhibitory receptors, renders NK cells anergic [23]. In mice, where only a fraction of the cells express MHC class I (either bone marrow chimeras generated from a mixture MHC class I sufficient and deficient bone marrow cells or in transgenic mice with an incompletely penetrant MHC class I expression, resulting in mosaic expression of an MHC class I molecule), all of the NK cells are hypo-responsive [24, 25]. These data suggest that continuous encounters with MHC class I-deficient normal self cells renders NK cells hypo-responsive, consistent with the disarming hypothesis. A slight modification of this hypothesis proposes that the number and affinity of self-MHC class I inhibitory receptors quantitatively alter NK cell responsiveness [26, 27]. Recent findings support this rheostat model [28, 29].

If NK cells that do not recognize self-MHC class I are non-functional, the question arises of why they are not selected against or deleted, much like T cells that do not recognize peptide-MHC class I in the thymus undergo death by neglect. T cells that become anergic due to chronic stimulation can regain functionality if stimulated via cytokines or if the anergizing signal is blocked [30, 31]. Similarly, hypo-responsive NK cells that lack self-reactive inhibitory receptors for MHC class I can regain responsiveness in a variety of ways. In vitro culture in high doses of IL-2, stimulation with IL-12 and IL-18, or in vivo infection with *Listeria monocytogenes* all restore responsiveness to disarmed or "unlicensed" NK cells [17, 18, 32].

4.3 NK Cell Control of MCMV

NK cells are critical for control of the mouse pathogen MCMV. In C57BL/6 (B6) mice NK cells recognize MCMV-infected cells via the activating Ly49H receptor [33]. Upon infection the MCMV MHC class I-like glycoprotein, m157 is expressed on the surface of infected cells and is directly ligated by Ly49H [34, 35]. In other mouse strains such

as 129, MCMV m157 binds the inhibitory Ly49I receptor, preventing NK cell control of MCMV [34]. The gene encoding Ly49H (*Klra8*) likely arose from the duplication and gene conversion of the gene encoding Ly49I (*Klra9)*, resulting in an activating NK cell receptor with the extracellular domain of Ly49I [36]. Upon recognition of MCMV-infected cells via Ly49H, NK cells secrete IFN- γ, kill infected cells via the release of lytic granules, and proliferate. During MCMV infection, Ly49H$^+$ NK cells expand from 50% of all NK cells to 90% of all NK cells [37].

B6 mice express four inhibitory Ly49 receptors; Ly49A, Ly49C, Ly49G2, and Ly49I [10]. Ly49C and Ly49I recognize the MHC class I molecule H-2K^b in B6 mice, whereas Ly49A and Ly49G2 do not bind to MHC class I molecules in B6 mice to any appreciable degree [38]. Ly49C and Ly49I (designated Ly49C/I because the antibody commonly used for their detection cross-reacts with both) are expressed on about half of all NK cells in B6 mice and confer responsiveness to these NK cells [17, 18]. Conversely, Ly49C/I$^-$ NK cells from B6 mice are hypo-responsive. When stimulated with MHC class I-deficient targets expressing m157, the Ly49C/I$^+$ subset of Ly49H$^+$ NK cells responded more readily than their Ly49C/I$^-$ counterparts, indicating licensing in Ly49C and/or Ly49I$^+$ NK cells in B6 mice. However, when stimulated with MHC class-I sufficient targets expressing m157, H-2K^b engagement with Ly49C/I inhibited NK responses such that the unlicensed cells that did not express H-2K^b reactive inhibitory receptors were more responsive than their licensed counterparts [39].

Upon MCMV infection, unlicensed Ly49C/I$^-$ Ly49H$^+$ NK cells proliferated much more than the licensed Ly49C/I$^+$ Ly49H$^+$ cells. Proliferation of licensed Ly49H$^+$ NK cells was impaired by interactions with MHC class I and the subsequent signaling via SHP-1 downstream of Ly49C/I engagement [39]. Although licensed cells account for half of all Ly49H$^+$ NK cells, depletion of these cells did not impair NK cell control of MCMV replication. Conversely, depletion of the 50% of Ly49H$^+$ NK cells that express Ly49G2$^+$ abrogated NK cell control of MCMV replication. Depletion of Ly49G2$^+$ NK cells removed both licensed and unlicensed cells, suggesting that only the unlicensed cells are sufficient to control MCMV replication [39]. NK cells from mice that lack MHC class I expression due to deletion of the gene encoding b2-microglobulin (*B2m)* are all unlicensed, yet NK cells from *B2m*$^{-/-}$ mice control MCMV replication better than their wild-type counterparts that include both licensed and unlicensed NK cells [17, 39]. Adoptive transfer of NK cells from adult mice into neonatal mice is sufficient to prevent MCMV-induced lethality. Transfer of unlicensed Ly49H$^+$ NK cells was sufficient to protect neonatal recipients, whereas transfer of an equal number of licensed Ly49H$^+$ NK cells provided no protection [39]. Thus, it is the unlicensed NK cells, which are uninhibited by self-MHC class I, that are able to protect against MCMV infection.

4.4 Unlicensed NK Cells in the Control of Leukemia

Several types of leukemias, including acute and chronic myeloid leukemias can be treated by lethal irradiation followed by bone marrow transplantation. Donor NK cells that are transferred along with the bone marrow are able to kill residual leukemic

cells, reducing the likelihood of leukemic relapse [40]. Donor NK cells also kill recipient antigen-presenting cells (APCs), limiting the priming of host vs. graft responses [40]. In HLA-mismatched transplantation, the number of mismatches between donor's inhibitory KIRs and the recipient's HLA correlated with fewer leukemic relapses and increased graft acceptance, suggesting that uninhibited NK cells are more efficient at eliminating leukemic cells and preventing rejection of the donor cells [40, 41]. In HLA-matched transplantation, the number of mismatches between donor's inhibitory KIR and HLA also correlated with fewer leukemic mismatches [42–45]. In this case, the donor and recipient's HLA are identical, suggesting that it might be the unlicensed NK cells that are killing the tumor cells and the recipient's APCs. These unlicensed NK cells appear phenotypically activated and express effector molecules after transplantation [46].

4.5 Consequences of Activation of Unlicensed NK Cells

The restoration of responsiveness to NK cells that are not inhibited by self-MHC class I during MCMV infection raises the possibility that the NK cells might also attack uninfected healthy cells. This might be prevented by inhibitory receptors that recognize self-ligands other than MHC class I. This possibility is supported by the increased expression of the inhibitory receptors KLRG1 and gp49B on NK cells during MCMV infection [47, 48]. Alternatively, the evolutionary advantage gained by unlicensed cells controlling infection may outweigh the cost of transient auto-immunity mediated by activated, unlicensed NK cells. In mixed bone marrow chimeras containing both MHC class I-deficient and sufficient hematopoetic cells, the MHC class I-deficient cells are tolerated and the NK cells are hypo-responsive [25]. After MCMV infection of these mixed bone marrow chimeras, NK cells rapidly kill the MHC class I-deficient cells, even if they are not infected with MCMV [49]. Under these conditions, NK cells can mediate auto-immune attack after viral activation if not inhibited by receptors for MHC class I. In wild-type animals it remains unclear whether unlicensed NK cells will attack normal-self during viral infections.

What is the biological importance of licensing? In the case of infection, inflammation reverses the hypo-responsiveness of unlicensed cells. In uninfected animals, licensed, but not unlicensed, NK cells are able to reject MHC class I-deficient cells. Although untested, licensed cells may be poised to control transformed cells that lose expression of MHC class I in the absence of inflammation. Similarly, the biological importance of MHC class I-reactive NK cell inhibitory receptors remains a mystery. Several viruses inhibit the expression of MHC class I as a means to avoid detection by $CD8^+$ T cells [50]. Although it has been proposed that MHC class I-reactive inhibitory receptors allow NK cells to detect this downregulation of MHC class I, there is currently no in vivo evidence to support this hypothesis. Alternatively, these inhibitory receptors may limit over-exuberant NK cell responses. In some settings, NK cells limit the magnitude and efficacy of $CD8^+$ T cell priming by APCs [51, 52]. Expression of MHC class I inhibitory receptors might limit NK cell killing of infected APCs, allowing the APCs to prime an effective T cell response.

Thus, MHC class I-reactive inhibitory receptors paradoxically enhance NK cell responsiveness, likely by preventing activation-induced anergy. During infection, these same receptors restrain the NK cell response in controlling infection. This restraint of NK cell activity might limit NK cell-mediated auto-immunity and allow for efficient T cell priming by infected APCs that may otherwise be eliminated by unrestrained NK cells.

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Chapter 5
Bridging Innate NK Cell Functions with Adaptive Immunity

Emanuela Marcenaro, Simona Carlomagno, Silvia Pesce, Alessandro Moretta, and Simona Sivori

Abstract Killer Ig-like receptors (KIRs) are major human NK receptors displaying either inhibitory or activating functions which recognize allotypic determinants of HLA-class I molecules. Surprisingly, NK cell treatment with CpG-ODN (TLR9 ligands) results in selective down-modulation of KIR3DL2, its co-internalization with CpG-ODN and its translocation to TLR9-rich early endosomes. This novel KIR-associated function may offer clues to better understand the possible role of certain KIRs and also emphasizes the involvement of NK cells in the course of microbial infections.

NK cells are involved not only in innate immune responses against viruses and tumors but also participate in the complex network of cell-to cell interaction that leads to the development of adaptive immune responses. In this context the interaction of NK cells with DC appears to play a crucial role in the acquisition of CCR7, a chemokine receptor that enables NK cells to migrate towards lymph nodes in response to CCL19 and/or CCL21. Analysis of NK cell clones revealed that KIR-mismatched but not KIR-matched NK cells acquire CCR7. These data have important implications in haploidentical haematopoietic stem cell transplantation (HSCT), in which KIR-mismatched NK cells may acquire the ability to migrate to secondary lymphoid compartments (SLCs), where they can kill recipient's antigen presenting cells (APCs) and T cells thus preventing graft versus host (and host vs. graft) reactions.

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5.1 Surface Receptors Regulating NK Cell Function

Natural killer (NK) cells represent a cellular component of the innate immune system able to exert a prompt cytolytic activity against infected and tumor cells without prior stimulation. In humans, NK cells express a large number of surface receptors that are involved in the regulation of the different NK cell functions. Some of these receptors sense the expression of HLA class I molecules on potential target cells and deliver negative signals that inhibit the NK-mediated killing [1]. These include the clonally distributed Killer Ig-like Receptors (KIR) (also referred to as CD158) that are able to distinguish among different HLA-C, B and A allotypes [1] and the CD94/NKG2A heterodimer that directly recognizes HLA-E, a non-classical MHC molecule characterized by a limited polymorphism [2].

In the absence of interactions between NK inhibitory receptors and self HLA class I molecules, a series of non-HLA specific receptors transduce activating signals that result in NK-mediated attack of allogeneic, tumor, or virus-transformed cells [3]. A number of triggering receptors responsible for NK cell activation have been identified and molecularly characterized in recent years. For example, NKp46, NKp30, and NKp44 (collectively termed Natural Cytotoxicity Receptors, NCR) [4] are mostly expressed by NK cells and represent crucial receptors for the recognition and killing of the majority of target cells. Other activating receptors involved in target cell recognition and lysis are 2B4, NTB-A [3, 5], NKG2D, and DNAM-1. NKG2D recognizes stress induced ligands such as MICA/MICB and ULBPs, whereas DNAM-1 recognizes PVR (CD155) and Nectin-2 (CD112) [6, 7]. Alternative pathways of NK cell activation can be induced by cytokines (e.g. IL-2, IL-15, and IL-12) [8, 9] or by toll-like receptors (TLRs) [10, 11]. These receptors, including TLR3 (that recognizes dsRNA of viral origin) and TLR9 (that recognizes microbial unmethylated CpG-DNA), are expressed in human NK cells, independent of their activation status, and allow NK cells to respond both to viral and bacterial products [11]. Frequently, the combination of different activating signals leads to optimal NK cell triggering.

5.2 KIRs as Receptors for HLA Class I

KIRs belong to the Ig superfamily and in most instances, recognize determinants shared by groups of HLA-A, HLA-B, or HLA-C allotypes [12–16]. The first identified KIRs were characterized by an inhibitory function and a long cytoplasmic tail containing two immune-receptor tyrosine-based inhibition motifs (ITIM) [1]. In addition, activating forms of KIRs, characterized by a short cytoplasmic tail lacking ITIM motifs, were also identified [17, 18]. Only for one of such activating KIRs (KIR2DS1) the specificity for HLA-class I molecules has been demonstrated [18–20].

From an evolutionary point of view, all KIRs appear to derive from an ancestral molecule constituted of three Ig-like domains (D0, D1 and D2) and a long cytoplasmic

tail, although the predominant human KIRs present in humans are characterized by two Ig-like domains (referred to as KIR2D) [21]. On the basis of domain content, two types of KIR2D can be determined. KIR2D of the first type are composed by D1 and D2 domains and include the majority of KIRs (KIR2DL1/L2/L3 and KIR2DS1/S2/S3/S4/S5) [22, 23] most of which recognize HLA-C molecules, whereas KIR2D of the second type are composed by D0 and D2 domains and include KIR2DL4/L5 [24, 25]. KIRs characterized by three Ig-like domains (KIR3D) are specific either for the HLA-Bw4 group of alleles (KIR3DL1) [26], or for some HLA-A alleles (KIR3DL2) [27, 28]. All KIRs, with the exception of KIR2DL4, are clonally distributed on NK cell surface [15, 29, 30]. Individual NK cell clones express different sets of inhibitory and activating KIRs; however, each NK cell clone expresses at least one self-reacting inhibitory receptor, either a KIR or CD94/NKG2A [31]. A small subset of NK cells may lack inhibitory receptors for self HLA class I molecules; but these cells are poorly functional due to their inability to become "educated" (or licensed) [32].

5.3 KIRs as Sensor of Microbial Products

Upon interaction with their specific ligands, TLR3 and TLR9 promote cytokine release and increment of cytotoxicity by NK cells [11]. Responses to these stimuli occur in both fresh and in IL-2-activated NK cells and are remarkably increased in the presence of IL-12. It has been demonstrated that prior to cell stimulation, TLR9 is localized in the endoplasmic reticulum and upon cell treatment with CpG-ODN, it translocates to the endosomal compartment where both CpG-ODN binding and initiation of signal transduction take place [33, 34].

One of the problems in TLR biology is represented by the delivery of ligands to endosomal/ER resident TLRs. It is likely that surface receptors could be involved in the process of ODN uptake and the delivery of ODN to TLR9-containing endosomes [35, 36] and that these surface receptors could be different depending on the cellular type considered. With this in mind, we have recently suggested that in human NK cells, some KIRs (primarily KIR3DL2) may function as sensors for microbial products and as chaperones for TLR9 ligands [37]. Indeed, KIR3DL2 can bind CpG-ODNs at the cell surface and shuttle them to endosomes where TLR9 is localized (Fig. 5.1). Interaction between KIR3DL2 and ODN results in sharp down-modulation of KIR3DL2 surface expression and in induction of cytokine release [37]. Moreover, KIR3DL2 molecules are associated with ODN not only at the cell surface but also intra-cellularly. In particular, KIR3DL2 and ODN are co-internalized in early (EEA-1+) endosomes (where TLR9 translocates upon ODN stimulation), but then they dissociate and ODN and TLR9 travel together toward the late (Rab7+) endosomes. TLR9 is stably associated with ODN during its residency in both early and late endosomes [37].

It is likely that ODN binding by KIR3DL2 does not require additional molecules associated with KIR3DL2 at the NK cell surface as suggested by the direct binding of ODN to KIR3DL2-Fc soluble molecules [37]. In addition to KIR3DL2-Fc, other

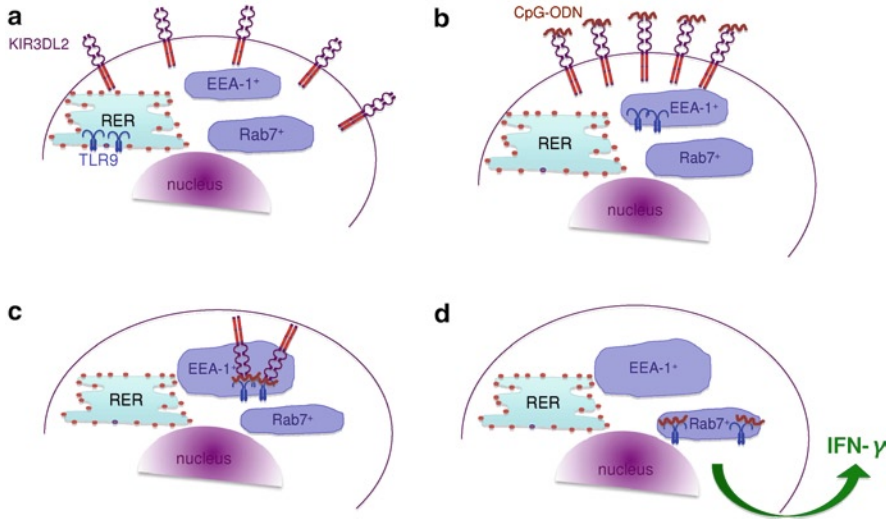

Fig. 5.1 KIR3DL2 as sensor for microbial products and as chaperones for TLR9 ligands. Unmethylated sequences rich in C and G (CpG) are typical of microbial DNA. CpG-ODN are specifically recognized by TLR9, a TLR expressed by different cell types of the innate immune system including NK cells. A subset of NK cells expresses KIR3DL2, an inhibitory KIR that binds certain HLA-A alleles. (**a**) Before stimulation with CpG-ODN, KIR3DL2 is expressed at the NK cell surface, whereas TLR9 resides in intracellular compartments (mostly in the RER). (**b**) In response to CpG-ODN, TLR9 translocates to EEA-1^{+} early endosomes. (**c**) Upon interaction with CpG-ODN, KIR3DL2 is modulated from the cell surface and internalized and shuttles CpG-ODN towards EEA-1^{+} early endosomes (where KIR3DL2, CpG-ODN and TLR9 co-localize). (**d**) Upon dissociation of KIR3DL2-ODN complexes, CpG-ODN interacts with TLR9 and they travel together towards Rab7^{+} late endosomes, inducing the NK cell functional program (increments of cytolytic activity and cytokine production)

KIR-Fc soluble proteins including KIR3DL1, KIR3DS1, and KIR2DL4 can bind ODN, whereas KIR-Fc specific for HLA-C alleles (KIR2DL1/S1 and KIR2DL2/L3/S2) do not. Remarkably, all the ODN-binding KIRs are characterized by a D0 domain. Analysis of the ODN-binding capability of individual Ig domains revealed a predominant involvement of D0 [37]. Previous studies showed that KIR3DL1-mediated recognition of HLA-class I takes place through D1 and D2 domains, while the D0 domain would only enhance the strength of KIR/HLA interaction [38]. Thus, our data provides evidence for a novel important function of D0, such as to mediate direct recognition of microbial CpG-ODN. CpG-ODN recognition may be based on the interaction between negative charges of DNA sequences and positive charges of binding KIR domains. CpG ODN-binding KIRs display a positive net charge whereas non-binding KIRs are characterized by a negative net charge [37].

Although soluble KIR3DL1, KIR3DS1, and KIR2DL4 could bind ODNs, they did not induce, in normal NK cells, modulation of the corresponding membrane

receptors comparable to that of KIR3DL2 [37]. Remarkably, IFN-γ production in response to ODN was mostly confined to KIR3DL2+ NK cell subsets suggesting that other ODN-binding KIRs do not mediate efficient ODN-shuttling into NK cells. This discrepancy may be explained by the existence of still undefined molecules necessary for the process of ODN internalization that associate with KIR3DL2. These molecules might be unable to associate to other ODN-binding KIRs; thus explaining their inability to internalize ODN.

It is of note that KIR3DL2 (together with KIR2DL4 and KIR3DL3) represents a framework gene (i.e. it is present in all KIR haplotypes) [39]. As a consequence, NK cells of all individuals can bind CpG-ODN. This novel functional capability of KIR3DL2 may provide an important clue to understanding the driving forces that led to conservation of KIR3DL2-encoding gene in all haplotypes, in spite of the low frequency in the human population of HLA-A3 or -A11 alleles (i.e. the HLA ligands of KIR3DL2). Indeed, the need of rapid NK-mediated responses to microbial products may represent an important factor of selective pressure. In this context, KIR3DL2 is characterized by a low inhibitory capability because of its low affinity for its HLA-A ligand. Therefore, it is possible that the prevalent role of KIR3DL2 in humans may be its ability to promptly sense microbial CpG-ODNs.

5.4 Selection of the Most Fitting DC for Antigen-Presentation by NK Cells

Recently it has been shown that NK cells not only represent the most efficient innate immune mechanism protecting an individual from viruses and tumors, but also participate in the complex network of cell to cell interaction that leads to the development of Ag-specific adaptive immune responses [40–42]. After recruitment and activation within inflamed peripheral tissues, NK cells acquire the ability to exert cytolytic activity against transformed or infected target cells (representing an early barrier against pathogen invasion) and the capacity to kill immature DC (editing process). Notably, this "editing process" is mediated by NK cells expressing the KIR-, NKG2A+phenotype which kill immature DC (that express low levels of HLA-E) while sparing those undergoing maturation. Mature DC become resistant to lysis [43] thanks to the upregulation of HLA-E surface expression. On the contrary, KIR+NK cells are unable to kill DC and do not participate to the "DC editing process." This reflects the interaction of the inhibitory KIR with classical HLA-class I molecules, expressed in sufficient amounts on DC.

On the other hand, in inflamed tissues, KIR+NK cells can kill target cells expressing low levels of HLA-class I as a result of viral infection or tumor transformation [44].

The NK cell-mediated "editing process" may serve to keep in check the quality of DC undergoing maturation, a process which is characterized by the acquisition of the expression of CCR7 and by the upregulation of HLA-class I/II and of co-stimulatory

molecules [45]. This appears to be crucial for the subsequent DC migration and priming of naïve T lymphocytes in secondary lymphoid organs [8, 46]. The final outcome of this process mediated by NK cells might be the selection of the most appropriate DC thanks to the removal of those DC that would fail to mediate optimal antigen presentation and T-cell priming [47].

5.5 Different Modes of NK Cell Migration to Lymph Nodes

The majority of peripheral blood NK cells are characterized by the CD56dull, CD16+, KIR+, and/or NKG2A + surface phenotypes and express chemokine receptors such as CXCR1, CX3CR1, and ChemR23. On the other hand, the minor peripheral blood CD56bright, CD16-, KIR-, and NKG2A+NK cell subset expresses CCR7 [48–51]. Based on their chemokine receptor profile it is conceivable that CD56dull, CD16+ cells may be mainly recruited into inflamed peripheral tissues, whereas CD56bright, CD16- cells should be attracted to secondary lymphoid compartments (SCLs) such as lymph nodes in response to CCL19 and CCL21 [42, 52]. In inflamed peripheral tissues NK cells were found to be mostly CD56dull and to express CXCR1 and ChemR23 [49]; whereas within normal, non-inflamed lymph nodes, NK cells were homogeneously characterized by the CD56bright, CD16dull/-, KIR-, NKG2A+ surface phenotype [40, 41, 48, 50]. These NK cells are localized in the para-cortical T cell areas and are high IFN-γ producers but display low cytolytic activity. In addition to these pathways of NK cell recruitment that reflect the constitutive expression of different sets of chemokine receptors, another mode of NK cell migration to lymph nodes has recently been described which allows the CD56dull, CD16+ NK subset to migrate to lymph nodes as well. When exposed to exogenous IL-18, that may be released in inflamed tissue by innate cells (such as Macrophages/Dendritic cells) they express surface CCR7 and respond to CCL19 and CCL21 [53].

On the basis of these observations, it has been proposed that CD56dull, CD16+ NK cells, recruited into inflamed tissues, in the presence of pro-inflammatory stimuli favoring their interaction with DC [44, 45, 54–57], may exert an instructive activity on naïve T cell priming. This could occur by one of the following mechanisms: (1) induction of DC polarization resulting in the production of high levels of IL-12 which, in turn, would induce type-1 T cell responses (this mechanism would not require tissue-activated NK cells to enter into lymph nodes) [9, 46] or (2) migration to lymph nodes of NK cells exposed to IL-18 (released by pathogen-activated antigen-presenting cells); their entry into the para-cortex area where they can interact with DC and T cells and regulate T cell responses [8, 53, 58].

Our recent data [59] demonstrates that KIR+, CD56dull NK cells may also express de novo surface CCR7 in an IL-18-independent manner [53]. Upon interaction with APCs or virus-infected cells, they express surface CCR7 and acquire migratory properties in response to SLC chemokines [59]. The acquisition of

CCR7 by these NK cells requires direct cell to cell contact and is detectable within few minutes and due to receptor uptake from CCR7+ cells, a mechanism termed trogocytosis [60–63].

The uptake of CCR7 is tightly regulated by KIR/HLA class I interactions. When KIR+NK cells interacted with autologous mDC, CCR7 was acquired only in the presence of anti-HLA class I antibodies capable of disrupting KIR/HLA interactions [59]. Alternatively, CCR7 was acquired when a KIR/KIR-ligand mismatch existed between NK and mDC. This condition is reproduced in vivo during KIR-mismatched haplo-identical HSC transplantation to cure high-risk leukemias [59, 63, 64].

5.6 How Alloreactive NK Cells Can Reach Lymph Nodes

In transplantation, the alloreactive NK cells are KIR+, NKG2A- cells characterized by the selective expression of given KIR that is not engaged by HLA class I alleles of recipient's target cells (KIR-mismatched alloreactive NK cells) [14, 65, 66].

Alloreactive NK cells kill KIR-ligand mismatched leukemic blasts and play a crucial role in eradicating high risk acute myeloid as well as lymphoid leukemias in the T-cell depleted haplo-identical HSC transplantation. It is of note that in this transplantation setting, all cases are at high risk of T cell-mediated alloreactivity both in the host vs. graft (HvG) and in the GvH direction. Remarkably, patients transplanted from an NK alloreactive donor benefit from higher rates of engraftment and reduced incidence of GvHD [66–69].

It has been proposed that the low rate of GvHD is consequent to the inefficient priming of (the few) alloreactive donor T cells due to the NK cell-mediated killing of recipient's APC [69]. In agreement with this concept, in vitro studies showed that alloreactive NK cell clones kill allogeneic mature DC [43].

Importantly, alloreactive NK cells predominantly attack the hematopoietic cells of the host (including DC, T cells, and leukemic cells) while sparing other tissues that are common targets for T-cell-mediated GvHD [66]. Some of these target cells might be killed within peripheral tissues, but the elimination of DC should occur primarily within SLC and plays a major role in preventing GvH reactions. Indeed, it is mainly at these sites that patient's DC would prime donor's allospecific naïve T cells.

However, since KIR+NK cells do not express CCR7, it has been difficult to explain how donor's KIR-mismatched NK cells may get in close proximity with recipient APCs and kill them within lymph nodes.

Our findings suggest a mode by which alloreactive KIR+NK cells upon interaction with mDC acquire the ability to reach lymph nodes [59]. Importantly, this DC-induced CCR7 expression may represent the major mechanism by which KIR-mismatched NK cells can migrate to lymph nodes, kill recipient's DC and prevent priming of alloreactive donor's T cells and induction of GvHD [59] (Fig. 5.2).

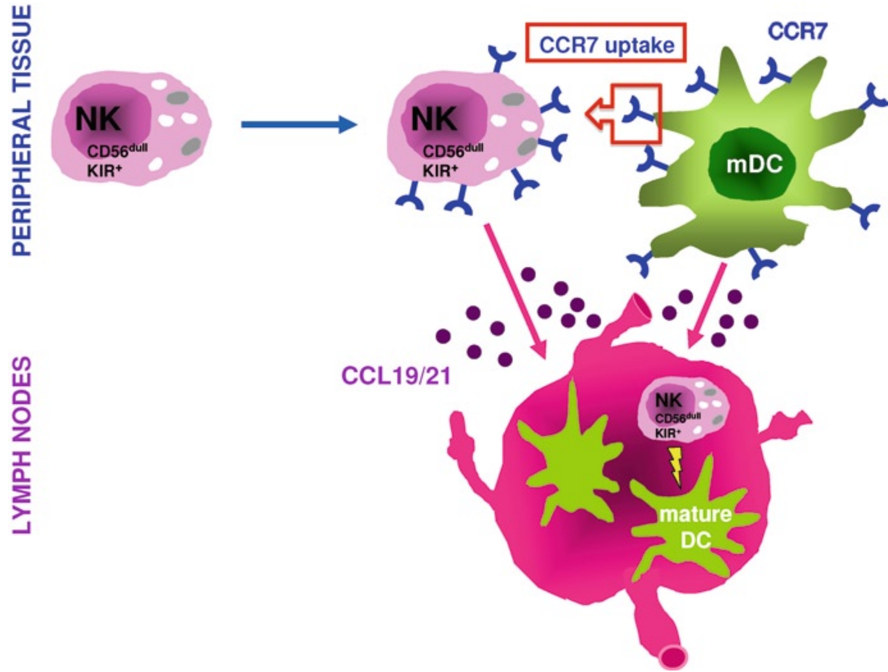

Fig. 5.2 How alloreactive NK cells can be recruited into lymph nodes. The interaction between NK and DCs may have an impact on the acquisition of migratory properties by NK cells. In pathological conditions such as transplantation, characterized by a mismatch between KIR expressed on donor NK cells and HLA-I expressed on recipient DC, NK cells may capture CCR7 molecules from mDC by a mechanism of trogocytosis. As a consequence, KIR+NK cells may migrate into lymph nodes in response to CCL19/21 chemokines. These alloreactive NK cells may prevent GvHD by killing recipient DC. This is important in order to prevent priming of donor alloreactive T cells

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Chapter 6
Control of Innate Immunity by Memory CD4 T Cells

Tara M. Strutt, K. Kai McKinstry, and Susan L. Swain

Abstract How memory CD4 T cells contribute to protection upon pathogen challenge is not fully understood. Beyond traditional helper functions for CD8 T cell and B cell responses, memory CD4 T cells can have a potent impact on the character and a magnitude of inflammatory responses. Here we discuss how memory CD4 T cell control of innate immunity at early time points after pathogen encounters can influence protective responses. We also discuss important aspects of the mechanism whereby memory CD4 T cells directly and indirectly impact the activation status of antigen-presenting cells and production of inflammatory cytokines and chemokines from multiple cell types. We suggest that control of innate immune responses by the adaptive immune system is a powerful protective mechanism associated with the memory state and represents an important fail-safe in the face of pathogens that fail to trigger robust inflammatory responses through conserved pattern recognition receptors.

6.1 Introduction

To successfully combat pathogens, elements of both the innate and adaptive immune system must be brought to bear as quickly as possible upon infection. The recognition of conserved pathogen-associated molecular patterns (PAMP) by germline encoded receptors expressed on the surface of, and within many different cell types represents a critical pathway for the initiation of inflammatory responses that can act to both limit initial infection and subsequently to enhance the generation of adaptive immune responses [1]. A better understanding of the importance of triggering the

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innate immune system has led to the successful incorporation of PAMP receptor ligands as powerful adjuvants in many vaccine formulations and therapies [2]. The ability of the innate immune system to exert a powerful level of control on antigen-specific T and B cell responses is well understood and has sometimes led to the paradigm that triggering of PAMP receptors is an obligate prerequisite for the generation of optimal adaptive immunity [3]. Whether the adaptive immune system can influence innate inflammatory responses is less studied.

While many important aspects of memory T cell immuno-biology have been described, a full understanding of the protective mechanisms employed by these populations during secondary challenges is lacking. This is especially relevant with regards to CD4 T cells, due at least in part to the difficulty of their study compared to memory CD8 T cells arising from the often dramatically lower numbers of the former that are maintained long term *in vivo* [4, 5]. A better understanding of how memory CD4 T cells contribute to protective immune responses beyond traditional "helper" functions is critical to the design of vaccine strategies against pathogens where neutralizing antibodies alone are unable to confer long term protection [6]. Here we discuss broad regulation of the innate immune system by memory CD4 T cells. Using influenza virus infection as an example, we discuss elements of the mechanism by which virus-specific memory CD4 T cells directly and indirectly activate cells of the innate immune system and lead to enhanced acute inflammatory responses. We propose that recruitment of the innate immune system represents an underappreciated protective mechanism employed by memory CD4 T cells during the early phases of pathogen challenge.

