Methods in Molecular Biology 1371

Springer Protocols

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Ignacio Anegon *Editors*

Suppression and Regulation of Immune Responses

Methods and Protocols
Volume II

Humana Press

METHODS IN MOLECULAR BIOLOGY

Series Editor
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School of Life and Medical Sciences
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Edited by

Maria Cristina Cuturi and Ignacio Anegon

INSERM UMR 1064-ITUN, Nantes, France

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ISSN 1064-3745 ISSN 1940-6029 (electronic)
Methods in Molecular Biology
ISBN 978-1-4939-3138-5 ISBN 978-1-4939-3139-2 (eBook)
DOI 10.1007/978-1-4939-3139-2

Library of Congress Control Number: 2010936185

Springer New York Heidelberg Dordrecht London

Printed on acid-free paper

Humana Press is a brand of Springer
Springer Science+Business Media LLC New York is part of Springer Science+Business Media (www.springer.com)

Dedication

Al Pepe Mujica, ejemplo de explorador de nuevos caminos.

Preface

New Roads for Immune Tolerance: Finding the Way

Caminante no hay camino, se hace camino al andar.

Antonio Machado

This book describes new ways to induce immune immunoregulation and tolerance. The epigraph underlines that the roads taken in research are not defined in advance and unwind whilst moving ahead. This may seem obvious but underscores some interesting points of present research. One is that basic research, without a final goal or a given objective but rather curiosity-driven, needs to be preserved and stimulated as a way to find new paths of progress. Another point is the need to have a very open mind to options that emerge during research and find the one that may seem the most appropriate to choose. Finally, once on the road to immunoregulation or immune tolerance as a goal, the trail may be long winded and dangerous.

The aim of this book is a guide to finding roads, walking along them whilst making choices finding objectives in immunoregulation and immune tolerance research and avoiding making the same mistakes. These aims are based on recent developments on cells and molecules of the immune system and their interactions. This volume II on "Suppression and Regulation of Immune Responses" follows the one published in 2009. All of the chapters of this volume II are entirely new and describe research on new areas from researchers with worldwide recognition and extensive experience in their topics.

We believe that the book will be of interest and inspirational for new research in immune tolerance, and we hope that future readers will find it useful. We are very grateful to the authors that made this book possible; they are the intrepid travelers on these research roads.

Nantes, France

Maria Cristina Cuturi
Ignacio Anegon

Acknowledgments

We gratefully acknowledge Mrs. Brenda Evans for her very efficient secretarial assistance.

We also acknowledge support by the project IMBIO-DCs from de Région Pays de la Loire, the Labex IGO project (n° ANR-11-LABX-0016-01) funded by the "Investissements d'Avenir" French Government program, managed by the French National Research Agency (ANR) and by the project Institut Hospitalo Universitaire-Centre Européen des Sciences de la Transplantation et d'Immunothérapie (CESTI) funded by the "Investissements d'Avenir" French Government program (n° ANR-10-IBHU-005) as well as by Nantes Metropole and the Pays de la Loire Region, the ONE Study funded by the European Union FP7 program and the Fondation ProGreffe.

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Part I

New Molecules and Cellular Mechanisms of Immunoregulation

Chapter 1

HLA-G as an Inhibitor of Immune Responses

Aifen Lin and Wei-Hua Yan

Abstract

HLA-G is a nonclassical human leukocyte antigen (HLA) class I molecule which plays important tolerogenic functions in various physiological and pathological situations such as fetus and transplant acceptance, and immune escaping of virus-infected and malignant cells. Here we describe a method, which allows for studying cell surface expression of HLA-G using specific antibodies with flow cytometry analysis.

Key words HLA-G, Flow cytometry, Antibody

1 Introduction

Human leukocyte antigen G (HLA-G) is a nonclassic HLA class I molecule that was initially observed to be restricted to the fetal-maternal interface on the extravillous cytotrophoblasts [1]. Unlike classical HLA class I antigens, HLA-G featured with limited polymorphism, restricted tissue distribution, slow turnover, limited peptide diversity, immunosuppressive properties, and seven isoforms, which include four membrane-bound (HLA-G1, -G2, -G3, and -G4) and three soluble isoforms (HLA-G5, -G6, and -G7) [2, 3].

The biological function of HLA-G is through binding to its receptors including ILT-2/CD85j, ILT-4/CD85d, KIR2DL4/CD158d, CD8, and CD160 expressed on different types of cells [4]. KIR2DL4 is expressed on NK cells, ILT-2 is expressed on B lymphocytes, some T lymphocytes and NK cells, and all dendritic cells and monocytes, ILT-4 is expressed by monocytes, dendritic cells, and recently reported in neutrophils. The CD8 is predominantly expressed on the surface of cytotoxic T cells, but can also be found on natural killer cells, CD160 is expressed by cytotoxic CD8+ T cells and NK cells, and a small proportion of CD4+ T cells [5]. By binding receptors expressed on various cells and the pathway of trogocytosis, HLA-G could inhibit the cytolytic function of NK cells and T lymphocytes, the alloproliferative response of CD4+ T cells, the ongoing proliferation of T cells and NK cells,

Maria Cristina Cuturi and Ignacio Anegon (eds.), *Suppression and Regulation of Immune Responses: Methods and Protocols, Volume II*, Methods in Molecular Biology, vol. 1371, DOI 10.1007/978-1-4939-3139-2_1,

the maturation of dendritic cells, the Ig secretion of B lymphocyte and phagocytosis of neutrophils, and induces regulatory cells [6–9]. In the context of clinical aspects, the significance of HLA-G expression has been extensively investigated in the fetal-maternal immune tolerance, the acceptance of solid organ transplants, and the immune escape of tumors and virus-infected cells [10].

In this chapter, we provide the protocol for a sensitive, quantitative, and simple method to measure cell surface expression of HLA-G on human cells with flow cytometry (or fluorescence-activated cell sorting, FACS). Please note that this protocol will allow detecting the certain types of HLA-G isoforms only according to the specificity of anti-HLA-G antibodies [11]. Other methods for the detection of HLA-G and HLA-G isoforms are available, such as quantitative RT-PCR, immunohistochemistry and Western blotting; however, these are not covered in this chapter.

2 Materials

Prepare all solutions using ultrapure water (prepared by deionized water to attain an electrical resistivity of 18 MΩ cm at 25 °C) and analytical or higher grade reagents. Store all reagents at 4 °C (unless indicated otherwise). Follow institutional guidelines for waste disposal.

2.1 Buffers

1. Cell detaching buffer: PBS (1×) containing 10 mM EDTA pH 8.0. We do not recommend using trypsin to detach adherent cells because it may strip/cleave the molecules expressed on the cell surface.
2. FACS buffer: PBS (1×) containing 1 % BSA and 10 mM EDTA (PBS-BSA) pH 8.0. Sterile filter solution and store at 4 °C or aliquot and freeze at –20 °C.
3. Cell fixing buffer: PBS (1×) containing 0.5 % paraformaldehyde (PBS-PFA). Store at room temperature.

2.2 Antibodies

1. We use anti-human HLA-G mouse monoclonal antibody MEM-G/9 (Exbio, Prague, The Czech Republic). This antibody targets native form of human HLA-G1 on the cell surface as well as with soluble HLA-G5 isoform in its beta2-microglobulin-associated form. For more information on the differences between anti-HLA-G antibodies please *see* **Note 1**.
2. If non-labeled primary antibody is to be used, subsequent staining with an appropriate fluorochrome-conjugated secondary antibody is required.

2.3 Other Materials and Equipment

1. 96-Well plates (U- or V-bottom) with lids or adhesive film.
2. Variable volume pipettes.

3. Sterile tips.
4. Centrifuge.
5. Flow cytometer.
6. Ice.

3 Methods

Grow cells as per protocol and treat cells as required for your experiment (*see* **Note 2**). We do not recommend using cells that are overgrown or exhibit excessive cell death (*see* **Note 3**). This protocol allows for detection of HLA-G expression on both adherent (such as JEG-3 cells) and on non-adherent cells (such as K562 cells). If adherent cells are used for the analysis of HLA-G expression, begin with **step 1** of the protocol and if non-adherent cells are used, omit **steps 1** and **2** below and begin directly with **step 3**. Prepare all buffers and solutions before starting the experiment. To be able to analyze the data obtained one needs to prepare and assess controls along with the experimental staining. The controls we routinely use are listed in Table 1.

1. Wash cells once in sterile 1× PBS by adding 10 ml sterile PBS to T75 flask and gently tap the flask. Be careful not to detach the cells at this point. Aspirate the PBS by decanting or by removing with a pipet.
2. After removing the PBS used for washing, add 5–10 ml of the detaching solution to T75 flask of cells, swirl slowly around so that all cells are covered with the detaching solution, and incubate for 5–10 min at 37 °C in incubator until the solution becomes cloudy.
3. Collect the detached cells or the non-adherent cells using a pipet and transfer into a fresh tube, such as a 15 or 50 ml conical centrifuge tube. Some cells may not detached completely just by incubation in detaching solution; detachment can then

Table 1
Suggested negative/positive controls for the detection of HLA-G expression by flow cytometry

Type of control	Notes
Unstained cells	To check for dead cells using FCS/SSC plots
Cells stained with secondary antibody or isotype control antibody	Control for nonspecific binding of secondary or isotype controls to cells of interest
Cells that does not express HLA-G (if desired)	Control for nonspecific binding of anti-HLA-G antibody
Cells that express HLA-G (if desired)	Control for specific binding of anti-HLA-G antibody

often be supported by gently tapping the side of the culture flask against one hand several times.

4. Resuspend cells with ice-cold PBS-BSA and adjust cell concentration to 2×10^6 cells/ml using hemocytometer or other suitable method.
5. Transfer 50 μl cell suspension per well in 96-well plate (U- or V-bottom). Prepare as many wells as you need to perform the experiment including all non-stained and/or isotype controls and/or secondary antibody only and/or positive controls (*see* Table 1).
6. Wash the cells once in 200 μl ice-cold 1× PBS by centrifugation at 300 × *g* for 5 min at 4 °C. Remove the supernatant after each wash by quickly flicking the plate upside down over a sink, and then carefully tapping it on clean paper towels to remove the remaining liquid.
7. While washing, prepare antibody solution by mixing ice-cold FACS buffer with the FITC-MEM-G/9 and isotype control (5 μg/ml final concentration) (*see* **Notes 4** and **5**).
8. Add 100 μl of the diluted antibody to each well and incubate with cells for 30–60 min at 4 °C. The final volume of the mix we typically use is 150 μl per well, but this could be scaled to from 50 to 250 μl if desired. *Do not* add the antibody to cells that are to be used as secondary and unstained cells control. Controls included negative (no stain added), isotype control (with similarly labeled, nonspecific primary antibody), and positive cell controls.
9. Pellet the cells by centrifugation (spin at 300 × *g* for 5 min at 4 °C).
10. Wash twice as in **step 6**. The samples are now ready for FACS analysis and you can proceed immediately to **steps 15** and **16**.
11. If you have used a non-labeled anti-HLA-G antibody in **steps 7** and **8** (indirect staining), dilute the secondary FITC-labeled anti-mouse IgG antibody to 0.25 μg/ml (or 1/100 from the stock) in FACS buffer. Vortex to mix.
12. Add the secondary FITC-labeled antibody to wells containing cells stained with the anti-HLA-G antibody and washed twice with 1× PBS. Also add the secondary FITC-labeled antibody to at least two wells containing isotype control-incubated and non-stained cells; these will be used as isotype control and a secondary antibody only control staining. *Do not* forget to leave some wells without any antibody addition as non-stained cell control wells.
13. Incubate for 20 min in the dark at 4 °C.
14. Wash twice as in **step 6**.

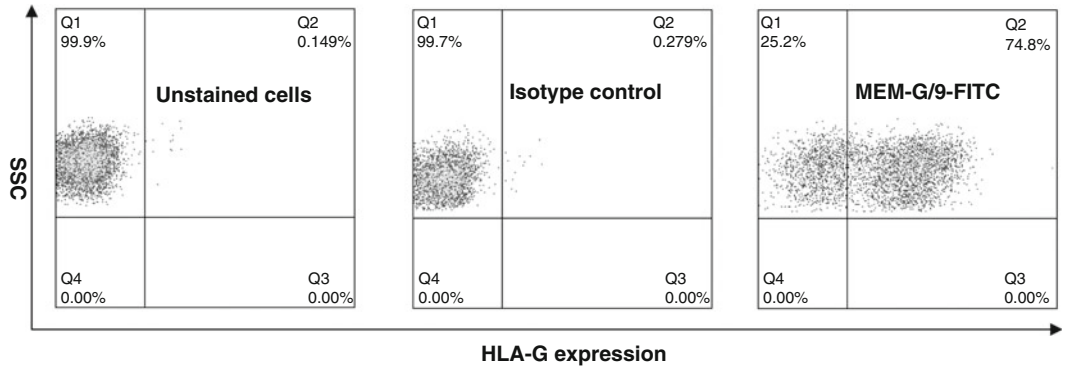

Fig. 1 Flow cytometry analysis of HLA-G expression on the surface of K562-G1 cells

15. If cells are to be analyzed the same day they can be resuspended with 300 μl FACS buffer and transferred cells to Falcon tubes (5 ml polystyrene round-bottom tube, 12 × 75 mm) and kept at 4 °C in the dark. Alternatively cells can be fixed with cell fixing buffer and stored in the fridge for 2–3 days prior to analysis.
16. Analyze on a suitable flow cytometer (e.g., FACS Calibur, FACS Canto II) acquiring the non-stained cells control first, then the secondary/isotype antibody controls, and the HLA-G-stained cells. Example FACS plots for HLA-G expression in K562-G1 cells (exogenous HLA-G1 expression in K562 cells) [13], normal peripheral blood CD14+ monocytes, and CD3+ T lymphocytes are shown in Figs. 1 and 2, respectively (*see* **Note 6**).

4 Notes

1. Excellent anti-HLA-G antibodies with HLA-G isoform specificity, that generate strong signals during flow cytometry analyses, can be purchased from most major vendors such as Exbio, Abcam, BD Biosciences, etc. There are six HLA-G isoform-specific antibodies that are often used by researchers in flow cytometry analysis to address the expression and functional of HLA-G in various conditions.

   MEM-G/9 reacts with HLA-G1 on the cell surface as well as with soluble HLA-G5 isoform in its beta2-microglobulin-associated form. This antibody is the standard reagent thoroughly validated during 3rd International Conference on HLA-G (Paris, 2003). 01G (IgG1) and MEM-G/11 (IgG1) recognize HLA-G1, but not soluble forms. 2A12 (IgG1) and 5A6G7 (IgG1) recognize HLA-G5 and HLA-G6 isoforms but not HLA-G1 isoform. 87G (IgG2a) recognizes both membrane-bound and soluble forms of HLA-G (HLA-G1 and HLA-G5) and can block the interaction

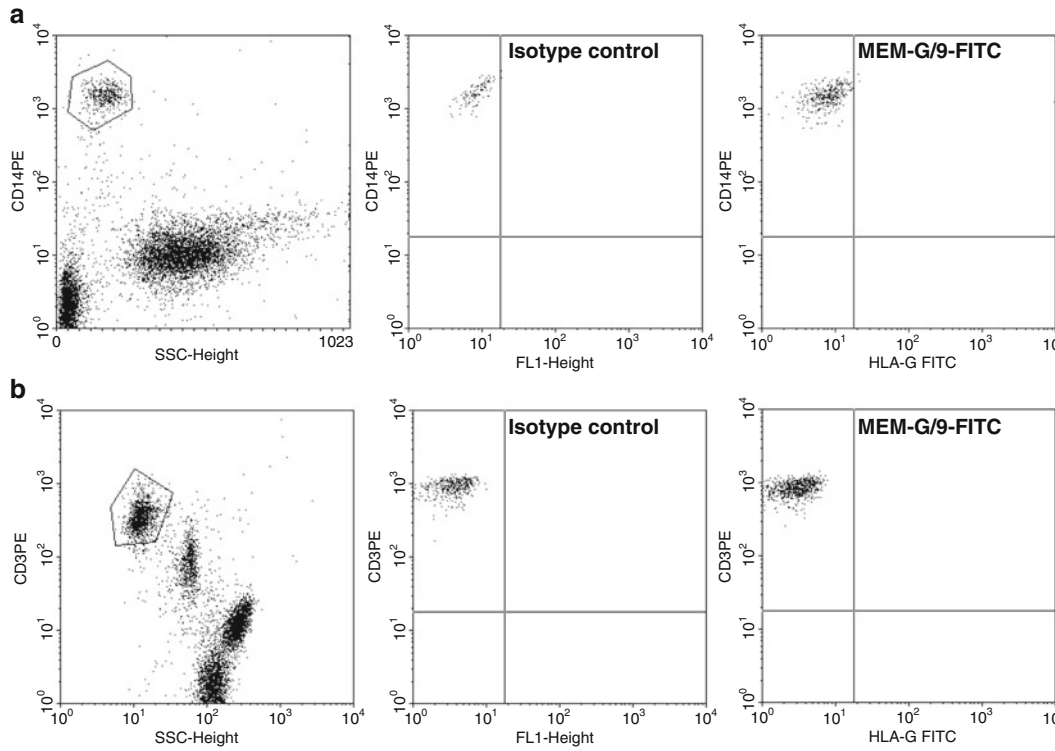

Fig. 2 Flow cytometry analysis of HLA-G expression in normal peripheral blood cells. (**a**) A representative flow cytometry diagram shows CD14+ monocytes gated by CD14 and its HLA-G expression. (**b**) A representative flow cytometry diagram shows CD3+ T lymphocytes gated by CD3 and its HLA-G expression

of HLA-G with inhibitory receptors, and thus can be used in functional assays evaluating the role of HLA-G [11, 12].