6.2 Pattern Recognition, Inflammation, and Innate Control of Adaptive Immunity

PAMP receptors have evolved to recognize a variety of targets expressed by different microorganisms. While the Toll-like receptors (TLR) constitute the most studied PAMP receptor family, many other distinct classes of PAMP receptors and their signaling pathways have been characterized and recently reviewed [7, 8]. Generally, these receptors are specific for conserved and unique elements shared by a broad class of potential pathogens such as the constituents of the cell walls of bacteria or fungi, or viral nucleic acids. It is also understood that elements of the innate immune system can be stimulated through the recognition of factors released by stressed, damaged, or dying host cells [9, 10]. Several of these substances, often termed damage-associated molecular patterns (DAMP), have recently been discovered, and their roles in the etiology of auto-immunity and potential as therapeutics offer exciting possibilities [10].

The immediate consequences of PAMP or DAMP receptor ligation are numerous, leading directly and indirectly to a complex cascade of events that together are rather vaguely termed "inflammation" [11]. Briefly, these triggers lead initially to the production of an array of pro-inflammatory cytokines and chemokines, often

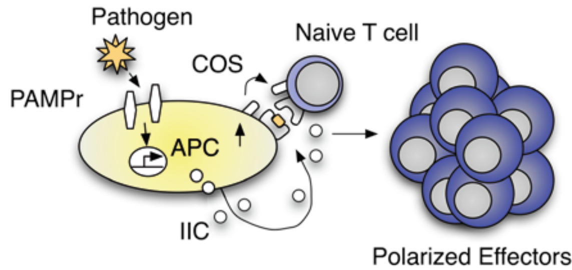

Fig. 6.1 Innate control of adaptive immunity. Uptake of pathogens or pathogen products by APC stimulates PAMP receptors (PAMPr) resulting in the upregulation of co-stimulatory molecules (COS) by APC as well as the production of innate inflammatory cytokines and chemokines (IIC). In addition to recognizing specific antigen presented by APC, COS and IIC are critical in driving naïve CD4 T cells to become properly polarized effector cells

including TNF, IL-1, and IL-6 by antigen presenting cells (APC) and other local cellular populations. These factors in turn lead to an increase in permeability of blood vessels and the influx of several classes of leukocytes to the inflamed site while local coagulation acts to constrain the dissemination of potential pathogens.

Another critical aspect of the inflammatory response is the activation of APC. Recognition of microbial products or DAMPs can lead to the upregulation of MHC:peptide complexes as well as important co-stimulatory markers, including CD40, that are critical to the full activation of naïve T cells. This aspect of the inflammatory response is beneficial when activated APC displaying peptides derived from pathogens migrate to draining lymph nodes, but can be potentially harmful if activated APC display immunogenic self-derived peptides. While microbial products have long been understood to enhance the development of antigen-specific immune responses (for example Freund's complete adjuvant and LPS), recent advances have led to the incorporation of other diverse PAMPs as components of vaccine formulations [2]. Such strategies are not only capable of enhancing the kinetics of antigen-specific T and B cell responses, but might also be able to direct the shape of adaptive immunity with regards to Th-polarization and antibody isotype. The paradigm of innate control of adaptive immunity, crystallized by Janeway in 1989 [12] highlights the interconnectivity of the innate and adaptive defense mechanisms, but has tended to overlook the ability of elements of the adaptive immune system to shape and regulate innate inflammatory responses (Fig. 6.1).

6.3 Functional Attributes of Memory CD4 T Cells

Following the resolution of a primary immune response, the majority of antigen-specific T cell effectors die leaving a small but relatively stable population of memory cells at frequencies higher than found in the naïve state [4]. While a quantitative gain in antigen-specific lymphocytes represents an important advantage of

the antigen-specific memory state, many studies have demonstrated important qualitative attributes that distinguish naïve from memory CD4 T cells [13]. First, and perhaps most importantly, memory CD4 T cells retain the ability to produce Th-associated cytokines associated with the effector cells from which they arise shortly after TCR stimulation – for example IFNγ, IL-4, and IL-17 for Th-1, Th-2, and Th17-polarized populations, respectively [14, 15]. In contrast, naïve CD4 T cells produce a far more restricted set of cytokines, predominantly IL-2, after TCR stimulation and only after a substantial lag period. Second, memory cells are less dependent on co-stimulatory signals for maximal responses than naïve cells [16, 17]. Third, memory cells respond maximally to substantially lower concentrations of antigen than naïve CD4 T cells bearing the same TCR [14]. Finally, while naïve CD4 T cells must be activated in lymphoid tissues by interacting with activated peptide-bearing APC, capable of providing strong co-stimulation, memory CD4 T cells can be activated in peripheral tissues [15, 18].

These properties and others are generally believed to endow memory CD4 T cells with superior helper capabilities as compared to naïve CD4 T cells in activating, and shaping, antigen-specific CD8 T cell and B cell responses leading to secondary responses that are "faster, larger, and better." CD4 T cells are generally referred to as "T helper cells," underscoring their specialized role in orchestrating immune responses, but the term is perhaps sometimes restrictive. We have recently demonstrated that the specialized qualities of memory CD4 T cells discussed above also endow this population with unique abilities to initiate production of broad innate inflammatory responses upon antigen recognition [15]. As we will discuss, the elaboration of enhanced innate inflammatory cytokines an chemokines (IIC) and the activation of innate populations at the site of infection mediated by memory CD4 T cells represents a novel protective mechanism operating during the early phases of pathogen challenge.

6.4 Memory CD4 T Cells Enhance Innate Inflammation upon Influenza Challenge

When influenza-primed mice are challenged with a heterosubtypic strain of virus (expressing surface proteins not recognized by neutralizing antibody specific for the priming virus), significantly higher levels of many IIC, including TNF, IL-1α, IL-1β, IL-6, IL-12, IFNγ, CXCL9, CXCL10, CCL2, and CXCL1, are observed in the lung and to a lesser extent in the serum at 40 hours post-infection compared to what is observed after challenge of naïve mice with the same dose of virus. The magnitude of the IIC response in primed mice can be significantly decreased by the depletion of CD4 T cells prior to challenge and increased IIC responses are observed in unprimed mice infected with influenza if virus-specific memory CD4 T cells are transferred prior to infection [15]. Not unexpectedly, the impact of memory CD4 T cells on the level of IIC requires recognition of specific antigen in an MHC II-restricted manner. Equivalent upregulation of IIC was observed after influenza

infection when memory cells were transferred to wild-type (WT) mice and mice that only express MHC II on CD11c+ cells. This is somewhat surprising given that influenza-infected lung epithelial cells dramatically upregulate expression of MHC II (unpublished observations).

Memory, but not naïve CD4 T cell transfer also dramatically upregulated expression of MHC II and co-stimulatory molecules on CD11c+ cells present in the lung at 40 h post-infection and similar effects were observed upon *in vitro* culture of memory CD4 T cells, specific antigen, and dendritic cells [15]. Furthermore, *in vitro* experiments utilizing both dendritic cells and alveolar macrophages cultured with memory CD4 T cells revealed substantial production of several IIC from both APC populations that required cell to cell contact. Interestingly, while previous studies found that co-culture of activated Th1 clones, adjuvant-free soluble antigen and APC *in vitro* drove IL-12 production in a CD40L-CD40-dependent manner [19], we observed substantial IIC upregulation driven by memory CD4 T cells in the absence of CD40L-, CD28-, ICOS-, OX-40 and CD70-dependent signaling (unpublished observations). We also found that IFNγ and TNF signals from memory CD4 T cells were not required to activate APC either *in vitro* or *in vivo* (unpublished observations).

In addition to enhanced IIC production from dendritic cells and macrophages, it is likely that several other cell types contribute to the inflammatory response mediated by memory CD4 T cells recognizing antigen in the lung. For example, we observed enhanced IFNγ production from lung-resident γδ T cells and NK cells [15]. These findings lead us to propose that the initial encounter between a flu-specific memory CD4 T cell and a peptide-bearing APC results in the activation of the APC and the production of an initial restricted set of IIC and the induction of a cascade of soluble and perhaps cell-surface signals that eventually recruit other innate populations into the early inflammatory response. Surprisingly, transfer of memory CD4 T cells specific for the protein ovalbumin (OVA) to unprimed mice that were challenged intranasally with LPS-free OVA drove enhanced IIC to similar levels as observed after influenza infection. This suggests that PAMP or DAMP signals are not involved in facilitating the enhanced inflammatory response mediated by memory CD4 T cells upon antigen recognition.

6.5 Regulation of Inflammation by T Cells

To test whether the enhanced inflammatory response mediated by memory CD4 T cells upon antigen recognition represents a general activity of memory cells, we transferred unpolarized (Th0), Th1-, Th2-, or Th17-polarized memory cells specific for influenza to unprimed mice and challenged them with virus. Correlating with the ability of Th1- and Th17-polarized CD4 T cell effectors to protect mice against lethal influenza challenge [20], Th1- and Th17-polarized memory cells induced enhanced IIC, while Th2 and Th0 populations did not, correlating with the inability of Th2 or Th0 effectors to protect mice against lethal challenge (unpublished observations). These findings demonstrate that differentially polarized memory CD4

T cells specific for the same antigen are capable of influencing acute inflammatory responses in unique ways.

In this respect it is interesting that other recent studies have found an ability of T cells to also suppress innate inflammatory responses. For example, T cell-deficient nude mice infected with mouse hepatitis virus (MHV) demonstrate a high mortality rate compared to infection of WT mice which does not correlate with increased viral load, but with much higher levels of IIC [21]. Transfer of naïve T cells to nude mice before MHV infection reduces the inflammatory response and rescues nude mice from morbidity [21]. Similarly, co-culture of memory phenotype CD4 T cells isolated from the spleen of un-immunized mice with dendritic cells has been reported to dampen production of IL-1β and IL18 [22]. Thus, it is possible that while naïve T cells or Treg populations [22] act to temper IIC upon antigen recognition, memory CD4 T cells can act to either enhance or otherwise regulate IIC upon antigen recognition, depending on their Th-polarization. It is likely that further variables, such as the site of infection and the route of exposure, might also significantly impact the character and magnitude of the acute inflammatory response.

This mechanism holds two advantages. First, upon initial encounter with a pathogen, the adaptive immune system acts to keep acute inflammation in check that otherwise itself may cause undue immunopathology [22]. Second, upon re-infection, memory CD4 T cells specific for the pathogen that were primed and polarized partly through signals delivered by the initial inflammatory milieu established through PAMP recognition, act to increase the tempo and magnitude of a similar protective inflammatory response. Memory CD4 T cells can remember the inflammatory environment that they were generated in and play an important role in rapidly re-establishing a similar inflammatory setting.

6.6 Protective Impact of Enhanced Inflammation Mediated by Memory CD4 T Cells

To directly test whether the enhanced inflammatory response mediated by memory CD4 T cells can contribute to a protective response against pathogen, we transferred naïve or memory CD4 T cells recognizing OVA (OT-II) to unprimed mice infected with A/PR8-OVA virus, which expresses the OVA peptide recognized by OT-II cells, and measured viral titers on days 2–4 post flu-infection. Memory cell transfer resulted in significantly lower virus detected in lungs as early as day 3 post-infection. Importantly, when LPS-free OVA was given together with A/PR8 virus, which does not express the OVA peptide recognized by OT-II cells, the mice which had received OVA-specific memory CD4 T cells viral titers were significantly lower affected when compared to mice that had received naïve OT-II cells. Finally, when administration of OVA protein preceded A/PR8 infection by 7 days, the protective impact of memory OT-II cells was lost [15]. This experiment reveals three important points. First, while the initiation of enhanced inflammation by memory CD4 T cells requires cognate antigen recognition, the protective impact of the response

does not. Second, as the OVA-specific memory CD4 T cells cannot recognize virally infected epithelial cells infected with A/PR8 virus, the protective impact of their response to lower viral titers presumably acts through elements of the enhanced inflammatory response induced and is unlikely to depend on direct antiviral actions of the memory CD4 T cells. This hypothesis is supported by recent studies in which the induction of enhanced inflammatory responses in mice prior to lethal influenza challenge significantly reduced viral titers and enhanced survival [23]. Finally, the protection afforded through enhanced inflammatory responses mediated by memory CD4 T cell antigen recognition is transient in nature and does not result in a long-term antiviral state.

6.7 How Does Memory CD4 T Cell-Mediated Enhanced IIC Contribute to Heterosubtypic Protection Against Influenza?

When mice previously primed with influenza are challenged with a dose of heterosubtypic virus that is supralethal for naïve animals, T cell responses against conserved internal influenza proteins can provide a strong degree of protection. Survival of primed mice is dependent on CD8 T cells, as depletion of this subset prior to heterosubtypic challenge abrogates protection [24]. In contrast, depletion of CD4 T cells before challenge results in more severe disease highlighted by earlier weight loss and significantly delayed recovery. We propose that the acute enhanced inflammatory responses mediated by virus-specific memory CD4 T cells contributes to protection in at least two distinct ways during heterosubtypic challenge.

First, direct control of viral titers through enhanced inflammation during the first few days of infection could provide an important restraint on influenza replication and spread during the development of maximal effector T cell responses that are ultimately responsible for viral clearance. It is likely that this early viral control dependent on memory CD4 T cells is due to elements of the inflammatory response acting through or on various innate populations as well as the lung epithelium [25, 26] (Fig. 6.2) The importance of individual components of the enhanced IIC response to viral control remain to be determined, but it is likely that several elements are involved. For example, IL-1, IL-6, and IL-12 have all been shown to influence protective responses against influenza [27–29]. Second, the earlier activation of APC populations in the lungs mediated through contact-dependent interactions with memory CD4 T cells could indirectly contribute to protection through enhancing the kinetics of and/or magnitude of virus-specific CD4 and CD8 T cell and B cell antibody responses [7, 30]. It is also possible that chemokine gradients established through enhanced IIC act to facilitate the more rapid influx of cellular populations important for viral control. Such a mode of action has been described by Nakanishi *et al.* in a herpes simplex virus infection model where CD4 T cells are required for the induction of CD8 T cell-recruiting chemokines in the infected tissue [31].

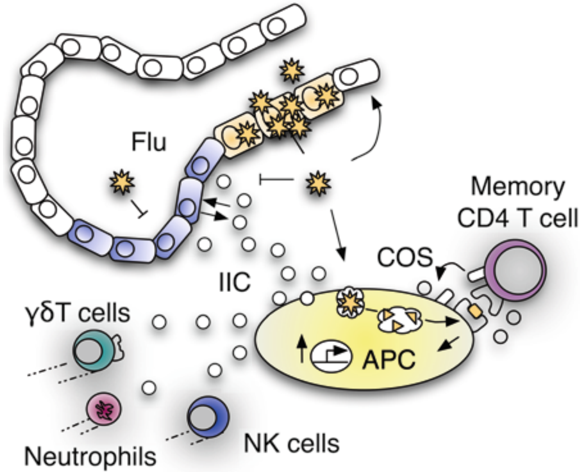

Fig. 6.2 Memory CD4 T cell control of innate inflammation during influenza infection. Memory CD4 T cells recognizing influenza (flu) presented by APC drive upregulation of COS and IIC independently of PAMP signals. IIC produced by the initial interaction between memory CD4 T cells and APC call in diverse elements of the innate immune system into the infected lung that also produce IIC. IIC and perhaps other aspects of the enhanced innate response mediated by memory CD4 T cells act on lung epithelial cells to drive further IIC production and to control virus during the initial days of infection

6.8 Adaptive Control of Innate Immunity

Memory CD4 T cell control of innate inflammation during pathogen challenge has several teleological benefits. First, this alternative mechanism of eliciting acute inflammation represents a fail-safe in situations where pathogens evade PAMP recognition [32]. Secondly, memory CD4 T cells by virtue of the capacity for maximal responses to very low concentrations of antigen and co-stimulation, may initiate innate effector mechanisms before pathogen levels are sufficient to drive maximal inflammatory responses through PAMP receptors. This may be of critical importance in the context of slowly replicating pathogens such as tuberculosis, trypanosomes, and hepatitis B virus [33]. Similarly, our results suggest that the "stealth" phase that characterizes infections such as flu, where the pathogens gain the upper hand through several rounds of replication before the initiation of innate or adaptive immunity [34], can be substantially abbreviated or perhaps eliminated through the action of memory CD4 T cells. It is possible that memory CD4 T cell triggering of earlier inflammation may also be beneficial in the context of vaccine strategies against HIV [35].

Thirdly, regulation of innate effector mechanisms by memory CD4 T cells may counteract direct suppression of inflammation mediated by pathogens such as influenza through the actions of the NS1 protein [36]. Fourth, beyond initial control of

infection through inflammatory responses, the establishment of Th-polarizing cytokine environments and the activation of APC populations by memory CD4 T cells could facilitate the development of optimal adaptive immunity at later stages of pathogen-specific responses. Indeed, increased production of TNF, IL-1, and IL-6 driven by memory CD4 T cells may be of particular importance in the context of orchestrating optimal adaptive responses in the elderly [37].

The adjuvant-like properties of CD4 T cells listed above have obvious implications in vaccine design and may help explain observations of strong immune responses to protein antigens in the absence of PAMP signaling [38]. However, many of the advantages of memory CD4 T cell control of innate immunity in situations of infectious disease may also be deleterious in situations of autoimmunity. Indeed, memory CD4 T cells are implicated in the etiology of several autoimmune diseases, both through helper functions and other mechanisms [39–42]. A recent study found that activated CD4 T cells were able to stimulate production of several IIC from human monocytes *in vitro* in a contact-dependent manner, suggesting that this function of auto-reactive CD4 T cells may play an important role in driving inflammation in rheumatoid arthritis [43]. A fuller understanding of the signals involved in regulating innate inflammatory responses by memory CD4 T cells may thus not only be harnessed for protective responses against pathogens or tumors, but could provide an early target in the treatment of autoimmunity.

6.9 Concluding Remarks

Innate inflammatory responses serve to combat initial infection and to optimize the subsequent generation of antigen-specific immune responses. The importance of early inflammatory responses to host protection is most clearly seen in the multiple evolutionarily conserved PAMP receptor systems. Control of adaptive immunity through triggering of PAMP receptors occurs both through the activation of APC, which increases the kinetic development of subsequent T cell responses, and through the release of important Th-polarizing cytokines, which help combat infection and also ensure the generation of appropriately polarized T cell responses. It is clear that in a naïve state, innate control of adaptive immunity represents a critical mechanism of regulation, without which invading pathogens would more routinely gain the upper hand in what is often a race between microbial replication on the one hand and clonal selection and expansion on the other.

In the memory state, the situation is inherently changed. Memory CD4 T cells, that are present in higher numbers than in the naïve state and that exhibit unique functional properties, can dramatically regulate innate inflammatory responses upon antigen recognition. Memory CD4 T cells, in the absence of PAMP receptor recognition, can activate APC and direct the secretion of Th-polarizing cytokines as well as a broad range of IIC that can play a key role in acute control of pathogens. We believe that this represents a critical advantage of the memory state and that this mechanism adds an important branch to the sometimes too linear view of "innate

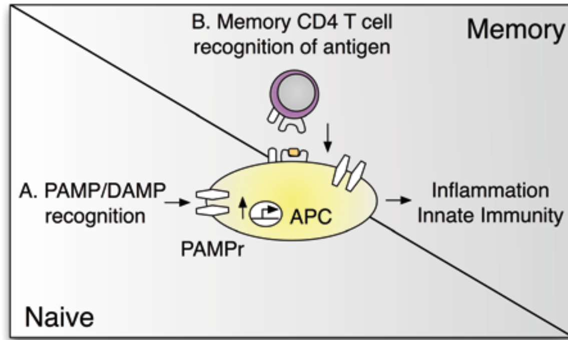

Fig. 6.3 Memory CD4 T cell control of innate immunity. Recognition of PAMPs and DAMPs (**A**) is a crucial trigger initiating innate immune responses in the naïve state. In a primed environment, in addition to pattern recognition receptors, antigen-specific memory CD4 T cells can initiate inflammatory responses and APC activation even in the absence of PAMP/DAMP recognition (**B**)

control of adaptive immunity" (Fig. 6.3). Harnessing the adjuvant qualities of memory CD4 T cells may provide a potent boost to vaccine strategies aimed against many prominent pathogens.

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Chapter 7
Strategies and Implications for Prime-Boost Vaccination to Generate Memory CD8 T Cells

Jeffrey C. Nolz and John T. Harty

Abstract Generating a large population of memory CD8 T cells is an appealing goal for vaccine design against a variety of human diseases. Indeed, experimental models have demonstrated that the overall number of memory CD8 T cells present at the time of infection correlates strongly with the ability to confer host protection against a range of different pathogens. Currently, the most conceivable approach to rapidly generate a large population of memory CD8 T cells is through the use of prime-boost vaccination. In addition, recent experimental findings have uncovered important principles that govern both the rate and magnitude of memory CD8 T cell formation.

Thus, this has resulted in novel prime-boost vaccination strategies that could potentially be used in humans to generate protective populations of memory CD8 T cells.

7.1 Introduction

The use of vaccination to protect against human diseases can be traced back to 1796, when Edward Jenner pioneered the field after successfully protecting his "test subjects" from smallpox by infecting them with the less virulent cowpox virus. Unknown to Jenner at the time, his method led to the formation of immune cell memory, thus protecting the individual from subsequent infection. Now, over two centuries later, our ability to manipulate the immune system through vaccination has allowed the human race to essentially eradicate a number of its most deadly

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diseases. It should come as no surprise that successful vaccinations are considered one of public health's greatest achievements [1].

Although a number of successful vaccines have been developed, many human diseases remain for which no vaccine is currently available. Because of this, efforts to maximize immunization strategies that elicit a strong CD8 T cell response are appealing because of this immune cell's unique ability to specifically kill cells infected with intracellular pathogens. In addition, CD8 T cells also have the potential to be utilized in the development of various "cancer vaccines" against tumor antigens that are either over-expressed or expressed in a mutated form. Thus, a thorough understanding of how memory CD8 T cells are generated and maintained has direct relevance in modern day medicine.

Recent studies have begun to elucidate many of the factors that contribute to the generation of memory CD8 T cells. Although qualitative differences in memory CD8 T cell populations may be relevant when targeting different pathogens, it is clear that the overall number of memory CD8 T cells correlates strongly with host defense [3, 5, 27, 33, 34, 54]. Because of this, the following will discuss how memory CD8 T cells are initially generated, strategies of effectively using prime-boost vaccinations to generate large memory CD8 T cell populations, and the impact that prime-boost vaccination has on the resulting memory CD8 T cell population.

7.2 Generation and Characteristics of Memory CD8 T Cells

Following their development in the thymus, naïve CD8 T cells enter the blood and lymphoid compartments of the body where they continue to re-circulate in their quest for foreign antigen. Because of the great diversity of T cell receptors (TCRs) required to ensure protection against a wide variety of pathogens, finding naïve CD8 T cells that are capable of recognizing a specific foreign peptide is an extremely rare event. In mice, the number of naïve precursors specific for a given foreign peptide ranges from as few as ten to as many as a couple thousand [11, 12, 35, 43, 45]. However, these naïve CD8 T cells have a tremendous proliferative capacity, and once activated, undergo clonal expansion resulting in a dramatic change in numbers, whereas one naïve CD8 T cell can give rise to 10,000 daughter cells (>13 divisions) [27, 34]. This amazing and dynamic biological process is the start of the CD8 T cell response that will eventually lead to the generation of a stable memory population and the fundamental basis of vaccinations.

Before a CD8 T cell can become activated and undergo proliferation, it must first receive appropriate stimulation from antigen-presenting cells of the innate immune system. It is widely accepted that a naïve CD8 T cell requires stimulation through the TCR, co-stimulatory molecules (such as CD28), and be exposed to inflammatory cytokines in order for an appropriate T cell response to occur [25, 42]. Following infection, dendritic cells (DCs) become activated through a variety of pattern recognition receptors (PRRs) such as toll-like receptors (TLRs), which recognize a wide assortment of molecules found on foreign pathogens including peptidoglycan, lipopolysachharide, double-stranded RNA, and unmethylated CpG DNA motifs.

Once activated, these professional antigen-presenting cells acquire foreign antigen, migrate into secondary lymphoid compartments, and present pathogen-derived peptides on major histocompatibility class I (MHC-I) to CD8 T cells [29, 57]. When naïve CD8 T cells encounter stimulatory peptide-MHC-I complex while concurrently receiving co-stimulation, they undergo massive proliferation, leading to the generation of a great number of effector cells. Effector CD8 T cells leave lymphoid organs, enter the peripheral tissues, and destroy infected cells using a variety of mechanisms, including the production of interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα) and through the expression of Fas Ligand (FasL) and perforin/granzymes [22]. During most acute infections, the invading pathogen is eliminated by the peak of the T cell expansion phase.

Once the number of T cells reaches a peak, most (90–95%) of the newly generated effector CD8 T cells then die. However, cells that survive this contraction phase of the immune response go on to form a stable memory pool of CD8 T cells (Fig. 7.1a). How to clearly define a memory CD8 T cell remains controversial, as no single molecular marker or functional property is represented by all cells within this memory population [32]. Although this makes it difficult to clearly label a CD8 T cell as "memory," the diversity and heterogeneity within the bulk memory population probably contributes to the high success rate of preventing re-infection of the pathogen. In addition, memory CD8 T cells are not static, and the phenotype and expression of effector molecules within this cell population changes over time (Fig. 7.1b–e). An understanding of the complexity of memory CD8 T cell populations is potentially critical when designing vaccinations against specific diseases.

One important distinguishing characteristic that separates memory CD8 T cells from naïve cells is the overall change in anatomical distribution. As mentioned, naïve CD8 T cell are confined mostly within the blood and spleen but also enter lymph nodes though the high endothelial venules (HEV) due to their high expression of the lymph node homing molecules L-selectin (CD62L) and CCR7. In contrast, memory CD8 T cells are able to traffic into peripheral tissues such as the liver, lung, and skin and can re-enter the lymph nodes and circulation via the afferent lymph (Fig. 7.2). In its most general characterization, memory CD8 T cells can be defined based on expression of CD62L and CCR7 [52, 60]. Memory CD8 T cells that express CD62L/CCR7 are defined as "central memory" (Tcm) and like naïve cells are able to enter lymph nodes through the HEV. Memory cells that lack expression of CD62L/CCR7, termed "effector memory" (Tem), are not able to enter lymph nodes via this route and tend to localize more efficiently into peripheral tissues [40]. This classification is likely an oversimplification of the diversity of trafficking patterns that occur in memory CD8 T cell populations and unique expression of chemokine receptors and integrins probably regulates distribution of specific memory cell subsets. In addition, recent evidence suggests that populations of non-circulating "resident memory" CD8 T cells can be found in certain organs such as the skin and gut [19, 38]. Thus, it is likely that future studies will uncover specific molecular mechanisms that regulate tissue distribution of memory CD8 T cells, which could potentially be used to maximize vaccine efficacy.

Survival of memory CD8 T cell populations does not require interactions between TCR and MHC-I [37, 44]. However, the cytokines IL-7 and IL-15 are critical

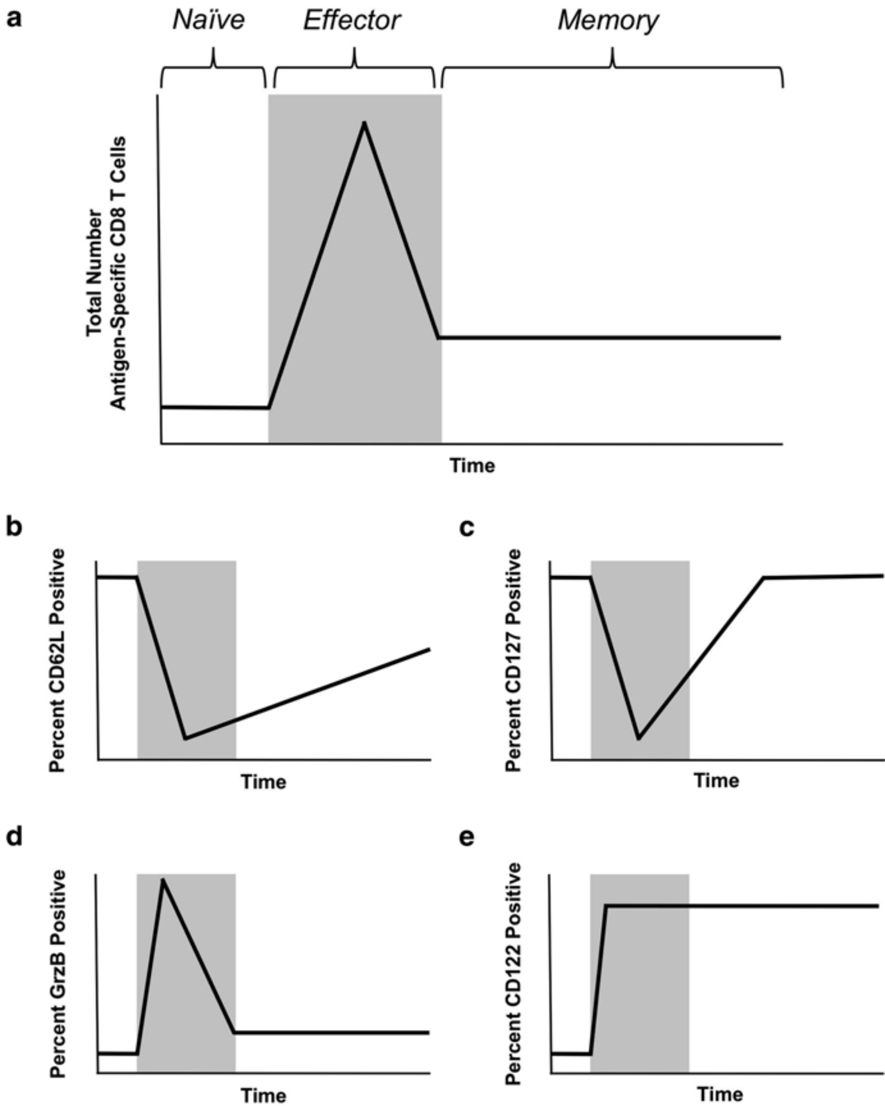

Fig. 7.1 Changes in CD8 T cell numbers and phenotype that occur following vaccination or infection. (**a**) Following activation, previously naïve CD8 T cells undergo expansion and contraction before forming a stable memory population. Based on this kinetic curve, CD8 T cells can be classified as either being naïve, effector (gray box), or memory. (**b–e**) Changes in expression of (**b**) CD62L, (**c**) CD127, (**d**) GrzB, and (**e**) CD122 that occur as CD8 T cell populations transition from naïve to effector to memory. (CD62L, L-Selectin; CD127, IL-7 Receptor α-chain; GrzB, Granzyme B; CD122, IL-2/15 Receptor β-chain)

regulators of memory CD8 T cell maintenance [58]. Both naïve and memory, but not effector, CD8 T cells express high levels of the IL-7 receptor α-chain (CD127) and this signaling pathway is necessary (but not sufficient) for the generation of memory CD8 T cells, possibly through the upregulation of anti-apoptotic molecules

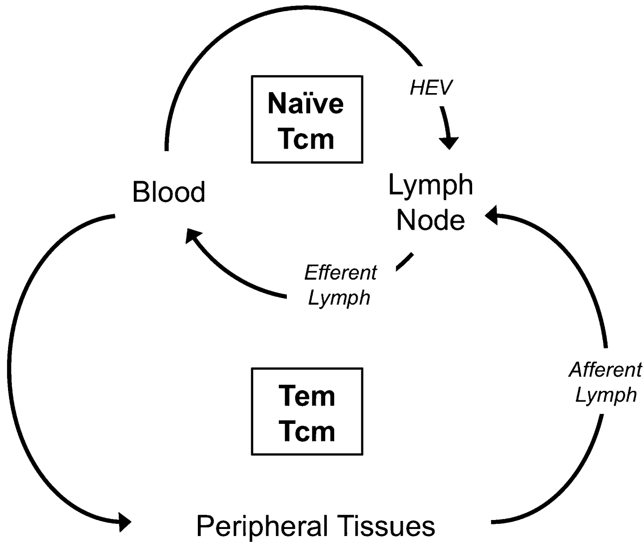

Fig. 7.2 Trafficking patterns of naïve, central memory (Tcm), or effector memory (Tem) CD8 T cells. Naïve CD8 T cells are primarily confined to the blood, spleen (not shown), and lymph node compartments of the body. Naïve and Tcm CD8 T cells are also able to enter lymph nodes via the high endothelial venules (HEV) due to high expression of CD62L and CCR7. In contrast, Tcm and Tem can also localize into peripheral tissues and then enter the lymph node through the afferent lymph. All CD8 T cells return to the circulation from the lymph nodes through the efferent lymph

including Bcl-2 [24, 26]. Interestingly, CD8 T cells express the IL-15/2 β-chain (CD122) following activation and this molecule is retained on memory populations. In contrast to the "pro-survival" impact of IL-7, signaling pathways activated in response to IL-15 drive a low level of homeostatic proliferation among memory CD8 T cells [9, 20]. Importantly, since primary memory CD8 T cell numbers remain relatively unchanged over time, this low level of homeostatic proliferation must also be accompanied by equivalent cell death. One possibility is the outgrowth of Tcm memory cells accompanied by Tem death, since the number of CD62L-expressing CD8 T cells increases with time and homeostatic proliferation is mostly confined to this subset. In contrast, there could be equal death between Tcm and Tem subsets and conversion of Tem to Tcm occurs during homeostatic proliferation. On the other hand, the eventual outgrowth of Tcm's could be the result of "out competing" their Tem counterparts for pro-survival cytokines such as IL-7 and IL-15.

In summary, following activation, naïve CD8 T cells undergo expansion and contraction resulting in the formation of a long-lived memory population. Through their differential expression of a number of trafficking molecules, memory CD8 T cells exhibit more diverse localization than naïve cells, which increases their ability to protect against invading pathogens. Importantly, following the generation of stable memory, these CD8 T cells are able to undergo a vigorous recall response in which they undergo a second round of expansion, contraction, and memory formation. This biologically relevant phenomenon is the basis for booster immunizations, where the overall goal is to maximize the number of memory CD8 T cells.

7.3 Methods of Prime-Boost Vaccination

High numbers of antigen-specific memory CD8 T cells are usually desired following vaccination, since this number strongly correlates with host protection [5, 27, 34]. Currently, the best approach known to generate these high numbers of cells is to utilize a system of prime-boost vaccination, which relies on the re-stimulation of antigen-specific immune cells following primary memory formation [51, 61]. Homologous booster immunizations that utilize re-administration of the same vaccination agent have essentially been used since the initial development of vaccines. Although this method is usually successful in boosting the humoral response to antigen, it is far less effective at generating increased numbers of CD8 T cells due to rapid clearance of the homologous boosting agent by the primed immune system [61]. Many of the pathogens for which no vaccine is currently available in humans such as human immunodeficiency virus (HIV), *Mycobacterium tuberculosis*, and *Plasmodium* species (causative agent of malaria) are highly resistant to humoral immunity generated by most traditional vaccines [55]. Thus, a successful vaccination strategy aimed at eliminating these intracellular pathogens could be aided by generation of a large memory CD8 T cell population.

In contrast to homologous prime-boost, use of heterologous prime-boost vaccination is much more effective at generating increased numbers of memory CD8 T cells (Fig. 7.3). The strategy implemented in heterologous prime-boost utilizes priming CD8 T cells with antigen delivered in one vector and then administering the same antigen in the context of a different vector at a later time point. Using this approach, the specific delivery of antigen to the CD8 T cell population and the generation of inflammatory signals during the booster immunization are maximized since the other arms of the immune system are not able to rapidly clear the vector as in homologous prime-boost vaccinations. This leads to not only a dramatic increase in the total number of antigen-specific CD8 T cells, but an enrichment of those T cells that have high affinity for antigen [18, 41]. Indeed, this strategy has been successful in generating protective immunity against a variety of pathogens in experimental models and is under evaluation in a number of human clinical trials [13, 14, 17, 30, 36, 61].

One important variable that impacts the "boosting-potential" of a CD8 T cell population is the length of time separating the primary and secondary antigen administration. In fact, following infection with common laboratory pathogens such as *Listeria monocytogenes* or lymphocytic choriomeningitis virus (LCMV), a time period of at least 40–60 days is required before optimal boosting of the CD8 T cell population is possible [27]. Indeed, this time frame is consistent with observed changes in overall gene expression of memory CD8 T cell populations, which begin to stabilize approximately 40 days after infection with LCMV [33]. Recent studies have begun to uncover the mechanisms that regulate the rate of memory CD8 T cell formation following infection or vaccination.

There is clear evidence that pro-inflammatory cytokines can dramatically impact the rate of memory formation. For example, antibiotic treatment to truncate infection following *L. monocytogenes* infection results in rapid generation of CD8 T cell memory without affecting the overall kinetics of the primary response [4, 7, 8]. This also decreases the interval that is required between initial infection and booster immunization to amplify

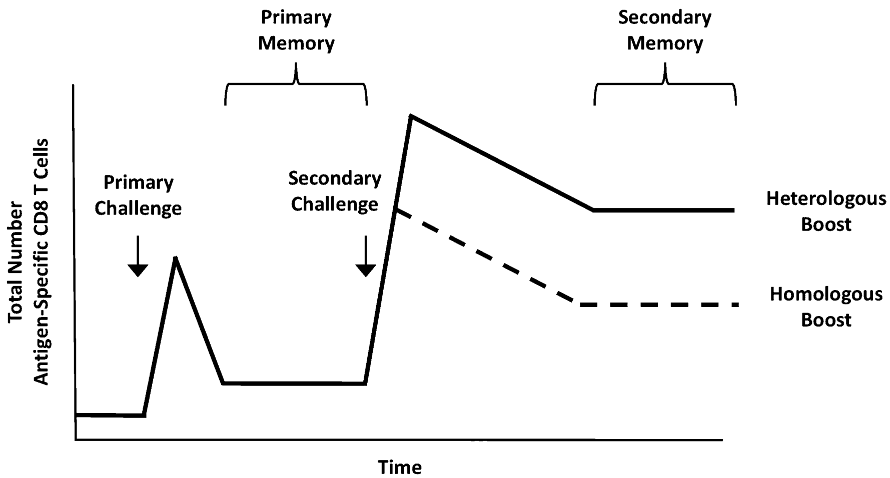

Fig. 7.3 Use of booster vaccinations to generate increased numbers of memory CD8 T cells. Following primary vaccine challenge, CD8 T cells undergo expansion, contraction, and form a primary memory population. When this primary memory population of CD8 T cells is exposed to a secondary challenge of the same vaccination (Homologous boost, dashed line), another round of expansion, contraction and formation of a larger, secondary memory population occurs. In contrast to a homologous booster vaccination, administration of a CD8 T cell antigen delivered in the context of a different vector (Heterologous boost, solid line) drives greater expansion of the primary memory CD8 T cells, resulting in a larger secondary memory population than what is seen with homologous booster vaccinations

CD8 T cell numbers. In agreement with the observation that pro-inflammatory cytokines have a negative impact on the rate of memory CD8 T cell formation, T cells genetically deficient for the IL-12 receptor progress to memory more quickly than wild-type cells [46]. Thus, limited exposure to pro-inflammatory cytokines allows for primary memory CD8 T cells to be boosted more quickly following initial antigen priming.

Another prime-boost method that leads to the rapid generation of memory CD8 T cells is by vaccinating directly with mature, peptide-pulsed DCs followed by a conventional booster immunization [6]. Using this technique, naïve CD8 T cells are exposed to antigen, co-stimulation, and localized inflammatory cytokines (from the DC), but are not exposed to overt systemic inflammation that occurs following infection. Although antigen-specific CD8 T cells still progress through a condensed effector phase, the transition to memory occurs within days without influencing the overall kinetics of the CD8 T cell response. In fact, DC-primed antigen-specific CD8 T cells can be boosted as soon as 4–7 days post infection. This protocol has been used in mice to establish lifelong CD8 T cell-mediated sterilizing immunity against the malaria parasite *Plasmodium berghei* [54]. This model has also been used to demonstrate the importance of inflammation in regulating the transition of CD8 T cells from effector to memory, as treatment with toll-like receptor agonists (such as CpG) following DC-peptide vaccination increases the length of time required for optimal boosting to occur [48]. Therefore, this form of heterologous prime-boost vaccination can be used to quickly generate a large, memory CD8 T cell population through

direct priming of the desired antigen-specific CD8 T cells in the absence of systemic inflammation followed shortly by a strong booster vaccination.

The biggest advantage of using peptide-pulsed DCs for vaccinations would be the rapid generation of large numbers of CD8 T cells following an adequate booster immunization. However, several hurdles exist that would make this strategy difficult to implement in an out-bred human population. Since this method utilizes direct priming with peptide, the appropriate immunization peptide(s) that elicit the desired CD8 T cell response will vary from individual to individual due to the diversity of MHC-I genes found within an out-bred human population. Therefore, this would require prior genetic screening of individuals, as well as the laborious task of determining immune-dominant CD8 T cell epitopes for different MHC haplotypes for any given pathogen. In addition, immature DCs would need to be isolated from the individual prior to vaccination in order to mature these cells in vitro. Utilizing DC vaccinations in humans would be both costly and time-consuming.

In order to circumvent the problems that would be encountered with individualized peptide-DC immunizations, recent studies have uncovered an alternative method of prime-boost vaccination by exploiting the immune system's ability to cross-present antigen to CD8 T cells. Uptake of apoptotic cells by antigen presenting cells occurs readily within the body every day, which will lead to the presentation of self-antigens by these cells [10]. However, no immune response is initiated to these self-antigens, most likely due to the mechanisms of both central and peripheral tolerance. When apoptotic cells are artificially coated with full length immunizing protein and used as a vaccination agent, a small primary CD8 T cell response can be detected in laboratory animals. When a conventional booster vaccination is then utilized shortly after this primary vaccination, antigen-specific CD8 T cell numbers explode to significantly higher numbers and establish long-term memory [49]. Using OVA-pulsed H-2K^{b}–/– apoptotic splenocytes, we verified that this priming step must occur through cross-presentation since these cells are unable to directly present peptide to CD8 T cells. These findings indicate that manipulating the immune system to cross-present foreign antigen in a low inflammatory environment leads to rapid generation of boostable, primary memory CD8 T cells.

Cross-presentation of antigen occurs much more effectively when introduced in a particulate, rather than soluble form [28]. In fact, encapsulating antigen in biodegradable particles such as poly(lactic-co-glycolic) acid (PLGA) microspheres has been explored as a potential mechanism for the delivery of antigen to the immune system [23, 53, 56]. However, it is widely accepted that use of adjuvants is required to generate any appreciable CD8 T cell response using this method. Our laboratory has also demonstrated that simply coating these microspheres with full-length protein and using them as a vaccination agents lead to the generation of a nearly undetectable CD8 T cell response. Regardless of this low starting number, these CD8 T cells undergo substantial boosting in response to secondary challenge and progress to long-lived memory cells. In fact, using this method, laboratory mice can be protected from both malaria and influenza [49]. Since the microbeads are coated using whole protein (not peptide), this strategy could be utilized in an outbred population and result in the rapid generation of boostable CD8 T cells, a technique that has the potential to be used as an effective "off-the-shelf" immunization method in humans.

Overall, the studies discussed here call into question the use of adjuvants during a primary vaccination when the ultimate goal is to quickly generate a large, memory CD8 T cell population. The methods described here that lead to the rapid generation of boostable memory CD8 T cells include primary immunizations that elicit an effector response dramatically smaller than the effector response that occurs following immunization with pathogen (Fig. 7.4). These forms of prime-boost vaccinations generate significantly higher memory CD8 T cell numbers compared to traditional

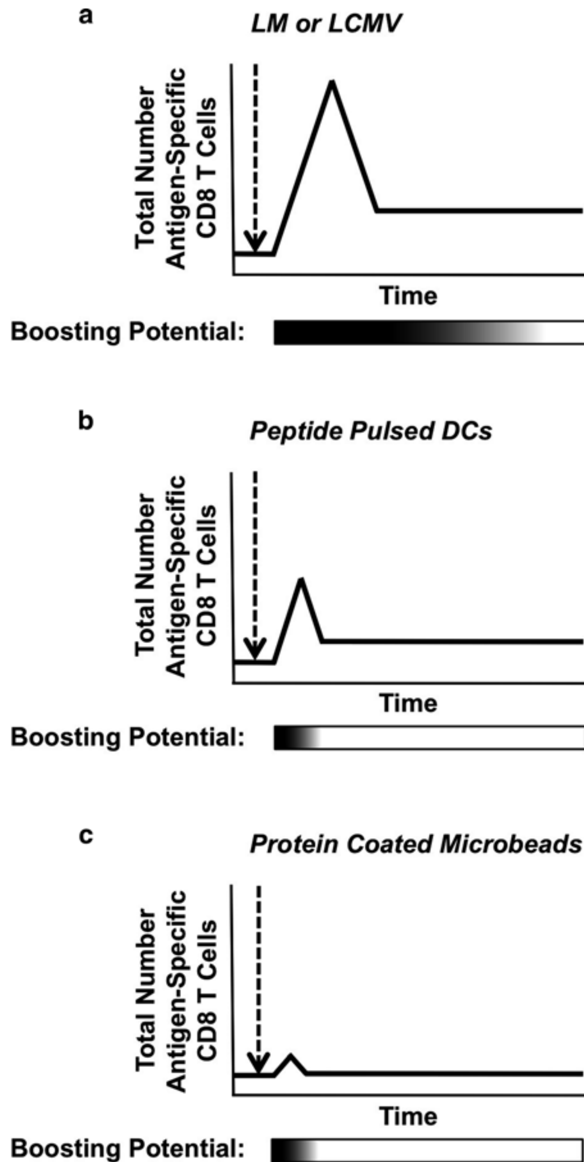

Fig. 7.4 Peptide pulsed DCs and protein coated microbead vaccinations rapidly generate boostable CD8 T cells. (**a**) Common laboratory pathogens such as *Listeria monocytogenes* (LM) and lymphocytic choriomeningitis virus (LCMV) stimulate strong primary CD8 T cell responses that are not able to undergo optimal boosting until 40–60 days following administration. (**b**) Peptide pulsed DCs and (**c**) protein coated microbeads stimulate a smaller primary CD8 T cell response, but generate memory CD8 T cells that can undergo boosting as soon as 4–7 days following administration. (Boosting Potential: *Black* – least boostable, *White* – most boostable)

primary vaccinations with laboratory pathogens. We would argue that rapidly progressing naïve CD8 T cell through a diminished effector phase and into an early primary memory phase followed by a strong booster vaccination is the most efficient way to rapidly generate a very large memory CD8 T cell population.

7.4 Impact of Subsequent Antigen Encounters on Memory CD8 T Cell Populations

Nearly all studies on the formation, maintenance, trafficking and overall characteristics of memory CD8 T cell populations have been performed by analyzing T cells during and following a primary infection or vaccination. This information has strengthened our understanding of the factors and mechanisms that influence the generation of memory CD8 T cells that originated from a naïve population. However, in all practicality, a population of memory CD8 T cells exposed to a single round of antigen rarely, if ever, exists outside of experimentally manipulated laboratory mice. Most successful vaccination regiments in humans require one or more rounds of booster immunizations in order to generate robust protective immunity against a number of pathogens [61]. In fact, recent studies demonstrate that multiple antigen encounters impact several aspects of memory CD8 T cell populations and are summarized in Fig. 7.5 [5, 21, 31, 39, 59]. Since prime-boost vaccinations would lead to the generation of "secondary" memory CD8 T cells, it is critical to determine how multiple antigen encounters change the ensuing memory populations, as this may lead to new ideas concerning ideal vaccine design and implementation.

	Naïve	Primary	Secondary
CD62L	+++	++	+
IL-2	-	++	+
CD122	-	+++	++
CD127	+++	+++	++
GrzB	-	+	++
Lymph Node Localization	+++	++	+
Peripheral Tissue Localization	-	+	++

Fig. 7.5 Impact of subsequent antigen encounters on the ensuing memory CD8 T cells. Naïve, primary memory (stimulated with antigen once), and secondary memory (stimulated with antigen twice) CD8 T cells exhibit differential phenotypes and anatomical localizations. (CD62L, L-Selectin; IL-2, Interleukin-2; CD122, IL-2/15 Receptor β-chain; CD127, IL-7 Receptor α-chain; GrzB, Granzyme B)

One of the most striking differences observed in memory CD8 T cell populations following multiple antigen encounters is the slower generation/formation of Tcm cells compared to a primary memory population [31, 39]. Because of this, secondary memory CD8 T cells localize poorly to lymph nodes and traffic much more efficiently to peripheral tissues including the lung and liver. Conceivably, this change in localization may enhance the ability of secondary memory CD8 T cells to provide immediate protection during the early stages of microbial infection. In addition to these changes in overall anatomical localization, secondary memory CD8 T cells also express increased levels of Granzyme B and exhibit more efficient in vivo cytolysis. In agreement with these findings, secondary memory CD8 T cells are more protective than an equal number of primary memory CD8 T cells during an acute infection with *L. monocytogenes* [31]. These studies suggest that boosted memory CD8 T cells take on a more “effector-like” phenotype rendering them more protective against acute infections in peripheral tissues.

As described earlier, the cytokines IL-7 and IL-15 are critical for both the survival and maintenance of a primary memory CD8 T cells population. Secondary memory CD8 T cells undergo less homeostatic proliferation and are less responsive to IL-15 than primary memory cells [31]. In addition, ensuing populations of memory CD8 T cells following multiple antigen challenges also express lower levels of CD127 [39]. These findings challenge the ideas of how pro-survival cytokines influence the generation of stable memory CD8 T cell populations. However, since contraction of T cell numbers is also protracted following secondary antigen stimulation, the observation of decreased homeostatic proliferation by this population may be due to prolonged survival of both effector and Tem cells since homeostatic proliferation is most robustly observed in the Tcm memory compartment. If this is the case, then how do Tem cells survive more efficiently following subsequent antigen encounters compared to a primary response? Could it be due to an overall resistance to apoptosis through both intrinsic and extrinsic pathways or to yet unidentified pro-survival cytokines? Clearly, our understanding of memory CD8 T cell maintenance following booster immunizations is far from complete.

Generally, booster immunizations will lead to increased memory CD8 T cell numbers with the majority of these cells exhibiting an effector phenotype and localizing to peripheral tissues. Another hurdle in utilizing prime-boost vaccination to generate memory CD8 T cells is the decreased representation of Tcm within these populations. Although the decreased localization into lymph nodes by boosted memory CD8 T cells allows for the priming of new naïve CD8 T cells during subsequent vaccinations or infection [31], in many cases, the presence of a large Tcm population may be optimal for host protection, especially against those pathogens that cause systemic or chronic infections. Many chronic/latent viruses, such as HIV and Epstein-Barr virus (EBV), undergo most re-activation and replication within lymph nodes. Since multiple antigen encounters drive memory CD8 T cells away from the lymph nodes, theoretically, this anatomical location would be most ideal for viral replication to occur in order to largely avoid the cellular arm of the immune system. In support of this idea, the majority of CD8 T cells in infected humans specific for HIV and EBV are CD62L^{Lo} [15, 16]. However, two recent studies have demonstrated that the rate of memory CD8 T cell formation may be regulated by

changes in cellular metabolism. Inhibition of mTOR (mammalian target of rapamycin), a key regulator of cellular energy homeostasis, not only impacts memory cell generation, but also augments the rate of Tcm formation following contraction [2, 47, 50]. Thus, prime-boost vaccination in combination with this type of drug treatment may be a conceivable approach to generate a large number of secondary memory CD8 T cells that exhibit a Tcm phenotype.

In summary, it is clear that multiple antigen encounters impact memory CD8 T cell populations in ways other than simply boosting the overall total numbers. Specifically, these phenotypic and functional differences that occur following booster-immunizations alter anatomic distribution, cytokine production, effector function, and protective capacity against different pathogens. It is clear that many aspects of memory CD8 T cell biology, as it relates to humans, may currently be unknown due to the lack of laboratory studies aimed at understanding key aspects of memory CD8 T cell populations that have gone through more than one antigen encounter. Future studies aimed at identifying key changes in both global and specific gene expression of multiply boosted CD8 T cells will undoubtedly provide tools for optimizing vaccine design in humans.

7.5 Conclusion

As described here, utilizing heterologous prime-boost vaccinations is a valid approach to generate very large numbers of memory CD8 T cells. Specifically, studies described here would argue that “weak” primary immunizations resulting in low inflammatory environments give rise to primary memory CD8 T cells that are able to undergo substantial boosting in response to a strong secondary immunization. Since major human pathogens including HIV and *Plasmodium* reside inside of cells, an effective vaccine against these diseases will most likely require the generation of protective memory CD8 T cells. It is also clear that learning and understanding methods that would manipulate memory CD8 T cell localization following multiple antigen encounters could potentially also increase efficacy of vaccine design. As more tumor antigens are discovered, efforts to develop “cancer vaccines” will also rely on the generation of memory CD8 T cells. By appropriately understanding the CD8 T cell biology using animal models and applying it to specific human diseases, the potential to continually develop and improve vaccinations is highly probable.

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Chapter 8
Blimp1: Driving Terminal Differentiation to a T

Annie Xin, Stephen L. Nutt, Gabrielle T. Belz, and Axel Kallies

Abstract B lymphocyte maturation-induced protein-1 (Blimp1) is a transcriptional repressor expressed in diverse cell types. In the adaptive immune system, Blimp1 is expressed in lymphocytes that have undergone effector differentiation. Blimp1 is a master regulator of plasma cell differentiation and plays important roles in controlling T cell homeostasis and effector differentiation. Blimp1 can be induced by a variety of cytokines including IL-2, IL-4, IL-12, and IL-21 in addition to TCR and co-stimulatory signals. Blimp1-deficient mice develop spontaneous inflammatory disease mediated by infiltration of activated T cells into tissues. During immune responses Blimp1 is required for the differentiation of plasma cells as well as short-lived CD8^{+} cytotoxic T cells. Mounting evidence suggests that Blimp1 plays a common role in the terminal differentiation of multiple cell subsets.

8.1 Discovery and Structure of Blimp1

In 1991, Maniatis and colleagues described a transcriptional repressor of the human β-interferon (*IFNβ*) gene [1]. The discovery came through a screen of a cDNA expression library for proteins that could interact with the positive regulatory domain I (PRDI) sites in the in the *IFNβ* promoter. The researchers identified a novel protein as a negative regulator of *IFNβ* expression in response to viral infection, which they named PRDI-binding factor 1 (PRDI-BF1). This was later discovered to be the homologue of the murine B lymphocyte maturation-induced protein-1 (Blimp1),

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the cDNA of which was cloned in Mark Davis' lab [2, 3]. Using B cell lines, Davis et al. had found Blimp1 transcripts to be enriched in Ig-secreting lymphoma BCL_1 cells and that over-expression of Blimp1 was sufficient to drive mature B cells into plasma or antibody-secreting cells (ASCs).

The gene encoding Blimp1, *Prdm1*, is located on chromosome 10 in mice and chromosome 6q12 in humans [4]. The murine *Blimp1* consists of eight exons, although exon 2 is absent in endogenous transcripts [5, 6]. The TATA-less, GC-rich promoter is immediately upstream of exon 1 [5]. Alternative transcriptional start sites and isoforms of Blimp1 have also been described [5–7] and recently, an alternative promoter and an additional exon located ~70 kb upstream of exon 1 have been reported [6].

The Blimp1 protein has a size of ~97 kD[3] and contains a N-terminal acidic domain, a PR domain, a proline-rich domain, a DNA binding domain, and a C-terminal acidic domain [1, 3]. The DNA-binding domain at the C-terminus consists of five C2H2 zinc-finger motifs, the first two of which are sufficient for PRDI-BF1 to bind to the *IFNβ* promoter [8]. The zinc finger domain has also been described to confer repressor activity by recruiting the histone methyl transferase G9a and histone deacetylases (HDACs) [9, 10]. The highly conserved proline-rich domain N-terminal to the zinc fingers also associates with HDACs and has a unique role in recruiting Groucho family co-repressor proteins as well as histone lysine demethylase 1 [10–12].

8.2 Blimp1 Mouse Models

Initial attempts to create viable Blimp1-deficient mice were unsuccessful but revealed a surprising role for Blimp1 in fetal development. On a mixed C57BL/6 × 129/SvEv background, embryos with deletions of either exons 1–3 or 6–8 died around embryonic day (E) 10.5 from loss of mesenchymal bronchial arches, placental defects, and severe hemorrhaging [13]. Mice on a C57BL/6 background with removal of only exons 7 and 8 showed a prolonged embryonic lifespan up to around E15 [14–16]. A more detailed examination showed that Blimp1 can be detected in embryos as early as E6.25 [17]. Its expression pattern is dynamic and is induced in the foregut pocket, anterior mesendoderm, primordial germ cells, limb buds, and myotomes in early gestation, and in chondroblasts, mesenchymal tissues, hair follicles, and sensory vibrissae in mid to late gestation [18]. Blimp1 deletion in epiblast-derived cells resulted in defects in the development of sensory vibrissae and posterior digits of the forelimbs [16], consistent with its expression pattern in these tissues. Blimp1 is also required for germ cell specification [13, 17], and the differentiation of epidermis and sebaceous glands [19–21]. Studies on the immune functions of Blimp1 have since utilized mice reconstituted with Blimp1-deficient fetal liver stem cell or conditional Blimp1 knock-out mice to bypass the embryonic lethality.

Examination of Blimp1 expression in vivo was made possible with the development of several Blimp1 reporter mouse strains [6, 17, 22, 23]. In particular, the

Blimp1^{gfp} knock-in allele, which was engineered by the insertion of the green fluorescent protein (GFP) expression cassette downstream of exon 6, was characterized in detail and found to be a precise reporter of Blimp1 expression at the single-cell level [14, 22]. The *Blimp1*^{gfp} allele expresses a truncated, DNA-binding deficient Blimp1 protein and GFP from the same RNA. Thus, heterozygous mice report Blimp1 expression *in vivo*, and homozygous mice lack functional Blimp1 protein but retain GFP expression from an activated Blimp1 gene locus.

8.3 Blimp1 Expression in the Immune System

Studies using the *Blimp1*^{gfp} reporter mouse demonstrated that Blimp1 is present in all ASCs independent of their locations and differentiation history, but not in naïve or memory B cells [22, 24, 25]. They further revealed that quantitative changes in Blimp1 expression are associated with distinct stages of ASC differentiation. Blimp1 expression is intermediate in cycling plasmablasts and high in mature splenic and bone marrow resident plasma cells [22]. Importantly, this finding was later shown to be relevant in human ASC [26]. Mice that lack Blimp1 in all hematopoietic cells or mice with B cell specific deletion of Blimp1 display normal early B cell development and unperturbed or even increased germinal center formation following immunization [22, 27]. However, in the absence of Blimp1, proliferating B cells arrest in their differentiation to plasma cells, leading to severe reduction in Ig production of all isotypes [22, 27]. In addition, Blimp1-deficient memory B cells cannot differentiate into ASCs, highlighting the obligatory role of Blimp1 in plasma cell formation during both primary and memory responses [27]. Ectopic expression of Blimp1 alone is sufficient to induce an ASC phenotype in B cells, further establishing Blimp1 as the master regulator of plasma cell formation [3, 28, 29].

In addition to plasma cells, Blimp1 was also found to be expressed in human peripheral blood monocytes and granulocytes, and Blimp1 overexpression in a monocytic cell line induced differentiation into cells with a macrophage phenotype [30]. Furthermore, Blimp1 was recently shown to be involved in osteoclastogenesis [31, 32], to modulate the functions of mature dendritic cells [33] and to drive maturation of natural killer (NK) cell [34, 35].

Importantly, over recent years it became evident that Blimp1 is an indispensable transcriptional regulator of peripheral T cell differentiation and homeostasis [14, 23, 36–38]. This article describes these results and discusses the functions of Blimp1 in T cell biology.

8.4 Blimp1 Expression and T Cell Function

In 2006, our and the Calame groups described for the first time that Blimp1 is expressed in $CD4^+$ and $CD8^+$ T cells with an effector or memory phenotype and required for their homeostasis [14, 36]. Mice lacking Blimp1 expression in all

hematopoietic cells and mice with a T cell-specific deletion of Blimp1 revealed a significant expansion of activated T cells and succumbed to inflammatory disease in the gastrointestinal tract, the liver, and the respiratory tract mediated by infiltrating $CD4^+$ and $CD8^+$ T cells [14, 36]. Chimeric mice reconstituted with wild-type and Blimp1-deficient bone marrow confirmed that the increase in cells with an activated phenotype was an intrinsic property of Blimp1-deficient T cells. However, the precise nature of the T cell accumulation was not fully understood. Blimp1-deficient T cells did not seem to possess an intrinsically enhanced ability to proliferate [14, 36], but Martins et al. reported better proliferation in low density T cell cultures, most likely to be due to increased secretion of interleukin (IL) 2 which enhances T cell survival and proliferation [36]. Furthermore, in vitro experiments demonstrated that Blimp1-deficient T cells were more resistant to activation-induced cell death compared to their wild-type counterparts [14]. In line with this, enforced expression of Blimp1 has also shown apoptotic effects in B and T cells [28, 39]. Therefore, it is tempting to speculate that Blimp1 regulates the contraction of the T cell pool by limiting the survival of activated cells.

Although Martins et al. [36] reported expression of Blimp1 in thymocytes, our own studies utilizing a GFP-reporter mouse failed to detect Blimp1 expression in immature or naïve T cells (unpublished results and Ref. [14]). This, in conjunction with the expression data discussed above implies a specific role for Blimp1 in the terminal differentiation of T cells similar to its role in B cells.

8.5 Blimp1 and $CD8^+$ T Cell Differentiation

The role of Blimp1 in T cell differentiation during an immune response is best characterized for $CD8^+$ T cells. This is due to the existence of well-established pathogen models and the availability of reagents to track antigen (Ag)-specific $CD8^+$ T cells. Three studies published in 2009 illustrated that Blimp1 could function as a molecular switch in effector and memory differentiation of $CD8^+$ T cells [23, 37, 38].

Several models have been proposed to describe the ontogeny of effector and memory $CD8^+$ T cells [40]. In the linear differentiation model, naive T cells after activation give rise to effector cells, which in a step-wise fashion further differentiate into memory cells during the contraction phase of an immune response [41]. In an alternative, early delineation model, effector and memory precursor cells emerge early during an immune response and differentiate along separate pathways into mature effector and memory T cells [42–44]. A third scenario integrates characteristics from the previous two models. According to this 'decreasing potential model' differentiating T cells while progressively acquire effector function loose their ability to give rise to memory T cells [45]. While this is a matter of great debate for T cells, it is clear that, B cell differentiation follows the early delineation model [24, 46].

Memory T cells are heterogeneous and distinct subpopulations have been described based on phenotypic and functional differences: (1) effector memory (T_{em}) cells, which show low expression of the chemokine receptor CCR7 and typically

low CD62L expression and are dominant in tissues at sites of infection, and (2) central memory T (T_{cm}) cells, which are high in CCR7 and CD62L expression and mainly reside in lymphoid organs [47, 48]. T_{em} cells are poorly proliferative and highly cytotoxic [47], potentially representing a subset of effector cells with prolonged lifespan. T_{cm} cells on the other hand, share many features of naïve T cells and are highly proliferative but weakly cytotoxic [47]. Regardless of the mechanism by which effector and memory cells are generated, it is clear that the fate of individual cells is influenced by their local environment (e.g. access to antigen, co-stimulation and cytokines), which induces a specific transcriptional program.

A number of phenotypic markers, most notably IL-7 receptor (IL7R), and the killer cell lectin-like receptor sub-family G member 1 (KLRG1) have been identified to distinguish effector and memory precursors in the Ag-specific $CD8^+$ T cell population as early as 4–5 days post-infection [49–54]. The $IL7R^{lo}$ $KLRG1^{hi}$ phenotype marks short-lived effector cells while memory precursor cells express high levels of IL7R and lack KLRG1 [53]. Strikingly, after viral infection formation of short-lived effector cells is blocked in the absence of Blimp1 and most Ag-specific $CD8^+$ T cells during the acute immune response resemble memory precursor cells [23, 37]. Blimp1-deficient Ag-specific $CD8^+$ T cells are intrinsically less cytotoxic than their wild-type counterparts [23, 37] and show severely reduced expression of granzyme B and reduced transcripts of granzyme K and perforin [37]. These cells also accumulate preferentially in lymphoid organs and are under-represented in sites of infection [37]. The reduced tissue migration is at least partially due to altered expression levels of chemokine receptors [23, 37]. Transcripts encoding CCR5, a chemokine receptor important for recruiting effector T $CD8^+$ T cells into lungs during influenza infections [55], are reduced in Blimp1-deficent Ag-specific $CD8^+$ T cells, whereas the transcripts of both *Ccr6* and *Ccr7* are elevated [23, 37]. The chemokine receptor CCR6 is expressed on effector and memory T cells with skin and mucosa-homing abilities and its heightened expression under inflammatory conditions [56]. CCR7 is a chemokine receptor predominantly expressed on naïve and memory T cell and important for their homing to lymphoid organs and its downregulation is essential for effector cells to migrate to tissues [57]. Thus, it may be speculated that Blimp1 regulates T cell differentiation not only directly but also by altering the migration patterns of Ag-specific cells and hence their accessibility to the milieu and signals that promote effector differentiation.