2. Grow cells as per protocol. Replace the media on the day before experiment. If cells growing in suspension are used skip **steps 1** and **2**. If adherent cells are to be analyzed start from **step 1**.
3. Note that dead cells can bind antibodies nonspecifically and often give false-positive results in flow cytometry analysis. It is therefore advisable to use cultures with a high proportion of viable cells. Cell viability can be assessed by Trypan blue staining before cell staining. Further, the forward scatter (FSC) vs. sideward scatter (SSC) pattern of the cells during FACS analysis after the FACS measurement of HLA-Gexpression staining procedures will give a second clue about the condition of the cells (dead cells have a distinctive FSC/SSC pattern).
4. Both primary and secondary antibodies must be tittered on known HLA-G-positive (Choriocarcinoma cell line JEG-3, ATCC Number: HTB-36) and HLA-G-negative (Choriocarcinoma cell line JAR, ATCC Number: HTB-144) cell populations prior to use in actual experiments.

5. The final concentration of the staining antibody and the isotype control should be the same. Optimal antibody concentrations are determined by titration. Always run in parallel an isotype-matched control antibody.
6. For two- and three-color analysis, compensation controls must be run to compensate for spectral overlap. These consist of one sample each stained with each fluorescent reagent separately and a control containing both colors; for example if two-color analysis is performed with FITC and phycoerythrin (PE) then samples stained with FITC alone and PE alone and a sample certain to be positive for both colors should be run.

Acknowledgements

This work is supported by grants from National Natural Science Foundation of China (31170879, 31370920, 81372247), Zhejiang Provincial Natural Science Foundation of China (LR13H160001), and by Zhejiang Provincial program for the cultivation of high-level innovative health talents.

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Chapter 2

New Molecular and Cellular Mechanisms of Tolerance: Tolerogenic Actions of IL-2

Louis Pérol and Eliane Piaggio

Abstract

Interleukin-2 (IL-2) is an old molecule with brand new functions. Indeed, IL-2 has been first described as a T-cell growth factor but recent data pointed out that its main function in vivo is the maintenance of immune tolerance. Mechanistically, IL-2 is essential for the development and function of $CD4^+$ $Foxp3^+$ regulatory T cells (T_{reg} cells) that are essential players in the control of immune responded to self, tumors, microbes and grafts. T_{reg} cells are exquisitely sensitive to IL-2 due to their constitutive expression of the high affinity IL-2 receptor (IL-2R) and the new paradigm suggests that low-doses of IL-2 could selectively boost T_{reg} cells in vivo. Consequently, a growing body of clinical research is aiming at using IL-2 at low doses as a tolerogenic drug to boost endogenous T_{reg} cells in patients suffering from autoimmune or inflammatory conditions. In this manuscript, we briefly review IL-2/IL-2R biology and the role of IL-2 in the development, maintenance, and function of T_{reg} cells; and also its effects on other immune cell populations such as $CD4^+$ T helper cells and $CD8^+$ memory T cells. Then, focusing on type 1 diabetes, we review the preclinical studies and clinical trials supporting the use of low-doses IL-2 as a tolerogenic immunotherapy. Finally, we discuss the limitations and future directions for IL-2 based immunotherapy.

Key words Interleukin-2, Immune tolerance, Immunotherapy, Autoimmunity, Regulatory T cells

1 Introduction

Interleukin-2 (IL-2) was the first cytokine to be identified, even before IL-1. In the 70s, the groups of Robert Gallo and Kendall Smith showed that activated T cells produced a soluble T-cell growth factor that allowed the long-term maintenance of T cells in vitro [1, 2]. The anecdote is that it was named IL-2, because Smith's experiments demonstrated that it was IL-1, produced by activated macrophages that induced IL-2 production by the activated T cells [3]. In 1983, the IL-2 gene was cloned by Tadatsugu Taniguchi's team [4] and its crystal structure was solved in 1992 [5]. IL-2 is an old molecule with brand new functions. Indeed, even if IL-2 has been administered for more than 20 years to cancer patients, based on its capacity to boost the immune response, recent results have pointed out that IL-2 can have impressive

Maria Cristina Cuturi and Ignacio Anegon (eds.), *Suppression and Regulation of Immune Responses: Methods and Protocols, Volume II*, Methods in Molecular Biology, vol. 1371, DOI 10.1007/978-1-4939-3139-2_2,

tolerogenic actions. This novel aspect has led researchers and clinicians to consider the potential of IL-2 as an immunosuppressor. Here, we will review the basic aspects of IL-2 biology, the contrasting effector and tolerogenic actions of the cytokine and we will focus on the lessons learned from mice and humans on the therapeutic use of low-dose IL-2 as inducer of immune tolerance.

1.1 The Biology of IL-2 and IL-2 Receptor (IL-2R)

1.1.1 IL-2

IL-2 is a 15,000 Da alpha-helical cytokine mainly produced by $CD4^+$ and also $CD8^+$ T lymphocytes after activation. In addition, IL-2 can also be produced at low levels by γδ T lymphocytes and by NKT cells after activation via their endogenous ligands [6, 7]. Finally, activated dendritic cells and mastocytes are also able to produce IL-2 [8, 9], although the precise relevance of this phenomenon remains to be elucidated. Upon activation, naïve T lymphocytes produce large amounts of IL-2 in a rapid and transient fashion. Indeed, different TCR signaling downstream molecules such as AP-1, NF-κB/p65, and NFAT are translocated into the nucleus and form a complex at the promoter of the *Il2* gene, inducing its transcription [10]. The engagement of co-stimulatory molecules on T lymphocytes is required for optimal IL-2 production, as it stabilizes IL-2 mRNA [11]. Then, IL-2 transcription is rapidly shutdown by termination of the *Il2* gene transcriptional activity and degradation of the IL-2 mRNA. Importantly, a negative feedback loop finely regulates IL-2 production: IL-2 acts in an autocrine way, inducing the expression of the transcription factor Blimp-1, a transcriptional repressor of *Il2* transcription [12, 13]. The importance of this negative feedback loop is shown by the observation that Blimp-$1^{-/-}$ T cells produce more IL-2 and that Blimp-$1^{-/-}$ mice die prematurely of an autoimmune syndrome marked by uncontrolled T lymphocyte activation [14, 15].

The rapid and transient nature of IL-2 production suggests that IL-2 mainly acts in an autocrine or paracrine fashion, therefore preventing widespread activation of the immune system. Furthermore, due to a very short half-life [16], IL-2 is highly difficult to detect in the serum of healthy individuals, even if non-negligible numbers of IL-2-producing T cells can be detected by flow cytometry. One hypothesis is that IL-2 is retained within the extracellular matrix in a biologically active form [17–19].

1.1.2 IL-2R

The IL-2R is composed of three non-covalently associating subunits: the alpha (IL-2Rα, CD25), the beta (IL-2Rβ, CD122), and the common cytokine receptor gamma (IL-2Rγ_c, CD132) chains.

Of note, the CD122 subunit is also a part of the IL-15R, along with IL-15Rα and the γ_c chains; and the CD132 subunit is common to the receptor complexes for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21.

The IL-2R exists in different forms, which display different affinities for IL-2 and different cellular repartitions. First, the

dimeric receptor composed of the IL-2Rβ and IL-2Rγ_c chains, forms the intermediate affinity IL-2R (dissociation constant (K_d) = 10^{-9} M). While the IL-2Rγ_c subunit is ubiquitously expressed on immune cells, the IL-2Rβ chain is expressed at high levels on NK cells, memory CD8$^+$ T cells and at lower levels on resting T cells, B cells and different innate immune cells such as dendritic cells, endowing them with the capacity to respond to IL-2 through this intermediate affinity IL-2R. Then, the association of the IL-2Rα to the intermediate affinity IL-2R forms the high affinity IL-2R ($K_d = 10^{-11}$ M). Importantly, CD25 by itself can bind IL-2 with low affinity ($K_d = 10^{-8}$ M) but does not induce any intracellular signal [20]. Crystallographic data revealed that CD25 binding to IL-2 is the first event in IL-2R formation, subsequently allowing the recruitment of CD122 and γ_c [21]. In addition, the binding of IL-2 to CD25 modifies the quaternary structure of IL-2, stabilizing it in its high affinity receptor [22]. The expression pattern of the high affinity IL-2R is much more restricted, with only regulatory T cells (T_{reg} cells) and type 2 innate lymphoid cells (ILC2) expressing it constitutively, making those extremely sensitive to IL-2. In addition, conventional dendritic cells express low levels of CD25 at steady state and could trans-present IL-2 to CD122/CD132 expressing naïve T cells, enhancing their activation [23]. Finally, CD4$^+$ Foxp3$^-$ effector T cells (T_{eff} cells), CD8$^+$ T cells but also B cells and NK cells transiently upregulate CD25 expression after stimulation with their respective ligands, giving them the ability to respond to low-doses IL-2.

Mechanistically, the α chain does not participate in signaling, but both the intermediate and high affinity IL-2Rs are functional as they induce the trans-phosphorylation of the intracellular domains of CD122 and CD132, allowing the recruitment of different intracellular signaling proteins. Namely, three main canonical pathways are activated after the interaction of IL-2 with its receptor: the JAK3/STAT5 pathway, the PI3K/Act/motor pathway and the MAPK pathway [24].

1.2 Effector Functions of IL-2

Besides its tolerogenic actions that are the subject of this review, one should not forget that historically IL-2 has been first described to play an important role in the homeostasis of different effector immune cells and particularly CD8$^+$ T cells.

1.2.1 IL-2 Actions Beyond CD4$^+$ T Cells

Indeed, after a viral infection, all activated antigen-specific CD8$^+$ T cells rapidly gain CD25 expression and upregulate CD122, making them able to respond to autocrine and paracrine IL-2 [25]. After a few days, CD25 becomes bimodal with only the short-lived effector cells maintaining high CD25 expression and the long-lived memory cells losing it, suggesting an important role of IL-2 in the differentiation of effector CD8$^+$ T cells [25]. In accordance, bone marrow chimera experiments then demonstrated that CD25$^{-/-}$ CD8$^+$ T cells fail to differentiate in fully competent

effector $CD8^+$ T cells, as they are present in reduced numbers and have less cytotoxic properties than their wild type counterparts [26–28]. Identical results were obtained with $CD122^{-/-}$ [29] and $STAT5^{-/-}$ mice [30]. Finally, even if the maintenance of memory cells in the host seems to mainly rely on IL-15 and not on IL-2 [31, 32], the presence of IL-2 during the priming of $CD8^+$ T cells is required for the normal expansion of memory $CD8^+$ T cells after a secondary infection [26–28].

In addition, as stated above, other cellular subsets such as T_{eff} cells, NK and B cells express the IL-2R at steady state or after activation. However, the extent of the effect of IL-2 in the homeostasis of these cells is not fully understood. For instance, the homeostasis of NK cells is strictly dependent on IL-15. Indeed, although both IL-15 and IL-2 signal via the intermediate affinity IL-2R, IL-2 is not able to compensate the profound NK defect observed in $IL\text{-}15^{-/-}$ and $IL\text{-}15Ra^{-/-}$ mice [33, 34]. Confirming those results, NK cell responses are only slightly reduced in $IL\text{-}2^{-/-}$ mice [35]. A recent study suggests that immature $CD127^+$ NK cells express low levels of CD25 and that IL-2 is necessary for the optimal expansion of this particular NK subset after an infection or in cancer [36]. Importantly, as NK cells express 10–100 times more CD122 than T lymphocytes, it makes sense that even low doses of IL-2 will boost NK cells in vivo [37]. The group of B. Bielekova demonstrated the heterogeneous pattern of CD122 distribution among human NK cells. Indeed, they showed that $CD56^{bright}$ "immunoregulatory" NK cells express ten times more CD122 than $CD56^{dim}$ NK cells, making them more sensible to IL-2 [38]. Also, B cells express the high affinity IL-2R after activation [39] and several studies reported that IL-2 can enhance B cell proliferation [39–41]. Furthermore, the frequencies and numbers of γδ T cells and intra-epithelial CD8αα T lymphocytes are reduced in $IL\text{-}2^{-/-}$ and $CD122^{-/-}$ mice, suggesting a role of IL-2 in their homeostasis [42–45]. Recent data demonstrated that type 2 innate lymphoid cells (ILC2) constitutively express the high affinity IL-2R [46, 47]. The contribution of IL-2 to the homeostasis of ILC2 remains to be determined, along with the contribution of ILC2 in the tolerogenic properties of IL-2. Recent results demonstrated that human mature dendritic cells could respond to IL-2 because they express the high affinity IL-2R. Of note, IL-2-stimulated mature dendritic cells secrete high quantities of interferon γ (IFNγ), enhancing $CD8^+$ T-cell activation in an in vitro co-culture model [48]. Finally, it is interesting to note that IL-2 action extends beyond the immune system as fibroblasts [49], neurons [50], pancreatic β-cells [51] and pulmonary endothelial cells [52] express the IL-2R and therefore can respond to IL-2. Of note, the fact that pulmonary endothelial cells express the functional IL-2 high affinity receptor could explain the vascular leak syndrome associated to high-dose IL-2 administration in a murine model of cancer [52].

1.2.2 IL-2 Modulates the Polarization of T_{eff} Cells

In vitro, $CD4^+$ T cells purified from an IL-$2^{-/-}$ mouse poorly proliferate after engagement of their TCR [53]. In mice, the immunopathology caused by the adoptive transfer of OVA-specific T_{eff} cells in OVA expressing lymphopenic recipients is reduced when the OVA-specific T_{eff} cells come from an IL-$2^{-/-}$ deficient donor [54]. These results clearly indicate that IL-2 participates in the initial proliferation of T_{eff} cells after a TCR/costimulation signal. In addition, other groups have demonstrated that IL-2 can also modulate the polarization of recently activated T_{eff} cells. Using in vitro models of T helper (Th) polarization, they showed that the absence of IL-2 signaling favors Th17 and follicular helper T cells (Tfh) polarization while it is detrimental to Th1 and Th2 polarization. Indeed, Th1 and Th2 polarization are strikingly reduced in naïve T_{eff} cells from a STAT$5^{-/-}$ or an IL-$2^{-/-}$ deficient mouse [55–57]. Similarly, addition of IL-2 neutralizing antibodies or its soluble receptor to cell cultures prevents Th1 and Th2 polarization [55–57]. Mechanistically, IL-2 acts in two ways: it induces the expression of the IL-12RB2 (in pro-Th1 conditions) or the IL-4RA (in pro-Th2 conditions) and it increases chromatin accessibility at the *Ifng* (in pro-Th1 conditions) or the *Il4* (in pro-Th2 conditions) loci [56, 58]. On the contrary, the polarization of activated T_{eff} cells in the presence of IL-6 and TGFβ (pro-Th17 conditions) and a neutralizing anti-IL-2 antibody dramatically enhances Th17 differentiation [59]. In accordance, IL-$2^{-/-}$ and STAT$5^{-/-}$ mice present elevated numbers of Th17 cells and abnormally high concentrations of IL-17 in their serum [59]. Mechanistically, the transcription factor STAT5 (induced by IL-2 signaling) directly binds the *Il17* promoter and represses its transcription [59]. In addition, IL-2 via STAT5 reduces the expression of the IL-6R subunits that are critical for optimal Th17 polarization [56]. Finally, the absence of IL-2 signaling in vivo reduces the Tfh polarization and therefore the amplitude of the germinal center reaction [60, 61]. IL-2 acts directly on Tfh cells, via the induction of Blimp-1 that represses Bcl6, the key transcription factor of Tfh cells [62].

1.2.3 Clinical Therapies Based on High-Dose IL-2 Administration

The initial results showing that IL-2 was an essential T-cell growth factor and that it could stimulate the effector functions of NK cells encouraged its administration in patients to boost effector immune responses. Indeed, the FDA approved the administration of recombinant human IL-2 at high doses in 1992 for the treatment of metastatic renal cell cancer and in 1998 for the treatment of metastatic melanoma. In addition, high-dose IL-2 therapy showed synergy with an antitumoral vaccine in metastatic melanoma [63] and with an anti-GD2 monoclonal antibody in neuroblastoma [64]. IL-2 at high doses has also been used in the clinic to boost effector immune responses in chronic infectious diseases, mainly in HIV-infected patients [65, 66]. Yet, high-dose IL-2 therapy is very toxic and the reached efficacy is not optimal [67, 68]. Indeed, IL-2 at high-doses fails to improve the survival of HIV-infected patients

[69] and induces only 5–15 % of tumor regression in cancer patients [70]. In both cases, although high doses of IL-2 can very efficiently stimulate the effector arm of the immune system, it also boosts T_{reg} cells and thus immune tolerance [71–73]. This phenomenon likely explains the relative failure of high-dose IL-2 therapy in cancer, as illustrated by the fact that the magnitude of the T_{reg} cell increment has been negatively correlated with the clinical outcome of the treated patients [74].