As mentioned above, Blimp1-deficient effector $CD8^+$ T cells phenotypically resemble memory precursors [23, 37]. These cells give rise to increased numbers of memory T cells that show enhanced longevity and increased expressing of CD62L, reminiscent of T_{cm} cells [23, 37]. However, conflicting results were obtained when Blimp1-deficient memory were re-challenged [23, 37]. In a non-competitive transfer experiment using lymphocytic choriomeningitis virus (LCMV), Blimp1-deficient memory cells showed equal proliferation upon recall in host mice compared to wild-type cells [23]. However, mice that received both wild-type and Blimp1-deficient memory cells and were re-challenged with influenza virus, showed a severely limited secondary expansion of Blimp1 deficient cells [37]. These results suggest that although strong stimuli are able to drive expansion of memory cells in

the absence of Blimp1, Blimp1 is required for optimal memory activation and differentiation to secondary effector cells under physiological conditions. This highlights the importance of Blimp1 in regulating effector differentiation during both primary and memory response, which is mirrored by the requirement of Blimp1 in plasma cell formation from both naïve and memory B cells [27]. Interestingly, during chronic virus infection, Blimp1 promotes the expression of inhibitory receptors, thereby exacerbating T cell exhaustion [38].

In agreement with its role in promoting effector cell fate, analysis of Blimp1 expression levels following acute viral infections revealed that Ag-specific $CD8^+$ T cells with an effector phenotype expressed high levels of Blimp1 both during the acute phase of the immune response and 60 days post-infection, while Blimp1 expression was lower in the T_{cm} cells [23, 37]. This is consistent with previous reports showing a high level of *Blimp1* transcripts in $CD44^{hi}$ $CD62L^{lo}$ cells and a reduced level in $CD44^{hi}$ $CD62L^{hi}$ memory cells [36, 58]. Interestingly, effector cells in chronic viral infection express Blimp1 even more strongly than those in acute infection [38], suggesting that Blimp1 level may be linked to the activation state of the cell.

Transcription profiles of Blimp1-deficient Ag-specific $CD8^+$ effector T cells showed altered expression of a wide range of genes [23, 37]. Decreased expression of effector molecules and increased expression of survival factors and homing chemokine receptors correlate with the phenotype associated with Blimp1 deficiency [23, 37]. Several transcriptional regulators including *Bcl6, Eomes, Tcf7, Id3, Pou6f1,* and *Blimp1* itself are upregulated in the absence of Blimp1 [23, 37], some of which (i.e. *Bcl6, Id3 and Blimp1*) are previously described target genes of Blimp1 [19, 59–61]. Tcf7 and Id3 are important regulators of thymic development and are highly expressed in naïve T cells [62–64], whereas both Eomesodermin (Eomes), a T-box family transcription factor, and Bcl6 play essential roles in the generation and maintenance of memory cells [65–68]. Therefore, Blimp1 function appears to inhibit the naïve and memory transcriptional programs of differentiating Ag-specific $CD8^+$ T cells, consistent with its role as a transcription repressor. This is paralleled by the role of Blimp1 in B cell differentiation during which Blimp1 suppresses key transcriptional regulators such as Pax5 and Bcl6 that are required for the identity of naïve and germinal centre B cells [69].

Collectively, this data supports a model in which Blimp1 acts a key regulator that programs terminal differentiation of Ag-specific $CD8^+$ T cells into short-lived, highly cytotoxic effector cells that preferentially migrate to sites of infection. In the absence of Blimp1, Ag-specific T cells default into memory precursors with limited effector function.

8.6 Blimp1 and $CD4^+$ T Cells

The function of Blimp1 in $CD4^+$ T cell differentiation is not well understood, which may be due to the heterogeneity and plasticity of T helper cells [70] and the lack of tools to track Ag-specific cells. Depending on the signals received, $CD4^+$ T cells can

differentiate into Th1, Th2, Th17, follicular T helper (T_{FH}) cells, adaptive regulatory T cells, or other T helper populations, each with distinct effector functions [71]. The role of Blimp1 in these helper subsets has been poorly characterized to date. Polarization of naïve $CD4^+$ T cells into Th1 or Th2 lineages in vitro is not generally perturbed in the absence of Blimp1 [14]. However, one study reported diminished humoral responses mediated by Blimp1-deficient Th2 cells and showed direct binding of Blimp1 to *Bcl6*, *Tbx21,* and *IFNγ* in $CD4^+$ T cells, suggesting that Blimp1 may be required for optimal Th2 differentiation [61].

Recently, Blimp1 was identified as a key repressor of T_{FH} development, which requires high expression of Bcl6 [72–74]. Bcl6-deficient $CD4^+$ T cells cannot differentiate into T_{FH} cells or drive GC formation. In keeping with the notion that Blimp1 might directly suppress *Bcl6* in T cells, T_{FH} cells express high levels of Bcl6 are low in Blimp1, and ectopic expression of Blimp1 inhibited *Bcl6* expression and T_{FH} formation [73]. This antagonistic relationship between Blimp1 and Bcl6 in T cell differentiation is reminiscent of the situation in B cell differentiation, where Bcl6 is not only believed to be a direct target of Blimp1 in B cells but also reciprocally represses Blimp1 thereby separating the genetic programs of GC B cells and plasma cells during B cell responses [59, 60, 75]. Thus, Blimp1 seems to target lineage specific as well as common genes in the immune system, which may help to explain how one transcription factor can regulate terminal differentiation of multiple cell lineages.

8.7 Blimp1 and Regulatory T Cells

Blimp1-deficient mice develop spontaneous inflammatory disease characterized by infiltration of T cells into various tissues, particularly the gastrointestinal tract and the lungs [14, 36]. This observation and the finding that $CD4^+CD25^+$ T cells, which contain Foxp3-expressing T_{reg} cells express high amounts of *Blimp1* transcripts, prompted examination of T_{reg} cells that are essential mediators of peripheral tolerance. T_{reg} cells develop independently of Blimp1, and Blimp1-deficient T_{reg} cells can suppress in vitro proliferation of wild-type effector cells [14, 36]. Although Blimp1-deficient T_{reg} cells could also suppress colitis induced by activated wild-type T cells in a T cell transfer model, they did not prevent disease caused by accumulation of Blimp1-deficient $CD4^+$ T cells, nor did they ameliorate colitis induced by dextran sulphate sodium [14, 36]. This data suggested that Blimp1 may regulate functional maturity of T_{reg} cells. Consistent with this hypothesis, production of IL-10, an immune-suppressive cytokine important for intestinal mucosal tolerance [76, 77], is markedly reduced in Blimp1-deficient $CD25^+CD4^+$ T cells [14, 36]. Our own recently published results [78], show that the T_{reg} population consists of both Blimp1-expressing and non-expressing cells. Blimp1-positive T_{reg} cells have an effector phenotype and IL-10 production is restricted to this sub-population. These cells also have limited proliferative capacity and may represent functionally mature T_{reg} cells. Indeed, Blimp1-negative T_{reg} cells can be induced to express Blimp1 both

in vitro and in vivo and become fully functional mature T_{reg} cells. Blimp1-deficiency impairs *Il10* transcription and homeostasis of T_{reg} cells, both of which are important for maintaining mucosal tolerance. Thus, Blimp1 is specifically required for the differentiation of IL-10+ effector T_{reg} cells. Interestingly, Blimp1 was recently also shown to be essential for IL-10 production by CD8 T cells [79].

8.8 Blimp1 Regulation

Productive T cell activation requires distinct signals from Ag recognition and engagement of TCR as well as co-stimulatory signals (e.g. CD28, ICOS, OX40, and CD27) [80]. Innate cells such as dendritic cells, macrophages, and natural killer cells act to integrate early signals with production of cytokines that provide additional cues to activated T cells. *Blimp1* is expressed late during a T cell response and TCR activation alone is insufficient to induce it [14, 81]. Indeed cytokine signals from both T cells and innate sources are thought to be key in the regulation of Blimp1 expression.

IL-2 is a classic trophic factor for T cell survival and growth in vitro [82], yet surprisingly, IL-2-deficient mice develop lymphadenopathy and rapidly succumb to auto-immunity [83]. It was later discovered that IL-2 is the key cytokine for the maintenance of T_{reg} cells [84, 85] and the absence of which in IL-2 and IL2R-deficient mice leads to massive accumulation of activated T cells and pathology. IL-2 induces *Blimp1* expression *in vitro*, and *Il2* and its activator *Fos* are repressed by Blimp1 in a negative feedback loop [39, 81, 86]. Recent studies showed that strong IL-2 signals drive the formation of effector $CD8^+$ T cells, marked by high expression of granzyme B and perforin, and downregulated IL-7R, thus favoring the development of short-lived effector $CD8^+$ T cells [87, 88]. Prolonged IL-2 signaling gives rise to $CD25^{hi}$ Ag-specific $CD8^+$ T cells that exhibit enhanced effector functions and decreased longevity, which represent terminally differentiated effector cells. $CD25^{lo}$ cells, on the other hand can upregulate IL7R and differentiate into memory precursor cells. The effects of IL-2 and IL-2R signaling on $CD8^+$ T cell differentiation can be at least partially attributed to its ability to induce or sustain high levels of Blimp1 that in conjunction with other factors such as T-bet, promote the formation of terminally differentiated effector cells.

IL-2Rα signaling has also been shown to be critical for secondary expansion of memory $CD8^+$ T cells in acute LCMV infections [89, 90]. In $CD4^+$ T cells, IL-2 also upregulates IL-4Rα expression, augmenting cellular responsiveness to IL-4 signals [91]. IL-4 itself is another inducer of Blimp1 and, in combination with IL-2, strongly upregulates Blimp1 expression [61, 81, 92, 93].

Another cytokine that induces Blimp1 is IL-21, which together with IL-2, IL-15 and IL-4 belongs to the family of four-α-helical-bundle type I cytokines that utilize common gamma chain cytokine receptors [94]. IL-21 is a regulator of Ig class switching and induces Blimp1 in B cells [95–98]. Furthermore, IL-21 stimulates T cell expansion in synergy with other activation signals [99, 100] and is a potent inducer of Blimp1 that can upregulate Blimp1 expression stronger and faster than IL-4 [93].

IL-12 is a pro-inflammatory cytokine and an inducer of T-bet, another T-box family transcription factor required for Th1 differentiation in CD4+ T cells and for the generation of short-lived effector cells in CD8+ T cells [53, 101, 102]. In addition to T-bet, IL-12 also induces Blimp1 [81], suggesting that T-bet and Blimp1 function together in promoting effector differentiation. IL-12 inhibits induction of Eomes, which is involved in memory T cell formation [65, 103]. This agrees with the observation that Blimp1-deficient CD8+ T cells exhibit a memory phenotype with heightened Eomes expression [37]. Interestingly, IL-15 was shown to suppress Blimp1 expression [81], which is consistent with its essential role in the maintenance of memory T cells [104] and the lower expression of Blimp1 in these cells [23, 37, 58].

The transcription factors that directly regulate Blimp1 expression are still not well understood. One exception, Irf4, has been shown to directly bind to regulatory regions of the *Blimp1* gene [93, 105]. Irf4 belongs to the interferon regulator factor family of transcription factors, but does not respond to IFNs or tumour necrosis factor α [106]. It is exclusively expressed in immune cells and is indispensable for Th2, Tfh and Th17 differentiation, and for T_{reg} functions [107–111]. During B cell responses, Irf4 is required for class switch recombination as well as plasma cell formation [105, 112]. Studies on the role of Irf4 in *Blimp1* induction in B cells yielded conflicting data. Although one study found unaltered Blimp1 levels in Irf4-deficient B cells [112], another study reported severely reduced induction of *Blimp1* and its target genes in *Irf4*$^{-/-}$ B cells and demonstrated that Irf4 directly binds to a conserved non-coding sequence of *Blimp1* gene and upregulates its expression [105]. A recent study examining IL-21-induced Blimp1 expression confirmed that Irf4 acts upstream of *Blimp1* in the transcriptional hierarchy in both B and CD4+ T cells [93]. Irf4 and STAT3 cooperatively induce *Blimp1* by directly binding to a 3′ sequence of *Blimp1* that is responsive to IL-21 signaling [93]. Another inducer of *Blimp1* in B cells is nuclear factor kappa B (NFκB), which binds directly to a region near the basal promoter of *Blimp1* and induces its expression in response to LPS stimulation [6]. NFκB is induced by TCR and co-stimulatory signals during T cell activation [113] and therefore may also serve as an upstream regulator of *Blimp1* in T cells.

8.9 Conclusion

It has been almost two decades since Blimp1 was first discovered. Over the years, a common model has emerged according to which Blimp1 regulates late differentiation of multiple cell lineages including B cells, NK cells, myeloid cells, and different T cell subsets. Blimp1 appears to be dispensable for the early development of lymphocytes and is exclusively expressed in fully activated and differentiated cells during an immune response. Cytokines in addition to Ag-receptor signaling and co-stimulation are potent inducers of *Blimp1*, which suppresses transcriptional signatures of naïve and memory cells and favors the formation of functional mature effector cells (Fig. 8.1). These cells are considered terminally differentiated and

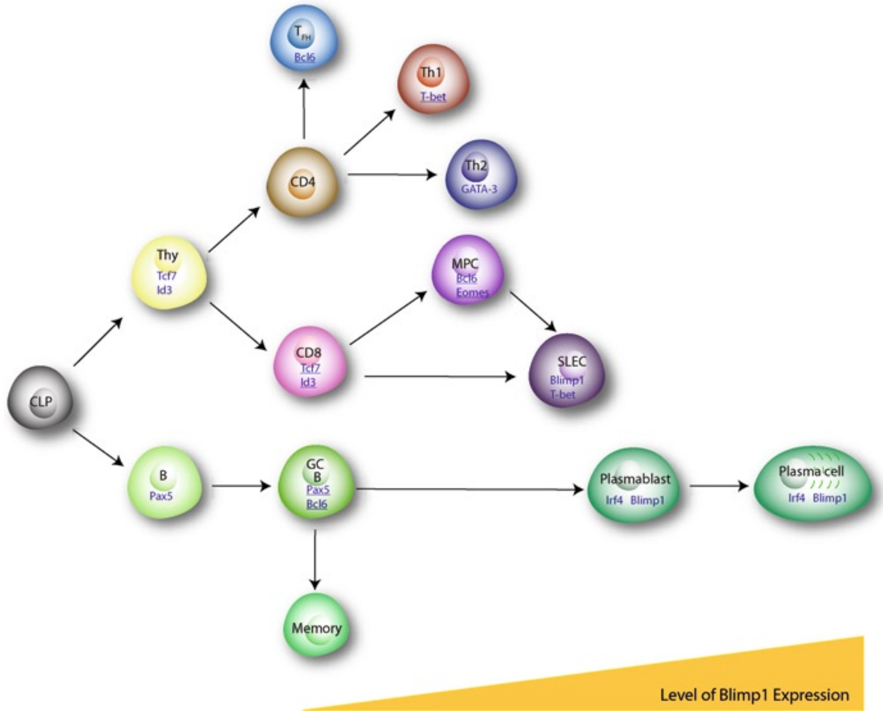

Fig. 8.1 Role of Blimp1 in lymphocyte differentiation. In B cells and CD8⁺ T cells, Blimp1 promotes terminal differentiation of effector cells by repressing transcriptional regulators that shape the naïve and/or memory cell identities. In CD4⁺ T cells, Blimp1 negatively regulates follicular T helper cell differentiation and may also dampen Th1 differentiation. Key transcriptional regulators of different subsets are shown in blue and genes directly or indirectly repressed by Blimp1 are underscored. The level of Blimp1 expression in each cell subset is presented schematically and not drawn to scale. *CLP* common lymphoid progenitor, *Thy* thymocyte, *GC* germinal centre, *MPC* memory precursor cell, *SLEC* short-lived effector cell, T_{FH} follicular T helper cell

their fate does not change under physiological conditions. A classic example of a terminally differentiated cell type is the plasma cell. Plasma cells have undergone significant morphological and biochemical changes to become effector cells that have lost their former B cell functions, such as antigen presentation, and have instead become specialized Ig-secreting cells. Although T cells appear more functionally plastic, some effector $CD8^+$ T cells adopt a highly cytotoxic and weakly proliferative phenotype and are short-lived, thereby appearing terminally differentiated. Blimp1 is crucial for the formation of plasma and short-lived effector $CD8^+$ T cells and for the function of effector T_{reg} cells, therefore playing a fundamental role in terminal differentiation of both B and T cells. Blimp1 mediates effector cell migration by changing chemokine receptor expression patterns and restricts the pool of effector cells. This is important for preventing excess tissue damage and auto-immunity. Blimp1 has been shown to control cell cycle by inhibiting *c-myc*

expression [19, 30, 114] and to restrict cell survival [14]. However, plasma cells and some T_{em} are long-lived and express high levels of Blimp1, indicating that niche-specific signals may modulate or override Blimp1-mediated apoptosis. It remains a challenge to understand how Blimp1 regulates unique target genes in different cell subsets. One possible mechanism would be through co-operation with other transcription factors or nuclear proteins. Another mechanism might involve the interaction of Blimp1 with epigenetic regulators. The role of histone modifications during cell differentiation has become an area of great interest over recent years. It has been shown that T helper cell differentiation is heavily influenced by chromatin accessibility and the action of histone modifying proteins such as G9a [115]. Therefore, the ability to interact with histone modifiers implies a role for Blimp1 in mediating changes in histone conformation and chromatin structure, and may be an important mechanism for Blimp1 to regulate gene repression and affect cell fate decision.

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Chapter 9
A New Role for Myeloid HO-1 in the Innate to Adaptive Crosstalk and Immune Homeostasis

Vasiliki Koliaraki and George Kollias

Abstract Increasing evidence supports the presence of a dynamic crosstalk between innate and adaptive immunity with a pivotal role played by pathways governing innate immune responses. TLRs (Toll-like receptors) and RLHs (retinoic acid-inducible gene I [RIG-I]-like helicases) are known to play a key role in these processes. A molecule of high significance in the protection against innate and adaptive immune aberrations is heme oxygenase 1 (HO-1). HO-1 is a microsomal enzyme that catalyses the degradation of heme to iron, carbon monoxide and bilirubin. These by-products appear to be the key mediators of its anti-inflammatory and cytoprotective action, mainly through the downregulation of pro-inflammatory and upregulation of anti-inflammatory molecules. Recent data from our lab support the presence of an additional direct effect of myeloid HO-1 on innate immune conditioning, and more specifically on the TLR3/TLR4/RIG-I pathway. In myeloid cells, HO-1 forms a complex with the transcription factor IRF3 (Interferon regulating factor 3) and is required for IRF3 phosphorylation and consequent type-I interferon and chemokine gene induction. Myeloid HO-1–deficient mice show reduced expression of IRF3 target genes and altered responses to infectious and organ-specific auto-immune diseases. This new frame of understanding HO-1 function should also be important for the future design of novel interventions differentially targeting the enzymatic versus the IRF3 modulating properties of HO-1.

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9.1 Innate Immunity: Induction of Type I Interferons

Innate immunity constitutes a host organism's first line of defense against invading pathogens. It relies on PRRs (pattern recognition receptors) that recognize specific PAMPs (pathogen-associated molecular patterns) leading to a cascade of events, which can ultimately result in microbial clearance. There are three types of PRRs; soluble, trans-membrane, and cytosolic [22]. From them, trans-membrane TLRs and cytosolic RLHs are further implicated in the induction of adaptive immune responses, both through their expression on APCs (antigen presenting cells) where they bridge microbe recognition and T-cell binding and through the induction of type I interferons, which are key molecules in augmenting and sustaining T cell responses [18, 30].

9.1.1 The TLR and RIG-I/Mda5 Pathways

TLRs constitute a large family of type I trans-membrane receptors, which are characterized by the presence of two domains, an extracellular LRR (leucine-rich repeat) and an intracellular TIR (Toll/IL-1 receptor) domain [52]. Mammalian cells possess at least 13 members of the TLR family, the first 9 of which are common between human and mouse. TLRs are further sub-categorized according to their subcellular localization in cell surface receptors. There are TLR1, 2, 4, 5 and 6, which detect lipoteichoic acid, bacterial lipoproteins, lipopolysacharide (LPS) and flagellin, and endosomal receptors TLR3, 7/8 and 9, which recognize double-stranded RNA (dsRNA), single-stranded RNA (ssRNA) and double-stranded DNA (dsDNA) [61].

Ligand binding leads to homo or in some cases hetero-dimerization of TLRs and the subsequent activation of two major pathways. The first, which is common for most TLRs is the activation of the transcription factors NF-kB (Nuclear Factor-kB), AP-1 (heterodimer of activating transcription factor 2 and c-Jun) and IRF7 through a complex consisting of MyD88 (Myeloid differentiation primary response gene 88), TOLLIP (Toll-Interacting Protein), IRAK (IL-1R-associated kinase), and TRAF6 (TNFreceptor-associated factor 6), which ultimately results in induction of pro-inflammatory cytokines and chemokines. The second pathway is downstream of TLR3 and 4 and leads to activation of the transcription factor IRF3 independently of MyD88. TLR3 and 4 utilize the adaptor molecule TRIF (TIR-domain containing adaptor inducing interferon-β, IFN-β) that leads to formation of a complex consisting of TRAF3 (TNFreceptor-associated factor 3), NAP1 (Nucleosome assembly protein 1), TANK (TRAF family member-associated NF-kappa-B activator), TBK1 (TANK-binding kinase-1), and IKKε (IkB kinase-ε), which in turn phosphorylates IRF3 in order to induce the production of type I interferons and antiviral chemokines [2, 37, 61].

The cytoplasmic RLHs, a family that consists of three proteins, RIG-I, Mda5 (Melanoma differentiation-associated gene 5), and LGP2 (laboratory of genetics and physiology 2) are also able to detect RNA and evoke antiviral responses, mainly through the production of type I interferons [63]. RLHs contain two repeats of the CARD (Caspase recruitment domain) like motif at their N terminus [77].

Upon ligand binding, RLHs interact through this domain with IPS-1 (interferon-beta promoter stimulator 1; alternatively termed MAVS, VISA, and Cardif), which is localized on the outer membrane of mitochondria, in order to activate the downstream signaling pathways [25, 58], mainly NF-kB and IRF-3 and −7. This is mediated by TRAF3 and IKKi (IkB kinase-i) or TBK1 [46, 56]. It is of note that different members of the RLHs family, RIG-I and Mda5 distinguish different RNA viruses [24].

9.2 Heme Oxygenases

Heme oxygenase (HO) is the rate-limiting microsomal enzyme that catalyzes the degradation of heme [65, 67]. With the use of cytochrome P-450, NADPH (nicotinamide adenine dinucleotide phosphate) and molecular oxygen, heme oxygenase catabolises heme to equimolar amounts of ferrous iron that is sequestered by ferritin, carbon monoxide (CO) and biliverdin, which is rapidly converted to bilirubin by biliverdin reductase (BVR) [34, 64, 66] (Fig. 9.1). Heme oxygenase is ubiquitously expressed and evolutionary conserved among organisms, highlighting the importance of the aforementioned pathway [73].

Two main isoforms have been described so far; HO-1 and HO-2, which are encoded by genes *HMOX1* and *HMOX2* [9, 35]. There is also a third isoform referred to as HO-3, however this has been identified only in rats and most likely represents a pseudogene [17, 36]. Both isoforms are expressed in most cell types and tissues, but HO-1 is inducible, while HO-2 is constitutively expressed. The function of HO-2 is not clearly established yet, although it has been suggested that it acts as an oxygen sensor [1]. As far as the degradation of heme is concerned, HO-1 is the key player. Besides its crucial role in heme metabolism, HO-1 also exhibits potent antioxidant and anti-inflammatory properties.

9.2.1 The Role of HO-1 in Inflammation and Auto-immunity

The only documented case of a human patient that did not express HO-1 was presented with generalized systemic inflammation, accompanied with intravascular hemolysis, abnormalities of the coagulation/fibrinolysis system, nephropathy,

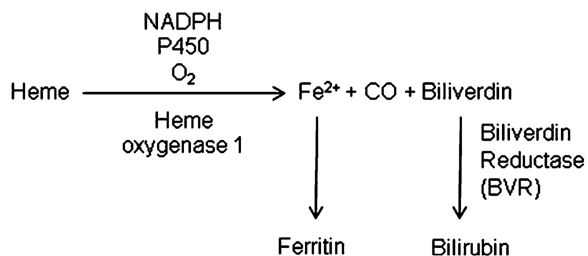

Fig. 9.1 Schematic representation of heme degradation by heme oxygenase

vascular endothelial injury and asplenia [26, 29, 74]. This case and the fact that polymorphisms in the human HMOX1 locus are implicated in the initiation and progression of several inflammatory disorders [10] have provided the necessary validation that the HO-1 system functions similarly in mice and humans.

The first direct proof of the immuno-regulatory role of HO-1 came from HO-1 knockout mice, which displayed increased embryonic lethality, anemia and chronic inflammatory disorders [48, 49, 76]. HO-1 deficient mice also developed a more severe disease phenotype after LPS injection [72] and CLP (cecal ligation and puncture) surgery [8], while overexpression of HO-1 under the ACLP (aortic carboxypeptidase-like protein) promoter, which is specific for the vasculature and the myofibroblast layer of the ileum and colon decreased death rate after CLP [8], suggesting a protective role during bacterial infection and sepsis. A similar function has also been reported in anti-viral and anti-parasitic responses [16, 44, 50].

Other inflammatory disorders, in which HO-1 plays a crucial role is atherosclerosis and graft rejection. More specifically, overexpression of HO-1 in LDL (low density lipoprotein)-receptor knockout mice [20] and Watanabe heritable hyperlipidemic rabbits [21] resulted in amelioration of the atherosclerotic lesions, while its absence led to exacerbation of the disease progression [23, 75]. Induction of HO-1 was able to protect the myocardium [39], the liver [3] and the kidneys [5] from ischemia-reperfusion injury, unveiling its crucial role in the regulation of immune tolerance during organ transplantation. It is of note that HO-1 overexpressing transplants displayed both reduced expression of pro-inflammatory cytokines and type I interferons [3, 68].

Pharmacological or genetic upregulation of HO-1 was further found to ameliorate disease symptoms in several auto-immune disorders, including type I diabetes, lupus, rheumatoid arthritis and EAE (experimental auto-immune encephalomyelitis). Systemic production of HO-1 or CO administration led to amelioration of type I diabetes in NOD (non-obese diabetic) mice which was accompanied by a reduced Th-1 response and increased pAkt and BcL-XL expression [19, 32, 53, 54]. Likewise, systemic chemical induction of HO-1 ameliorated lupus nephritis by suppressing nitric oxide (NO) dependent inflammatory responses and attenuating production of pathogenic auto-antibodies [62]. In rheumatoid arthritis induction of HO-1 in vivo or in vitro in human synovial cells led to amelioration of disease phenotype and decreased release of pro-inflammatory molecules [4, 28, 45], a function possibly implicating effects of carbon monoxide [11]. In EAE, the mouse model for multiple sclerosis, genetic ablation in knockout mice or chemical inhibition of HO-1 was shown to severely enhance disease progression [7], while pharmacological induction ameliorated disease symptoms [33].

9.2.2 The Role of Myeloid Specific HO-1 in the Modulation of Adaptive Responses in Auto-immunity

In organ-specific auto-immunity as experimental auto-immune encephalomyelitis, recent work from our lab showed that specific genetic ablation of HO-1 in macrophages led to increased severity and chronicity of the disease. Interestingly,

exacerbation of the disease phenotype was characterized by increased activation of APCs, enhanced infiltration of Th17 cells within the CNS, and a non-regressing myelin-specific T cell reactivity. More specifically, conditional knockout mice displayed increased numbers of inflammatory cells, including CD4+ and CD8+ T cells, macrophages, and granulocytes, than did control mice. Moreover, infiltrating macrophages (CD45high CD11b+Gr1$^-$) showed increased expression of CD40, CD80, CD86, H-2Kb, and I-Ab as compared with controls. In the CNS of HO-1M-KO mice, a fivefold increase in the numbers of IL-17–producing CD4+ T cells and a twofold increase in the numbers of IFN-β producing CD4+ T cells with an IL-17/IFN-β ratio of >2:1 was also observed. In the absence of HO-1 in macrophages, treatment with poly I:C (Polyinosinic:polycytidylic acid) failed to suppress the development of the disease, as happened in control mice, while administration of IFN-β ameliorated these symptoms in both cases. This fact was indicative of the link between HO-1 and TLR3 signaling in auto-immunity and suggested a relationship between innate and adaptive immune response orchestrated by HO-1 in this model of disease [70].

9.2.3 Heme Oxygenase-1: Mechanisms of Action

9.2.3.1 The Enzymatic Activities of HO-1

Numerous studies have highlighted the important role of HO-1 byproducts in the control of inflammatory responses [55]. Carbon monoxide (CO) has been shown to modulate innate immune responses mainly by inhibiting TLR2, 4, 5, and 9 signaling [40], with more studies focusing on its role in the TLR4 mediated pathway. More specifically, HO-1 appears to act through the p38 MAPK pathway, the NFkB pathway or the induction of cGMP in order to inhibit the LPS-induced production of iNOS, COX2 and pro-inflammatory cytokines, such as TNF, IL1b, and MIP-1a in macrophages [41, 59, 74] and epithelial cells [38]. HO-1 also acts to downregulate HMGB1, a TLR4 ligand, which contributes to inflammation and lethality in endotoxemia models [14,60], a function which appears to be mainly mediated by carbon monoxide. Furthermore, CO has been implicated in the suppression of the type I IFN pathway downstream of TLR4, which leads to protection against hepatic ischemia/reperfusion (I/R) injury [68, 69]. In macrophages, CO was further shown to induce the production of the anti-inflammatory cytokine IL-10 [41]. It is of note that HO-1 is considered to be a mediator of IL-10 action [31]. Additional mechanisms of the cytoprotective function of CO include an anti-apoptotic mechanism in several cell types, such as hepatocytes [27], fibroblasts [47], endothelial cells [6], and pancreatic β-cells [15], which can depend on the NF-kB, the p38 MAPK, or the cGMP pathway according to the cell type. Finally, CO has also been found to be able to suppress the production of IL-2 in CD4+ T cells and inhibit their proliferation through interaction with the ERK pathway [43].

The Biliverdin/Bilirubin system also exerts some of the anti-inflammatory properties of HO-1. More specifically, it reduces the production of pro-inflammatory cytokines and induces the expression of anti-inflammatory cytokines [57], while biliverdin therapy is able to protect the liver of rats from ischemia and reperfusion injury [12] and polymicrobial sepsis [42].

9.2.3.2 Direct Binding and Modulation of the IRF3 Pathway by HO-1 in Myeloid Cells

Apart from the previously described mechanisms, recent data from our lab supports the presence of an additional direct regulation of innate immune pathways by HO-1. Tzima et al. [70] showed that HO-1 can regulate the function of TLRs and RLHs by directly interfering in their downstream signaling pathway. HO-1 could directly bind to IRF3, the transcription factor downstream of both TLR3/4 and RIG-I/Mda5 and thus modulate the type I IFN production in macrophages. In particular, HO-1–deficient macrophages showed reduced expression of IFN-β and of primary IRF3 target genes encoding RANTES, CXCL10 (Chemokine (C-X-C motif) ligand 10), and MCP1 (Monocyte chemotactic protein-1). This defect caused exacerbation of EAE and enhanced bacterial clearance and survival after infection with Listeria monocytogenesis, which is dependent on IFN-β production. The fact that carbon monoxide seems not to play a role in the TLR3 pathway [40] could be evidence that direct interaction between HO-1 and IRF3 might be the prevailing mechanism of its action in response to viral infections.

Interestingly, pharmacological induction of HO-1 in primary mouse macrophages has the same effect as its genetic ablation, reducing CXCL10 production in response to LPS or poly I:C stimulation (Fig. 9.2a).

A plausible explanation for this is although HO-1 promotes signaling via the TLR3/4 and the RIG-I/Mda5 pathways, in conditions of HO-1 chemical induction, the consequent increased enzymatic activities of HO-1 predominate and exert their suppressive function on the type I IFN pathway. For example, carbon monoxide has already been shown to inhibit the LPS-mediated production of pro-inflammatory cytokines and type I IFN pathway in macrophages [71] and dendritic cells [51]. In the case of dendritic cells, Remy et al. [51] specifically showed that induction of HO-1 led to inhibition of IRF3 phosphorylation and downregulation of IRF3 expressing genes. This was mediated through the direct effect of carbon monoxide on the IRF3 pathway downstream of both TLR3 and TLR4. Alternatively, possible binding of the CoPP enhancer on HO-1 may hinder its interaction with IRF3 leading to reduced type I IFN and chemokine production (see also Fig. 9.2b–d).

9.2.3.3 Further IRF3 Modulating Properties of HO-1 in Fibroblasts

Besides macrophages, studies have demonstrated the presence of a relationship between HO-1 and type I interferon pathway in other cell types, such as beta

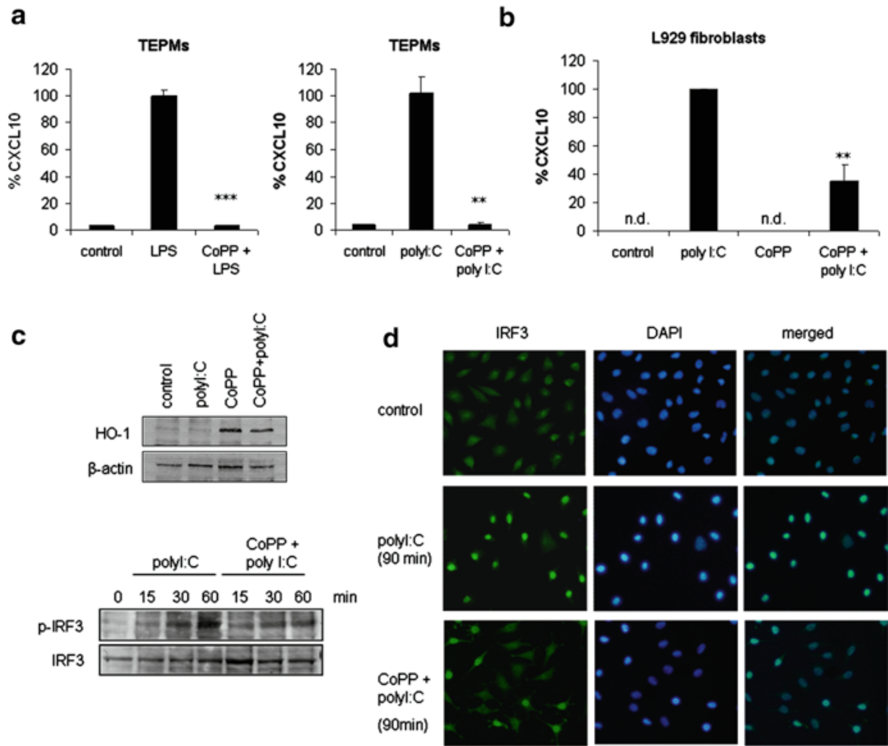

Fig. 9.2 HO-1 induction by CoPP causes a reduction in the TLR3/4-mediated production of CXCL10 in macrophages and fibroblasts. (**a**) TEPMs (thioglycolate elicited peritoneal macrophages) were pre-incubated with CoPP (50 μM, Frontier Scientific, UT) for 24 h before addition of LPS (1 μg/ml, Amersham, UK) or poly I:C (100 μg/ml, Amersham, UK) for another 24 h. CXCL10 was measured with a commercial ELISA kit purchased by R&D Systems (Minneapolis, MN). $^{**}p<0.01$, $^{***}p<0.001$. (**b**) L929 fibroblast cell line (ATCC) was pre-incubated with CoPP (50 μM, Frontier Scientific, UT) for 24 h before transfection with poly I:C (50 ng/ml, Amersham, UK) using the TransIT-293 transfection reagent (Mirus, WI, USA) for 24 h. CXCL10 was measured as described above. $^{**}p<0.01$ (**c**, **d**) L929 cells were pre-incubated with CoPP for 24 h before transfection with poly I:C for the time points indicated. Western blot analysis was performed using primary antibodies against HO-1 (Stressgen, Victoria, Canada), pIRF3 (Cell Signaling, Boston, MA), IRF3 (Santa Cruz Biotechnology, Santa Cruz, CA) and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). IRF3 and β-actin were used as loading controls. Immunofluorescence was performed using the anti-IRF3 antibody (*green*) and DAPI (*blue*). DAPI-stained, IRF3-stained, and merged images are shown

pancreatic cells [13] and dendritic cells [51]. Based on recent data, a similar mechanism seems to also exist in fibroblasts (Fig. 9.2b–d). Here we used the mouse fibroblast cell line L929 and we pre-treated cells with Cobalt Protoporphyrin XI (CoPP) for 24 h before transfecting them with poly I:C. Upregulation of HO-1 in this cell line is shown in Fig. 9.2c. Cells treated with the HO-1 inducer exhibited reduced production of the chemokine CXCL10 (Fig. 9.2b). This was accompanied by the

reduced phosphorylation and subsequent nuclear localization of the transcription factor IRF3 (Fig. 9.2c, d). Therefore, also in fibroblasts, chemical induction of HO-1 results in inhibition of the pathway downstream of TLR3/RIGI/Mda5 that leads to production of type I interferons and antiviral chemokines.

9.3 Conclusions

Heme oxygenase 1 displays potent cytoprotective, antioxidant and anti-inflammatory properties along with its crucial role in the heme degradation pathway. This function has been shown to be extremely important in the manifestation of a number of inflammatory disorders, such as infection, auto-immunity, atherosclerosis and graft rejection. A prevailing mechanism of action of HO-1 has been through its byproducts, CO and bilirubin, which act to reduce pro-inflammatory and induce anti-inflammatory responses. A new concept, supported from work by our lab using conditional ablation of HO-1 in macrophages suggests that many of the functions of HO-1 in immunity are mediated through its direct binding to the transcription factor IRF3 and consequent induction of type-I Interferon and chemokine gene expression. Differential modulation of the enzymatic versus IRF3-inducing properties of HO-1 may prove useful in the treatment of conditions such as lupus, where blockade of the type I IFNs and the concomitant enhancement of the anti-inflammatory, anti-oxidant and cytoprotective properties of HO-1 would be desirable.

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Chapter 10
Impact of Differential Glycosylation on IgG Activity

Anja Lux and Falk Nimmerjahn

Abstract Immunoglobulin G (IgG) molecules are glycoproteins with dual functionality. While participating in the destruction of virally infected cells or healthy tissues during autoimmune disease, IgG antibodies are also used as a therapeutic agent to suppress IgG-triggered autoimmune disease and inflammation. Research of recent years has put the IgG-associated sugar moiety in the spotlight for regulating these opposing activities. This review will focus on how certain IgG glycovariants impact different IgG-dependent effector functions and how this knowledge might be used to further improve the therapeutic effectiveness of this class of molecules.

10.1 Introduction

In humans and mice five different isotypes of immunoglobulins (IgA, IgD, IgE, IgM and IgG) exist. In addition, there are several subclasses of IgA (IgA1 and IgA2) and IgG (IgG1-4 in humans and IgG1, IgG2a, IgG2b, and IgG3 in mice) building up a complex repertoire of molecules for the defense against microbial pathogens [1]. Despite this array of antibody isotypes and subclasses, antibodies of the IgG isotype are most frequently used as a platform for immune-therapeutic approaches [2–4]. This does not only include full-length antibody molecules but also a great variety of proteins fused to the IgG fragment crystallisable (Fc-fragment) to confer enhanced stability and serum half-life. Within the last 20 years, more than 20 monoclonal

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antibodies were approved for the use in human therapy of cancer and autoimmune disease. The success story of the human CD20-specific antibody Rituximab and the Her2/neu specific antibody Trastuzumab has fueled the interest in therapeutic IgG antibodies and several strategies are employed to further enhance the activity of this class of molecules. Besides improving the affinity of therapeutic IgGs for their cognate antigen or the generation of IgG molecules for novel target antigens, many strategies focus on enhancing IgG dependent effector functions such as release of pro-inflammatory mediators, antibody-dependent cellular phagocytosis (ADCP), antibody-dependent cellular cytotoxicity (ADCC), and complement-dependent cytotoxicity (CDC) [5–8]. Although CDC is a potent way to kill target cells in vitro, results by many groups obtained in different mouse in vivo model systems of autoimmune disease and antibody-dependent tumor immunotherapy argue for a limited involvement of this pathway in destruction of target cells [9–17]. In contrast, mice lacking functional Fc-receptors for IgG (Fcγ-receptors, FcγR) were protected from IgG-dependent destruction of healthy tissues during autoimmune disease or were unresponsive to anti-tumor antibodies in different tumor models [11, 13, 15, 16, 18]. There is clear evidence however, for a complement C5a mediated enhancement of FcγR activity through regulation of activating versus inhibitory FcγR expression [19, 20]. Therefore, many strategies to improve IgG activity are focused on the IgG-FcγR interaction. The family of FcγRs consists of several members with distinct features. There is one high affinity FcγR (FcγRI or CD64) which has the capacity to bind monomeric IgG similar to the high affinity receptor for IgE, FcεRI. All the other members have a much lower affinity for IgG and can only recognize antibodies in the form of multimeric immune complexes (Fig. 10.1). The second distinguishing feature is the signaling pathways initiated by the different family members. Thus, there are three activating (FcγRIA, IIA and IIIA in humans and FcγRI, III and IV in mice) and one inhibitory member (FcγRIIB) in mice and man. Whereas the activating FcγRs signal cell activation through immune-receptor tyrosine based activation motifs (ITAM) present in their intracellular domains or in signaling adaptor molecules such as the common FcR gamma chain (γ-chain), the inhibitory FcγRIIB contains and immune-receptor tyrosine based inhibitory motif (ITIM) and therefore initiates inhibitory signaling pathways [15, 16, 21, 22]. One exception to this rule is the GPI-linked FcγRIIIB, which is selectively expressed on human neutrophils and has no signaling capacity (Fig. 10.1). As activating and inhibitory FcγRs are co-expressed on the majority of innate immune effector cells, including basophils, eosinophil mast cells, neutrophils, monocytes, and macrophages immune complexes will trigger both, activating and inhibitory signaling pathways. Thus, co-expression of FcγRs sets a threshold for cell activation preventing unwanted activation of the powerful effector functions that can be unleashed by the innate immune system. On B cells, the inhibitory FcγRIIB is a crucial regulator of activating signaling pathways triggered by the B cell receptor, thereby preventing unwanted activation of B cells and generation of low affinity and potentially cross-reactive antibodies [15, 21, 22]. Mice deficient in FcγRIIB develop an autoimmune disease very similar to human systemic lupus erythematosus characterized by auto-antibodies specific for double stranded DNA, development of glomerulonephritis and a reduced life expectancy [23–26].

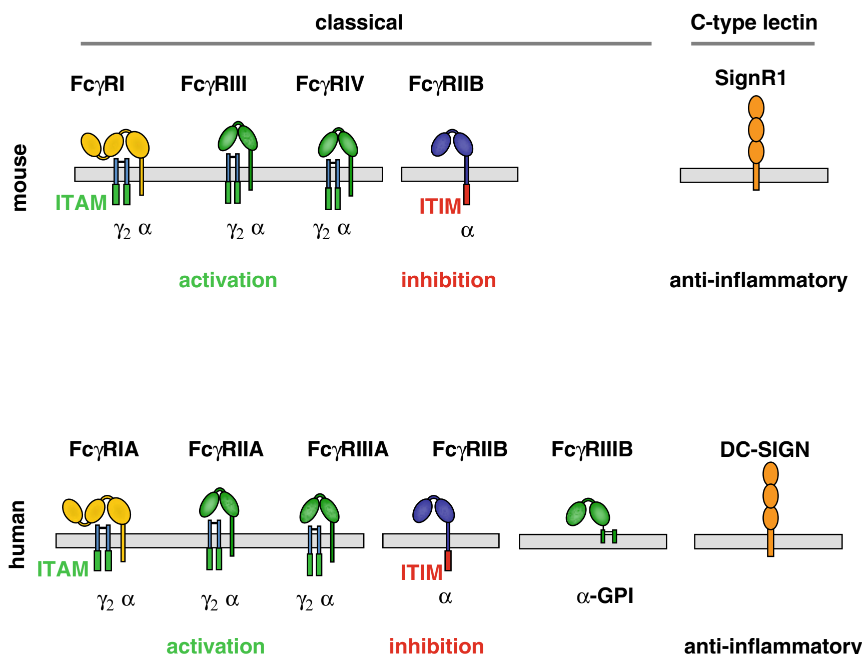

Fig. 10.1 The extended family of human and mouse Fcγ-receptors. Shown here are the members of the mouse (*upper panel*) and human (*lower panel*) FcγR family. Only the high affinity FcγRI has the capacity to interact with monomeric IgG, whereas all the other receptors can only bind to IgG in the form of an immune complex. There is one inhibitory Fc-receptor (FcγRIIB) which regulates activating signals initiated via FcγRIA, FcγRIIA, FcγRIIIA, and their respective mouse orthologous receptors. The GPI-linked human FcγRIIIB has not been identified in mice. In addition to the canonical FcγRs, mouse SIGN-R1 and human DC-SIGN have recently been shown to interact with IgG glycovariants rich in terminal sialic acid residues. See text for further details

Another interesting result from these studies was that the low affinity FcγRs were the major contributors to IgG dependent effects in mice and humans [27]. Human lymphoma patients carrying allelic variants of FcγRIIA and FcγRIIIA with enhanced binding to human IgG1 showed improved anti-tumor responses under RituxiMab therapy or after vaccination against the lymphoma idiotype [28–30]. In a similar fashion, the mouse orthologous low affinity receptors FcγRIII and FcγRIV were dominantly involved in triggering IgG1, IgG2a and IgG2b dependent effects [5, 12, 14, 31–34]. One explanation for the relatively small contribution of the high affinity FcγRI to these antibody-dependent effects might be that due to the high affinity of this receptor immune complex, binding might be compromised in the presence of high levels of serum IgG. Indeed, a role for the high affinity receptor was detected in solid tumor models where the tumor is localized in tissues [35, 36].

Besides this pro-inflammatory activity, IgG is long known to have an active anti-inflammatory activity [37, 38]. The injection of high doses of pooled IgG fractions derived from thousands of donors (intravenous IgG or IVIg therapy) is

an efficient means to suppress a variety of autoimmune diseases including immune-thrombocyotpenia (ITP), chronic inflammatory demyelinating polyneuropathy (CIDP), and rheumatoid arthritis [37, 38]. It is an unsolved mystery as to how IgG can mediate these opposing functions. A possible explanation for this conundrum was afforded by the finding that the IgG-associated sugar moiety is essential for both activities. Thus, IgG deglycosylation impairs both the pro and anti-inflammatory activity [39–42]. Using this Achilles heel of IgG, it was demonstrated that novel endoglycosidases that selectively remove the sugar moiety of IgG are a potent means to interfere with autoantibody induced inflammation in vivo [43–45]. In the following paragraphs we discuss which individual sugar residues participate in the modulation of IgG activity.

10.2 IgG Glycosylation is Essential for IgG Functionality

As is true for all antibody isotypes, IgG is a glycoprotein with a sugar moiety attached to each of the asparagin 297 (N297) residues in the CH2-domains of the two Fc-fragments. In contrast to other Ig isotypes, the IgG-associated sugar domain is not exposed on the IgG surface but rather buried within the hydrophobic core between the two Fc-fragments and impacts Fc-structure [1]. Removal of this sugar moiety impairs Fc-dependent effector functions such as binding to FcγRs and C1q [40, 42]. The core of this sugar moiety consists of a bi-antennary heptameric structure consisting of mannose and N-acetylglucosamine (GlcNAc), further decorated with terminal and branching residues including galactose, sialic acid, fucose, and N-acetylglucosamine (Fig. 10.2). Depending on the presence of terminal galactose residues, IgG glycovariants are termed IgG-G0 (no terminal galactose and sialic acid residues), IgG-G1 (one terminal galactose residue with or without an additional sialic acid residue), and IgG-G2 (two terminal galactose residues with or

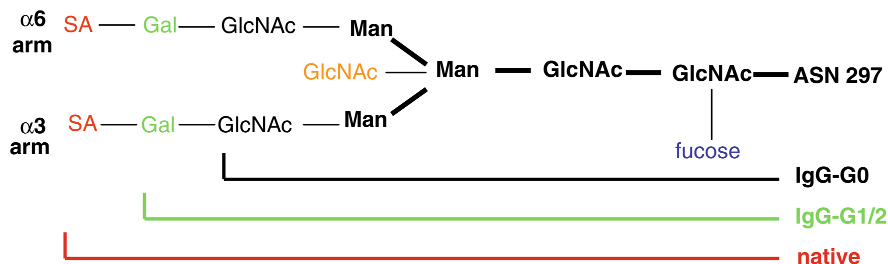

Fig. 10.2 The sugar moiety of IgG. Shown is the asparagine 297 (ASN 297) attached sugar moiety of IgG. Depending on the presence of none, one, or two terminal galactose residues IgG-G0, IgG-G1, and IgG-G2 glycovariants can be distinguished. All colored sugar residues including sialic acid (SA), galactose (Gal), fucose, and N-acetylglucosamine (GlcNAc) are variable, whereas the black colored residues of the heptameric core sugar structure consisting of mannose (Man) and GlcNAc are always present

without sialic acid residues) [15, 46]. In contrast to IgG produced in tissue culture, serum IgG is heterogeneous with respect to the exact composition of these terminal and branching sugar residues [47]. Thus, between 30–40 different IgG glycovariants can be identified in the serum of healthy individuals [46]. Early studies in patients with arthritis, osteoarthritis and spondyloarthropathy demonstrated that the glycosylation pattern of serum IgG can be altered dramatically and correlate to disease activity [48–50]. Similar results were obtained in a variety of mouse strains naturally prone to autoimmune disease development or induced to develop autoimmune symptoms [39, 51, 52]. Apart from inflammatory diseases, changes in serum IgG glycosylation were noticed during pregnancy and aging, suggesting that an active process modulating the composition of the IgG linked sugar moiety might exist [53–55]. Interestingly a differential regulation of IgG glycoforms was noticed between pregnancy and autoimmune diseases. Thus, during autoimmune disease (representing a pro-inflammatory state), the level of IgG-G0 glycovariants lacking terminal galactose and sialic acid residues were increased, whereas during pregnancy (representing an anti-inflammatory state with suppression of certain autoimmune disease symptoms), the IgG-G0 forms were decreased [48, 49, 53, 54]. These observations support the hypothesis that some of these variable branching or terminal sugar residues might be involved in modulating antibody activity, which we will discuss in the next paragraphs.

10.3 The Role of Branching Fucose Residues

It is widely accepted that branching fucose residues are crucially involved in modulating the pro-inflammatory activity of IgG [56]. Several studies have shown that removal of fucose residues enhances the ADCC activity of therapeutic antibodies in vitro and in vivo [14, 56–59]. Although largely studied for the IgG1 subclass, which is most widely used in clinical applications, it was recently shown that all IgG subclasses show enhanced activity upon removal of fucose [60]. Of note, fucose removal seems to selectively enhance the affinity of IgG for human activating FcγRIIIA and its mouse orthologue FcγRIV. The binding to all other activating FcγRs was unchanged, regardless of the presence or absence of fucose. A possible explanation for this selective enhancement was afforded by a study that showed that yet another sugar side chain, this time attached to activating FcγRIIIA (and mouse FcγRIV), might explain this result [61]. All FcγRs are glycoproteins that contain multiple sugar domains. FcγRIIIA has five asparagine-linked glycosylation sites. Removal of the sugar moiety attached to the asparagine 162 (N162) residue of FcγRIIIA resulted in the inability to bind to IgG without fucose with enhanced affinity. Modeling the FcγR-associated sugar domain on the available co-crystal structure of FcγRIIIA bound to IgG1 suggested that the sugar domains of FcγRIIIA and the IgG molecule come in close contact if fucose residues are present, which might lead to a sterical hindrance effect [61]. In the absence of fucose, however, this inhibitory effect was removed, offering a possible explanation for the affinity data.

A more recent study confirmed these results and showed that another sugar moiety attached to the asparagine 45 (N45) residue of FcγRIIIA further modulates the binding to IgG1. Thus, in the absence of the N45 linked sugar moiety, an increase of N162-dependent binding of FcγRIIIA to IgG without fucose could be observed [62]. It is currently unclear whether the level of FcγR glycosylation is stable or is subject to modulation during immune responses or cell activation. Regardless of these open questions, many companies are in the process of manufacturing and testing fucose-deficient glycovariants of antibodies already used successfully in clinical applications [56]. It should be kept in mind that in cases where FcγRIIA is the dominant triggering activating Fc-receptor, no increased antibody activity might be expected. In this scenario, the generation of optimized FcγRIIA binding antibodies generated through introduction of amino acid mutations into the IgG backbone that additionally enhance binding to this activating receptor might be the method of choice. Moreover, the combined engineering of both the sugar moiety and the amino acid backbone might be useful to generate more powerful antibody variants.

10.4 The Role of Branching N-Acetylglucosamine Residues

In contrast to the relative abundant presence of fucose in the sugar moiety of IgG, branching N-Acetylglucosamines (GlcNAc) are rather rare and even absent if the antibodies are produced in cell lines such as Chinese hamster ovarian (CHO) cells. In contrast, rat myeloma cell lines do add significant amounts of branching GlcNAcs, and it was observed that a humanized CAMPATH-1H (anti-CD52) antibody produced in this rat myeloma line had a higher ADCC activity [63]. Similar results were obtained by other studies that generated cell lines overexpressing the enzyme b [1, 4]-N-acetylglucosaminyltransferase III (GnTIII) which adds branching GlcNAc residues to the bi-antennary sugar moiety of IgG [64, 65]. One effect of GnTIII over-expression and addition of branching GlcNAcs is that other consecutive glycosylation enzymes such as Golgi-mannosidase II, galactosidase, and fucosyl transferases no longer recognize this sugar moiety efficently, resulting in lower levels of core fucosylation, which may explain the enhanced activity [65, 66]. Shinkawa and colleagues compared the effects of either enhanced levels of GlcNAcs or the lack of fucose, and found that compared to the more than 50-fold ADCC enhancement seen for IgG without fucose, the enhancement of activity for IgG with high levels of GlcNAcs was much smaller [58].

10.5 The Role of Terminal Galactose Residues

There are several conflicting reports about the role of terminal galactose residues in modulating IgG activity. Initial studies indicated that a highly galactosylated anti-D IgG1 antibody has a 2–3 fold higher ADCC activity [67, 68]. Others could not

confirm these results when using anti-CD52, anti-IL5R and anti-CD20 antibodies [58, 69]. Complicating the situation further studies found IgG molecules lacking terminal galactose residues (the IgG-G0 glycovariant) are more active than their IgG-G1 or IgG-G2 counterparts [70]. These results were supported by the notion that patients with rheumatoid arthritis, primary osteoarthritis or spondyloarthrophathy and several autoimmune prone mouse strains showed an altered serum IgG glycosylation pattern with higher levels of the IgG-G0 glycoform lacking terminal sialic acid and galactose residues [48–50, 71, 72]. In vitro studies showed that a potential mechanism for this enhanced activity might be the exposure of the high mannose core sugar structure which could acquire the capacity to bind to mannan binding lectin (MBL), the first component of the lectin pathway of complement activation [73]. More recent studies in MBL knockout animals, however, argue against a significant involvement of this pathway and show that the activity of IgG-G0 glycovariants is still fully dependent on activating FcγRs and independent of the complement pathway [74]. Taken together, current evidence does not support an important role of terminal galactose residues in either enhancing or attenuating the activity of IgG in vivo.

10.6 The Role of Terminal Sialic Acid Residues

In addition to the absence of terminal galactose residues, IgG-G0 glycoforms also lack terminal sialic acid residues (Fig. 10.2). Whereas many of the previous studies had anticipated that the reduction of terminal galactose residues in patients with autoimmune disease would enhance their pro-inflammatory activity, an alternative explanation could be that IgG antibodies lacking these terminal sialic acid or galactose residues loose an active anti-inflammatory activity. There is long standing evidence that IgG molecules can have an anti-inflammatory activity. The infusion of large amounts of the pooled fraction of serum IgG obtained from thousands of donors is a well established and efficient treatment for many autoimmune diseases including immune-thrombocytopenia (ITP), chronic inflammatory demyelinating polyneuropathy (CIDP), and rheumatoid arthritis (RA) [37, 38]. Removing the sugar moiety from this pooled IgG fraction abolishes the anti-inflammatory activity [39]. A comparable level of reduction in IVIg activity was seen if terminal sialic acid residues were removed by treatment with neuraminidase. Consistently, enriching IVIg for terminal sialic acid residues increased its anti-inflammatory activity in models of arthritis and nephrotoxic nephritis [39]. Further support for this concept was provided by data showing that only 2,6-linked sialic acid residues (the predominant type of linkage for sialic acid in the sugar moiety of IgG) were responsible for the anti-inflammatory activity, enabling the generation of a recombinant IVIg product produced in tissue culture [75]. Importantly, the sugar structure itself was not sufficient to provide this anti-inflammatory activity, as other serum proteins containing the same sugar moiety with high levels of terminal sialic acid residues had no anti-inflammatory activity [39]. This indicates that both the IgG amino acid

backbone and the sialic acid residues in the sugar moiety were essential for the anti-inflammatory activity. When terminal sialic acid residues come in close contact with the amino acid backbone, an altered tertiary structure of the IgG molecule imposed by the negatively charged acidic residues might be one mechanistic explanation. As human and mouse IgG glycovariants rich in terminal sialic acid residues show a reduced affinity for activating FcγRs, it seems clear that other receptors might be involved in recognizing sialic acid rich IgG [39, 76]. Indeed, a recent study showed that mouse SIGN-R1 or its human orthologue, DC-SIGN, have the capacity to recognize this IgG glycovariant [77]. Knock-out mice lacking SIGN-R1 expression showed an abrogation of IVIg activity in a model of serum transfer arthritis, strongly arguing for an important role of this receptor in the anti-inflammatory activity of IVIg. Nonetheless, it seems clear that the inhibitory FcγRIIB is also essential for IVIg activity. IVIg lost its therapeutic activity in FcγRIIB knock-out animals in models of ITP, nephrotoxic nephritis, and serum transfer arthritis [39, 78, 79]. A detailed analysis of FcγR expression on innate immune effector cells in the course of IVIg therapy showed that in mice and humans, IVIg induces an upregulation of the inhibitory FcγRIIB and a downregulation of activating FcγRs, resulting in an enhanced threshold for innate immune effector cell activation [78–80]. At present, it is unclear how this change in FcγR expression is achieved. It is tempting to speculate that anti-inflammatory cytokines might be involved in this pathway, although a recent study using several mouse strains deficient in a variety of cytokines could not detect a reduced anti-inflammatory activity [81]. Taken together, we begin to get a better picture of the mechanisms underlying the anti-inflammatory activity of IgG, although many open questions remain. Moreover, we have to point out that depending on the autoimmune disease, other anti-inflammatory pathways might exist. There are excellent reviews providing a more complete overview over this exciting field [38, 82].

10.7 Conclusion

Research during the last few years has provided convincing evidence that for understanding the many activities of IgG more than just protein-protein interactions have to be taken into consideration. The importance of certain sugar residues for the pro and anti-inflammatory functions of IgG have highlighted that the immunoglobulin attached sugar moiety is much more than just a scaffold for the correct three dimensional structure. The presence or absence of distinct sugar residues such as fucose or sialic acid can dramatically alter IgG activity and the change in serum IgG glycosylation during age, autoimmune disease, and pregnancy suggests that active regulatory mechanisms might exist that could be envisaged as a molecular switch keeping the humoral immune system in an active pro-inflammatory or a more anti-inflammatory state. We are clearly just at the beginning of understanding the mechanisms involved in fine-tuning IgG glycosylation and which functions the different glycoforms play during the steady state and infection.

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Chapter 11
Murine Model of Chronic Respiratory Inflammation

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Abstract The respiratory mucosa is exposed to the external environment each time we breathe and therefore requires a robust and sophisticated immune defense system. As with other mucosal sites, the respiratory mucosal immune system must balance its response to pathogens while also regulating inflammatory immune cell-mediated tissue damage. In the airways, a failure to tightly control immune responses to a pathogen can result in chronic inflammation and tissue destruction with an over-zealous response being deleterious for the host. Chronic obstructive pulmonary disease (COPD) is the fourth most common cause of death in the US and both the prevalence of and mortality rate of this disease is increasing annually. COPD is characterized by intermittent disease exacerbation. The causal contribution of bacterial infections to exacerbations of COPD is now widely accepted, accounting for at least 50% of all exacerbations. Non-typeable *Haemophilus influenzae* and *Moraxella catarrhalis* (both gram-negative bacteria) along with *Streptococcus pneumoniae* (a gram-positive bacterium) are the three most common bacterial pathogens that cause respiratory tract infections in COPD patients. The colonization of bacteria in the lower airways is similar to a low-grade smoldering infection that induces chronic airway inflammation. Chronic low-grade infection can induce a persistent inflammatory response in the airways and parenchyma. Inefficient removal of bacteria from the lower respiratory tract is characteristic of chronic bronchitis. Inflammation is believed to be central to the pathogenesis of exacerbations, but a clear understanding of the inflammatory changes during an exacerbation of COPD has yet to emerge. As bacterial colonization of the lung in COPD patients is a chronic inflammatory condition highlighted by frequent bouts of exacerbation and clearance, we sought to reproduce this chronic pathogen-mediated inflammation in a murine model by repeatedly delivering the intact, whole, live bacteria intra-tracheally to the lungs.

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11.1 Immune Defenses in the Respiratory Tract

The respiratory mucosa represents a very large surface area that is exposed to the external environment each time we breathe and therefore requires a robust and sophisticated immune defense system. Respiratory infections represent the leading cause of mortality worldwide [1]. The architecture of the respiratory system itself provides the initial level of defense against both the entry of particulate matter and airborne pathogens. Additionally, the columnar-ciliated epithelium of the trachea and bronchi form the mucociliary escalator into which pathogens are also trapped. For pathogens that escape past these anatomical defenses, the next line of non-specific defense is provided by anti-microbial peptides, mucosal IgA and surfactants. Motifs on the surface of the intact organism are recognized by innate immune cells using pathogen recognition receptors, thereby initiating an inflammatory response via production of chemokines and cytokines. Therefore, the immune system is poised to launch an effective attack against pathogens in the respiratory tract, but the response must be limited in order to avoid collateral damage to airway tissue. The inflammatory response that occurs in response to infection is beneficial, but can also have pathological consequences for the lung. As the mucosa is a major portal of entry and the replication site for many pathogens, studying the interaction between microbes and the host mucosa is critical to our understanding of heterologous immunity. Education of the immune system at respiratory surfaces is crucial for efficient protection against many human pathogens.

The respiratory mucosal immune system (as other mucosal sites) must balance its response to pathogens while also regulating inflammatory immune cell-mediated tissue damage [2]. Ineffective immune responses as well as an overzealous responses are deleterious for the host. In the airways, a failure to tightly control immune responses to a pathogen can result in chronic inflammation and tissue destruction. There is also the growing realization that chronic inflammation can affect subsequent responses to future infection by the same pathogen, and to an unrelated pathogen [3–6]. The effect of infection history on the immune response cannot be ignored, as repeated exposure to pathogens shapes the immune system in its ability to mount protective responses later in life. Experimental and epidemiological evidence show that infection with one pathogen and the ensuing immune response can influence the way the host responds to a second unrelated pathogen.

Conventional wisdom has ascribed a memory phenotype only to adaptive immunity. However, a body of evidence is emerging to show that innate immune pathways may also remain altered long after infection resolution. These findings are helping to support the notion that the innate immune system is in fact shaped by a history of infection of the host. To this end, we had generated a mouse model to define immune responses in the respiratory mucosa resulting from repeated exposure to a pathogen and the ensuing inflammation. This model is utilized to define immune mechanisms that protect the respiratory mucosal surface from infection and the inflammatory consequences of infection. To aid in our understanding of how infection in the lung alters both innate and adaptive immune responses, we have

developed a mouse model of both acute and chronic infection with bacteria that colonizes in the airways of adults with stable chronic obstructive pulmonary disease (COPD) and has been identified in at least 30% of acute exacerbations of the disease.