1.3 The Main Function of IL-2 Is the Maintenance of Immune Tolerance

The paradigm of IL-2 as the T-cell growth factor was questioned by the generation of IL-2 or IL-2R deficient mice. Indeed, those mice are not lymphopenic as it could have been expected, but die prematurely of a lymphoproliferative autoimmune syndrome [75–77]. Different groups then showed that the immunopathology observed in IL-2 and IL-2R deficient was not due to an intrinsic defect of the T_{eff} cell compartment but to the absence of $CD4^+$ $CD25^+$ $Foxp3^+$ T_{reg} cells that are critically dependent on IL-2 for their homeostasis [78, 79]. Indeed, the adoptive transfer of T_{reg} cells rescues the immunopathology caused by IL-$2^{-/-}$, $CD25^{-/-}$ or $CD122^{-/-}$ T lymphocytes, demonstrating that the main function of IL-2 is the maintenance of immune tolerance [80–82].

Two seminal works then showed that IL-$2^{-/-}$ and $CD25^{-/-}$ mice present a substantial reduction of the T_{reg} cell frequencies and numbers in the periphery but not in the thymus, suggesting that IL-2 was mandatory for T_{reg} cell homeostasis in the periphery but not for thymic generation [83, 84]. However, $CD122^{-/-}$ mice and IL-$2^{-/-}$ IL-$15^{-/-}$ mice present an important depletion of thymic T_{reg} cells [85, 86]. In addition, thymic-restricted re-expression of CD122 in $CD122^{-/-}$ mice is sufficient to restore T_{reg} cell differentiation and to prevent the lymphoproliferation normally observed in this strain [81, 87, 88]. Consequently, CD122 signaling is required for T_{reg} cell differentiation in the thymus. At the molecular level, CD122 signaling induces STAT5 phosphorylation, the latter directly binding to the *Foxp3* gene promoter and conserved noncoding DNA sequence 2 (CNS2), enhancing its transcription [85, 89, 90]. Subsequently, the identification of the immediate precursors of thymic T_{reg} cells ($CD4^+$ $CD8^-$ $CD25^+$ $Foxp3^-$ thymocytes) allowed precise delineation of the role of CD122 signaling cytokines in the differentiation of T_{reg} cells [91]. In vitro, the culture of these precursors with IL-2 is sufficient to induce the expression of key T_{reg} cell molecules as Foxp3, GITR, or CTLA-4 [91]. However, IL-2 alone is not able to drive demethylation of the CNS2 of the *Foxp3* locus, a hallmark of T_{reg} cell stability, indicating that other factors are still required for the proper differentiation of T_{reg} cells [92]. Interestingly, IL-15 and IL-7 but not IL-4, IL-21, or TSLP (the other γ_c signaling cytokines) are also able to induce Foxp3 expression in T_{reg} cell precursors, but to a lesser extent than IL-2 [91, 93, 94]. Altogether, these results show that

IL-2 plays an essential role in the differentiation of T_{reg} cells. Indeed, IL-2 via CD122/STAT5 induces the expression of Foxp3 in the $CD4^+$ $CD8^+$ $CD25^+$ $Foxp3^-$ T_{reg} cell precursors. In the absence of IL-2, IL-15 but also IL-7 can compensate, certainly explaining the absence of thymic T_{reg} cell deficit observed in IL-$2^{-/-}$ and $CD25^{-/-}$ mice.

The key role of IL-2 in the homeostasis of T_{reg} cells outside the thymus has been extensively studied. Early work showed that T_{reg} cells fail to survive and proliferate in the periphery if they are adoptively transferred in an IL-$2^{-/-}$ host [81]. In accordance, IL-$2^{-/-}$, $CD25^{-/-}$ and $CD122^{-/-}$ mice have less T_{reg} cells in the periphery, the reduction being more important in $CD122^{-/-}$, thus suggesting that IL-15 can partially compensate for lL-2 absence [83–85]. Also, the administration of a neutralizing monoclonal anti-IL-2 antibody results in the rapid depletion of T_{reg} cells and in the appearance of various autoimmune diseases [95, 96]. IL-2 is required for the maintenance of Foxp3 protein and mRNA expression both in vitro and in vivo [95, 97–99]. As T_{reg} cells cannot produce IL-2 due to direct Foxp3-mediated repression of IL-2 transcription [100], they are crucially dependent on paracrine IL-2 and their number is indexed to the number of IL-2-producing T_{eff} cells [101, 102]. In addition, polymorphisms in *Il2*, *Cd25* or downstream adaptor genes are associated with impaired T_{reg} cell function or homeostasis and higher susceptibility to various autoimmune diseases as type 1 diabetes (T1D) or multiple sclerosis [103–107]. Mechanistically, IL-2 via STAT5 induces the anti-apoptotic molecules Bcl2 and Mcl1 and therefore prevents T_{reg} cell apoptosis [102, 108–110]. IL-2 is also important to maintain the high T_{reg} cell proliferation rate in vivo, as the transcriptome of T_{reg} cells purified from IL-$2^{-/-}$ mice reveals several metabolic and cell cycle alterations [84]. Recently, the group of D. Campbell showed that only a subset of T_{reg} cells ("central" T_{reg} cells, $CD44^{low}$ $CD62L^+$) is dependent on IL-2 for its survival, partially explaining why T_{reg} cells are not totally absent in IL-$2^{-/-}$ and $CD25^{-/-}$ mice [111]. Finally, in addition to thymic T_{reg} cells, T_{eff} cells can be educated in the periphery to become peripheral T_{reg} cells [112]. IL-2 also plays an important role in the differentiation and stability of pT_{reg} cells in vivo (for a recent review, see ref. [113]) [114–120].

T_{reg} cell suppressive function relies on multiple mechanisms such as anti-inflammatory cytokine production, direct cytotoxicity, cytokine deprivation, and inhibition of dendritic cell maturation [121]. Of note, T_{reg} cells by constitutively expressing the high affinity IL-2R, they can capture the IL-2 produced by recently activated T_{eff} cells in their vicinity, inducing their apoptosis [122]. In addition, using this IL-2-dependent suppression mechanism, T_{reg} cells restrain both $CD8^+$ T cell and NK cell immune responses [36, 123–126]. Interestingly, T_{reg} cells also promote Th17, Tfh

and memory CD8+ differentiation, as IL-2 negatively regulates the differentiation of these cellular subtypes [127–130].

To conclude, contrary to what was initially proposed, IL-2 is now viewed as a gatekeeper of immune tolerance. Thus, the aforementioned studies led to the emergence of the concept that IL-2 at low doses could represent a novel class of immunosuppressive drug, acting by specific T_{reg} expansion/activation.

1.4 IL-2 as Inducer of Immune Tolerance: Lessons Learned from Mice and Humans

Animal studies have demonstrated the potential of T_{reg} cell based cellular therapies for the treatment of autoimmune diseases [131, 132], hematopoietic stem cell transplantation [133, 134] and solid organ transplantation [135]. However, although probably feasible [136–138], translating this strategy to humans is technically challenging and not applicable to large numbers of patients. Alternatively, IL-2 administration could represent an easier and more affordable strategy to boost the patient's own Tregs compartment.

1.4.1 The Specific Case of Type 1 Diabetes

T1D is a prototypic organ-specific autoimmune disease in which the immune system destroys the insulin producing β-cells of the pancreas. At disease onset, there are still residual β-cells, offering a window for therapeutic intervention to stop the autoimmune destruction and rescue insulin production. Major knowledge on the physiopathology of T1D has been obtained from studies in the nonobese diabetic (NOD) mice [139], which shares many similarities with human disease. In these mice, T_{reg} cells control the autoimmune reaction [140–142], and adoptively transferred Tregs recognizing a pancreatic antigen can reverse T1D [131]. In this specific pathological setting, low doses IL-2 treatment could be particularly adapted. Interestingly, genome-wide association studies have shown that multiple T1D susceptibility genes are related to the IL-2 pathway: *Il2*, *Cd25*, *Cd122*, and *PTPN2*, potentially affecting T_{reg} cell homeostasis [104, 143]. Furthermore, in NOD mice, insufficient IL-2 amounts in the pancreas seem to be responsible for poor Treg survival in the islets, and the loss of the T_{reg}:T_{eff} balance, which could lead to progressive breakdown of self-tolerance [108]. Indeed, administration of low doses of IL-2 to young pre-diabetic NOD mice prevents diabetes development [108, 144] and more impressively, only 5 days of low-dose IL-2 administration to new onset diabetic NOD mice induces long-lasting disease remission [145]. These results are impressive because very few drugs are able to revert new-onset clinical diabetes [146]. The most striking feature of low-dose IL-2 therapy in NOD mice is that even though different cell types can potentially respond to IL-2, low dose IL-2 acts specifically on Tregs in the pancreas, inducing an increase of their frequency and reinforcing their function as suggested by the decrease in IFNγ production by pancreas infiltrating Teffs and CD8+ T cells. A recent randomized double blind, placebo-controlled phase I/II dose-finding clinical

trial demonstrated that IL-2 at low-doses was well tolerated in T1D patients [147]. In addition, low-doses IL-2 induced a dose-dependent increase in the frequency of T_{reg} cells, without expansion of potentially pathogenic T_{eff} cells. A precise analysis of the effect of low-doses IL-2 on glucose metabolism and on residual insulin secretion in new-onset T1D patients awaits the results of the phase II clinical trials that are currently launched (NCT01862120).

1.4.2 Low-Dose IL-2 Therapeutic Potential in Other Autoimmune/Inflammatory Situations

Besides the specific case of T1D, different studies have demonstrated that administration of low doses of IL-2 can have an important therapeutic potential in various autoimmune/inflammatory pathologies. In mice, low doses of IL-2 (alone or complexed with an anti-IL-2 monoclonal antibody that increases its half-life and redirects its action towards CD25-expressing cells (i.e., T_{reg} cells)) can reduce inflammation and improve the clinical outcome in various pathologies such as systemic lupus erythematosus [148], atherosclerosis [149], renal proteinuria [150], or solid organ graft [151]. In addition, when combined to the immunosuppressive drug rapamycin, low doses of IL-2 can strikingly reduce the gravity of experimental autoimmune encephalomyelitis [151], acute graft-versus-host disease (GVHD) [152], and allogeneic solid graft rejection [153].

Interestingly, in humans, low-dose IL-2 therapy induces a dose-dependent increase in the numbers of T_{reg} cells in the peripheral blood of patients suffering from autoimmune vasculitis secondary to infection with hepatitis C virus [154], or from active chronic GVHD refractory to glucocorticoids [155]. In the latter situation, low-dose IL-2 administration selectively acts on T_{reg} cells, increasing their survival, proliferation and thymic export, while maintaining their suppressive function [156].

1.4.3 Limitations of Low-Dose IL-2 Therapy

We have shown that in the NOD mice, therapy based in the administration of low doses of IL-2 has a long-term curative effect in only 30 % of the treated animals [145]. Improving the capacity of low-doses to revert T1D in new-onset diabetic NOD mice represents an important step before the clinical application of this therapy in patients.

Administration of higher IL-2 doses in pre-diabetic NOD mice induces a higher expansion of T_{reg} cells in the pancreas [41]. Despite this dramatic T_{reg} cell expansion, high doses of IL-2 were highly toxic and rapidly precipitated diabetes onset. Disease occurrence could be explained by the concurrent activation of pathogenic T_{eff}, $CD8^+$ and NK cells in the pancreas, which actively divided and produced IFN-γ. In the context of human therapy, these results indicated that IL-2 therapeutic window is narrow, highlighting the need to perform thorough immunomonitoring of the broad effects of IL-2 so as to determine the dose that would uniquely act on Treg and other regulatory populations.

Another strategy would be to combine low doses of IL-2 with other therapies that have shown therapeutic potential in the NOD model [157]. In the specific case of rapamycin, although the combination IL-2/rapamycin can prevent T1D onset in NOD mice [158], we showed that rapamycin abolished IL-2 curative effect [41]. Indeed, rapamycin partially counteracts the IL-2 induced T_{reg} cell expansion/activation and induces rapid glucose intolerance. Interestingly, these results may explain the recent failure of a clinical trial aiming at administrating low-dose IL-2 combined to rapamycin in patients suffering from T1D [159].

Finally, low-dose IL-2 therapeutic potential may critically depend on the underlying immunopathology. Indeed, during solid organ transplantation, complexed IL-2 can delay graft rejection only in minor-HLA mismatched [160]. On the contrary, in the very aggressive clinical scenario of HLA-mismatched transplant, IL-2 complexes slightly boost T_{reg} cells, without any clinical benefit. Similar observations have been made in the case of acute GVHD. Indeed, while old studies suggested that low-dose IL-2 alone could prevent acute GVHD [161–163], we recently reevaluated the potential of this therapy. Using different models of allogeneic or xenogeneic GVHD, we demonstrated that low doses of IL-2 do not have a specific effect on donor T_{reg} cells. Indeed, in the inflammatory context of GVHD, allogeneic T_{eff} cells and $CD8^+$ T cells upregulate CD25, endowing them with the capacity to respond to low doses of IL-2 and explaining the observed loss of T_{reg} cell specificity [164]. Interestingly, in the case of acute GVHD, combination of IL-2 with rapamycin may represent a valid approach [152].

1.4.4 Future Directions for Low-Doses IL-2 Based Immunotherapy

The results previously discussed indicate that IL-2 has a promising future as a tolerance-inducing therapy. However, its application in patients may require previous optimization to define new strategies to selectively enhance the regulatory compartment versus the effector compartment. One strategy could be to prolong low-doses IL-2 administration in order to maintain the T_{reg} cell boost in time. This could be achieved through repeated administration schemes or through the use of lentiviral vectors coding for IL-2, allowing constant release of IL-2. In this line, two groups demonstrated that the injection of an adenovirus coding for IL-2 in pre-diabetic NOD mice could prevent autoimmune diabetes, through a specific boost of endogenous T_{reg} cells [165, 166]. Another strategy could be to increase IL-2 half-life in vivo, for instance via the use of IL-2-albumin or IL-2-Fc fusion proteins, or through the administration of complexed IL-2. Indeed, when IL-2 is complexed with an anti-IL-2 antibody, depending on the antibody, this complex can redirect IL-2 action to T_{reg} cells or to cells expressing the intermediate affinity IL-2R [167]. Finally, future preclinical studies will require precise evaluation of low-dose IL-2 therapeutic potential using appropriate preclinical models, as the effect of low doses of IL-2 cannot be extrapolated from one pathology to another.

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Chapter 3

Expansion of Regulatory T Cells In Vitro and In Vivo by IL-33

Benjamin M. Matta and Hēth R. Turnquist

Abstract

Thymic-derived, regulatory T cells (Treg) represent a subset of $CD4^+$ T cells that are required for normal immune homeostasis and suppression of unwanted responses against self-antigens (Ags) that prevent autoimmunity. Their role as immune regulators and potent ability to suppress T cell responses has been the focus of intense investigations aimed at utilizing these cells therapeutically, particularly in the settings of autoimmunity and transplantation. Many methods for expanding Treg have been described; however, efforts to generate large numbers of Treg for use in vivo often compromise their suppressor function or rely on the induction of Treg rather than their expansion. Our recent studies have focused on the barrier tissue-derived cytokine IL-33, a recently described IL-1 family member. IL-33 has emerged as a multifunctional protein, with reported roles in driving potent Type 1 and Type 2 immunity, as well as facilitating profound Treg expansion in vitro and in vivo. IL-33-expanded Treg express the IL-33 receptor (R) ST2, and express classical markers associated with Treg phenotype and suppressor function. They suppress both $CD4^+$ and $CD8^+$ T cell proliferation and effector functions in vitro, and Treg expressing ST2 have been identified as important regulators of detrimental immune responses in vivo. In the present chapter, we detail methods for expanding significant numbers of Treg using IL-33 both in vitro and in vivo that may potentially be used to promote/maintain organ transplant tolerance or suppress autoimmunity.