11.2 Chronic Obstructive Pulmonary Disease

COPD is a disease of the lungs in which the airways become narrow, thereby limiting the airflow and causing shortness of breath. In contrast to asthma, the limitation of airflow is poorly reversible and usually gets progressively worse over time. Chronic bronchitis and emphysema commonly co-exist in the lungs of COPD patients. This condition is caused by respiratory exposure to noxious particles or gas, most commonly tobacco smoke, which triggers an inflammatory response in the lung. The inflammatory response in the airways is known as chronic bronchitis. In the alveoli, the inflammatory response causes destruction of the tissues of the lung, a process known as emphysema. COPD is the fourth most common cause of death in the US [7–10]. In 1995, the National Heart, Lung, and Blood Institute estimated that 16.4 million people in this country suffer from COPD and both the prevalence and mortality rate of this disease have been increasing [11–13]. This disease is responsible for the expenditure of approximately $15 billion in direct costs of medical care. The impact of this disease to the health care system can be fully appreciated by noting that COPD results in ten million physician office visits and two million hospitalizations annually (in the US alone), and a large percentage of these are caused by infection. Tobacco smoke is recognized as a crucial factor in the development and pathogenesis of COPD. Tobacco smoke results in increased vulnerability to many infections and predisposes to airway diseases [14, 15]. Epidemiological, laboratory, and clinical studies have provided good evidence that tobacco smoke alters immunity. For example, soluble components extracted from cigarette smoke suppress some human dendritic cell functions and favor the development of skewed Th2 immunity [16]. In addition to its direct inflammation-inducing effect on the airways, chronic smoke inhalation (direct and second-hand) is also characterized by profound immunosuppressive changes which impair host defenses and increase susceptibility to infection. Smoking may also influence the extent of pulmonary inflammation, as there is mounting evidence for the presence of bacterial lipooligosaccharides (LPS) in tobacco [17]. This known bacterial inflammatory mediator and the carcinogens present in inhaled cigarette smoke (CS) create damage in the lung, setting up the organ for recurrent bacterial infections. Cigarette smoke is a strong inflammatory stimulus that induces pro-inflammatory cytokines (e.g. IL-6 and TNF-α) and recruits activated macrophages and neutrophils to lung tissue [18, 19]. Tobacco smoking suppresses the immune system and smoking is an established risk factor for several diseases including lung cancer [20]. It is also noteworthy that a person with COPD has four to six times greater risk of developing lung cancer compared to smokers without COPD.

COPD is characterized by intermittent disease exacerbation. Exacerbations in COPD patients can vary from mild to severe, sometimes requiring hospitalization and involving respiratory failure associated with significant mortality. Bacteria are always present in the upper airways, but in the absence of lung disease, the tracheobronchial tree is maintained sterile. Tobacco smoke causes airway inflammation and impairment of mucociliary clearance. Impaired mucociliary clearance can be the result of disruption of normal ciliary activity, enhanced mucus secretion, and airway epithelial injury. Once there is impaired mucocilliary clearance, potential respiratory pathogens gain a foothold in the lower respiratory tract and then persist in the respiratory tract by further impairing mucociliary clearance as a result of their products (including proteins, peptidoglycans, lipooligosaccharide, and nucleic acids). Even though the potential contribution of bacterial infection to the etiology, pathogenesis, and clinical course of COPD could be identified, the precise role of bacterial infection was a source of some controversy for several decades [21, 22]. So, while some argued that bacterial infection played a crucial role in the pathogenesis of COPD, others believed it to be merely an epiphenomenon (i.e. bacteria as innocent bystanders). However, with the improvements in PCR detection of pathogen gene sequences, the causal contribution of bacterial infections to exacerbations of COPD is now widely accepted, accounting for at least 50% of all exacerbations. Non-typeable *Haemophilus influenzae* and *Moraxella catarrhalis* (both gram-negative bacteria), along with *Streptococcus pneumoniae* (a gram-positive bacterium) are the three most common bacterial pathogens that cause respiratory tract infections in COPD patients. Bacteria, by invading and persisting in respiratory tissue, induce inflammation of the lower airway, promote hypersensitivity, cause immigration of neutrophils, and activate eosinophils and macrophages. Bacteria and bacterial products serve as the most effective activators of inflammatory conditions characterized by the abundance of local and systemic mediators (chemokines and cytokines). The key initiators of these reactions are lung macrophages, resident dendritic cells, and bronchial epithelial cells. Viral infections also have substantial clinical consequences in COPD patients. A range of viruses have also been shown to cause exacerbations, infection with rhinovirus being the most common. However, in more severe exacerbations that require hospitalization, influenza is more common. Understanding the nature of the immune response made by COPD patients to the respiratory pathogens is instructive in understanding the mechanism underlying their recurrent infections.

The majority of COPD exacerbations are infectious in etiology. Infection is detected in up to 78% of exacerbations [23], but is likely to be implicated in most exacerbations. The use of newer diagnostic techniques in microbiology has led to increased recognition of the importance of individual infections and combined bacterial and viral infection in exacerbations. Bacteria are detected in 50% of exacerbations and polymerase chain reaction techniques have established that in half, to two thirds of exacerbations, viruses are causative pathogens. Combined bacterial and viral infection (dual) can be identified in 25% of exacerbations, and these are often more severe. In patients with more severe exacerbations who required mechanical ventilation, *Pseudomonas aeruginosa* and gram-negative *Bacilli* are also recognized [23]. The colonization of bacteria in the lower airways of patients with stable COPD is

similar to a low-grade, smoldering infection that induces chronic airway inflammation. Chronic low-grade infection can induce a persistent inflammatory response in the airways and parenchyma. Inefficient removal of bacteria from the lower respiratory tract is characteristic of chronic bronchitis. This allows prolonged interaction between airway lymphoid tissue and bacterial antigens. Despite occurring frequently, the mechanisms by which infection with these pathogens causes exacerbations are not completely understood. Inflammation is believed to be central to the pathogenesis of exacerbations, but a clear understanding of the inflammatory changes during an exacerbation of COPD has yet to emerge.

Non-typeable *Haemophilus influenzae* (NTHI) is frequently present in the airways of adults with COPD [24]. Besides colonizing the airways when the patient is clinically stable, the acquisition of new NTHI strains is considered an important cause of lower respiratory tract infection and is associated with disease exacerbations [25]. Many, but not all individuals who suffer from COPD have a history of smoking. It is believed that inhaled smoke and particulates destroy the respiratory mucosa, leaving the individual susceptible to frequent viral and bacterial infections.

11.3 Immunological Consequences of Cigarette Smoke Exposure

An extensive body of literature has detailed the impact of CS exposure on the development of airway diseases such as COPD and smoking-related malignancies [26–28]. Apart from these devastating lung diseases, smoking has also been associated with the worsening of asthmatic symptoms [29]. The overall result of CS exposure is the progression and exacerbation of a host of lung diseases. In most cases, the immune cells and soluble mediators produced by the immune cells play an important role in the pathology of these diseases [30]. Although the effects of CS on the immune system directly *in vivo* are not as well studied, it is clear that the components of CS compromise various immune cell types and thus may compromise the immune system's overall ability to fight off infections and possibly also affect the COPD patient's ability to respond appropriately to vaccines [31].

Defects in the immune response as a result of smoking are due to the multitude of gaseous and particulate compounds which assault the cells in the respiratory tract [32]. Localized exposure to these chemicals also has a systemic effect on immunity due to the production of soluble mediators by cells in the lung [33–35]. There is convincing evidence that the effects of CS can be simultaneously pro-inflammatory and immunosuppressive. Diseases such as COPD are a result of the continued and heightened pro-inflammatory state of the CS-exposed lung, whereas increased susceptibility to respiratory microbial and viral infections can be attributed to the tobacco-mediated immunosuppressive milieu in which the lymphocytes attempt to clear pathogens. Thus, the true damage associated with CS exposure extends beyond susceptibility to airway diseases and infections; it also has broad and significant implications on the patient's ability to mount protective immune responses following routine vaccinations against pathogens to which the smoker is at increased risk [36].

Published reports on the effects of smoking on innate immune and adaptive immune responses are quite varied. Studies in humans, either population or *ex vivo*, utilize parameters such as smoking history and socio-economic status in order to stratify and control for confounding factors. Animal models in which exposure to cigarette smoke can be controlled and modified are also not exempt from these issues as the frequency, duration, manner, and type of smoke exposure have a direct impact on the outcome of immune responses measured by *in vitro* assays. Despite all these complexities, immunological responses of phagocytic cells (e.g. alveolar macrophages, neutrophils, and dendritic cells) and lymphocytes (i.e. T and B cells) to cigarette smoke have been studied in both human and rodent models to understand the overall effect on respiratory immunity.

The alveolar macrophage is positioned within the lung to capture particulate pollutants along with sensing and eliminating pathogens. Exposure to CS is known to increase the number of alveolar macrophages in the broncho-alveolar lavage fluid (BALF) of smokers [37, 38] and increased bronchio-locentric macrophages are seen commonly in smokers as part of respiratory bronchiolitis. Though this would appear to be beneficial for the removal of pathogens, the exposure to CS diminishes the phagocytic function of these cells while simultaneously decreasing the secretion of key pathogen-fighting cytokines such as IL-1, IL-6, and TNF-α [39–41]. Analysis of signaling pathways in primary human lung macrophages and macrophage cell lines suggests that this response is due to impairment in NF-κB phosphorylation. Consequently, the defective macrophage is unable to clear debris and phagocytose bacteria to prevent their colonization in the respiratory tract. Although alveolar macrophages are unable to secrete pro-inflammatory cytokines that clear pathogens, CS exposure promotes the production of reactive oxygen species (ROS) and proteolytic enzymes such as matrix metalloproteinases from the alveolar macrophage which then promote detrimental inflammation in the lung that leads to parenchymal cell destruction and emphysema [42, 43]. The impairment of phagocytic function can be attributed to the decreased surface expression of pattern recognition receptors. Droemann *et al.* demonstrated that TLR2 expression is diminished on alveolar macrophages from smokers with COPD, thereby preventing the immune system from sensing bacterial infections and initiating inflammatory responses to clear the infection [44]. These findings were confirmed by an mRNA microarray comparison of macrophages from smokers versus non-smokers. In addition to diminished expression of pathogen sensing molecules such as TLR2 and TLR4, there was a clear upregulation of adhesion molecules and extracellular matrix proteins [45].

Expression profile studies of macrophages from smokers suggests that there is a skewing in their function attributable to CS exposure and could therefore provide an explanation for why the host remains highly susceptible to respiratory infections. Along with the alterations in the gene expression profile, smoke exposure and the milieu it generates in the lung skew the macrophage towards a M2 macrophage [46]. Alveolar macrophages are thought to differentiate into the M1 phenotype as a default pathway, characterized by their ability to eliminate pathogens by nitric oxide production. M2 macrophage differentiation is generated by the alternative activation with IL-4 and is characterized by arginase production. The M2 phenotype is more

closely associated with tumor promotion and has been shown to be present at greater levels in smokers compared to non-smokers. Thus, the skewing of the alveolar macrophage phenotype by exposure to CS prevents the cell from clearing pathogens.

An alteration of T cell responses by CS exposure also has significant consequences on the pathogenesis of COPD and emphysema. The dual nature of immune-activating and immune-suppressing phenotypes generated by CS smoke leads to a skewed immune response that promotes airway disease and prevents pathogen clearance. This is an important facet of how CS alters T cell function. T lymphocytes are increased in COPD patients [26–28]. Finkelstein *et al.* showed a correlation between numbers of T lymphocytes/mm^3 and the extent of emphysema [47]. Increased CD8$^+$ T cells were found in the airways and lung parenchyma of COPD patients compared to non-smokers. Their presence is associated with progression and worsening of emphysema, as these cells induce MMP12 production from alveolar macrophages [42, 43]. In more severe cases of COPD, Hogg *et al.* showed the presence of lymphoid follicles which were comprised of B cells surrounded by T cells [48]. Though normal CD8$^+$ T cells are critical for clearing virally-infected cells, exposure to CS renders them unable to function by diminishing their secretion of IFN-γ. Instead of generating pathogen-clearing Th1 responses (characterized by IFN-γ production), CS exposure leads to Th2 differentiation (characterized by IL-4 and IL-13 production) [16, 49]. The absence of Th1 responses impairs pathogen elimination, whereas Th2 responses hasten the COPD and asthmatic phenotype. The CD4$^+$ helper T cells from smokers secrete the cytokine IL-17, which is involved in the maintenance of chronic inflammation. The presence of T reg cells and Th17 cells (IL-17 secreting CD4$^+$ T cells) is a debilitating combination that is likely the result of the diminished production of IL-12 (Th1 promoting) and increased secretion of IL-23 (Th17 promoting) and TGF-β (T reg promoting) from DCs exposed to tobacco smoke [50, 51].

Along with diminished production of IFN-γ (the cytokine most directly involved in bacterial and viral clearance), the T cells of COPD patients are also highly susceptible to anergy (antigen non-responsiveness) and apoptosis. Kalra *et al.* have demonstrated in their animal model that T cells display signs of anergy, such as diminished intracellular Ca^{2+} mobilization and proliferation, and this could hinder the generation of effective vaccine responses [52]. Additionally, the increased rate of apoptosis in CD4$^+$ and CD8$^+$ T cells in COPD patients is due to the enhanced surface expression of Fas, a pro-apoptotic marker [53, 54]. Together, it would suggest that the profile of immune responses in COPD patients is affected by their current or previous long-term exposure to CS.

11.4 Models of Pulmonary Inflammation

Animal models have been frequently used over the years to understand the pathogenesis of COPD. In fact, using animal models over 40 years ago, Gross *et al.* instilled elastases into rodents which then developed emphysema and led to the

elastase – anti-elastase theory [55]. Over the years, a number of transgenic mice have been utilized to interrogate the role of specific genes or their products in the pathogenesis of COPD. Transgenic mice which overexpress MMP-1, IFN-γ, IL-13, and integrin β6 have all been utilized to explore specific aspects of disease manifestation in patients with COPD [56–59]. A recent report described a model aimed at understanding some aspects of the contribution of NTHI to COPD progression. In this model, mice were exposed (for up to 50 weeks) to an aerosolized lysate of NTHI. The treatment involved exposing the mice to 4 ml of bacterial lysate while the mice were placed in an AeroMist nebulizer [60]. The limitation of this study, as recognized by the authors themselves, was that the mice were only exposed to dead bacterial products, in contrast to the airways of patients with COPD who are exposed to live bacteria. This crucial difference will impact on the interpretation of findings from their model. Finally, many groups have utilized the "smoking mouse model" as a surrogate to understand the effect of smoking on lung pathology in COPD.

To understand which aspects of COPD are the result of frequent infections, we have established a mouse model of non-surgical repeated instillation of live bacteria over a prolonged time period and analyzed the resulting changes in the lungs of the mice. It is now generally accepted that COPD is an inflammation-mediated disease. The inflammation that occurs in the lung contributes to the destruction and pathology seen in the lung at autopsy. Equally important is the notion that chronic inflammation in the lung (as in other organs and tissues) can markedly influence the outcome of innate and adaptive immune responses in the host. We further contend that such chronic perturbations in immune function can impact on the outcome of both therapeutic agents and on vaccine efficacy in the treated host.

In order to generate a model of chronic lung inflammation, we began with considerations of the potential inflammatory stimuli that are relevant in the setting of NTHI infection in humans. Our previous work on the stimulatory nature of the key outer membrane protein (OMP), lipoprotein P6, had given us substantial experience to project potential in vivo immune response outcomes. We initially considered utilizing P6 as the model stimuli, as it would provide a single antigenic source from which the resulting inflammation could be measured. We reasoned that although a single antigenic source might prove beneficial in elucidating the true contribution of an individual bacterial component to inflammation, this system would not fully reflect the manner in which the patient's lungs are exposed to bacterial infections. Rather than develop a model of P6-mediated inflammation, we chose to utilize the entire live bacterial cell to initiate infection and subsequent lung inflammation. As the entire bacterial cell is present in the lung during an episode of COPD exacerbation, we reasoned that all the components of the cell would be integral to the initiation of inflammation. Though our studies have highlighted the pivotal role and importance of the P6 lipoprotein, it represents only a small percent of total membrane proteins of the intact bacteria. The other components of NTHI outer membrane include P1, P2, P4, and P5. Several groups have also evaluated the potential of these proteins to induce immune responses. In addition to these proteins, the NTHI outer membrane also expresses lipooligosaccharides (LOS) which are also known to stimulate inflammatory responses in *in vitro* assays. Combined with these

known components and the immunogenic nature of the P6 lipoprotein, we believe that utilization of the live bacterial cell was the most relevant choice for induction of inflammation in the lung.

Specifically, our model involves a non-surgical method of intratracheal administration of live pathogens to mice. The instillation is administered a single time to observe inflammatory and immune changes resulting from acute infection or repeatedly to establish short-term chronic (4 consecutive weeks) or long-term chronic (16 consecutive weeks) inflammatory changes in the mouse lung. We have also adapted the basic model to mimic alternating periods of infection and rest (stable disease) to allow an understanding of the attempts at tissue repair that may occur.

11.5 Characterization of a Murine Model of COPD

Bacterial colonization of the lungs of COPD patients is a chronic inflammatory condition highlighted by frequent bouts of exacerbation and clearance. We sought to model this chronic pathogen-mediated inflammation in our murine model by delivering the intact live bacterial stimulant directly into the lungs. The presence of NTHI (and other bacteria or viruses) in the lower respiratory tract is a hallmark of the disease in COPD patients. In order to recreate this feature in the mouse model, we developed a non-surgical method of consistently, reliably, and repeatedly delivering NTHI deep into the animal's lung lobes for several months. Mice received 1 x 10^6 colony forming units (cfu) of live NTHI intratracheally (i.t.) in a volume of 50 μl twice a week. This schedule of bacterial instillation was maintained for 4 or 16 consecutive weeks in order to generate varying degrees of lung inflammation and pathology, but could be tailored according to the model pathogen or inflammatory insult. In our model, the length and frequency of the inflammatory insults demanded the development of a non-surgical method of i.t. instillation. Under deep inhaled anesthesia, the tongue of the mouse is pulled forward in order to prevent delivery of the innoculum down the esophagus (thereby avoiding delivery to the stomach and intestines). The i.t. instillation method we have developed is ideal for eliciting pulmonary inflammation in the mouse lung that require multiple instillations of pathogen over several months time without adverse damage to the animal's throat and trachea. In addition to the ease of i.t. instillation, the inflammation that results in the mouse lung has pathological characteristics that recapitulate the hallmarks of several human lung diseases. Although we have developed this system for delivering NTHI, the tongue-pull i.t. method can be easily adapted to evaluate the delivery of other pathogens, vaccines, drugs, and small molecules. This non-surgical method of i.t. instillation has been adapted by us for use in infant and aged mice to study the effects that aging has on infection-mediated pathology and immune responses.

Following chronic exposure to the bacteria, we evaluated the effects of the inflammatory stimuli on lung pathology. Control mice exposed to sterile PBS displayed no accumulation of lymphocytes in the airway, nor were there any signs of bronchio-epithelial cell damage (Fig. 11.1, top row). However, there were striking

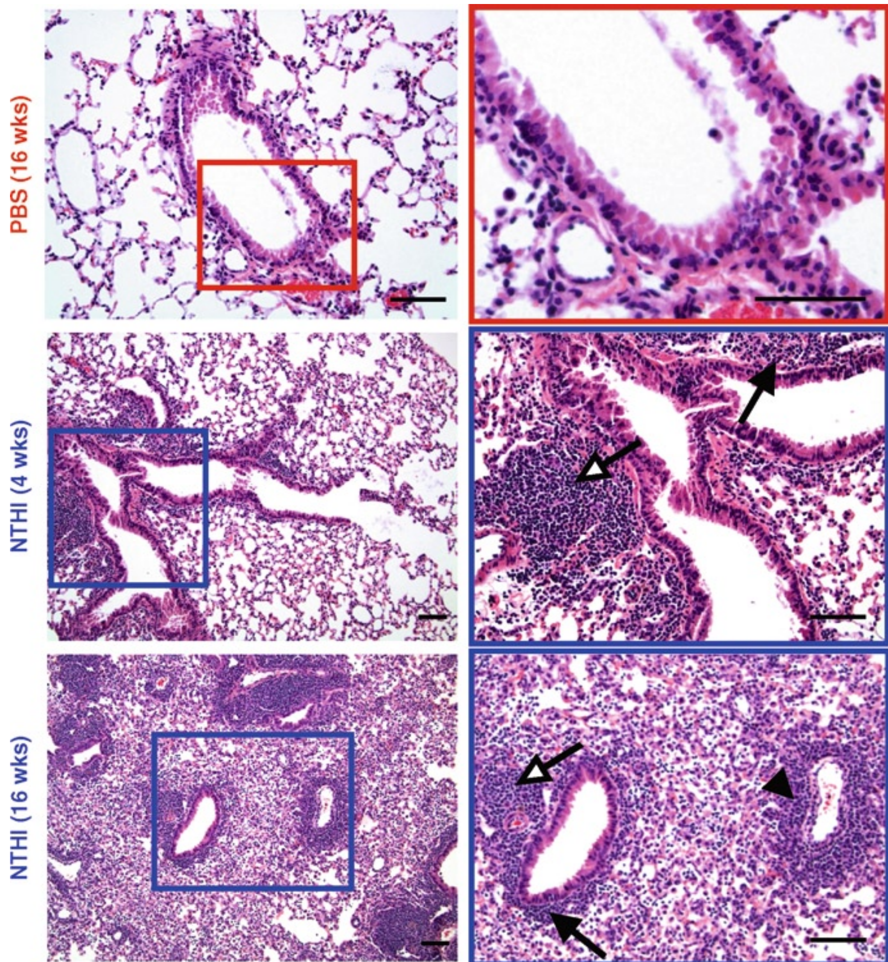

Fig. 11.1 Histological evaluation of inflamed lungs. Chronic bronchiolitis in lungs of mice exposed to NTHI for 4 or 16 consecutive weeks. Lungs were fixed in formalin, embedded in paraffin, and stained with H&E. Boxed areas in left column are magnified and the corresponding region is shown in the right column. Lymphocyte cuffing is evident in peribronchial (*arrows*) and perivascular (*arrowheads*) spaces of inflammed lungs from mice exposed to NTHI. Lymphoid follicles with BALT-like structures are visible (*open arrows*). Bars represent 100 μm

contrasts in the histology of mice chronically exposed to the bacteria for either 4 weeks or 16 weeks. The lungs of mice exposed to NTHI for 4 weeks exhibited peribronchial inflammation highlighted by cuffs of immune cells (closed arrow) (Fig. 11.1, middle row). The severity of lung inflammation comprised lymphocytic infiltration around airway and vascular spaces (closed arrow). The severity of inflammation increased markedly during the course of the 16 week exposure to NTHI (Fig. 11.1, bottom row). An increased number of bronchi and vasculature were surrounded by infiltrating immune cells and the pattern of infiltration extended further

into the lung space. Interestingly, we noted that the lymphocytes appeared to be organized into aggregates, which resemble the morphology commonly observed with bronchial-associated lymphoid tissue (BALT) (open arrows) and were distributed throughout the lung, including some regions in which lymphocytes infiltrated the bronchio-epithelia. The lymphoid aggregates also strongly resembled those observed in the lungs of advanced stage COPD patients (GOLD Stage 3–4) [48].

The NTHI-mediated inflammatory damages to the lung were scored in a blinded fashion by a clinical pulmonary pathologist. A scoring schema was developed to quantify and monitor in a blinded manner the effects of bacterial instillation in the lungs of mice (Table 11.1). In comparing the features commonly observed in human lung diseases, the scoring schema in the murine model was used to account for the severity and location of the inflammatory foci. Patterns of inflammation in the bron-

Table 11.1 Scoring schema to evaluate lung inflammation

I. Bronchovascular bundles		
(a) Inflammation severity		
	0	No significant inflammation
	1	Minimal (<5 inflammatory cells in most inflammatory cuffs and no evidence of peribronchial lymphoid follicles)
	2	Marked (5–15 cell average cuff thickness and 2–3 peribronchial lymphoid follicles present)
	3	Exuberant (>15 cell cuff thickness and >4 well developed peribronchial lymphoid follicles present)
(b) Plasma cell frequency		
	0	Rare to no plasma cells
	1	<10% plasma cells
	2	10–50% plasma cells
	3	>50% plasma cells
(c) Hemorrhage		
	0	No hemosiderin laden macrophages
	1	Clusters of hemosiderin laden macrophages
II. Pleural Inflammation		
	0	No inflammation of pleura
	1	Mild focal inflammation without pleural thickening
	2	Patchy thickening of pleura by inflammatory cells and/or more diffuse involvement by inflammatory cells
	3	Marked thickening or significant pleural inflammatory nodules
III. Interstitial Inflammation		
	0	No interstitial inflammation
	1	Focal interstitial inflammatory infiltrate without nodules
	2	Patchy interstitial inflammatory infiltrate with occasional inflammatory nodules
	3	Diffuse interstitial infiltrate or marked nodular aggregates of interstitial inflammatory cells
IV. Alveolar Contents		
	0	No increase in alveolar macrophages
	1	Rare to focal alveolar macrophages
	2	Patchy increase in alveolar macrophages

Table 11.2 Scoring of inflammation in the lungs[a]

	Bronchovascular inflammation[b]		Plasma cell frequency		Pleural inflammation[c]		Interstitial inflammation[d]	
	Marked	Minimal	10–50%	<10%	Patchy	Focal	Patchy	Focal
4 weeks	4/4	0/4	0/4	4/4	1/4	3/4	1/4	3/4
16 weeks	4/4	0/4	2/4	2/4	0/4	4/4	2/4	2/4

[a]Frequency of mice scored with lung inflammation. Blinded consensus scoring of lung histology by a pathologist. For scoring schema refer to Table 1.
[b]Severity of inflammatory cell accumulation around bronchioles. Marked = score 2: 5–15 cell cuff thickness and 2–3 peribronchial lymphoid follicles present. Minimal = score 1 : <5 cell cuff thickness and no evidence of peribronchial lymphoid follicles.
[c]Severity of inflammation in lung pleura. Patchy = score 2: pleural involvement of inflammatory cells with pleural thickening. Focal = score 1: inflammatory cells without pleural thickening.
[d]Severity of inflammation in interstitial space. Patchy = score 2: interstitial involvement of inflammatory cells with occasional inflammatory nodules. Focal = score 1: interstitial involvement of inflammatory cells without inflammatory nodules.

chovascular bundles, pleura, parenchymal interstitium, and alveolar spaces were assigned an ordered scoring system, and a consensus score of at least two separate blinded evaluations was used in the final assessment of lung inflammation (Table 11.2). Inflammatory foci accumulate around the bronchovasculature as early as 4 weeks of exposure and the extent of inflammation increases to further include the interstitial and pleural sites, which are commonly observed in the lungs of COPD patients. Thus, in our model we have elicited and scored the inflammatory responses that are generated in response to NTHI.

11.6 Additional Applications of the Model

While thus far our studies have been restricted to the use of NTHI, our model can easily be extended to include assays for measuring inflammatory responses elicited against *Moraxella catarrhalis* and *Streptococcus pneumoniae*, two additional pathogens isolated from sampling distal airways of COPD patients with protected specimen brush [24]. Similarly, we envision that the model will have considerable utility in the study of respiratory viruses, such as influenza and respiratory syncytial virus (RSV), and in studying the pathogenesis resulting from polymicrobial infections. Although bacteria are detected in 50% of exacerbations [61], polymerase chain reaction techniques have established that, in half to two-thirds of exacerbations, viruses are causative pathogens [62]. Interactions between these viral and bacterial pathogens have direct relevance to diseases in both pediatric and adult populations [7–10, 25].

Influenza virus causes enormous morbidity and mortality on an annual basis and is a disease with global impact. It primarily infects the respiratory tract and causes

a broad range of illness ranging from symptomless infection to fulminant primary viral and secondary bacterial pneumonia. The mortality associated with influenza is concentrated in the elderly and those with co-existing disease such as COPD. According to the CDC, the main aim of vaccination in elderly individuals is to reduce the risk of complications in those who are most vulnerable [63, 64]. It is hoped that influenza can be reduced by the increasing use of vaccination and the development of new antiviral drugs. However, the constant emergence of new influenza strains and the risk of avian influenza pandemic suggest that influenza will remain a serious public health concern for the near future. Our mouse model can also be adapted for studying responses to bacterial infection that are associated with influenza infection in aged mice.

Cigarette smoke is a strong inflammatory stimulus that induces pro-inflammatory cytokines (IL-6 and TNF-α) and recruits activated macrophages and neutrophils to lung tissue. Tobacco smoking suppresses the immune system and smoking is an established risk factor for several diseases including lung cancer, cardiovascular disease, COPD and recurrent respiratory infections. Nicotine, a major component of cigarette smoke, has been demonstrated to inhibit influenza virus-induced leukocyte accumulation in the mouse lung, attenuate inflammation, and thereby promote viral burden in the lung [65]. Nicotine has also been shown to increase the reactivation of herpes simplex virus type I (HSV) and decrease the expression of pro-inflammatory cytokines allowing replication of *Legionella pneumophila* in lung macrophages [66]. Exposure to tobacco smoke in our mouse model is ideal for evaluating the long-term impact on immune responses to viral and bacterial infections.

The effect of infection history on the immune response cannot be ignored, as repeated microbial exposure, particularly during childhood, shapes the immune system in its response later in life. Experimental and epidemiological evidence shows that infection with one pathogen and the ensuing immune response can influence the way the host responds to a second unrelated pathogen. A previous infectious event could significantly impact responsiveness to the next, depending on when the first infection occurred, how long the first influence lasted, and whether subsequent infection with another pathogen triggers similar signaling pathways. The impact of infection history on the immune response is of paramount importance as it helps to shape the immune system. Both epidemiological and experimental data provide support for the notion that infection with one pathogen and the ensuing immune response can markedly influence the immune response to infection by a subsequent, even unrelated pathogen [67–70]. In the pathophysiology of respiratory tract infections, it is now well recognized that simultaneous or sequential viral-bacterial infections often occur [3, 5, 71]. In a recent study, inhibition of bacterial clearance from the lungs was observed in mice that had been previously infected with mouse-adapted influenza virus [72]. This correlated with the production of IFN-γ from T cells stimulated during the viral infection, which later impacted on the ability of resident alveolar macrophages to clear a subsequent *S. pneumoniae* infection [72]. These studies highlight the ability of innate responses provoked by viral infection to modulate innate protection against unrelated pathogens in the

lung. As an example, our mouse model could be utilized to study the interaction between anti-viral and anti-bacterial immune responses in the lung using mouse adapted RSV and NTHI. In a broader context, the interactions between viruses and bacteria are particularly important as they impact many diseases such as pneumonia, sinusitis, otitis media, COPD, and gastroenteritis. Identifying the molecules/pathways involved in increased susceptibility or protection against subsequent infections may have a significant impact on therapeutic strategies to tackle infectious diseases.

The mouse model of chronic respiratory inflammation that we have developed has been most thoroughly characterized in its responses to NTHI. Efforts are underway to adapt this model in several other models of respiratory inflammation and in sequential pathogen infections. The adaptability of this model to various inflammatory stimuli has the potential to provide mechanistic understanding of how inflammation impacts adaptive immune responses, especially in the case of eliciting protective responses in vaccine testing.

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Chapter 12
Incorporation of Innate Immune Effector Mechanisms in the Formulation of a Vaccine Against HIV-1*

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Abstract The realization of a major role for events that occur during acute viremia that dictate the course of disease both in HIV-1 infected humans and susceptible SIV infected non-human primates has prompted an intense interest in studies of the contribution of innate immune effector mechanisms. It is reasoned that findings from such studies may be important and need to be incorporated into the design and formulation of potential candidate vaccines against HIV-1. This review serves to outline the various non-human primate models that can best serve to address this issue, a summary of our knowledge on the various subsets of NK cells (one of the major innate immune cell lineage) that have an impact on the course of disease, the potential pathways that regulate their function and the potential role of the KIRs on SIV-induced disease course. Finally, the major points from this report and the data presented on similar subjects by other investigators is utilized to provide a summary of the potential future directions that we need to take in efforts to move this field forward.