Key words Regulatory T cells, Interleukin-33, ST2, Dendritic cells, Transplantation tolerance, Interleukin-2

1 Introduction

The name Treg is a broadly used connotation that encompasses multiple subsets of functionally suppressive $CD4^+$ T cells, each with unique phenotypic and functional characteristics. The most widely characterized and studied are thymic-derived, $CD4^+$ T cells that undergo the T cell selection process and are released into the periphery. These cells express the transcription factor Forkhead box P 3 (Foxp3) and constitutively express high levels of the high-affinity IL-2R, CD25. A mutation in the Foxp3 gene in mice results in a disorder that closely resembles the fatal disorder IPEX (immune dysregulation, polyendocrinopathy, enteropathy, and

Maria Cristina Cuturi and Ignacio Anegon (eds.), *Suppression and Regulation of Immune Responses: Methods and Protocols, Volume II*, Methods in Molecular Biology, vol. 1371, DOI 10.1007/978-1-4939-3139-2_3,

X-linked inheritance) in humans and revealed a critical role for Foxp3 in the reported suppressive function of $CD4^+CD25^+$ regulatory T cells that control immune homeostasis and suppress autoimmunity [1–3]. Given their potent ability to suppress T cell responses, intense investigation has centered on harnessing these cells for therapeutic applications, particularly in the settings of transplantationand autoimmunity [4–6]. Many protocols have been described for expanding Treg, including polyclonal and Ag-specific cells [7]. Likewise, significant effort has been made to maintain or enhance their suppressor function, especially in the presence of inflammatory stimuli that could counter their regulatory mechanisms [8].

High constitutive expression of CD25 by Treg indicates that they rely heavily on IL-2 [9] and accordingly, culture of $CD4^+CD25^+$ T cells with IL-2 and TCR stimulation has been used as a method of Treg expansion in vitro [10]. Our recent published findings have revealed that IL-33, a pleiotropic cytokine with inflammatory and regulatory functions, stimulates IL-2 secretion by $CD11c^+$ DC (Fig. 1; [11]) that supports robust Treg expansion (Fig. 2; [11]). Additionally, IL-33 acting on DC in vitro results in selective expansion of IL-33R/$ST2^+$ Treg over $ST2^-$ Treg, as well as $Foxp3^-$ effector T cells (Fig. 2c; [11]). Administration of IL-33 in vivo also significantly expands Treg (Fig. 3; [11]) that suppress T cell responses (Fig. 4; [11]) and critically mediate fully MHC-mismatched experimental cardiac allograft survival [12, 13]. IL-33-expanded $ST2^+$ Treg express classical Treg phenotypic markers, and have recently emerged as key regulators of immune function [14]. Thus, the ability to selectively expand this unique Treg subset for potential therapeutic application is an important immunologic tool and is described in detail herein.

The observation that IL-33 expands Treg [11–13] was an important finding that added to the known pleiotropic functions

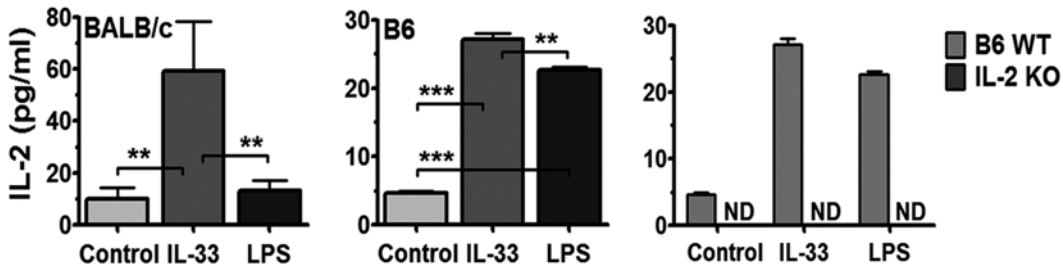

Fig. 1 IL-33 stimulates IL-2 secretion by $CD11c^+$ BMDCs. BALB/c, B6 wild-type (WT) or IL-$2^{-/-}$ bone marrow (BM)-derived $CD11c^+$ DC were generated in 7-day culture and cultured for 18 h in medium alone or supplemented with IL-33 (20 ng/ml) or LPS (100 ng/ml). Supernatants were harvested for cytokine quantitation by ELISA. Data are representative of $n=4$ (LPS) or $n=5$ (control and IL-33). ND = Not detectable, $^{**}p<0.01$, $^{***}p<0.001$. Figure and legend were modified and reproduced with permission from *The JournalofImmunology* [11]. (Copyright 2014. The American Association of Immunologists, Inc.)

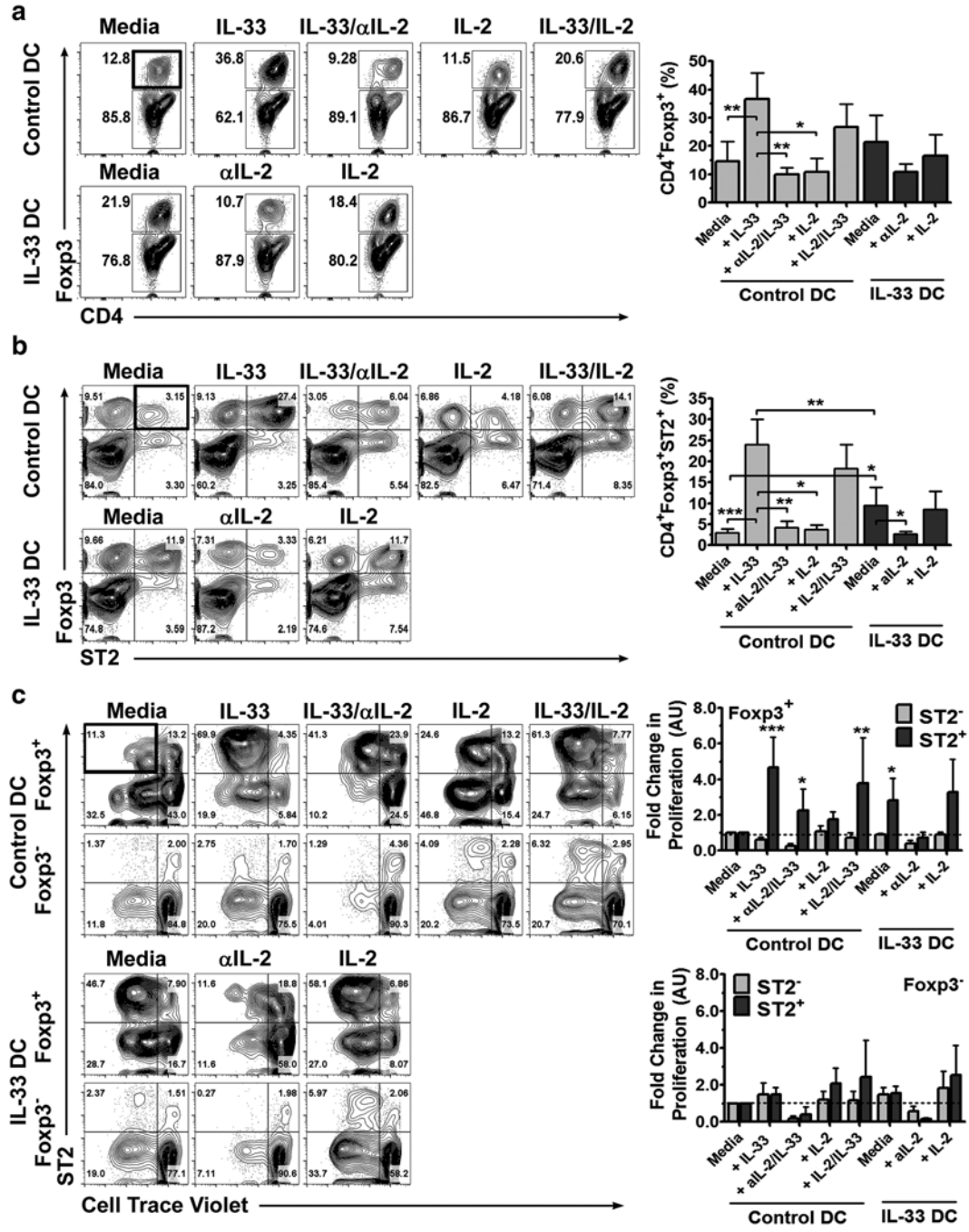

Fig. 2 CD11c⁺ BMDC-derived IL-2 promotes selective expansion of ST2⁺ Treg following IL-33 exposure. CD11c⁺ BALB/c BMDC were cultured overnight in media alone or supplemented with IL-33 (20 ng/ml). BMDC were cultured in MLR with Cell Trace Violet (CTV)-labeled B6 Foxp3-IRES-mRFP (FIR) CD4⁺ T cells for 5 days. Some wells were supplemented with IL-33 (10 ng/ml), neutralizing IL-2 antibody (10 μg/ml), rhIL-2 (50 U/ml), or a combination as indicated. After 5 days, cells were harvested and stained for flow cytometric analysis. Representative flow plots and an average of (**a**) Foxp3 and (**b**) Foxp3 and ST2 expression on CD4⁺-gated cells or (**c**) ST2 expression vs. CTV on CD4⁺ Foxp3⁺ (*top panels*) and CD4⁺Foxp3⁻ (*bottom panels*) cells. Results in were averaged from $n=4$ independent experiments. *Black boxes* on flow plots indicate populations used to generate corresponding graphs. *AU* Arbitrary Units for fold change reported in graphs in (**c**). *$p<0.05$, **$p<0.01$, ***$p<0.001$. Figure and legend were modified and reproduced with permission from *The Journal of Immunology* [11]. (Copyright 2014. The American Association of Immunologists, Inc.)

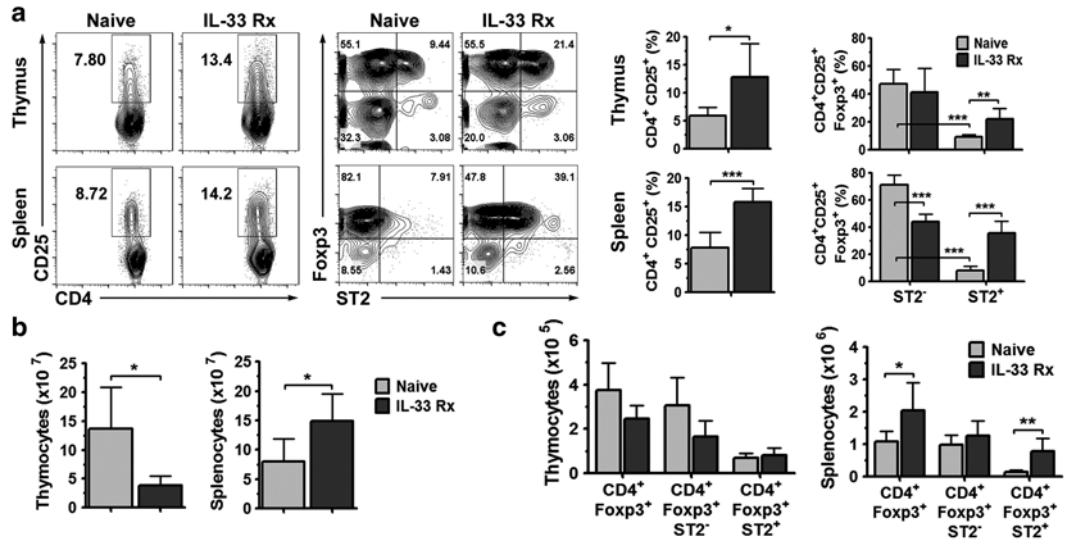

Fig. 3 IL-33 administration expandsan ST2$^+$ subset of CD4$^+$CD25$^+$Foxp3$^+$ T cells originating in the thymus. (**a**) Representative flow cytometry plots and frequency of CD4$^+$CD25$^+$ and CD4$^+$CD25$^+$Foxp3$^+$ cells from CD3$^+$CD4$^+$-gated total thymocytes or splenocytes from naïve or IL-33-treated (IL-33 Rx) C57BL/6J (B6) mice. (**b**) Absolute number of total cells and (**c**) indicated cell populations in the thymus and spleens averaged from $n=5$ mice. $^*p<0.05$,$^{**}p<0.01$,$^{***}p<0.001$. Figure and legend reproduced with permission from *The Journal of Immunology* [11]. (Copyright 2014. The American Association of Immunologists, Inc.)

already ascribed to this IL-1 family cytokine [15]. IL-33 was initially found to promote potent Type 2 responses [16] through actions on ST2-expressing immune cells [17]. Through stimulation of IL-4, IL-5, and IL-13 production, these downstream effects of IL-33 are important in protective responses during parasitic infection [18], but conversely can severely exacerbate disease pathology associated with Type 2 responses [15]. It was later discovered that IL-33 could also act synergistically with proinflammatory stimuli, such as IL-12, to drive robust Type 1 immune responses in CD8$^+$ T cells [19], and NK and NKT cells [20], and stimulate potent antiviral immunity [21].

With the current use of Treg therapy already in clinical trials, studies continue in an effort to develop Treg expansion protocols tailored to specific uses. ST2$^+$ Treg may offer a therapeutic advantage over ST2$^-$ Treg in their ability to consume potentially detrimental IL-33 released from damaged tissues under pathologic conditions. We have demonstrated an ability for ST2$^+$ Treg to significantly suppress IL-12/IL-33-driven CD8$^+$ T cell IFN-γ production in vitro (Fig. 4; [11]) compared to ST2$^-$ Treg; however, this effect has not yet been reported in vivo. Given the pleiotropic effects of IL-33 in vivo, the capacity to selectively expand ST2$^+$ Treg ex vivo using IL-33-exposed CD11c$^+$ BMDC may be a promising way to capitalize on these important immunoregulatory cells. The following protocols describing robust expansion of ST2$^+$

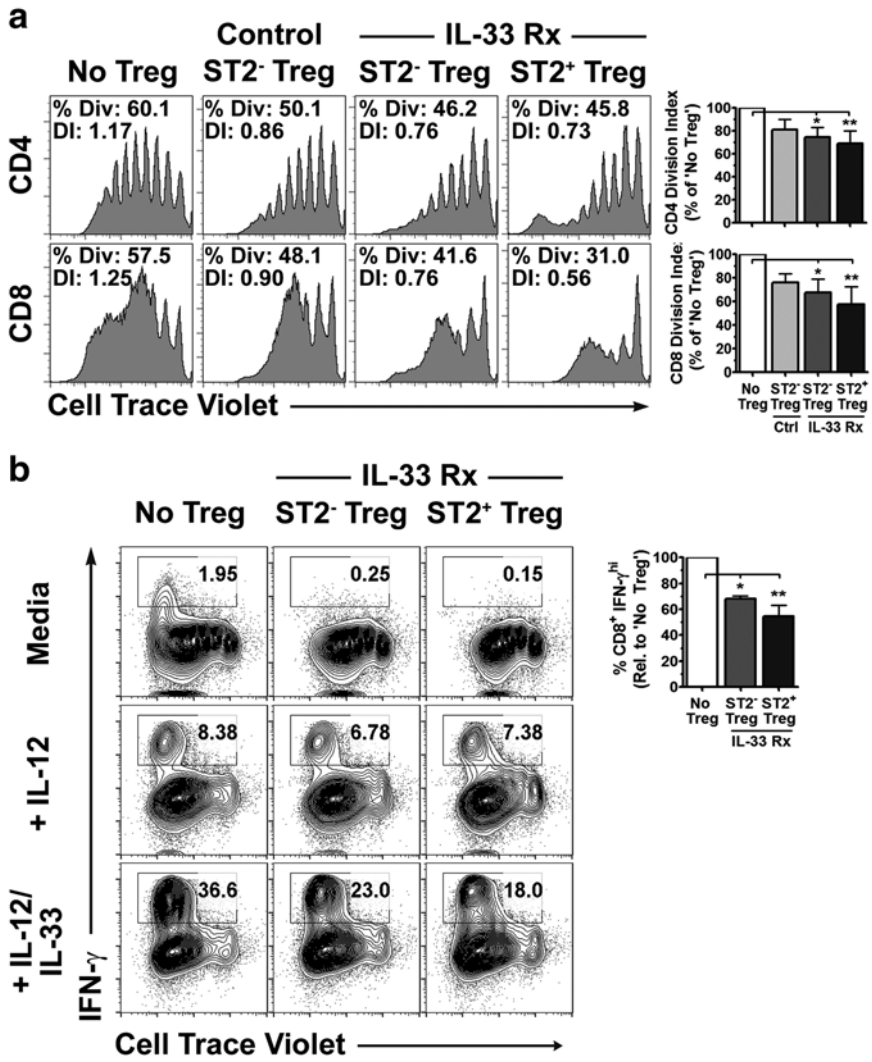

Fig. 4 IL-33-expanded Treg suppress CD8$^+$ effector T cell function. B6 FIR mice were administered IL-33 (0.5 μg/day; for 10 day). CD4$^+$ cells were purified on d 11 from total splenocytes and stained for flow sorting of CD4$^+$ RFP(Foxp3)$^+$ST2$^-$ or ST2$^+$ Treg. Sorted Treg were cultured with purified CTV-labeled, B6 CD4$^+$, or CD8$^+$ T cells at a Treg:Teffector ratio of 1:8 with CD3/CD28 T-activator beads for 4 days. Cultures were harvested and stained for flow cytometric analysis of T cell proliferation. (**a**) Representative flow plots and Division Index Relative to "No Treg" group of T cell proliferation based on CTV dilution profile. (**b**) Representative flow plots and an average of IFN-γ^{hi}CD8$^+$ T cells unstimulated or stimulated with 5 ng/ml IL-12 alone or in combination with 10 ng/ml IL-33. Data were generated from $n=3$ independent experiments. Statistical significance in A and B were determined by one-way ANOVA. DI = Division Index. *$p<0.05$, **$p<0.01$. Figure and legend reproduced with permission from *The JournalofImmunology* [11]. (Copyright 2014. The American Association of Immunologists, Inc.)