12.1 Introduction

Data from several lines of study has prompted the HIV/AIDS research community to re-evaluate the potential mechanisms of HIV-1 infection in humans and the various non-human primate models of SIV infection. One of the major impetus for this call for re-examination has been the failure of the HIV-1 vaccine STEP trial

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which led to the realization that considerable more basic knowledge is needed for the formulation of an effective vaccine against HIV-1 [1]. Coincidentally, during the last few years, a growing body of data indicates that events that occur during the period of acute infection prior to the generation of mature and effective adaptive immune responses appear to control major differences in the kinetics of viremia. These differences in the rate of viremia appear to dictate the rate of disease progression which suggest that innate immune mechanisms clearly contribute in executing such events [2] and their definition needs to be incorporated in vaccine formulation. A more detailed understanding of the mechanisms involved that dictate the occurrence of an eclipse phase following infection, the potential reason(s) why the virus replicates at such a rapid rate shortly thereafter (log phase), and importantly, what mechanisms dictate the "spontaneous" establishment of variable rates of viral load set points (see Fig. 12.1) during the acute infection period. They have been reasoned to provide knowledge that may be important for the formulation of effective anti-HIV vaccines. The variable outcomes (ranging from marked spontaneous control of plasma viremia to uncontrolled viremia and rapid disease course) of infection have been clearly shown in non-human primates infected with aliquots of the same virus and administered by the same route suggesting that there are unique aspects of virus–host

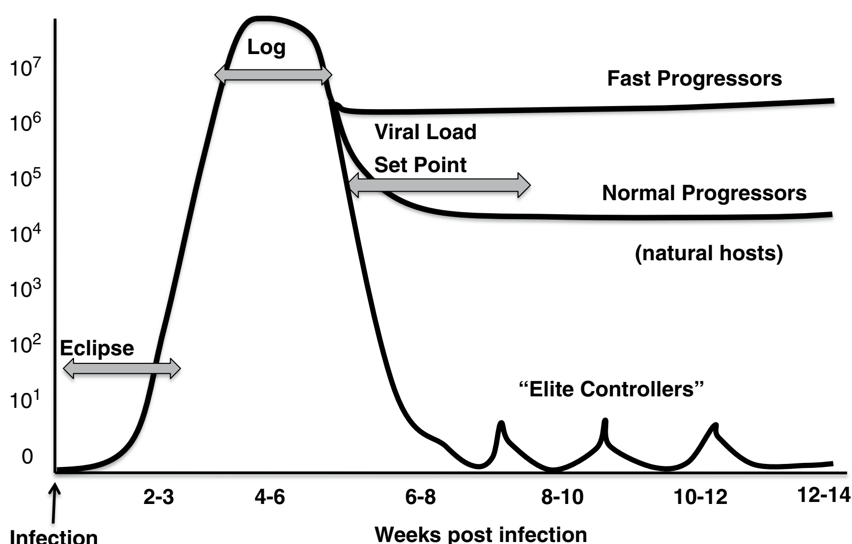
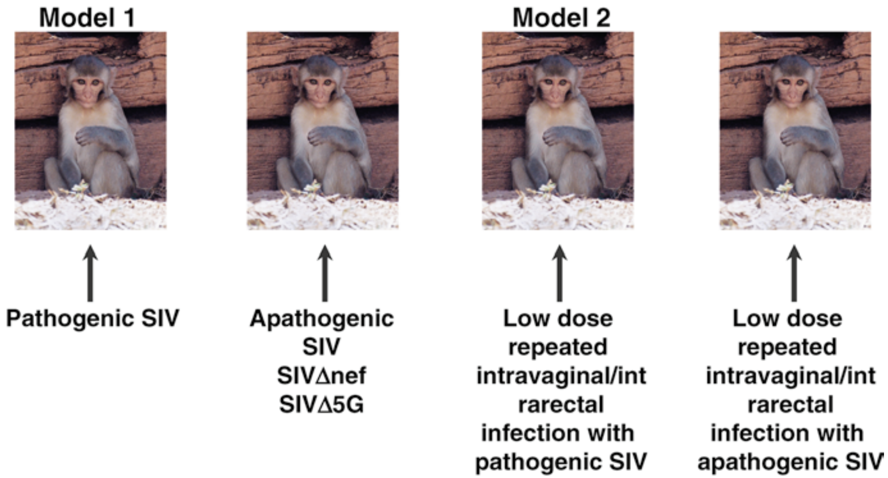


Fig. 12.1 A schematic that highlights the various phases of viremia in rhesus macaques infected with Simian immuno-deficiency Virus (SIV). The first phase is the eclipse phase during which plasma viremia is low or difficult to detect followed by a log phase during which there is a robust level of readily detectable plasma viremia. This is followed by a stage when the plasma viral load appears to stabilize to varying levels (viral load set point phase) with 5–10% of the animals showing relatively high plasma viral loads (fast disease progressors), 5–20% that demonstrate spontaneous viral control (elite controllers), and the rest showing variable levels of plasma viremia (normal progressors). The plasma viremia profile of the natural hosts of SIV such as sooty mangabeys resembles that of normal progressors

interactions during the acute infection period that lead to different outcomes during persistent viremia. The reader is referred to a general review on the subject of "new concepts" on persistent viremia that has recently been published [3]. Finally, the realization that the gut-associated lymphoid tissues are the major target of both HIV-1 and SIV infection during this acute infection [4] period suggests that the contribution of the innate immune system that leads to differences in viral loads must be focused directly and/or indirectly on the G.I. tissues. The natural question that emerges from the above is this: What do we know about the role of the innate immune system in humans and non-human primates during acute infection and how do these effector mechanisms execute their function within the G.I. tissues? Relevant to the overall thrust of this mini-symposium, how can this knowledge be exploited for the formulation of an effective vaccine against HIV-1?

In addition, the reasons for our laboratory's interest in studying the role of the innate immune system in SIV infected non-human primates was prompted by the results of a series of studies aimed at identifying the mechanisms by which natural hosts of SIV (such as sooty mangabeys) remain to a large extent disease free. This disease resistant status is maintained despite the fact that these natural hosts develop plasma and cellular viral loads that often exceed levels seen in the non-natural hosts such as rhesus macaques who develop an immuno-deficiency disease remarkably similar to HIV-1 infected humans [5]. Thus, in vivo depletion of either $CD4^+$ or $CD8^+$ cells in SIV infected disease-resistant sooty mangabeys failed to lead to any detectable signs of disease, despite significant depletion of these cell populations [6, 7]. The finding that the sooty mangabeys have a higher frequency of NK cells and demonstrate a significantly higher level of NK cell function [8, 9], combined with our failure to demonstrate any detectable disease following in vivo depletion of either CD4 or CD8 expressing cells led us to postulate a role for the innate immune system in contributing to the disease resistant state in sooty mangabeys [10]. Thus, the rationale outlined at the beginning of the introduction and the results from our own studies strongly suggest that the role of the innate responses in lentivirus infection needs to be explored and the results utilized for the formulation of an effective vaccine against HIV-1.

An overriding issue is whether the animal models being utilized at present are amenable to study these concerns and are the right ones to provide the answer. The latter issue has been raised primarily because most investigators utilizing the SIV infected non-human primate models to study pathogenic mechanisms have to a large extent utilized a single large bolus of virus for establishing infection and has been administered intravenously. This is clearly not reflective of the dose and route of infection that mimics the natural transmission of HIV-1 in the vast majority of new cases worldwide, which most likely involves low dose repeated exposure via the mucosal route. These thoughts have led our lab to identify a series of SIV infected non-human primate models which we submit would provide the most informative knowledge with regards to the potential role of innate immune mechanisms on influencing the different outcomes of infection during acute infection (see Fig. 12.2 a and b). Model 1 involves the comparative analysis of innate immune responses during acute infection in rhesus macaques infected with pathogenic versus apathogenic viruses and should provide some important



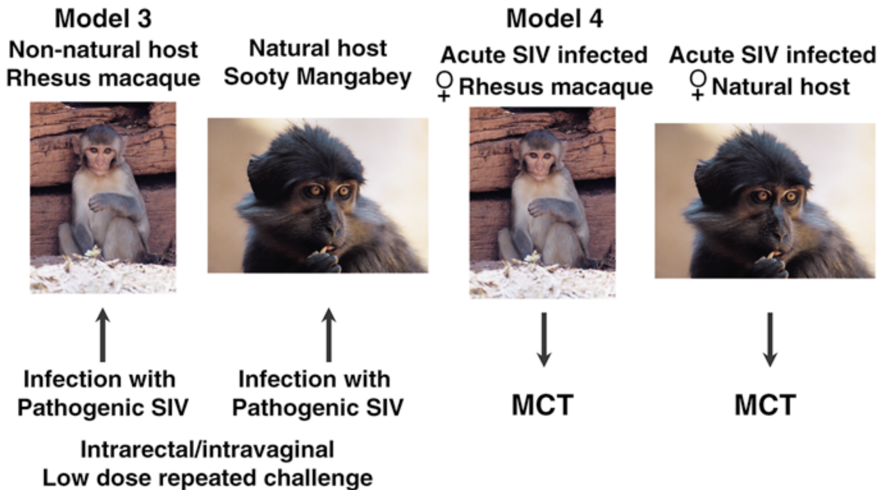


Fig. 12.2 Non-human primate models of SIV infection that we advocate as ideal for the study of the role of innate immunity. In efforts to rule out the role of Mamu-A01, B08, and B17, it is best to utilize monkeys that do not express these MHC alleles. For analysis of the genetic role of KIR polymorphisms, it would be ideal to have the animals keyed into KIR haplotypes. They would be: (1) models 1 and 2 and (2) models 3 and 4 as outlined in the text. *MCT*=Mother to Child transmission studies

clues to distinguish the polarized clinical outcomes in the same species. As noted, whereas the dose and route of infection do not mimic natural infection in model 1, the model does lead to polarized clinical outcomes of SIV infection. Its major positive attribute is that there is a considerable amount of data already available using these

models that can be exploited for the interpretation of the data to be gained by studying the role of innate immune mechanisms in more detail with this and other models. Model 2 includes the use of either rectal or vaginal infection of rhesus macaques with low doses (10 TCID50 or 1–2 million copies) of the viruses applied atraumatically twice a week for up to 20 weeks or until infection is confirmed [11]. In this model, a comparison of innate immune responses of rhesus macaques infected with pathogenic SIVmac239 or 251 can be compared with rhesus macaques infected with to a large extent apathogenic SIVδnef or SIVδ5G [12, 13]. In order to minimize the potential role of MHC and KIR genotypes in conferring spontaneous protection, the animals to be selected should exclude Mamu-A01, B08 and B17 and select KIR genotype and alleles. Model 3 requires the repeated low dose challenge via intra-rectal or intra-vaginal infection of natural hosts of SIV such as sooty mangabeys or African green monkeys and its comparison with the non-natural hosts such as rhesus macaques. In the case of the natural hosts, whereas a considerable amount of data has already been obtained using the IV route, there has been limited data using intra-rectal or intra-vaginal experimental infection of natural hosts of SIV. The last model involves mother to child transmission (MCT) studies. The importance of this model is based on the finding that while the rate of MCT in non-natural hosts such as rhesus macaques is about the same as in untreated HIV-1 infected humans, there is markedly lower MCT in the natural hosts such as sooty mangabeys and mandrills [14]. Thus, it is likely that innate immune effector mechanisms are involved during the acute phase of infection in this model and their delineation may provide some important new clues that can be implemented for preventing MCT in countries where such transmission continues to be a cause of major morbidity and mortality.

Having outlined the various models that one can utilize to ferret out the potential pathways and mechanisms of innate immunity that contribute to decreasing viremia and the rate of degree progression, it is the objective of this review to summarize what we have learned so far. The study of the cell lineages that comprise the innate immune system in non-human primates can be potentially exploited for the formulation of an effective HIV-1 vaccine. The studies to be summarized include kinetic changes in the frequencies of select lineages of innate immune cells during acute infection, studies of the heterogeneity of NK cells (the major cell lineage of the innate immune system), our current understanding of the potential role of the KIR molecules that regulate NK cell function, the potential role of plasma levels of soluble HLA-E and G molecules that influence NK cell function and our view of the important issues that need to be addressed in order to move this field forward.

12.2 Kinetic Changes in the Frequencies of NK Cells and PDCs During Acute SIV Infection

One of the first changes identified with regard to innate immune cells was the rapid increase in frequencies of total NK cells in the PBMCs of both non-natural (rhesus macaques) and natural hosts (sooty mangabeys) of SIV during acute infection.

Table 12.1 Kinetics of pDC in PBMC of SIV infected rhesus macaques and sooty mangabeys [a]

	% pDCs in whole blood	
Sample time	R. M. (n=6)	S. M. (n=4)
Baseline	0.13 +/– 0.05	0.15 +/– 0.07
Week 1	0.67 +/– 0.027 [b]	0.14 +/– 0.08
Week 2	0.11 +/– 0.07	0.17 +/– 0.09
Week 3	0.06 +/– 0.04	0.19 +/– 0.07
Week 4	0.03 +/– 0.02	0.14 +/– 0.04
Week 8	0.03 +/– 0.01	0.18 +/– 0.09
Week 12	0.02 +/– 0.02	0.15 +/– 0.05
Week 24	0.03 +/– 0.01	0.17 +/– 0.08

[a]Whole blood staining technique was used for the enumeration of the frequency of Lin$^-$, HLA-DR$^+$, CD123$^+$ pDCs in rhesus macaques and sooty mangabeys prior to and post experimental infection with SIVmac251

[b]$p<0.001$

Thus, as reported elsewhere (8) there are marked increases in the frequencies of CD3$^-$NKG2a$^+$ cells in rhesus macaques and sooty mangabeys following experimental infection with SIV. It is important to note that the kinetics of increase was most rapid in sooty mangabeys followed by rhesus macaques, who later on maintained low plasma viral loads (<10,000 copies/ml) followed by rhesus macaques who maintained high viral loads (>50,000 copies/ml) after viral load set point. Thus, the difference in the kinetics by which the NK cells are mobilized appears to correlate with disease resistance in the natural hosts and rate of disease progression in the non-natural hosts. Other changes that have been noted are a sharp early increase followed by a decline in the frequencies of plasmacytoid dendritic cells (pDCs) in the blood following SIV infection (see Table 12.1). In the case of pDCs, this increase appears to occur in most, if not all rhesus macaques experimentally infected with SIV, but not sooty mangabeys. The rise occurs prior to a noticeable increase in the NK cells, suggesting that the increase in the pDCs in rhesus macaques may be inducing the increase in NK cells. The significance of this very early increase in the pDCs of SIV infected rhesus macaques is not clear at present and we speculate that these pDCs are being mobilized to either regional lymph nodes or to the gut tissues wherein SIV is actively replicating. The chemokines that lead to such mobilization and trafficking are important to identify and could serve as an important tool for future manipulation. In addition, the reasons why this mobilization of DCs does not occur in sooty mangabeys are also important and the target of current study.

12.3 Heterogeneity of NK Cells and Differences in Natural Versus Non-natural Hosts of SIV

Several laboratories have identified polychromatic flow cytometry-based strategies for the identification of subsets of NK cells in the blood from non-human primates, including ours [8, 15]. While there is no perfect strategy yet identified, the most optimal

strategy has been to open up the SSC and FSC gates wide to accommodate for large granular lymphocytes that also belong to the NK cell lineage and then gate on CD3−, CD8α/α$^+$ cells, which are NKG2a$^+$. This is followed by the use of monoclonal antibodies against CD16 and CD56 which facilitates the identification of at least three major subsets of NK cells in non-human primates. These include the CD16$^+$/CD56^{dim}, CD16$^-$/CD56^{bright}, and the CD16$^-$/CD56$^-$ populations. The CD16$^+$/CD56^{dim} population appears to be the major population in the blood and is also the subset that shows degranulation (CD107$^+$) upon co-culture with NK sensitive target cells. The CD16$^-$/CD56^{bright} is the population that primarily synthesizes a series of cytokines and is present at a much higher ratio in secondary lymphoid organs than peripheral blood. There is paucity of data at present with regards to the CD16$^-$/CD56$^-$ (DN) subset from normal healthy animals. There are clearly differences in the frequencies and absolute numbers of these subsets in rhesus macaques and sooty mangabeys as published elsewhere [8]. Our lab has also utilized the HLA-E tetramer reagent to determine the frequency of HLA-E tetramer positive cells among the NKG2a$^+$ cells. In addition, the frequency of HLA-E tetramer positive cells among the three major subsets of NK cells has been investigated to further define the differences in the NK cell subsets in the two species. As seen in Fig. 12.3, there is a marked increase in the frequencies of CD16$^-$/CD56$^+$ subset in sooty mangabeys as compared with rhesus macaques. Furthermore, there were also clear differences in the frequencies of

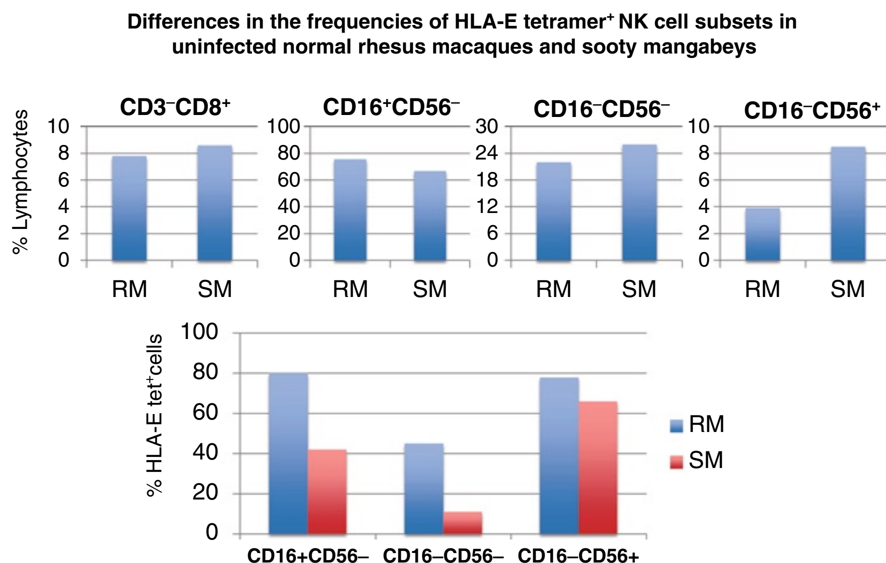


Fig. 12.3 The top panel reflects the frequencies of total NK cells (CD3$^-$, CD8α/α$^+$), and the percentage of its three subsets (CD16$^+$, CD56$^-$; CD16$^-$, CD56$^-$; and the CD16$^-$, CD56$^+$) in the PBMCs from 16 uninfected rhesus macaques (RM) and nine uninfected sooty mangabeys (SM). The bottom panel reflects the frequencies of HLA-E tetramer positive cells among the three NK cell subsets of RM and SM. Data on the differences in the frequencies on HLA-E tetramer positive cells were highly significant ($p < 0.0001$)

HLA-E tetramer positive cells within the $CD16^+$/ $CD56^-$ and $CD16^-$/ $CD56^-$ subsets in rhesus macaques as compared with sooty mangabeys. The significance of these differences has yet to be determined and is a subject of current study. With an interest in gut homing cells, our lab has also conducted preliminary studies on the frequencies of NK cells and its subset that express the heterodimeric gut homing marker α4β7 integrin. Results of these studies indicated that NK cells in general expressed low mean densities of the marker, unlike $CD4^+$ T cells that clearly comprised a subset with medium to relatively higher mean densities of α4β7. While incubation of $CD4^+$ T cells with retinoic acid led to a noticeable increase in the frequencies and densities of α4β7 expressing cells, similar incubation of NK cells did not show any detectable changes in the expression of α4β7. Overall, while about 50% of the NK cells from rhesus macaques expressed α4β7, only about 30% of the NK cells from sooty mangabeys expressed α4β7. Once again, the significance of these differences is not known at present and are a subject of study. It is important to note that recent studies have ascribed a regulatory role for the β7 integrin on the expression of the α4β7 heterodimer and in gut homing of lymphoid cells [16]. Whether it is β7 integrin that is differentially affecting the expression of α4β7 expression on NK cells of sooty mangabeys as compared with rhesus macaques needs to be explored. It would also be important to determine whether such decreased frequency of a4b7 expressing NK cells in sooty mangabeys as compared with rhesus macaques leads to decreased homing of the NK cells to the gut tissue in mangabeys, thereby contributing to decreased inflammation and rapid gut tissue recovery following acute infection needs address.

While it is becoming clear that differences exist in the frequencies of NK cell subsets that are present in the blood as compared with lymph nodes, it is also becoming increasingly evident that there are NK-like cell lineages in other tissues that are distinct from the subsets described above. Clearly, NK cells are present in placental and decidual tissues and play an important role in pregnancy and conception [17, 18]. In addition, there are NK cells that are present in the gut-associated lymphoid tissues that are distinct in terms of phenotype and function highlighted by the finding that they synthesize IL-22 and express p44 NCR [19]. Whether these tissue-localized NK cells have the same progenitor cell as the conventional blood and lymph node NK cells remains to be determined. These tissue-specific NK cells add to the complexity of our understanding of NK cell heterogeneity. It is also important to recognize that the role of gut flora is important, particularly in the generation of NK cells. It is known that gram negative bacteria in the gut via their expression of peptidoglycan induce the generation of intestinal lymphoid follicles (ILF's) by binding to the nucleotide binding oligomerization domain 1 innate receptor on epithelial cells and beta defensin 3 and CCL20 mediated signaling through the chemokine receptor CCR6. These interactions are hypothesized to contribute to the generation of the NK-22 cells in the gut. What is of further interest is the discovery of some discrete but important differences between the colon and small intestine with regards to gut-associated lymphoid tissue (see Table 12.2). First of all, there are more ILFs in the colon than the small intestine. Secondly, there are also more $TH17^+$ cells in the colon than the small intestines, plus the latter tissue contains more Tregs than the former. Thirdly, while small intestinal tissues demonstrate

Table 12.2 Differences between colon and small intestinal tissue

Small intestinal tissues	Colon tissues
Lower bacterial content	Higher bacterial content
Lower levels of ILF's [a]	Higher levels of ILF's [a]
Lower levels of TH17	Higher levels of TH17
Higher levels of Tregs	Lower levels of Tregs
Readily detectable levels of CCL25 [b]	Absence of CCL25 [b]

[a] *ILF's* Intestinal Lymphoid Follicles
[b] CCL25 Ligand for CCR9 homing marker

readily detectable levels of CCL25 (ligand for the gut homing molecule CCR9), there is a virtual absence of CCL25 expression in colonic tissues. Thus, differences do exist in the composition of the various immune cells in the colon versus the small intestine. These variances may contribute to changes in the role of the innate immune system localized to each of these tissues.

12.4 Role of the Killer Cell-Like Immunoglobulin Receptors (KIRs) in HIV and SIV Infection

While NK cells are decorated with a large number of cell surface markers and receptors [20], the KIRs have received considerable attention because they consist of both activating and negatively signaling molecules and several genome wide association studies have shown a strong link between the region of the chromosome encoding the HLA-C (ligand for select KIRs), HLA complex P5 (HCP5), and slow disease progression in HIV-1 infected individuals [21]. This data confirms previous results of studies that have documented a strong association between inheritance of select MHC class I alleles such as the HLA-*B5701 and HLA-B27 MHC class I alleles and slow disease progression [22–24]. A more recent genome wide association study on the other hand, has shown a stronger association with the PROX1 (negative regulator of IFN-γ in T cells) and HIV-1 disease [25]. Studies carried out by the laboratories of Drs. Altfeld and Galit have made a strong case for the involvement of NK cells and select KIR genes in the control of HIV-1 infection [26, 27]. These views are supported by the finding that a high frequency of HIV-1 infected individuals who were classified as slow disease progressors expressed the KIR3DS1 NK cell receptor or the KIR3DL1 receptor in association with its putative ligand HLA-B allele with isoleucine at position 80 [28, 29].

These findings prompted our laboratory to initiate studies on developing techniques for KIR genotyping of rhesus macaques with the aim of determining if similar to HIV-1 infected humans, we could also identify the role of KIR and MHC allele associations in rhesus macaques. A search of the literature on KIR gene analysis of rhesus macaques made us realize that there was very limited knowledge with regard to this subject area. Pioneering studies of the KIR genes in rhesus macaques were carried out by the laboratory of Dr. Norman Letvin [30]. Studies performed in

his laboratory utilized expression cloning which led to the characterization of five families of KIRs in rhesus macaques that were termed KIR1D, KIR2DL4, KIR2DL5, KIR3DH, and KIR3DL (KIRs are classified based on the number of immunoglobulin-like extracellular domains such as 1D, 2D, and 3D and whether they are associated with a long (L) or short (S) intracytoplasmic domain. The DH is designated for KIRs, which are hybrids and DP as those that are pseudotypes). These studies were soon followed by the description of KIR receptor expression in other non-human primate species [31–33]. Studies of KIR genes at the genomic level led to the identification of KIR haplotypes in chimpanzees, rhesus macaques and orangutans [34], and the discovery that on an evolutionary level, there has been very rapid diversification of the KIR genes. The availability of a unique, relatively inbred population of Mauritian cynomolgus macaques were elegantly exploited by Bimber et al. [35] who reported the existence of at least eight distinct loci which code for KIRs and included some of the loci previously published [30]. Studies from our lab were focused on defining associations between alleles of the various KIR loci and SIV infection-induced disease progression in rhesus macaques which led us first to identify an association between specific alleles encoded by the KIR3DL locus [20] and the development of high plasma viral loads. An extension of these studies led us to define a much stronger association between alleles 13 and 14 of the KIR3DL locus, a set of alleles encoded by the KIR3DH locus and the development of high plasma viral loads in a cohort of 38 rhesus macaques [36]. Very recently, there have been two reports that utilized a combined approach consisting of both cDNA analysis and genomic DNA analysis in families of a large cohort of colony-bred rhesus macaques at the German and Netherland Primate Centers [37, 38]. Results of these studies have led to the identification of 14 and 21 putative haplotypes in rhesus macaques. These haplotypes included both activating and inhibitory KIRs, with each haplotype including 5–11 KIR genes with the result that a given KIR gene may be present in one haplotype, but not the other. This adds to the complexity of KIR gene analysis. These new findings provide a foundation for future studies of KIR haplotype-association with disease progression in not only SIV infected rhesus macaques but in a variety of other studies that employ rhesus macaques. The findings from these studies also emphasize the need for a more detailed analysis of the KIR gene loci in rhesus macaques. In addition, it also highlights the fact that the precise role of these KIR gene products can only be realized when their respective putative ligands are identified. It should be noted that whereas our laboratory carried out studies of KIR gene and allele identification using highly enriched population of NKG2a^{+} cells, the other studies reported above either utilized unfractionated PBMC's for mRNA studies or whole genomic DNA. This is an important issue, since the expression of KIRs has also been shown to occur on other cell lineages, including CD8^{+} T cells. The identification of monoclonal antibodies against KIRs specially activating and inhibitory KIRs could also be very useful for characterizing their relative expression of different NK cell subsets and CD8^{+} T cells. Unfortunately, none of the monoclonal antibody reagents against human KIRs that we have tested appear to cross-react with rhesus macaque KIR gene products. It would be ideal to produce some that at least distinguish between the activating and inhibitory KIRs as recently shown [39].

It is important to note that there are a variety of other receptors that have been shown to play a role in the cytotoxicity of NK cells. These include the p30, p44, and p46 molecules expressed by NK cells. More detailed studies of the role of these molecules are needed in rhesus macaques prior to and post SIV infection. In this regard, it is also important to mention that the relatively less polymorphic MHC loci such as HLA-C, HLA-E and G have been thought to serve as ligands for KIRs [40]. Soluble forms of the HLA-G molecules have been shown to exist in plasma [41] and be released by mettalloproteinases. Increased mettalloproteinase activity has previously been shown to cleave CD16 from NK cells leading to the concept that such activity could be contributing to decreased NK cell function in HIV-1 infected individuals [42]. Thus, it was reasoned that soluble plasma rhesus homologue of human HLA-G could play a role in modulating NK cell activity in SIV-infected rhesus macaques in vivo. Preliminary studies by our lab on quantitating soluble HLA-E and G rhesus macaque homologue have failed to show any association with plasma or cellular viral loads. However, the effect SIV-infection has on the density of expression on cell membranes is currently under evaluation.

12.5 Regulatory Mechanisms Involved in Innate Immunity

While our knowledge of regulatory mechanisms and the intracellular molecular pathways used by cell lineages involved in such processes have focused primarily on how such mechanisms control adaptive immune responses, there is a paucity of data on the regulatory mechanisms involved in the control of innate immune responses. The groundbreaking discovery that the majority of innate immune responses are initiated by the cellular detection of pathogen-associated molecular patterns (PAMPs) expressed by micro-organisms has paved the way for advancing our knowledge of innate immune regulatory pathways. There are several pathogen recognition receptors (PRRs) that recognize these PAMPs [43] and include Toll like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors (NOD-like), retinoic acid inducible gene-1 (RIG-1) receptors, and C-type lectin receptors. Each of these receptors when ligated transduces signals via distinct pathways such as MyD88 for TLRs and recruitment of MAVS by RIG-1 resulting in activation of NFkB and the type I interferon genes. It is clear that uncontrolled activation of the innate immune system can have lethal consequences. Thus, these activation pathways must be tightly controlled for the maintenance of homeostasis in the host. While these studies are still in their infancy, certain issues appear to be clear. The importance of the IKK complex as the central transducer that integrates signaling from diverse pathways to activate NFkB and the important role of the RLR in activating type 1 interferon genes is well recognized, but the detailed mechanisms for each remain poorly understood. The NLRs represent a relatively large family of intracellular pathogen-recognition receptors which consist of a conserved nucleotide-binding and oligomerization domain (NOD) and a leucine-rich repeat region (LRR). These NLRs such as NOD1, NOD2, and NALP3 have all been

documented to be involved in transducing a diverse set of signaling pathways upon recognition of signature pathogen-associated molecular patterns. Examples of the involvement of these NLRs include the finding that the adjuvant effect of agents such as aluminum and asbestos function by activating the NALP3 inflammasome and NLRX1 functions by interacting with the mitochondrial adaptor MAVS. This results in the inhibition of RIG-1 induced signaling in addition to triggering the generation of reactive oxygen species. It is important to recognize that while some of the NLRs can transduce activating signals, other have been shown to deliver inhibitory signals. Delineation of the various pathways that promote or inhibit each of these pathways continues to be defined. It is clear that knowledge by which these signals are transmitted can be exploited to promote and/or inhibit innate immune system-mediated pro-inflammatory responses depending on the immune modulation that is needed. Such is the case as in malignancies and inhibition in the auto-immune disease scenario. The recent discovery of specific small molecule inhibitors of intracellular signaling molecules suggest that the pathways involved in signaling the activity of innate immune cells can be modulated for therapeutic purposes. Hence, the inclusion of this subject herein. It should be noted that there is now a growing acceptance for the existence of not only memory [44–48], but also regulatory [49] function for NK cells. The nature of the intracellular signals involved in the activation of the memory NK cell response and those involved in conferring inhibitory regulatory signals have yet to be defined.

It is also important to be cognizant of the fact that there are marked differences in the types of NK cells and their subsets within various organs and tissues. Their localization to these tissues may impart upon them specific mechanisms that control their function. Thus, NK cells present in placental tissues may be governed by a completely different regulatory network as compared with those in lymph nodes and blood. Similarly, the recently described NK-22 cells in the gut-associated lymphoid tissue are likely to utilize distinct pathways for their activation and regulation. The study of the regulation of NK cells from peripheral blood may not in fact provide clues as to how the NK cells function and are regulated in mucosal tissues which warrants a note of caution in terms of data interpretation.

12.6 Future Directions

The overall question of how can we harness the knowledge of the mechanisms by which innate immune systems operate for the formulation of a more effective vaccine against HIV-1 remains to be defined. The fact that some individuals infected with HIV-1 and some rhesus macaques infected with otherwise pathogenic SIV both spontaneously control viremia and become either “elite” controllers or “long term non-progressors” coupled with the finding that natural hosts of SIV even when experimentally infected rarely, if ever, develop disease, suggests that these cohorts have developed an unique viral control and disease resistance mechanisms. It is also seems clear that this mechanism must execute its function during the acute infection

period since this phenotype in these groups develop by 5–6 weeks post-infection prior to the development of mature adaptive immune responses. It seems reasonable to assume that innate immune mechanisms are thus likely the major contributors to this phenotype. Within this concept, it is important to keep in mind that the reverse may also be true. A dysfunctional and/or over reactive innate response could be the basis for high viral loads and rapid disease progression. In either case, it is clear that unraveling these events in the models described above may provide some unique insights into what needs to be incorporated with the formulation of a more efficient vaccine against HIV-1. Some attempts have already been made and data so far has led us to speculate on the avenues that need to be pursued to move this field forward. These avenues are summarized in Fig. 12.4.

Three major issues emerge:

1. Limit the ability of the virus to traffic to the gastrointestinal tissues and devise strategies for the protection and if needed reconstitution of the gut epithelial cell architecture.
2. Provide an alternative pathway for the generation of required helper T cell responses.
3. Incorporate therapeutic strategies that would induce an anti-inflammatory immune response in the GI tissues.

Attempts to devise strategies that would limit the availability of target cells within the gut-associated lymphoid tissues seem to be a reasonable approach.

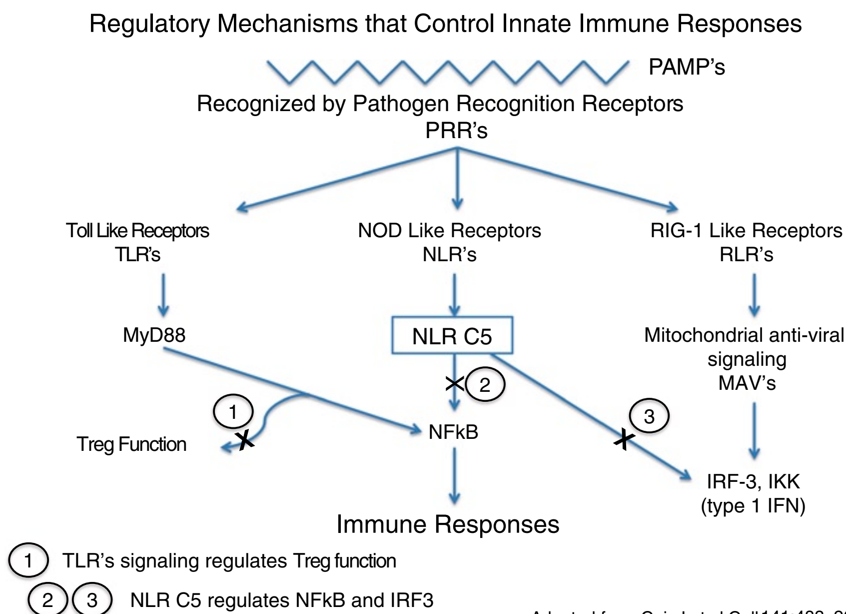


Fig. 12.4 A schematic that summarizes some of the intracellular pathways that have been shown to regulate the activity of innate immune effector cells

Inhibition of trafficking newly minted $CD4^+$ T cells to the GALT may limit gut pathology and allow for the gut innate immune system to contain whatever level of viremia that does occur and provides a necessary time period for the generation of effective adaptive immune responses. Use of inhibitors of homing molecules such as $\alpha4\beta7$ integrin and CCR9 are clearly important candidates for such a targeted approach. This strategy should include methods to increase the life span of GI tissue epithelial progenitor cells or include inducers of increased GI epithelial progenitor cells mobilization and localization to the intestinal crypts. The recent discovery of such GI epithelial stem cells indicates the potential feasibility of this approach. It also seems appropriate to limit the degree of inflammation within the gut. Thus, we reason that inflammation within the gut must provide a perpetual source of highly susceptible target cells but more importantly, increases gut pathology which we submit is a major contributing factor to the generalized immunosuppression that is characteristic of human HIV infection. This is also recapitulated in rhesus macaques experimentally infected with SIV. Devising strategies to induce an anti-inflammatory milieu in the gut would be an important avenue to pursue and could include upregulation of molecules such as PD-1, CTLA-4, and synthesis of TGF-β. Our preliminary data also suggests that high levels of IFN-alpha within the gut tissues by innate immune cells are a likely contributing factor to the GI tissue pathology. Since pDCs are the major source of IFN-α, it seems that regulating their function or transient depletion of such cell lineages in the gut tissue may be a worthwhile approach. Consistent with this view, is the idea that an overactive NK cell function within the gut could, in fact, be detrimental to the host and that avenues aimed at detuning NK cell function may be in order. While the precise mechanism by which the $CD4^+$ $CCR5^+$ T cells contribute to pathogenesis is far from clear, we hypothesize that it is not the number of this cell subset that is playing a role, but its function that is likely the major contributor for HIV pathogenesis. The function of the CCR5 molecule in regulating immune responses would be an important new avenue of approach. Since helper $CD4^+$ T cell function becomes severely compromised, it seems finding an alternate source of non-CD4 expressing helper T cells for autotransfusion therapy may be an important immune reconstitution approach. The feasibility of this approach is highlighted by the finding of a few non-human primates who lack any detectable $CD4^+$ T cells, but demonstrate normal helper T cell responses and appear healthy throughout their life. Therefore, a significant number of sooty mangabeys that are natural hosts for SIV appear to have very low detectable levels of circulating $CD4^+$ T cells, but appear to mount readily detectable $CD4^+$ T cell dependent helper T cell responses. The origin of such helper $CD4^+$ T cell response is at present unknown. Defining how this is executed and then applying this to the generation of helper T cells that do not express CD4 or the co-receptors CCR5 and CXCR4 would be a worthwhile endeavor and would have important therapeutic implications. Other approaches worth considering are avenues that limit bacterial translocation while maintaining gut tissue integrity.

Overall, it is our working hypothesis that limiting gut tissue pathology during acute infection is a worthwhile endeavor and that methodologies can serve this purpose

if incorporated into a putative vaccine against HIV. It may not eliminate viremia, but could lead to the generation of "elite" controllers or LTNP. We believe this is the correct first step for the formulation of an effective vaccine against HIV-1.

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Chapter 13
Influenza Vaccines: What Do We Want and How Can We Get It?

Felix Geeraedts and Anke Huckriede

Abstract Influenza vaccines have been in use for more than 60 years and have proven to be efficacious in protecting from influenza infections during epidemics and the recent H1N1 pandemic. The development of influenza vaccines has so far been largely based on empirical grounds, which leaves room for vaccine improvement by implementation of recent insights in innate and adaptive immunity. Also, evaluation and approval of new vaccines rely on rather broad correlates of protection such as the hemagglutination inhibition titre, thereby neglecting qualitative aspects of the immune response. Here we discuss how current inactivated influenza vaccine formulations differ in the type of immune response they elcit, their protective capacity, and what causes these differences. Finally, we will discuss how this knowledge can guide the development of new adjuvants that optimize the protective efficacy of influenza vaccines.

13.1 Introduction

Influenza A virus is a highly infectious agent responsible for 500,000 deaths during annual epidemics and excess deaths during sporadically occurring pandemics [1]. The most dramatic pandemic so far was the Spanish flu in 1918, with an estimated death toll of over 50 million worldwide [2]. Infection with influenza virus can be asymptomatic but usually presents with symptoms, such as the fever, cough, runny nose or nasal congestion, sore throat, myalgia and headache. During pandemics more atypical and severe symptoms may be seen, like diarrhea and life threatening viral pneumonia [3, 4].

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Influenza virus infections induce vigorous immune responses comprising antibodies of the IgA and IgG subclass, T helper 1 (Th1) cells, and cytotoxic T lymphocytes [5–7]. Nevertheless, re-infections are common due to rapid mutational changes of antigenic epitopes on the major influenza virus surface antigen, hemagglutinin (HA). Such mutations, caused by the poor fidelity of the viral RNA polymerase, lead to "antigenic drift" and are considered the reason for yearly influenza epidemics. The genome of influenza A virus is built up of eight segments and antigenic variation may take a leap when whole segments of the viral genome change. Such a change of the HA-encoding segment is associated with "antigenic shift" and may lead to new virus strains which can spread easily in an immunologically naïve human population, causing a pandemic. Segment changes may be the result of reassortment of genomic segments between circulating viruses and other subtype viruses from different species, like avian influenza. Such reassorted viruses caused the 1957 Asian flu and the 1968 Hong Kong flu [8]. Alternatively, a virus subtype circulating in animal species and carrying an HA subtype that differs strongly from those of human virus strains may cross the species barrier and start a pandemic if sustained human-to-human transmission evolves. Such zoonoses were the cause of the Spanish flu and the 2009 H1N1 pandemic [9, 10]. The avian H5N1 virus is a zoonotic virus which regularly crosses the species barrier. Due to its high mortality rate (about 60% of laboratory confirmed cases), H5N1 poses a serious threat [11], yet so far human-to-human transmission has occured only very sporadically, if at all [12].

Vaccination has proven to be highly efficacious in preventing influenza during epidemic periods and is considered as the most promising mitigation strategy in case of a pandemic [13]. Current epidemic vaccines still suffer from limited immunogenicity in important risk populations like the elderly and immuno-compromised individuals. Pandemic vaccines, on the other hand, need to be highly immunogenic at low antigen doses in order to protect a very large population against a new virus, with the limited vaccine production capacities available. So far, vaccine design has been largely empirical. A more rational approach to influenza vaccine development starts with a good understanding of immune responses evoked by current vaccines and the immune mechanisms involved in raising these responses. These issues and their impact on the development of new vaccines will be discussed below.

13.2 Current Influenza Vaccines

Current inactivated influenza vaccines consist of either preparations of whole virus (WIV), detergent-treated virus (split-virus), isolated viral surface proteins (subunit) or reconstituted viral membranes (virosomes) (Fig. 13.1). With few exceptions, these vaccines are used without adjuvant. Because of their lower reactogenicity split-virus (SV) and subunit (SU) vaccines have largely replaced the archetype WIV vaccines in the 1960s and 1970s [14]. Modern production technology might alleviate the reactogenicity problem of WIV vaccines and therefore these vaccines have received renewed attention as (candidate) formulations in the context of a putative H5N1 pandemic and the recent H1N1 pandemic [15–18].

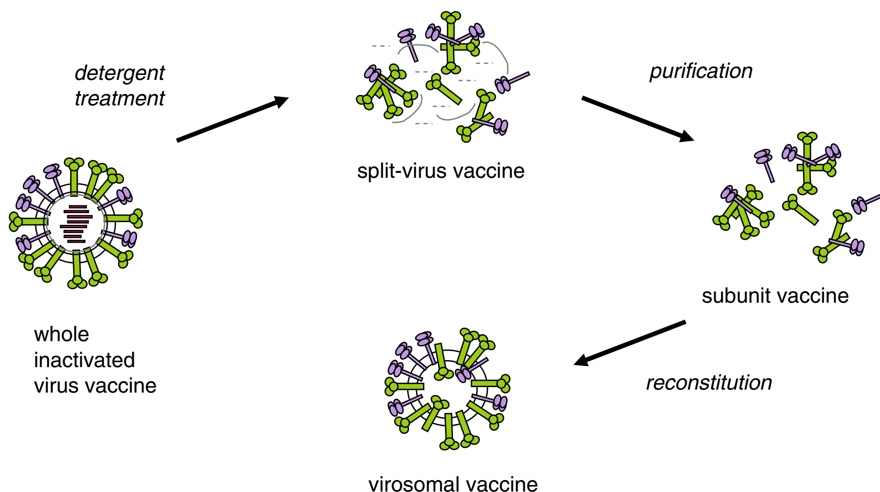

Fig. 13.1 Current inactivated influenza vaccines. Egg- or cell-grown virus is chemically inactivated to obtain WIV vaccine. Solubilization of the viral membrane by detergent treatment results in SV vaccine. By further purification steps SU vaccine is obtained which contains only the viral membrane proteins. These can be reconstituted with natural or synthetic lipids to empty virus envelopes, so-called virosomes

Vaccine efficacy is primarily defined by levels of antibodies induced against HA, which is considered the minimally required constituent of an influenza vaccine. Antibody levels are typically measured by determination of the hemagglutination inhibition (HI) titre, the reciprocal of the dilution at which serum of a vaccinee is still capable of inhibiting hemagglutination of erythrocytes by the virus. An HI titre of 40 is estimated to be associated with a 50% reduction of the risk of contracting influenza. This titre is used as the basis for approval of influenza vaccines by the European Medicines Agency (EMEA) and the Food and Drug Administration (FDA) [19]. The 50% protective titre was calculated from a number of clinical studies in which immunity was achieved by either natural infection or by vaccination with inactivated or live-attenuated influenza vaccines. Since infection, as well as vaccination, will induce a plethora of immune reactions, it is unclear whether HI antibodies themselves provide protection or whether their presence is simply an indication for the immune status to influenza virus [20].

In primed individuals, non-adjuvanted WIV, SV, and SU vaccines induce similar immune responses in terms of HI titres (for a meta-analysis of 24 studies see [21]). However, in individuals that have not been exposed to the vaccine antigens before, WIV vaccines are more immunogenic than SV and SU vaccines [21–23]. Among inactivated, non-adjuvanted, H5N1 candidate vaccines, only WIV (derived from wild-type virus) evoked antibody titres which met the EMEA and FDA criteria [24]. A cell culture-derived WIV mock-up vaccine, Celvapan®, was licensed in Europe in 2009 and an H1N1 Celvapan® vaccine was used during the 2009 H1N1 pandemic.

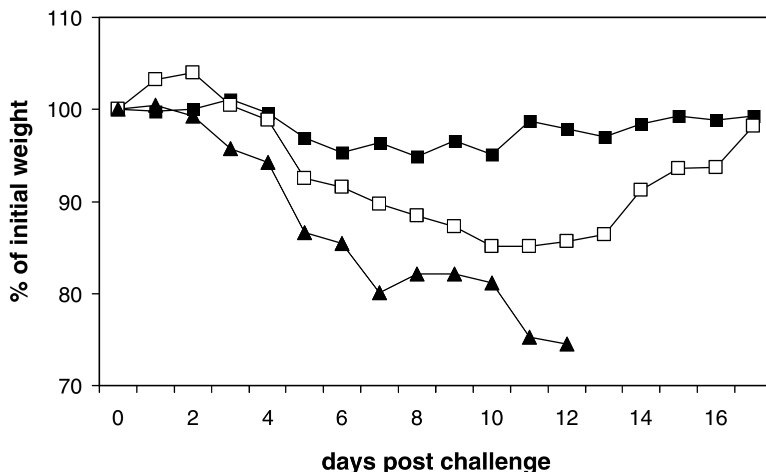

Fig. 13.2 Protection from influenza challenge by different vaccines. Mice were immunized intramuscularly on day 0 with 5 μg HA formulated as WIV vaccine (*filled squares*) or SU vaccine (*open squares*), or were injected with buffer (*filled triangles*). On day 28 the mice were challenged with 100 TCID50 of A/PR8 virus and weight was measured for 16 days. Mice were euthanized when weight loss exceeded 20%

No data is available on the relative *protective* efficacy of WIV, SV and SU influenza vaccines in humans. A head-to-head comparison of different vaccines in terms of their protective capacity can, however, easily be performed in animal models. Challenge studies in mice show that a single immunization with WIV protects more efficiently from weight loss (Fig. 13.2) and leads to a stronger decrease in nose or lung virus titre than immunization with other vaccines [25, 26]. Moreover, WIV prevents weight loss and reduces viral loads in the lung already at low antigen doses, whereas substantially higher doses of SV and SU vaccine are needed to achieve the same effect [27].

Detailed comparison of the immune response in vaccinated mice revealed that WIV induces higher serum HI titres than SV and SU vaccine especially after a first immunization [27–30]. This data is in line with the superior immunogenicity of WIV in unprimed individuals mentioned above. Interestingly, the different vaccine formulations also induce different types of responses. WIV vaccine elicits an overt Th1 type immune response characterized by high IgG2a/c titres and high numbers of IFNγ-producing T cells. In contrast, SV and SU vaccines induce a Th2-dominated response with large amounts of IgG1 and high numbers of IL4-producing T cells [27–30].

The relative contribution of IgG subtypes to protection from influenza attack has been investigated in detail by Huber et al. [31]. Mice were vaccinated either with an HA DNA vaccine or with a viral replicon system encoding HA, or with a combination of both to induce IgG1-dominated, IgG2a-dominated, or mixed IgG1/IgG2a responses. IgG2a alone protected mice as efficiently from an outbreak as a mixture of IgG1 and IgG2a and could contain both mild and severe virus challenges. In contrast, IgG1 alone only protected from mild virus challenge, but provided insufficient protection

upon a high-dose challenge. Thus, antibody subclasses differ in their capacity to neutralize virus. The strong affinity of IgG2a/c antibodies for complement factors and Fc receptors has been proposed as possible reason for the superiority of this antibody subclass since both mechanisms contribute to efficient clearance of virus by phagocytosis and antibody-mediated cellular cytotoxicity [31]. In contrast, IgG1 does not lead to complement activation and has only weak affinity for Fc receptors.

Thus, current non-adjuvanted vaccine formulations differ substantially in the magnitude, but also in the phenotype of the immune reaction they evoke. This has profound effects on their protective capacity, at least in animal models. Data on the phenotype of the immune response to influenza vaccination and the relevance of the immune phenotype for protection in humans is urgently needed. Moreover, for a rational design of improved vaccines, we need to understand which vaccine properties determine the differential immune reactions.

13.3 Mechanisms Involved in Vaccine-Induced Immunity

In order to reveal which mechanisms are responsible for the superior immunity of WIV, we and others have evaluated the effects of influenza vaccines on dendritic cells (DC) in vitro. Similar to live virus, WIV induced the production of IL12 and a variety of pro-inflammatory cytokines in conventional DC (cDC) generated from bone marrow by culture in the presence of GM-CSF [29, 32]. In contrast, SV and SU vaccine had little effect. Plasmacytoid DC (pDC) were either generated from bone marrow by culture in the presence of Flt3-ligand or directly purified from spleen. When incubated with live virus or WIV, these cells responded with production of type I interferons (IFNα and IFNβ), while neither SV nor SU vaccine had any effect [29, 30, 33].

Type I interferons are antiviral cytokines produced immediately upon viral infection. They have been shown to augment antibody responses in vivo and to steer the phenotype of the response to a Th1 type by direct interaction with B cells, (but also T cells) [34–37]. The role of type I interferons in the immune response to WIV was recently studied by Koyama and co-workers using IFNα/β receptor knock-out mice [33]. In contrast to wild type mice, IFNα/β receptor knock-out mice immunized with WIV showed little induction of antibodies, no induction of influenza-specific IFNγ-producing Th cells, and died quickly upon lethal virus challenge. Since pDC had been identified as an important source of type I IFN in vitro, the role of this cell type for the immune responses in vivo was investigated. Depletion of pDC by antibody treatment prior to intranasal immunization with WIV largely abolished IgG induction and significantly reduced induction of Th1 cells. Interestingly, pDCs were essential only during primary immunization, while depletion prior to a booster immunization had no effect [33]. These results show that type I IFNs have an important role in the strong antibody and T cell response to immunization with WIV and that pDC as the major producer of type I IFNs are important, at least in the context of intranasal immunization. The role of pDC in the response to parentally administered vaccines remains to be elucidated.

In the in vitro experiments described above, WIV but not SV or SU vaccine induced IFNα production by pDC [29, 30]. WIV differs from the other vaccine formulations by retaining the structure of the virus envelope and by containing the viral RNA (which is degraded in SV and removed in SU vaccines). Although an intact envelope structure may enhance antigen uptake, it do not by itself trigger production of IFNα, since pDC incubated with reconstituted viral envelopes (virosomes) do not secrete this cytokine [29]. Single-stranded RNA (ssRNA) is known as the ligand of Toll-like receptor (TLR) 7 which is expressed in pDC [38]. Indeed, bone marrow-derived Flt3-ligand pDC from TLR7 knock-out mice do not produce IFNα when incubated with WIV [30, 33]. On the other hand, transfection of pDC with viral RNA purified from WIV leads to similar IFNα production as incubation with WIV itself [30]. Thus, binding of the viral ssRNA in WIV to TLR7 is the trigger for activation and IFNα production in pDC. TLR7 is also essential for immune responses to WIV in vivo. In TLR7 knockout mice antibody induction to intramuscularly or intranasally administered WIV is significantly reduced or completely abolished, and Th1 induction is largely lost [30, 33].

Not only pDC, but also B cells express TLR7 [39]. In fact, B cell-intrinsic TLR signaling was recently found to be essential for class switching to IgG2a/c in immunization and infection [40]. Moreover, B cells also possess IFNα/β receptors and can react to viruses and vaccines carrying TLR7 ligands in a direct way by intrinsic TLR7 signaling, as well as in an indirect way imposed by pDC-derived IFNα [41]. Additionally, pDC-derived IFNα can upregulate the expression of TLR7 in B cells, thereby increasing TLR7 sensitivity [42]. A third signaling route can be provided by interactions between B cells and T cells via CD40-CD40L binding.

B cells receive a variety of signals, either via direct contact with the antigen, via cell-cell contact with T cells, or via cytokines derived from pDC and cDC. Heer and co-workers suggested a model illustrating how the different signaling mechanisms might be integrated and affect antibody production by B cells [41]. In this model, triggering of either TLR7 or CD40 on B cells alone induces cell proliferation and production of IgG1, whereas stimulation of both receptors together induces production of IgG1 and IgG2a/c antibodies. Additional signaling through the IFNα/β receptor further polarizes the subtype ratio in favor of IgG2a. Figure 13.3 illustrates how WIV and SU vaccine would activate B cells in mice according to this model. WIV can activate B cells directly via cross-linking of B cell receptors and engagement of B cell-intrinsic TLR7. Through triggering of TLR7 in pDC, it induces secretion of IFNα which can further activate B cells. WIV also strongly stimulates cDC, which produce cytokines like IL-12 and activate Th cells. By direct cell-cell contact between B and T cells involving CD40-CD40L binding and by cytokines derived from cDC and Th cells, B cells are further activated. Integration of all these signals leads to B cell proliferation, class switching to IgG2a/c and strong production of antibodies. In contrast, SU vaccine does not activate B cells directly and does not involve pDC. Instead, SU-activated cDC stimulate Th2-skewed Th responses via direct contact and cytokines. Cell-cell contact between B cells and T cells and cytokines derived from cDC and Th cells induce B cell proliferation and production of antibodies of the IgG1 subclass. To what extent this model applies to the working mechanisms of WIV and SU vaccines in humans remains to be elucidated.

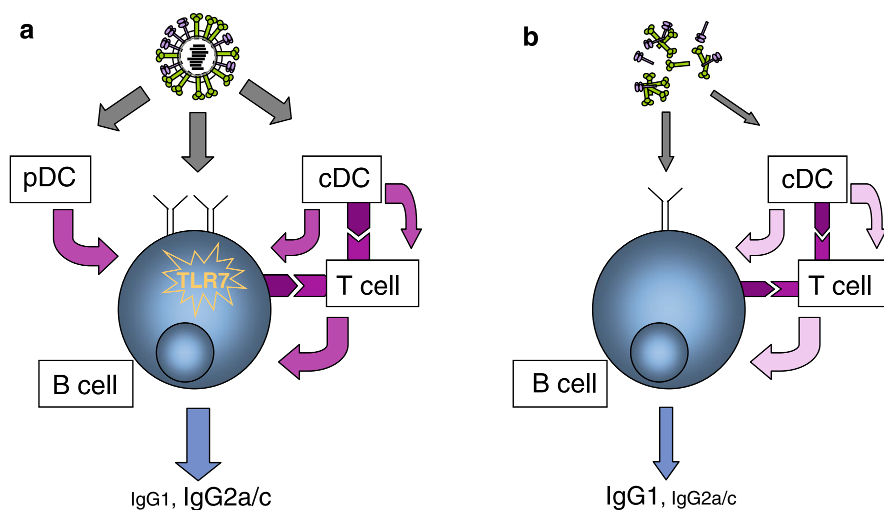

Fig. 13.3 Model of B cell activation by WIV (**a**) or SU (**b**) vaccine. See text for explanation

13.4 Adjuvants for Influenza Vaccines

The high immunogenicity of WIV, carrying TLR ligands, as compared to SV or SU vaccines, being devoid of TLR ligands, indicate that synthetic TLR ligands might be promising adjuvants for influenza vaccines. Indeed, the Koyama study shows that the lack of IFNα induction by and the poor immunogenicity of intranasally administered SV vaccine can be completely restored by addition of a CpG-based adjuvant [33]. Other TLR ligands investigated in the context of influenza vaccines include MPLA, flagellins, Poly I:C, and peptide-modified CpG (IC31) [33, 43–46]. Of these adjuvants, IC31 consisting of CpG oligomers bound to an immune-stimulatory peptide has already successfully passed Phase I clinical testing [47]. However, no influenza vaccine adjuvanted with TLR ligands has obtained registration so far.

Adjuvants currently licensed for use with influenza vaccines are comprised of aluminium salts and the oil-in-water emulsions MF59 and AS03. Neither of these falls into the category of TLR ligands. These adjuvants work via other, only partly elucidated mechanisms.

Aluminium salts (phosphate or hydroxide) were introduced as adjuvants for human vaccines in 1932. Being components of diphtheria, tetanus, pertussis, and some *Haemophilus influenzae*, hepatitis A and hepatitis B vaccines, aluminium salts are currently the principle adjuvants in clinical use [48]. Originally, aluminium adjuvants were thought to stimulate immune responses by providing a depot from which antigen is released slowly during an extended time period. Currently, activation of inflammasomes is considered as the primary mechanism of action [49]. Aluminium salts are known to stimulate predominantly Th2-type responses characterized by IL-4 and IL-5 production and the generation of antibodies of the IgG1 subtype in mice [49].

Clinical trials with aluminium-adjuvanted influenza vaccines in the past showed low to modest enhancing effects of the adjuvant on HI titres. More recently, aluminium adjuvants have been tested in the context of H5N1 pre-pandemic vaccines. These studies, which were performed with WIV as well as subvirion vaccines, rendered inconsistent results varying from modest enhancement, to a decrease in antibody titres when non-adjuvanted and adjuvanted vaccines were compared side-by-side [50, 51]. Nevertheless, WIV influenza vaccines adjuvanted with aluminium salts were registered for use during the 2009 H1N1 pandemic in Hungary and China [52]. No data is available on the effect of aluminium adjuvants in the protective efficacy of influenza vaccines in humans.

Influenza immunization studies in mice confirmed the known Th2-skewing properties of aluminium-based adjuvants. When combined with subunit vaccine, which by itself raised an IgG1-dominated antibody response, aluminium hydroxide increased HI and IgG1 titres. When combined with WIV, the IgG2a/c response usually evoked by this formulation was completely blocked. Instead, a strong IgG1 response was elicited which correlated with enhanced HI titres. However, the efficacy of the vaccine to protect from severe virus challenge was not improved and even decreased for aluminium-adjuvanted WIV [26]. The situation might be different for a mild virus challenge as others reported improved protection after immunization with aluminium-adjuvanted WIV [53]. The inconsistent results might thus be related to the fact that IgG1 alone, as evoked by aluminium-adjuvanted WIV, can protect from mild virus challenge, but that IgG2a is essential to contain the virus upon severe challenge as discussed above [31]. In any case, these results underline that the HI titre alone is insufficient as correlate of protection. Qualitative parameters such as the phenotype of the elicited response also need to be evaluated and adjuvants used in combination with influenza vaccines should preferably support a mixed Th1/Th2 response or a Th1-dominated response.

Other adjuvants used clinically in combination with influenza vaccines are the water-in-oil emulsions MF59 and AS03. A seasonal MF59-adjuvanted SV vaccine (Fluad®) is licensed in Europe for use in elderly and has been administered to more than 27 million subjects without causing significant side effects [54]. In clinical trials of H5N1 pre-pandemic vaccines, MF59 proved to enhance antibody titres, allow antigen dose reduction, and induce cross-reactive antibodies against drift strains [55–57]. The latter is explained by the fact that MF59 broadens the antibody response such that a larger number of HA epitopes is recognized [58]. An MF59-adjuvanted vaccine, Focetria®, was used during the 2009 H1N1 pandemic.

The working mechanism of MF59 is not entirely clear yet. Obviously, it involves enhanced recruitment of mononuclear cells to the injection site, promotion of differentiation of monocytes to dendritic cells with concomitant upregulation of co-stimulatory molecules, and enhanced antigen uptake [59]. In mice, MF59 induces a Th2-type immune response dominated by IgG1 and IL5-producing Th cells. Combination of MF59 with Th1-skewing adjuvants like CpG or synthetic TLR4 ligands might allow skewing of the response into the desired Th1 direction [60]. In ferrets, MF59 modestly improved the cross-protective potential of a WIV H5N1 vaccine, yet no further analysis of immune parameters was done [61]. In a clinical study, MF59 had little effect on the phenotype of the Th response to a subunit H5N1 vaccine, the response consisting mainly of $IL2^{+}IFN\gamma^{-}$ cells [62].

AS03, another oil-in-water adjuvant, was explored extensively in the context of H5N1 pre-pandemic vaccines (for recent review see [19]). AS03 has been used on a large scale as adjuvant in the 2009 H1N1 pandemic vaccine Pandemrix®, with a very high estimated efficacy of 98.6% in 14–59 year olds and 83.3% in 60>year olds [63]. Similar to MF59, it enhances antibody titres, allows antigen dose sparing and elicits protection against homotypic and heterotypic influenza strains [64]. So far, the mechanism of action of AS03 and the immune phenotype elicited by AS03-adjuvanted influenza vaccines remain elusive.

Thus, currently used adjuvants were included in influenza vaccines with the aim of enhancing HI titres, the only correlate of protection generally accepted so far. Other parameters, like the phenotype of the response induced and the effects of adjuvants in naïve versus primed individuals have only been investigated sporadically. Taking these parameters into consideration might greatly enhance the benefits of future adjuvants.

13.5 Concluding Remarks

So far, development of vaccines, including influenza vaccines, has taken place largely by a trial and error approach. Undoubtedly, this tactic has been successful in the sense that influenza vaccines with high efficacy are available at least for adolescents and young to middle-aged adults. However, implementation of recent insights in innate and adaptive immune response has the potential to significantly improve current vaccines in their protective efficacy with possibly lower amounts of antigen needed than used today. Such a rational approach to vaccine design has only recently found its way into vaccine development.

Clues for vaccine improvement come from the detailed study of the immune responses to current vaccines and the immune mechanisms involved. Animal experiments indicate that the value of HI titres as correlate of protection may be limited and knowledge of the immune phenotype is needed to judge the potential of influenza vaccines. In this context, immunological experience may be relevant. The majority of adolescents and adults will have developed a Th1 memory response to influenza virus caused by natural infection. In these subjects, vaccination against seasonal influenza will probably boost this response without changing its phenotype. Things are different when young children need to be vaccinated or when vaccination against a new virus strain is necessary, as in the case of a pandemic. Results from mouse experiments imply that the phenotype imposed by priming might govern the response during later infection. Thus, a vaccine-induced Th2 response leads to an unnatural and sub-optimal Th2 reaction during later influenza infection (A. Huckriede, unpublished observations). If this would hold true for the human situation, special care should be taken to induce the appropriate immune response in individuals who are immunologically naïve for the antigen in question. These observations indicate that it is highly important to learn more about the relevance of the immune phenotype in humans. Clinical evaluation of influenza vaccines should therefore include determination of immunoglobulin subclasses and/or Th cell profiles.

Regarding current knowledge, influenza vaccines should activate DC to secrete Th1-skewing cytokines like type I IFNs and IL-12. In this light, vaccines based on intact virus particles are interesting, as they contain ssRNA as an intrinsic natural adjuvant with Th1-skweing properties. Live attenuated vaccines in use in the US and the former Soviet Union and recently approved in Europe fall into this category. However, such vaccines have raised safety concerns related to the possibility of reassortment between vaccine strain and circulating strains. WIV vaccines can be an alternative, provided that reactogenicity, associated with early WIV vaccines, can be controlled. Post-marketing surveillance is now possible for the 2009 H1N1 WIV vaccine Celvapan® and should shed light on this issue.

Our knowledge about innate immunity and the interaction between innate and adaptive immune responses has made enormous progress during the last 10 years. Moreover, much has been learned about influenza vaccines since the threat of an H5N1 pandemic boosted research activities. Implementation of this knowledge will be decisive for the development of safe and highly efficacious influenza vaccines needed to control seasonal influenza in an aging population and future influenza pandemics.

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