Treg using IL-33 in vitro and in vivo provide the avenue for further exploration, including investigation of ST2$^+$ Treg in humans, of the function and potential therapeutic use of this unique Treg subset.

2 Materials

2.1 Administration of Recombinant Mouse IL-33 and Spleen Harvest

1. Phosphate-buffered saline (PBS; Lonza, Walkersville, MD).
2. Recombinant mouse IL-33 (BioLegend, San Diego, CA) is resuspended in PBS at a final concentration of 5–10 μg/ml. IL-33 is administered by intraperitoneal (i.p.) injection at 100 μl/mouse (0.5–1 μg/mouse) daily for 10 days.
3. Fetal bovine serum (FBS)/EDTA solution: 500 ml PBS, 0.5 % heat-inactivated FBS, 2 mM EDTA (Sigma, St. Louis, MO).
4. Complete RPMI 1640 media: RPMI 1640 (Gibco by Life Technologies, Grand Island, NY) supplemented with 10 % heat-inactivated FBS, 50 μM 2-mercaptoethanol, 2 mM l-glutamine, 1 M MEM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethane-sulfonic acid).
5. Red blood cell (RBC) lysis buffer: 1 L MilliQ water, 0.1 mM EDTA, 1 g potassium bicarbonate ($KHCO_3$), 8.29 g ammonium chloride (NH_4Cl). Adjust pH to 7.2–7.5 and filter.
6. Sterile needles, 27 gauge, 1/2 in.
7. Sterile syringes (1 and 5 cc).
8. Nylon mesh cell strainers, 70 μm (BD Biosciences, Franklin Lakes, NJ).
9. Sterile Petri dish, 100 mm × 15 mm (Fisher Scientific, Pittsburgh, PA).

2.2 Expansion of Treg In Vitro

2.2.1 Propagation of $CD11c^+$ Cells from Mouse Bone Marrow

1. Recombinant murine IL-4 (R&D Systems, Minneapolis, MN).
2. Recombinant murine GM-CSF (R&D Systems, Minneapolis, MN).
3. Mouse Anti-CD11c immunomagnetic beads (Miltenyi Biotec, Auburn, CA).
4. MACS® Separation Columns (Miltenyi Biotec, Auburn, CA).
5. MACS® Separator Magnet (Miltenyi Biotec, Auburn, CA).
6. Bright-Line™ Hemacytometer (Fisher Scientific, Pittsburgh, PA).
7. Cell Culture Dish, 100 mm tissue culture-treated polystyrene (BD Biosciences).
8. 0.4 % (w/v) trypan blue in 0.81 % sodium chloride and 0.06 % potassium phosphate, dibasic (Sigma-Aldrich, St. Louis, MO).
9. Needles, syringes, cell strainers, PBS, FBS/EDTA, and RPMI 1640 medium—*see* Subheading 2.1.

2.2.2 Expansion of Treg In Vitro by IL-33 and CD11c+ BMDC

1. *See* Subheading 2.1 for necessary materials for isolation of total splenocytes.
2. T1/ST2 (IL-33R) FITC-conjugated mAb (MD Bioproducts, St. Paul, MN), Anti-mouse CD4 (Clone RM 4-5) APC-conjugated mAb (Affymetrix/eBioscience, San Diego, CA).
3. 5 % FBS: 500 ml PBS, 25 ml heat-inactivated FBS.
4. Dynabeads® Untouched™ Mouse CD4 Cells Kit (Life Technologies).
5. DynaMag™-15 Magnet (Life Technologies).
6. Recombinant mouse IL-33 (BioLegend).

2.3 Flow Cytometric Analysis of IL-33-Expanded Treg

1. 5 ml Round-bottom polystyrene tubes (BD Falcon).
2. Cell stain buffer (CSB): 500 ml PBS, 5 % heat-activated FBS, 0.1 % (w/v) sodium azide, pH 7.4–7.6.

2.3.1 Surface Staining

3. 5 % Normal goat serum (GS) in CSB.
4. Appropriate monoclonal antibodies (mAbs).
5. 4 % Paraformaldehyde (PFA): 4 g of PFA per 100 ml of PBS. Can be diluted to 1–2 % working solution with PBS to fix cells.

2.3.2 Intracellular Staining

1. Permeabilization buffer (PB): 500 ml PBS, 5 % heat-inactivated FBS, 0.1 % sodium azide, 0.1 % saponin.
2. Fixation/permeabilization buffer (Fix/Perm): Foxp3/Transcription Factor Staining Set (Affymetrix/eBioscience).
3. 5 % Normal goat serum in PB.
4. Appropriate mAbs.
5. 4 % Paraformaldehyde (PFA): 40 g PFA in 1 L PBS. Store at 4 °C protected from light.

3 Methods

3.1 Expansion of Treg In Vivo by Administration of Recombinant Mouse IL-33

1. Dissolve recombinant mouse IL-33 in PBS to a final concentration of 5–10 μg/ml.
2. Using a 1 cc syringe with a 27G, ½″ needle, administer 100 μl by intraperitoneal (i.p.) injection once daily for 10 days to C57BL/6 (B6) mice. Foxp3 reporter mice may be used to permit flow sorting of expanded Treg for functional assays (Foxp3-IRES-mRFP (FIR) on a C57BL/6 background) following IL-33 administration.
3. One day after the final injection, sacrifice animals using approved methods of euthanasia. Harvest the spleen using aseptic techniques and place in FBS/EDTA on ice.

4. Transfer the spleen to a sterile Petri dish and mechanically dissect into small pieces no larger than 2–3 mm. The dissected spleen should be forced through a 70 μm nylon mesh cell strainer into a 50 ml conical tube using a sterile 5 ml syringe. Wash the dish and cell strainer with 20–30 ml cold FBS/EDTA.
5. Centrifuge at 500 × *g* for 5 min at 4 °C, and resuspend the cells in RBC lysis buffer (3–5 ml per spleen) for 5 min.
6. Following lysis, add PBS to bring the total volume to 50 ml, and centrifuge the cells again under the same conditions. Splenocytes are ready for purification or immunostaining for flow sorting or phenotypic analysis. Keep cells in RPMI 1640 medium on ice until ready to use.

3.2 Expansion of Treg In Vitro Using IL-33 and CD11c+ Dendritic Cells

3.2.1 Generation of CD11c+ BMDC

1. From 8- to 12-week-old mice, dissect the femurs and tibiae, taking care to remove as much muscle as possible, and place in a 50 ml tube with FBS/EDTA on ice.
2. Under sterile conditions in a laminar flow hood, place the bones in a sterile Petri dish with 10–15 ml FBS/EDTA. Sterile scissors and forceps are used to tease away and remove any remaining muscle tissue. Place cleaned bones ina second Petri dish with 10–15 ml fresh FBS/EDTA.
3. Using scissors, carefully cut the bones and flush the BM into a clean dish with FBS/EDTA using a 5–10 cc syringe and 27G needle.
4. Once all the bones have been flushed, remove the needle and use the syringe to transfer the cells to a 50 ml tube, passing them through a 70 μm nylon cell strainer. Wash the dish and strainer with 5–10 ml FBS/EDTA.
5. Centrifuge at 500 × *g* for 5 min at 4 °C.
6. Carefully aspirate the supernatant, taking care not to disrupt the pellet. Resuspend the cells in 3–5 ml RBC lysis buffer for 5 min.
7. Add cold FBS/EDTA to 50 ml and centrifuge as described above.
8. Resuspend the cells in RPMI 1640 and count viable cells using Trypan Blue and a hemacytometer.
9. Culture cells in a sterile, 100 mm tissue culture-treated dish at $3–4 \times 10^6$ cells in 15 ml RPMI 1640 supplemented with GM-CSF and IL-4 at 1000 U/ml (day 0). Cells will be cultured for a total of 7 days.
10. On day 2, remove 10 ml of tissue culture media per dish and centrifuge at 500 × *g* for 5 min at 4 °C. Pour off the supernatant and resuspend cells in 15 ml fresh RPMI 1640 per dish with GM-CSF and IL-4.

11. On day 4, gently swirl the plates and remove 15 ml media containing floating cells. Discard this media and add 15 ml fresh RPMI 1640 per dish with GM-CSF and IL-4.
12. On day 6, repeat process for day 2, removing 15 ml, spinning down cells, pouring off the supernatant and resuspending cells in 15 ml fresh RPMI per dish with GM-CSF and IL-4.
13. On day 7, gently swirl plates and harvest all media/non-adherent cells into a 50 ml tube. Add 5 ml FBS/EDTA per dish and pipette up and down to wash thoroughly. Collect these cells and add to 50 ml tube.
14. Centrifuge at 500 × *g* for 5 min at 4 °C.
15. Resuspend cells in FBS/EDTA and count using hemacytometer. Proceed with CD11c isolation using mouse anti-CD11c immunomagnetic beads (Miltenyi Biotec) according to the manufacturer's protocol.
16. Resuspend positively-selected CD11c$^+$ cells in RPMI 1640 medium and count. Bring cells to a concentration of 1×10^5 cells/ml and keep on ice. Cells are ready for culture.

3.2.2 Expansion of Treg In Vitro by CD11c$^+$ BMDC and IL-33

1. Prepare total splenocyte single-cell suspension from untreated/naïve wild-type (WT) B6 or FIR B6 mice as described in Subheading 3.1.
2. Following RBC lysis, isolate untouched bulk CD4$^+$ T cells by negative selection using Dynabeads® Untouched™ Mouse CD4 Cells Kit (Life Technologies) according to the manufacturer's instructions.
3. Remove non-CD4$^+$T cells using the DynaMag™-15 Magnet (Life Technologies).
4. Wash the isolated CD4$^+$ T cells in FBS/EDTA. Centrifuge at 500 × *g* for 5 min at 4 °C.
5. Pour off the supernatant and resuspend cells in RPMI 1640 medium. Count cells using trypan blue and a hemacytometer.
6. Dilute CD4$^+$ T cells to a concentration of 1×10^6 cells/ml in RPMI 1640 medium.
7. In a 96-well U-bottom plate, add purified CD11c$^+$ BMDC (100 µl from Subheading 3.2.1) and bulk CD4$^+$ T cells at a DC:T cell ratio of 1:10 in 200 µl total volume (*see* **Note 1**). In a syngeneic system when B6 CD11c$^+$ BMDC are used with B6 FIR CD4$^+$ T cells, anti-CD3 is added at a final concentration of 0.25 µg/ml (*see* **Note 2**).
8. Recombinant IL-33 is added to each well at a final concentration of 10 ng/ml (*see* **Notes 3** & **4**).
7. Cells are cultured for a total of 5 days at 37 °C, in a humidified 5 % CO_2 incubator.

8. On day 5, cells are harvested by pipetting up and down and transferred to a 50 ml tube.
9. Centrifuge at 500 × *g* for 5 min at 4 °C.
10. The cells are ready for immunostaining for flow sorting or phenotypic analysis.

3.3 Flow Sorting of IL-33-Expanded Treg for Functional Assays

3.3.1 Immunostaining and FlowSorting of IL-33-Expanded Treg

1. Utilizing Foxp3-IRES-mRFP (FIR) reporter mice, Treg expanded by IL-33 in vivo (Subheading 3.1) or in vitro (Subheading 3.2) may be flow-sorted for testing of their functional suppressive capacity, and comparison of IL-33R/ST2$^+$ vs. ST2$^-$Foxp3$^+$ Treg subsets.
2. Total splenocytes from FIR mice (Subheading 3.1) or cells harvested 4 °C from culture (Subheading 3.2) are resuspended in 5 % FBS and stained using ST2-FITC (1:200 dilution) and CD4-APC (1:800 dilution). Cells are incubated at 4 °C for 30 min.
3. Wash cells with 10× volume 5 % FBS and centrifuge at 500 × *g* for 5 min at 4 °C.
4. Resuspend cells in 5 % FBS and filter through cell strainer cap into 5 ml round bottom tube.
5. Cells are sorted using a FACSAria™ (BD Biosciences). Foxp3$^+$ (RFP$^+$) cells are detected using the PE/Texas Red channel. Gating on lymphocytes (FSC-A vs. SSC-A) and single cells (FSC-H vs. FSC-W), ST2$^+$ and ST2$^-$Foxp3$^+$ cells are sorted from the CD4$^+$-gated population. Sorted cells are collected in sterile 5 ml snap-cap round-bottom tubes containing approximately 1 ml cold RPMI 1640 medium.
6. Sorted cells are washed in RPMI 1640 and centrifuged at 500 × *g* for 5 min at 4 °C.
7. Cells are resuspended in RPMI 1640 and counted using trypan blue and a hemacytometer. Cells are ready for in vitro suppressionassay or phenotype analysis by flow cytometry. For in vivo use, wash cells with PBS to remove traces of FBS.

3.3.2 Testing the T Cell Suppressive Capacity of IL-33-Expanded Treg In Vitro

1. The T cell suppressor function of flow-sorted Treg populations (Subheading 3.3.1) can be tested in vitro. CD4$^+$CD25$^-$ T effector (T_{eff}) cells are used as responders and purified from total splenocytes of a naïve B6 mouse. To do this, purified rat anti-mouse CD25 (clone PC61 at 1:100) is included in the antibody cocktail during the CD4$^+$ T cell purification process (described in Subheading 3.1).
2. Purified CD4$^+$CD25$^-$ T cells are labeled with CellTrace™ Violet (CTV) Cell Proliferation kit (Life Technologies) according to the manufacturer's instructions, taking care to keep cells in the dark during and after the labeling process.

3. Resuspend CTV-labeled cells in RPMI 1640 medium and count. Adjust the concentration to 1×10^6/ml.
4. CTV-labeled T_{eff} cells (1×10^5/well) are stimulated in 96-well U-bottom plates with Dynabeads® Mouse T-Activator CD3/CD28 (1×10^4/well; Life Technologies). Flow-sorted Treg populations are added at different Treg:T_{eff} ratios in a final volume of 200 μl per well and cells are cultured for 3 days at 37 °C, in a humidified 5 % CO_2 incubator.
5. At the end of 3 days, cells are harvested from culture and stained for flow cytometric quantification of T_{eff} cell proliferation by CTV-dilution analysis. Flow-sorted $CD4^+$ Treg can be distinguished from $CD4^+$ T_{eff} by staining for CD90.1/Thy1.1 and exclusion of $CD90.1^+$ cells from final analysis.
6. Cells are acquired using an LSRFortessa (BD Biosciences) and data is analyzed using FlowJo software (v10; Tree Star, Ashland, OR).

3.4 Phenotypic Analysis of IL-33-Expanded Treg by Flow Cytometry

1. Surface Ag expression can be analyzed by multicolor flow cytometry on total splenocytes or CD4-purified cells following 10 day administration of IL-33 or cells harvested from DC:T cell co-culture following in vitro expansion.

3.4.1 Surface Staining

2. Specifically, fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, allophycocyanin (APC)/AlexaFluor647-, Pacific Blue-, PerCP-Cy5.5-, and PE-Cy7-conjugated antibodies are used to stain Treg.
3. Typically, 5×10^5 cells are blocked with 5 % normal goat serum (GS) in Cell Stain Buffer (CSB) (10 min; 4 °C), then stained with appropriate mAbs (30 min; 4 °C) in CSB. Appropriate isotype-matched IgGs should be included as negative controls.
4. The mAbs (clones) used for Treg surface staining are: CD3 (17A2), CD4 (RM 4-5), CD25 (PC61), ST2 (DJ8), CD304/Neuropilin-1 (3E12), CD44 (IM7), and CD278/ICOS (7E.17G9).
5. Wash cells twice in CSB. Data should be acquired immediately if using Foxp3 (FIR) reporter mice (*see* **Note 5**).
6. Data are acquired with a LSRFortessa flow cytometer (BD ImmunoCytometry Systems; San Jose, CA) and analyzed using FlowJo software (v10; Tree Star).

3.4.2 Intracellular Staining

1. To determine expression of transcription factors (from cells stained in 3.4.1 generated using WT mice and not FIR reporter mice), perform intracellular staining immediately following surface staining. The mAbs (clones) used for intracellular staining include: Foxp3 (FJK-16 s), GATA-3 (16E10A23), T-bet (4B10), and Helios (22F6).

2. After the second wash with CSB, resuspend cells in 100 µl Fix/Perm buffer and immediately vortex. Incubate at room temperature (RT) for 45 min, or alternatively, up to 16 h at 4 °C.
3. After the incubation in Fix/Perm buffer, wash the cells with 400 µl permeabilization buffer (PB) and centrifuge at 500 × *g* for 5 min at 4 °C. Aspirate the supernatant and resuspend in PB + 3 % GS and desired mAbs. Incubate for 30 min at 4 °C.
4. Wash the cells (2×) with PB.
5. Resuspend cells in CSB and acquire, of fix with 1–2 % paraformaldehyde (PFA).

4 Notes

1. For optimal yield of ST2$^+$Foxp3$^+$ Treg, bulk CD4$^+$ T cells should be used as the starting population during in vitro expansion with CD11c$^+$ BMDC and IL-33. Although they still undergo expansion, we have found that the yield of ST2$^+$ Treg is actually lower when starting with purified CD4$^+$CD25$^+$ T cells.
2. For applications where MHC-mismatch is a factor, in vitro expansion of Treg by IL-33 can be achieved using allogeneic (BALB/c) CD11c$^+$ BMDC in place of syngeneic (B6) CD11c$^+$ BMDC + anti-CD3, with comparable results.
3. ST2$^+$ Treg can be generated in vitro in 5 day culture with CD11c$^+$ BMDC that were exposed to 20 ng/ml IL-33 for 18 h, without the addition of exogenous IL-33 (Fig. 2; [11]). The overall yield of Treg may be lower compared to cultures with IL-33 directly added to BMDC:T cell co-culture; however, the resulting phenotype and suppressor function of Treg generated by either method are comparable.
4. Recombinant IL-2 is not required during in vitro Treg expansion using CD11c$^+$ BMDC and IL-33. We have reported that adding exogenous IL-2 does not augment Treg expansion above levels achieved with CD11c$^+$ BMDC and IL-33 (Fig. 2; [11]), and IL-33 stimulates IL-2 secretion by DC to support Treg expansion (Fig. 1; [11]).
5. For phenotypic analysis of surface markers only on Treg, FIR reporter mice can be used to identify Treg to avoid intracellular staining (identification of Foxp3$^+$/RFP$^+$ cells during flow acquisition). Cells must be acquired immediately following staining, since fixation with PFA will quench RFP fluorescence. For analysis of intracellular proteins, including cytokines and transcription factors, WT mice are used with intracellular Foxp3 staining.

Acknowledgements

Many of these protocols were developed in studies with the support National Institutes of Health (NIH) grants to B.M.M. (T32 AI074490) and H.R.T. (R00 HL097155). H.R.T. was also supported by the American Society of Transplantation (AST) Basic Science Faculty Grant, Roche Organ Transplantation Foundation Grant (ROTRF; 58911427) and the American Heart Association Grant-in-Aid (AHA; 14GRNT20400004).

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Chapter 4

Standardization, Evaluation, and Area-Under-Curve Analysis of Human and Murine Treg Suppressive Function

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Abstract

FOXP3+ T-regulatory (Treg) cells have important roles in immune homeostasis, and alterations in their number and function can predispose to diseases ranging from autoimmunity to allograft rejection and tumor growth. Reliable identification of human Tregs remains a persistent problem due to a lack of specific markers. The most definitive Treg characterization currently involves combined assessment of phenotypic, epigenetic and functional parameters, with the latter typically involving in vitro Treg suppression assays. Unfortunately, suppression assays are frequently performed using differing methods and readouts, limiting comparisons between studies. We provide a perspective on our experience with human and murine Treg suppression assay conditions, including Treg data obtained in clinical transplant studies, Tregs isolated from healthy donors and treated with epigenetically active compounds, and Tregs from standard murine strains (C57BL/6 and BALB/c). We provide detailed descriptions and illustrations of typical problems, shortcomings and troubleshooting; describe new modifications and approaches; and present a new method for calculation of suppressive assay data using a modified area-under-curve (AUC) method. This method allows us to directly compare Treg suppressive function between multiple patients (such as in clinical transplant studies), to reliably track changes in Treg function from the same person over time, or compare effects of Treg-modulating compounds tested with different healthy donors Tregs in separate or combined experimental settings.

Key words FOXP3+ regulatory T cells, Tregs, Suppression assay

1 Introduction

FOXP3+ T-regulatory (Treg) cellscomprise a small subset of CD4+ T cells with bystander suppressor activity. Since their initial characterization as CD4+ CD25+ T cells by Sakaguchi and colleagues in 1995 [1], Tregs have been a focus of interest for the development of new diagnostic and therapeutic strategies for immunologically mediated diseases. FOXP3 is a key marker for use in the identification of murine Tregs [2], and the development of YFP/GFP-Foxp3 knock-in mice facilitated isolation of live murine Tregs for in vitro and in vivo functional studies [3]. However, in addition to Tregs, activated human effector T (Teffs) cells can upregulate FOXP3 [4],

Maria Cristina Cuturi and Ignacio Anegon (eds.), *Suppression and Regulation of Immune Responses: Methods and Protocols, Volume II*, Methods in Molecular Biology, vol. 1371, DOI 10.1007/978-1-4939-3139-2_4,

such that phenotypic evaluation of human Tregs using FOXP3 alone is not sufficiently specific for clinical studies. To overcome this problem, various additional Treg-associated markers with supposed preferential expression by FOXP3+ cells have been proposed, but in most cases direct validation of their Treg specificity in human clinical samples was not shown. Indeed, FOXP3 is an indispensable, but not sufficient marker of Treg cell fate. Thus, to maintain their Treg function and phenotype, an additional set of FOXP3-independent genes, including *CTLA4* and *IKZF2*, need to be demethylated [5]. Likewise, a quintet of transcription factors, namely IRF4, Eos, Gata1, Satb1, and FOXP3, but not FOXP3 alone, can reproduce Treg-like gene expression in Teff cells [6]. As a result, a "gold standard" for reliable phenotypic Treg evaluation is still lacking, and functional studies remain the most reliable way to confirm that CD4+CD25+FOXP3+ cells under study are indeed Treg cells.

The first in vitro suppression assays were described over a decade ago [7, 8]. Since then, many modifications of activation and co-stimulation conditions, time in culture, methods for readout of T cell proliferation, and calculations of results were developed. These modifications have led to controversy due, in part, to poor reproducibility between different research groups. An "ideal" Treg suppression assay would be sensitive enough to show alteration in suppressive function, and, at the same time, should not be prone to generate misleading results due to small variations in experimental conditions such as different lots of sera, stimulation reagents, etc., altered resistance of Teffs to Treg suppression, or differing characteristics of antigen-presenting cells (APC). For this purpose, each key assay component should be standardized, have minimal assay-to-assay variability, and provide the researcher with data for standardization controls in each experiment.

In this chapter, we discuss various technical considerations that must be taken into account especially when using human Treg suppression assays, since these more challenging and difficult to normalize than murine Treg suppression assays, as well as additional features of Tregs we have noted in ongoing clinical studies. We also discuss the most common pitfalls and drawbacks of this method, and illustrate issues using our data accumulated over 6 years of work with human Tregs, and a decade of work with murine Tregs. Finally, we suggest a new sensitive and reproducible method for calculation of Treg suppressive function that can be used to compare experimental data.

2 Materials

1. Ficoll-Paque PLUS Medium (GE Healthcare).
2. ACCUSPIN™ Tubes Sterile 50 mL (Sigma) or SepMate 50 mL tubes (Stemcell Technologies), or BD Vacutainer CPT tubes, 8 mL.

3. Ca^{2+} and Mg^{2+}-free DPBS.
4. 10× Ca^{2+} and Mg^{2+}-free DPBS or red blood cell lysis buffer.
5. Heat-inactivated fetal bovine serum (FBS).
6. Trypan blue (Sigma).
7. For murine cells and human tissue samples: Cell Strainers 70 μm or Nylon Mesh 70 μm.
8. Cell isolation buffer: DPBS supplemented with 2 mM EDTA and 0.5 % FBS.
9. For human cells: CD4+CD25+ Regulatory T cell isolation kit, human (Miltenyi) and for murine cells: CD4+CD25+ Regulatory T Cell Isolation Kit, mouse (Miltenyi).
10. For human cells: CD3 MicroBeads, human (Miltenyi) and for murine cells: CD90.2 MicroBeads, mouse (Miltenyi). Both reagents are optional.
11. LD columns and LS columns (Miltenyi).
12. T-cell media: RPMI1640 (Invitrogen), supplemented with 10 % heat-inactivated FBS, penicillin and streptomycin, and 2-mercaptoethanol (100 μM).
13. CFSE or CellTrace (Invitrogen).
14. For human cells: MACS GMP CD3 antibody (Miltenyi) and Dynabeads® M-450 Tosylactivated beads (Invitrogen). For murine cells: anti-mouse CD3 functional grade purified antibody.
15. 96-Well U-bottom plates or 96-well V-bottom plates for low cell numbers.
16. (Optional) CryoStor® CS5 cell cryopreservation media (Sigma) and cryovials 1–2 mL.
17. Flow cytometry buffer: DPBS supplemented with 2 % heat-inactivated FBS.
18. Fixable LIVE/DEAD (Invitrogen) or fixable Zombie (Biolegend) or fixable Viability Dye (eBioscience).
19. Transcription Factor Buffer Set (BD Biosciences) or Foxp3/Transcription Factor Staining Buffer Set (eBioscience).
20. Anti-human or anti-mouse CD4, FOXP3, CD8 (optional), CD25 (optional), CD127 (optional) and CTLA4 (optional) antibodies for evaluation of human or murine cells, correspondingly. Ki-67 antibody (optional) from BD Biosciences works for both species.

3 Methods

3.1 Human PBMC Isolation

For blood samples that were collected the same day, use ACCUSPIN, SepMate or CPT tubes according to corresponding manufacturer's instruction. Fill ACCUSPIN or SepMate with

Ficoll-Paque PLUS. You may also isolate PBMC using standard sterile 50 or 15 mL tubes, according to protocol in Ficoll-Paque PLUS package.

If blood has to be kept overnight or was shipped overnight («old» blood), then maintain the room temperature of blood until PBMC is isolated, and follow this modified procedure:

1. Warm, and then keep all reagents for PBMC isolation from «old» blood, at room temperature. It is also important to set up centrifuge for room temperature. Cold may provoke massive hemolysis of «old» erythrocytes which decreases PBMC yield and quality.
2. Evaluate blood volume. Use ACCUSPIN tubes for 15–21 mL of undiluted «old» blood, or SepMate for 16–26 mL of 2×-diluted blood. Dilute blood with DPBS + 2 % FBS. Combine both types of tubes if needed. Alternatively, you may use 2×-diluted blood and standard sterile 50 mL or 15 mL plastic tubes, according to protocol in Ficoll-Paque PLUS package, or combine all types of tubes.
3. Fill ACCUSPIN (by centrifugation at 800 × *g*, 30 s) and SepMate (by pipetting) tubes with room-temperature Ficoll-Paque PLUS, 15–15.5 mL in each 50 mL tube. Avoid air bubbles under the membranes.
4. Add corresponding volume of blood and spin down tubes at room temperature, 400 × *g*, 35 min. Higher speed with «old» blood samples may destroy red blood cells and lead to massive hemolysis. Make sure that centrifuge is set up as «no brake» (no rapid deceleration).
5. Remove plasma layer and collect PBMC cells within mononuclear band.
6. Wash PBMC with room temperature DPBS, 10 min at 300 × *g*.
7. Remove supernatant and evaluate whether red blood cell lysis is required. If not, continue with **step 9**.
8. Proceed with erythrocytes lysis if needed. Human PBMC sustain well the hypotonic shock. For that, tap tube to loosen cell pellet, add 18 mL of sterile DI water, mix for 5–10 s, and add 2 mL of 10× Ca^{2+} and Mg^{2+}-free DPBS. Mix, add sterile DPBS to 50 mL, and wash for 10 min at 300 × *g*.
9. Resuspend cells in cell isolation buffer, and evaluate cell numbers and viability using Trypan blue staining.

3.2 Human Tissue Cell Isolation and Murine Cell Isolation

1. Obtain spleen, peripheral, and mesenteric lymph modes of sacrificed mice and collect them into tubes with 4 °C sterile DPBS. Obtain human lymph modes, collected into tubes with 4 °C cell culture media.
2. Use a plunger of a 5 mL syringe and cell strainer or mesh cuts to prepare single-cell suspension in Petri dishes. Collect cells

into 50 mL tubes with inserted cell strainer (or mesh cuts) to filter cell clumps. Use DPBS for murine cells and cell culture media for human cells.

3. Wash cells at 300 × *g* for 10 min, remove supernatant, tap tube to loosen the pellet, and proceed with red blood cell lysis. Murine cells sustain well hypotonic shock. For that, tap tube to loosen cell pellet, add 18 mL of sterile DI water, mix for 5–10 s, and add 2 mL of 10× Ca^{2+} and Mg^{2+}-free DPBS. Mix, add sterile DPBS to 50 mL, and wash for 10 min at 300 × *g*. For human tissue cells, use homemade or commercial red blood cell lysis buffer according to the manufacturer's instruction.
4. Wash cells at 300 × *g* for 10 min, remove supernatant, resuspend cells in cell isolation buffer, and filter them if needed (using cell strainer or mesh cuts), and/or dissociate clumps by intensive pipetting. Calculate cell numbers and evaluate their viability using Trypan blue staining.

3.3 Human and Murine Treg, Teffs, and APC Isolation

Avoid using samples if more than 10–15 % of dead cells are observed prior to Treg isolation. Such levels require troubleshooting to improve cell isolation techniques and may seriously compromise the purity of isolated cells, especially Tregs. You may apply the Dead cell removal kit (Miltenyi) or dead cell isolation method by Ficoll using corresponding standard protocols (not detailed here), but in most cases it leads to insufficient cell numbers for Treg isolation.

There are three options of experimental setup: first one is to isolate the CD4+CD25+ subset as Tregs, CD4+CD25– as Teffs and CD4– cells as APC. This modification may be performed for both human and murine cells, and requires just a corresponding CD4+CD25+ Regulatory T cell isolation kit (Miltenyi) for human or mouse cells. Follow the manufacturer's instructions and wash out CD4– depleted cells to use them as APC. Then, obtain CD4+CD25– Teffs and CD4+CD25+ Tregs on the second step of isolation.

Second option is to use an additional kit with CD3 MicroBeads (Miltenyi) for human cells, or mouse CD90.2 MicroBeads (Miltenyi) for murine cells. Follow the manufacturer's instructions. In that case APC will be depleted of CD3+CD8+ cells, which are active dividers. As a result, use of CD3-depleted APC instead of CD4-depleted APC will provide with better Treg suppression within the same Treg/Teff ratios. Serious drawbacks of this approach are the need for additional cells that cannot be used for Treg isolation, and the more expensive isolation procedure. However, for most murine experiments starting cell number is not an issue.

In both cases, when CD3- or CD4-depleted APC are used, they may be irradiated (100 Gy) prior to suppression assay. Irradiation of APC cells will help to stop their divisions and therefore will help to improve suppression by Tregs in the given Treg/Teffs ratios. Another way to obtain a better suppression is to

use slightly less APC if they are CD4-depleted, and about 1.3–1.5 times more APC if they are CD3-depleted.

Third option is to use CD4+CD25+ Regulatory T cell isolation kit exclusively to obtain Tregs, and use a bulk of allogeneic or autologous splenocytes or lymph nodes (mouse) or PBMC (human) cells as responders and APC. There are different advantages of this strategy. First of all, it allows to standardize suppression assay by using an aliquoted standardized responders from the same healthy donor (*see* Subheadings 4.2 and 4.3 in Results). Second, the suppression effect of Tregs on CD4+ and CD8+ T cell divisions can be evaluated within the same assay. The drawback of this approach is to higher risk to receive a bad shape of CFSE peaks (*see* Subheading 4.5 in Results). Another drawback is the tendency of bulk cells to be more resistant to Treg suppression in comparison with CD4+CD25− Teffs and APC setup. To overcome this, increase Treg to responders ratio and adjust stimulation to the level of 0.6–0.9 times less from Teffs + APC setup levels (also *see* Subheading 4.3 in Results).

3.4 Labeling of Responder Cells

CD4+CD25− Teffs or PBMC cells should be labeled with CFSE or its analogs according to the manufacturer's instructions. Avoid light exposure of cells. By the end of labeling, no changes in cell viability should be observed. After culture, labeled cells should demonstrate the same number and viability as non-labeled cells within the same experiment. If cells demonstrate decreased viability, then increase concentration of protein in labeling and/or washing buffers. You may also decrease concentration or time of labeling.

3.5 Suppression Assay

1. Calculate number of isolated Tregs, Teffs, and APC (or Tregs and PBMC). Numbers of Teffs and PBMC should be evaluated after CFSE labeling. Teffs and PBMC should be kept in dark after labeling and until plating. Dilute cells to 1×10^6/mL concentration in cell culture media. If low number of cells used, prepare them in 0.5×10^6/mL concentration to decrease pipetting errors (also *see* Subheading 4.3 in Results).
2. In advance, prepare CD3-coated magnetic beads to stimulate human cells by coupling of M-450 Tosylactivated beads (Invitrogen) with MACS GMP CD3 antibody (Miltenyi) according to the manufacturer's instructions in the bead package. Keep sodium azide-preserved sterile aliquots of the CD3-beads at 4 °C. Do not apply vortexing and vigorous pipetting for these beads. Such beads are stable for at least 12 months. In preliminary experiments, find the best stimulation for current experimental conditions (*see* Subheading 4.3 Results for details). Prior to cell culture, take the required volume of CD3 beads and wash them 2–3 times using magnet and cell culture medium. Prepare stock

of anti-mouse CD3 functional grade antibodies (4 μg/mL) in cell culture medium.

3. Plate cells into 96-well U-bottom cell culture plate (if $35–80 \times 10^3$ Tregs used in 1/1 ratios) or into V-bottom plate (if $20–35 \times 10^3$ Tregs used in 1/1 ratios). Prepare serial 2× dilution of Tregs, starting with 2/1 or 1/1 Treg/Teff ratio and until 1/16 to 1/32 ratio. Do not use single Treg/Teff ratio for the experiment (*see* in Subheading 4.3 in Results). Then plate APC and CFSE-labeled CD4– Teffs or CFSE-labeled PBMC, each well should have the same number of labeled cells as the number of Tregs in 1/1 ratio. It is convenient to mix human responder cells (CD4+ Teffs or PBMC cells) with corresponding number of washed CD3-coated beads prior to platting. For murine cells, add corresponding volume of pre-diluted anti-mouse CD3 antibodies to have final concentration about 1 μg/mL. Add cell culture media to have equal total volume in each well, 200 or 250 μL. Also plate cells for positive control (responder cells without Tregs) and negative control (responder cells without Tregs and without stimulation).
4. Incubate cells for 3–4 days (murine) or 4–6 days (human) at 37 °C.

3.6 Purity Control of Isolated Tregs

Immediately after platting of cells for suppression assay, stain leftover Tregs for LIVE/DEAD (Invitrogen), then stain for superficial markers: CD4, CD25, and CD127, and after fixation and permeabilization step, stain for CTLA4 and FOXP3, according to the manufacturer's instruction for FOXP3 antibody. Alternatively, freeze the small aliquots of isolated Tregs, consisting of 2000–10,000 cells, to stain them for listed markers later. This is convenient for clinical trials, when bulk of patients' Tregs evaluation is preferable. Evaluate phenotype of isolated Tregs with flow cytometry. These data serve as Treg purity controls in interpretation of suppression assay results (*see* Subheading 4.1 in Results).

3.7 Cryopreservation of Human and Murine Cells

Use CryoStor® CS5 cell cryopreservation media (Sigma) according to manufacturer's instructions for freezing, storage and thawing [9]. For healthy donor PBMC, the viability just after thawing should be about 98–99 %. Cells should also preserve the same phenotype and same division rate as they had before freezing. As an internal quality control, we use healthy donor PBMC cells in cell culture experiments only if they demonstrated less than 5 % of dead cells by Trypan blue evaluation after CFSE-labeling.

However, in clinical trials, some patients' cells may demonstrate notable levels of apoptosis (10–30 % after thawing), probably due to hyperactivation in vivo or due to the effects of immunosuppressive therapy in transplant recipients.

For Treg cryopreservation using very limited cell numbers, you may exclude all optional washing steps. Thus, 1 mL of CryoStor

media, pre-cooled on ice, can be used directly to wash out all leftover Tregs (it is about 10–50 μl in cell culture media) from their 15 mL tube, and to transfer washouts into cryotube. After thawing, Tregs may be stained in the same 15 ml tubes, to exclude cell losses by transfer. If a washing step could not be excluded, the supernatant should be removed by pipetting instead of pouring. With such modifications, we usually receive at least 500–1000 events in live cell gates for Treg evaluation from any leftover clinical Tregs (*see* Subheading 4.1 in Results).

3.8 Evaluation of Suppression Assay Results

Stain cells with CD4 and (optional) CD8 antibodies, fix them in 1 % formaldehyde if required, and proceed with flow cytometry. Set up FS-SS gate on live cells. CFSE-negative cells are Tregs and APC, and CFSE-labeled cells are responders. Gate on CFSE+CD4+ subset to evaluate CD4+ Teffs divisions, and on CFSE+ CD8+ (or CFSE+CD4−) subset to evaluate CD8+ Teffs divisions. If needed, use additional LIVE/DEAD staining prior to fixation. *See* Subheading 4.5 in Results for details.

3.9 Statistics and AUC Calculation

In Results, data are shown as min-max and mean ± SEM. For continuous variables with normal distribution, statistical significance was assessed by Student's *t*-test to compare two groups, ANOVA with Newman-Keuls multiple comparison post-test to compare three groups, and Pearson correlation assay to test correlations. For data not normally distributed, two groups of continuous variables were compared with the Mann Whitney *U* test, and three groups were compared with the Kruskal-Wallis test with Dunn's multiple comparison post-test. A two-tailed p value of <0.05 was considered statistically significant.

For AUC calculations (*see* details in Subheading 4.6, in Results), we used GraphPad Prism v.5.00. After calculation of standardized or normalized suppression (described in the main text), we chose "XY" table format, and placed numbers of Teffs per Treg as values on X axis and corresponding percent of standardized suppression as Y values column(s) (*see* corresponding examples in Table 3 and Figs. 8 and 9). For the subsequent calculations, we excluded all zero values of suppression in "no Tregs" ratios, and decreased variability of data by replacing all minor suppression data that were 3 % and less, to zero values. Therefore, nonsignificant and biologically irrelevant cellular variability such as 86 % vs. 89 % of divisions giving 1–2 % of standardized suppression had no effect on resulting data. Then we used an option "XY analyses, area under curve" with standard settings to obtain AUCs numbers, listed as "total peak area."

3.10 Ethics Statement

Pediatric liver and kidney allograft clinical studies were approved by the respective Institutional Review Board of each Center: CHOP-08-006311_CR1, CCHMC-2009-0018, and HSC-1000014523.

Adult liver allograft studies were approved by the Institutional Review Board of the Hospital of the University of Pennsylvania (#810878). Animal studies (C57BL/6 and BALB/c mice, The Jackson Laboratory, Bar Harbor, ME) were approved by the Institutional Animal Care and Use Committee of The Children's Hospital of Philadelphia (#2010-6-561).

3.11 Processing of Human Cells from Healthy Donors and Transplant Recipients

PBMC-enriched apheresis product was obtained by leukapheresis of healthy volunteer donors, via the University of Pennsylvania Human Immunology Core. Specimens were collected according to the protocol approved by the Hospital of the University of Pennsylvania Institutional Review Board, and informed consent was obtained from each donor. For pediatric liver and kidney allograft studies, PBMC were isolated from 32 mL of blood and shipped overnight at 4 °C to the Children's Hospital of Philadelphia (CHOP). For standardization, CHOP samples were also kept at 4 °C overnight. For adult liver allograft studies, PBMC were isolated from 45 to 50 mL of blood within a few hours of venipuncture, and kept overnight at 4 °C in cell culture media plus EDTA.

For all human Treg studies in this chapter, Tregs were isolated from PBMC using CD4+CD25+ regulatory Treg kits (Miltenyi Biotec). CD4+CD25– cells (Teffs) and CD4-depleted cells (APC), isolated with the same kit, were used in experiments with autologous cells. For some experiments, Teffs and APC were further cryopreserved to study combinations of autologous vs. allogeneic responders, or pre-transplant vs. post-transplant responders. In addition, for some experiments with healthy donor Tregs and responders, we used CD3-depleted and irradiated APC, isolated with CD3 MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions. For all clinical studies, we did not irradiate APC.

CD4+CD25– Teffs, or aliquots of cryopreserved healthy donor PBMC, were CFSE-labeled, and stimulated with CD3-coated beads (OKT3 clone from Miltenyi, Dynabeads® M-450 tosylactivated beads from Invitrogen) using a 3.5:1 bead:cell ratio in most cases. With some responder cells, the optimal stimulation was adjusted in additional experiments, as described in Results, Subheading 3.3. Tregs and responder cells were cultured for 3–5 days (for clinical studies, we used 4 days) in cell media, consisting of RPMI-1640 plus 10 % heat-inactivated FBS, penicillin and streptomycin, and 2-mercapto-ethanol (100 μM). CD4+ and CD8+ cell divisions were determined by CFSE dilution. When noted, we co-stained Tregs and responder cells with additional markers of interest after suppression assay or labeled Tregs with CellTrace (Invitrogen) prior to suppression assay. We used 96-well U-bottom plates, and for experiments with low cell numbers (35,000 and less of human Tregs in 1/1 ratio) we used 96-well V-bottom plates.

4 Results

4.1 Treg Isolation and Purity Control

4.1.1 Need to Evaluate FOXP3 in Isolated CD4+CD25+ as Treg Purity Control

For many laboratories, the most common method of Treg isolation involves use of magnetic bead kits with CD4+ and CD25+ (high) markers. In normal murine samples, this method provides a stable and relatively pure (71–81 %, mean 77.1 ± 1.3) population of FOXP3+ cells, and CD4+CD25+ Treg isolation from healthy human donor PBMC can also be performed with satisfactory results (49–79 %, mean 64.7 ± 2.5), as detailed [10]. However, in murine models of autoimmune or inflammatory diseases, a significant percent of CD4+CD25+ cells are FOXP3– activated Teffs. In murine models, use of fluorescent tags, such as YFP/GFP-FOXP3 mice [3], can address this problem, but isolation of Tregs from clinical samples is restricted by use of nonspecific surface markers. Despite this, most human Treg studies do not provide data of FOXP3 expression in isolated human CD4+CD25+ cells, and are thus of limited value. Figure 1a shows two human Treg samples isolated sequentially, as indicated, from the same liver allograft recipient. In both cases, CD4+ and CD25+ purity after isolation was reasonably high and comparable (78–82 %), but the first sample had no suppressive function at all due to a very low percentage of FOXP3+ Tregs within isolated CD4+CD25+ cells (see below). Of note, this patient was diagnosed with acute allograft rejection 7 days after the blood sample collection. This example illustrates the need for human clinical Treg samples to be evaluated for not only CD4+CD25+, but, at least, for FOXP3 purity after isolation, to allow interpretation of suppression assay findings.

The Treg number is usually claimed to be one of the most limiting factors for such additional tests, but in our studies, we found that it is possible to freeze, thaw and evaluate Treg aliquots of just 2000–10,000 cells, and to perform good quality staining for CD4, CD25, and FOXP3 as well as any other Treg-associated markers of interest (*see* Subheading 3.3, and Fig. 1a and [10] for examples). This number of cells typically requires just a washout from tubes after the majority of Tregs were used for suppression assays.

4.1.2 Role of CTLA4 and an Absence of CD127 as Treg-Associated Markers

To improve Treg/FOXP3+ purity in human samples after isolation, some additional and potentially useful markers have been suggested. In our experience, CTLA4+ and CD127– had the most significant correlation with suppressive function of corresponding isolated Tregs (Fig. 1b and [10]), but use of these markers in clinical studies is not reasonable, due to extremely low Treg yield and inconsistent results for corresponding FOXP3+ expression (described in detail [10]). As a compromise, we included CD127 and CTLA4 expression in our current panel evaluating purity of Tregs after isolation. In a study liver transplant recipients, CD127 expression in isolated Tregs inversely correlated with all other Treg-associated markers, including MFI of FOXP3 (Fig. 1b).

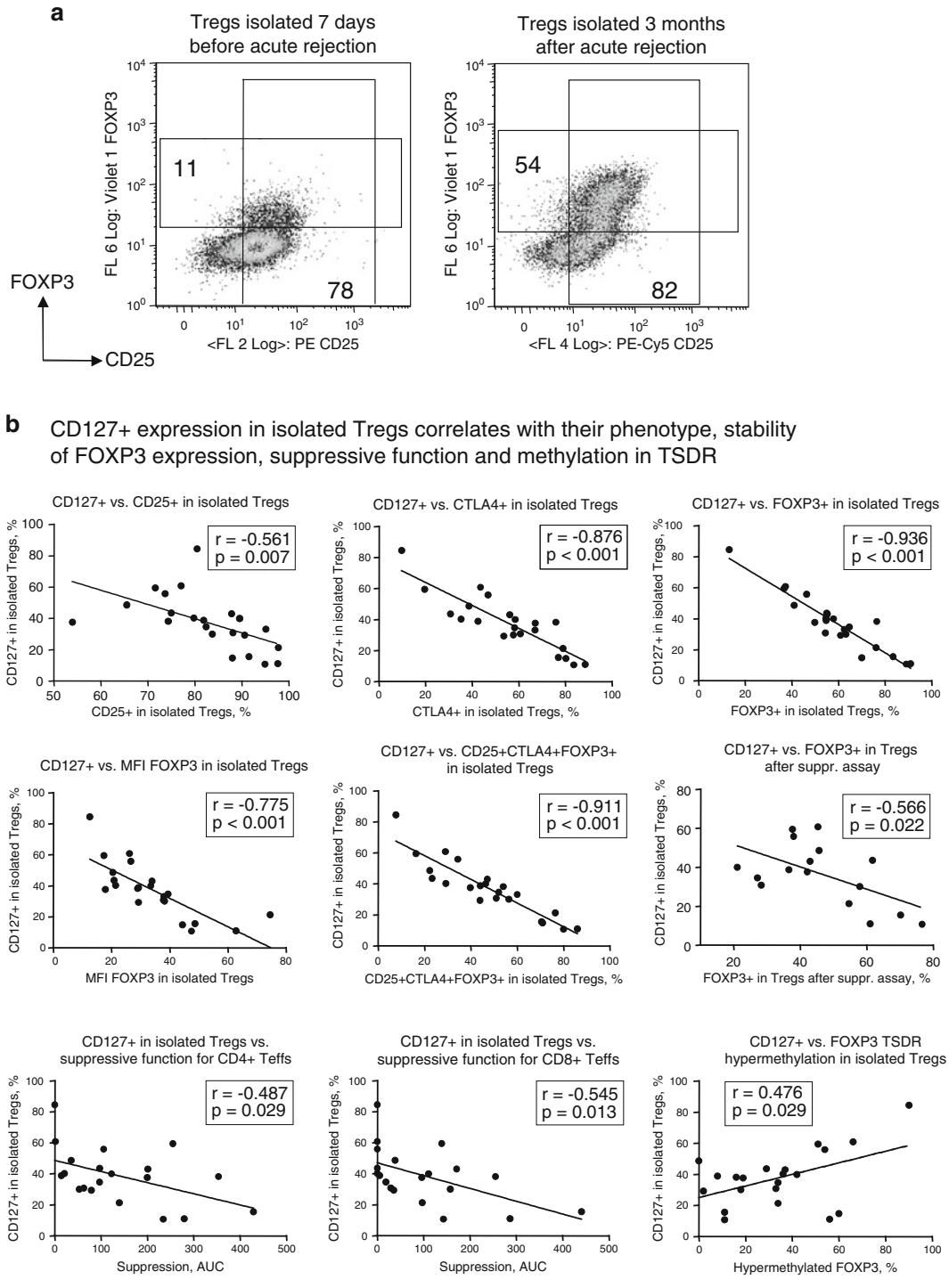


Fig. 1 Treg phenotypes after isolation and assays of their suppressive function. (**a**) Tregs were isolated from the same liver allograft recipient 7 days prior (*left*) and 3 months after an acute rejection episode (*right*) and stained for live/dead, CD4, CD25, and FOXP3. Gated CD4+ cells demonstrate comparable CD25+ expression, but very low FOXP3 level in pre-rejection sample. (**b**) Tregs were isolated from 8 liver allograft recipients pre-transplant, and then at 7–14 days, 3 months, and 1 year post-Tx. Aliquots of isolated Tregs were cryopreserved and stained for live/dead, CD4, CD25, CTLA4, FOXP3, and CD127, while freshly isolated Tregs were used for suppression assays with healthy donor's responder cells (PBMC). CD127 expression in isolated Tregs shows inverse correlations with many important Treg-associated markers and with suppressive function of these cells

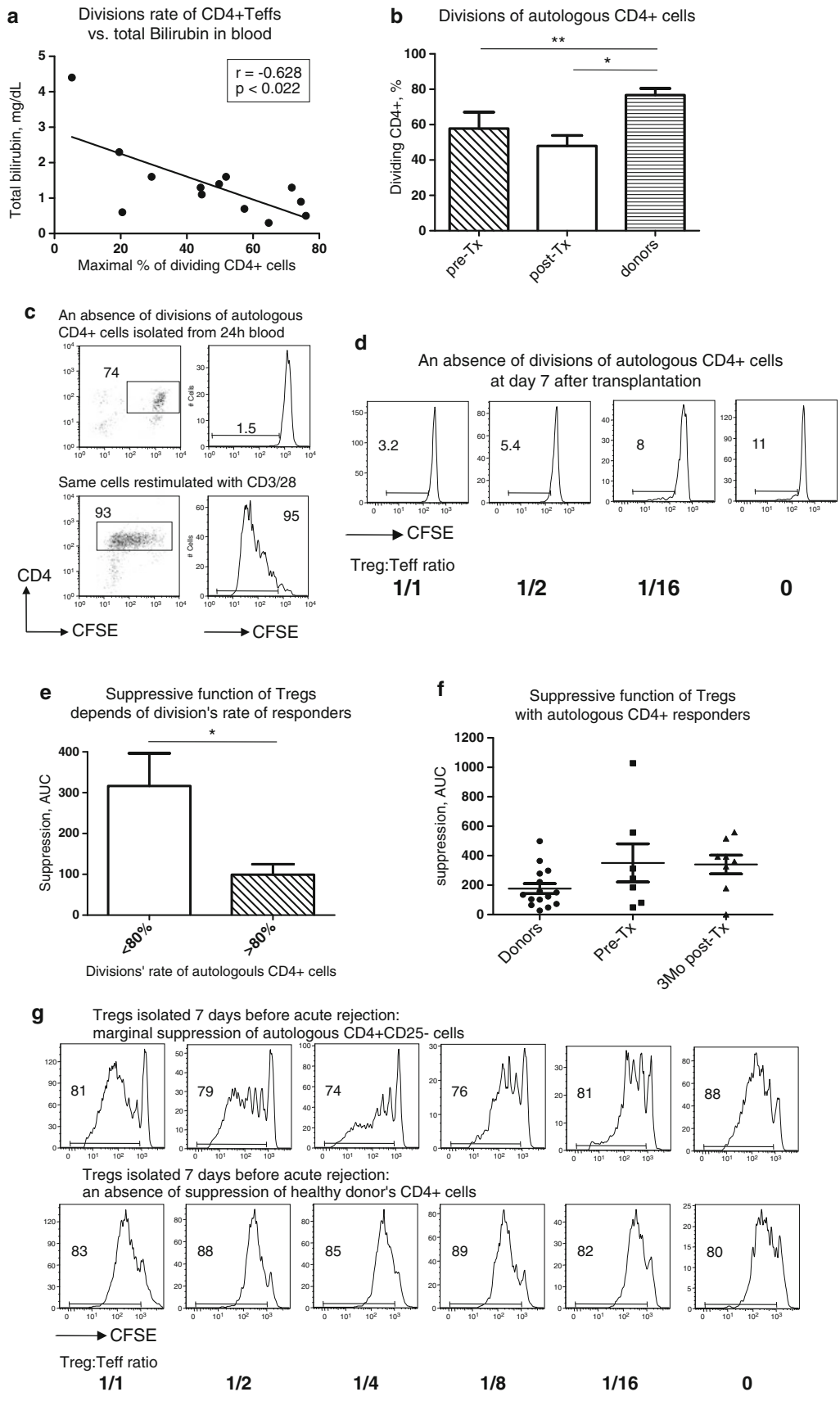
Moreover, CD127 also inversely correlated with stability of FOXP3 expression, evaluated as FOXP3+ in Tregs after 4 days of suppression assay, with suppressive function for CD4+ and CD8+ responders and with FOXP3 demethylation in the Treg-specific demethylated region (TSDR) (Fig. 1b).

4.2 Choice of Responder Cells for Suppression Assay

4.2.1 Problems with Use of Autologous Responder Cells in Clinical Samples

Use of autologous responder cells is often thought to be more physiologically appropriate, and avoids potential alloreactivity. However, autologous responders in Treg suppression assays have grave shortcomings that may lead to unsatisfactory results. First, a disadvantage of autologous responders is their dependence on pre-existing in vivo conditions. Multiple inflammatory and metabolic factors, medications and unidentified factors may affect the ability of CD4+ and CD8+ T cells to divide, as well as their resistance to Treg suppression, and also the costimulatory function of autologous APC. For example, we have noticed in our studies of liver transplant recipients that CD4+ Teff cell division was inversely correlated with total bilirubin levels in corresponding blood samples (Fig. 2a). We also observed a tendency to impaired cellular division in autologous CD4+ Teffs stimulated with CD3 and autologous CD4-depleted irradiated APC in liver transplant patients with hepatitis C, in comparison with the non-hepatitis group (data not shown). As a result of multiple complex factors, CD4+ Teff cellular division from liver transplant recipients did not reach levels obtained with healthy donor controls, before or after transplantation (Fig. 2b). Another problem may arise from

Fig. 2 Problems with use of autologous responder cells in Treg suppression assays. (**a**) Bilirubin levels in blood inversely correlated with Teff proliferation. CD4+CD25– Teffs were isolated from the same liver allograft recipients as in Fig. 1b (7–14 days and 3 months post-Tx), and stimulated with autologous CD4-depleted APC and anti-CD3 beads at 3.5/1 beads/Teff cell ratio for 4 days. Data of Teff proliferation was plotted against corresponding total bilirubin levels obtained at the same time point. (**b**) Allograft recipient CD4+ Teffs demonstrated impaired divisions in comparison with healthy donors cells. CD4+CD25– cells isolated from the same patients as shown at Fig. 1b (pre-Tx or 7–14 days and 3 months post-Tx) were stimulated as in (**a**), and their rates of division were compared with healthy donor CD4+CD25– Teffs stimulated under the same conditions. ANOVA with Newman-Keuls multiple comparison test was used for comparison. (**c**) Whole blood sample from a patient listed for lung transplant was shipped overnight at room temperature, and CD4+CD25– cells were isolated and stimulated as in *panels* (**a**) and (**b**). Four days later, Teffs were alive, but showed a lack of proliferation (*top*). At day 4, CD3/28 beads were added at 1/1 bead/cell ratio, and 4 days later Teffs demonstrated restored proliferation. (**d**) Treg suppression assay was performed with Tregs from liver transplant allograft recipient, with autologous Teffs and APC, stimulated as in *panel* (**a**). Autologous cells, collected 7 days post-Tx, exhibited a lack of proliferation, making evaluation of Tregs suppressive function impossible. (**e**) Results of autologous suppression assays of healthy donors and liver transplant patient Tregs (pre-Tx) were grouped according to maximal rate of CD4+CD25– divisions. When Teffs had impaired proliferation, corresponding Treg function seemed to be higher. Data were analyzed by Mann–Whitney test. (**f**) Results of autologous suppression assays of healthy donors and liver transplant patient Tregs (pre-Tx and 3 months post-Tx) were compared. Healthy donor Tregs tended to demonstrate worse suppressive function than patient Tregs, although data did not reach significant p value (Kruskal-Wallis test). (**g**) Tregs depicted in Fig. 1a on *left*, demonstrated marginal suppression in assay with autologous (*top*), but not with healthy donor responders (*bottom*). For all data, $*p<0.05$ and $**p<0.001$


a
Divisions rate of CD4+Teffs
vs. total Bilirubin in blood
r = -0.628
p < 0.022
Total bilirubin, mg/dL
Maximal % of dividing CD4+ cells
b
Divisions of autologous CD4+ cells
Dividing CD4+, %
pre-Tx
post-Tx
donors
c
An absence of divisions of autologous
CD4+ cells isolated from 24h blood
74
1.5
Same cells restimulated with CD3/28
93
95
CD4
CFSE
CFSE
d
An absence of divisions of autologous CD4+ cells
at day 7 after transplantation
3.2
5.4
8
11
CFSE
Treg:Teff ratio
1/1
1/2
1/16
0
e
Suppressive function of Tregs
depends of division's rate of responders
Suppression, AUC
<80%
>80%
Divisions' rate of autologouls CD4+ cells
f
Suppressive function of Tregs
with autologous CD4+ responders
suppression, AUC
Donors
Pre-Tx
3Mo post-Tx
g
Tregs isolated 7 days before acute rejection:
marginal suppression of autologous CD4+CD25- cells
81
79
74
76
81
88
Tregs isolated 7 days before acute rejection:
an absence of suppression of healthy donor's CD4+ cells
83
88
85
89
82
80
CFSE
Treg:Teff ratio
1/1
1/2
1/4
1/8
1/16
0

technical factors, such as loss of co-stimulatory activity in autologous APC, isolated from overnight-shipped whole blood in multicenter clinical studies (Fig. 2c). The in vivo effects of immunosuppressive therapy in freshly isolated early post-transplant samples can become so prominent that autologous responder cells may become unable to divide with or without the additional impact of Tregs (Fig. 2d).

Importantly, differences in responder cell division rates are problematic in Treg suppression assays, and not off-set by standardization formulas that subtract responder cells divisions in the presence of Tregs from responder cell division in the absence of Tregs, divided by divisions without Treg. By this calculation, the suppressive function of Tregs becomes substantially overestimated in assays with low baseline responder cell division, and vice versa (Fig. 2e and reviewed in ref. 11). Therefore, use of autologous responder cells in clinical Treg studies can be misleading. Any condition that affects the ability of the responder cells to divide can skew the measured suppressive Treg function and lead to overestimation of Treg suppressive function (Fig. 2f).

4.2.2 Use of Healthy Donor Responders

In contrast to autologous Teffs, healthy donor responder cells are convenient for standardization, when multiple small aliquots ($1–3 \times 10^6$ cells per tube, 100–200 vials for study) of PBMC from the same donor may be preserved in liquid nitrogen. In preliminary experiments, we tested PBMC from different donors and the same Tregs, and found that any effect of HLA mismatch for human suppression assays with polyclonal CD3 stimulation was negligible. In other words, "strong" Tregs remained very suppressive with any healthy donor's responder cells, and vice versa. Therefore, to assess patient Treg suppressive function objectively, the suppression assay should be standardized as efficiently as possible, excluding potential confounders from autologous Teffs, leaving patient Tregs to be the only remaining variable in the experimental conditions.

We have noted the practical value of using healthy donor PBMC responder cells in our pediatric allograft cross-sectional study, given the impaired Treg function in patients with higher calcineurin inhibitor (CNI) levels in comparison with low-CNI levels, and correlations of Treg suppressive function with their CTLA4+ and CD127– phenotypes [10]. In addition, ongoing studies of adult liver transplant recipients indicated additional benefits of using standardized, healthy donor PBMC responder cells, Thus, these patients' Tregs showed a marginal suppression with autologous responder cells, but an absence of any suppression, or even some stimulation, with healthy donor PBMC responder cells at 7 days prior to an episode of acute rejection (Fig. 2g). The phenotype of these cells after isolation is shown in Fig. 1a. We found that 10 % of remnant FOXP3+ cells were still able to suppress proliferation of in vivo immunosuppressed autologous responders,

while normal responder cells provided far more plausible and clinically relevant results, namely decreased Treg suppressive function 1 week ahead of an acute rejection episode.

4.2.3 Use of Combinations of Autologous Cells to Obtain Additional Data

Using combinations of a patient's Treg with cryopreserved autologous responder cells can be useful to study the effects of immunosuppression protocols, including responder cell resistance to Treg suppression. Thus, 3 months after the episode of acute rejection, Tregs from the patient depicted at Figs. 1a and 2f were 54 % FOXP3+ after isolation, and restored their suppressive phenotype with healthy donor responder cells (Fig. 3a, top row). A hyper-activated phenotype of cryopreserved pre-rejection CD4+ Teffs from this patient led to substantial apoptotic cell death and impaired cell division using autologous, post-rejection APC (seemingly, due to shortage of co-stimulation for alloreactive clones). However, use of pre-rejection APC in combination with pre-rejection CD4+ Teffs not only improved Teff viability and partially restored their division rate, but also showed enhanced resistance to Treg suppression post-rejection (Fig. 3a, middle and bottom rows, respectively).

Cryopreservation of non-hyper-activated autologous cells is usually free from additional pro-apoptotic effects. These cells survive freezing and thawing well and exhibit neither signs of impaired viability nor defects in proliferation. Thus, comparison of suppression curves of post-transplant CD4+ Teffs with their pre-transplant counterparts (Fig. 3b, top and second rows) clearly shows that post-transplant CNI- and steroid-treated Teffs have diminished capacity for cell division. At the same time, pre-transplant Teff cells do not demonstrate enhanced resistance to post-transplant autologous Treg suppression (i.e., 1/1 and 1/2 ratios are the same despite enhanced proliferation at low Tregs ratios). If post-transplant APC are replaced by pre-transplant APC, free of in vivo immunosuppression effects (Fig. 3b, top vs. third rows), suppression curves show evident increases in cellular resistance to Treg suppression with the same divisional capabilities of post-transplant CD4+ Teffs. This effect can be further studied, e.g., using co-stimulation blocking antibodies, analysis of pre- vs. post-transplant APC phenotypes and their cellular composition, and by investigation of soluble factors released into the cell media. Finally, comparison of suppression curves with post-transplant APC and Teffs vs. pre-transplant APC and Teffs verified these observations, namely that pre-transplant responders had enhanced Teff division and increased resistance to post-transplant autologous Treg suppression. To study these mechanisms in detail, healthy donor Tregs can be used in parallel with patient Tregs and the same combinations of patient responder cells. At the same time, use of healthy donor responder cells can serve as an internal control, providing standardized conditions for evaluation of a patient's "polyclonal" Treg suppressive function.

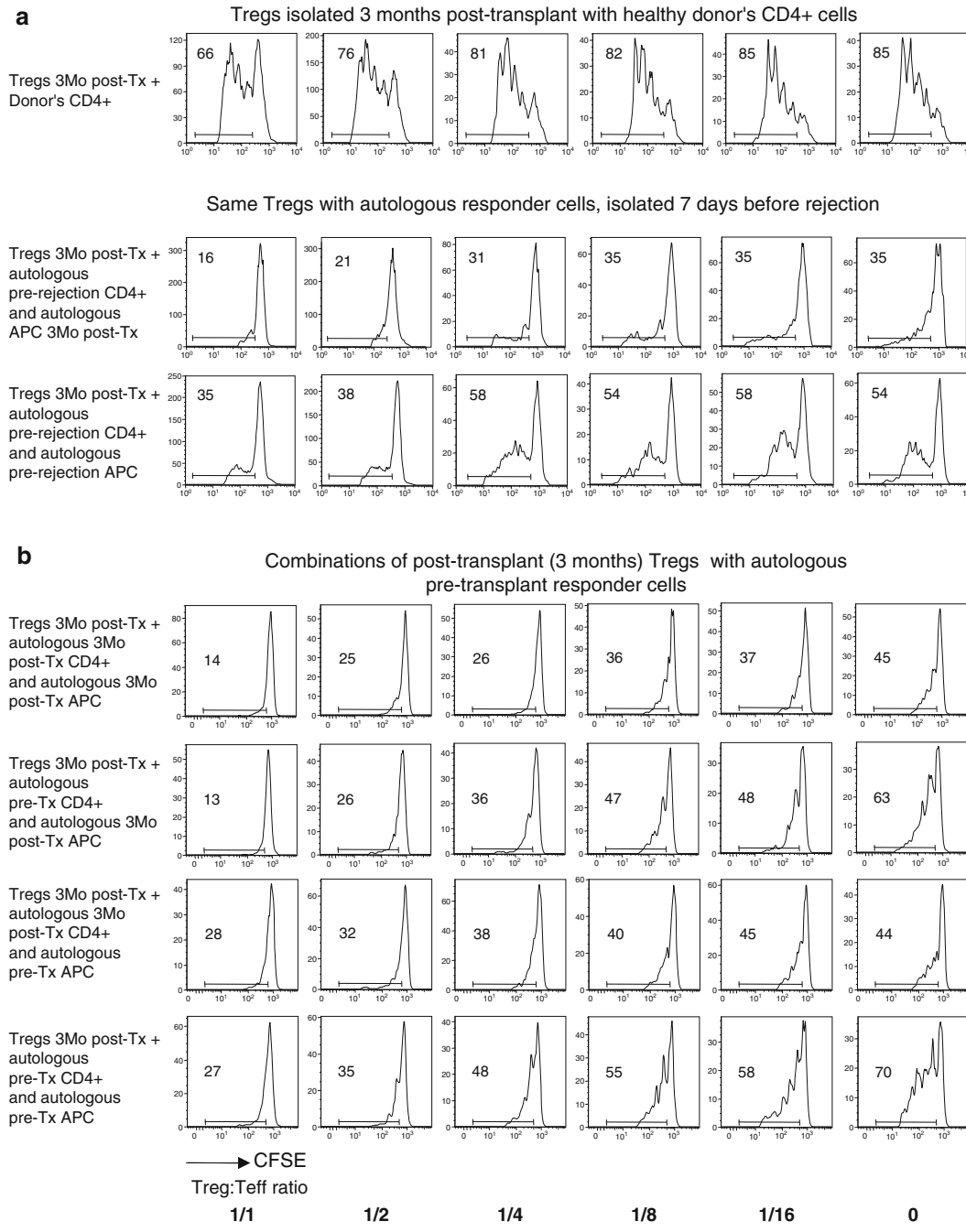


Fig. 3 Use of different combinations of autologous or allogeneic responders to evaluate Treg function (**a**) Tregs shown in Fig. 1a (*right*) demonstrated suppressive function with healthy donor responders (*top*). Combination of pre-rejection autologous Teffs (*middle*) or pre-rejection autologous Teffs and APC (*bottom*) showed that pre-rejection APC provided better co-stimulation for Teffs and some resistance to Treg suppression in comparison with 3 months post-Tx APC. (**b**) Combination of 3 months post-Tx Tregs either with autologous responders collected the same time (*top*) or pre-Tx. Pre-Tx Teffs demonstrate better divisions (*second row*), pre-Tx APC confer increased resistance of Teffs to Treg suppression (*third row*), and combination of both pre-Tx responders (Teffs and APC) demonstrate sufficiently enhanced Teff cell proliferation, as well as increased resistance to Tregs (*bottom row*)

4.3 Standardization of Conditions to Receive Comparable Results of Suppression Assays

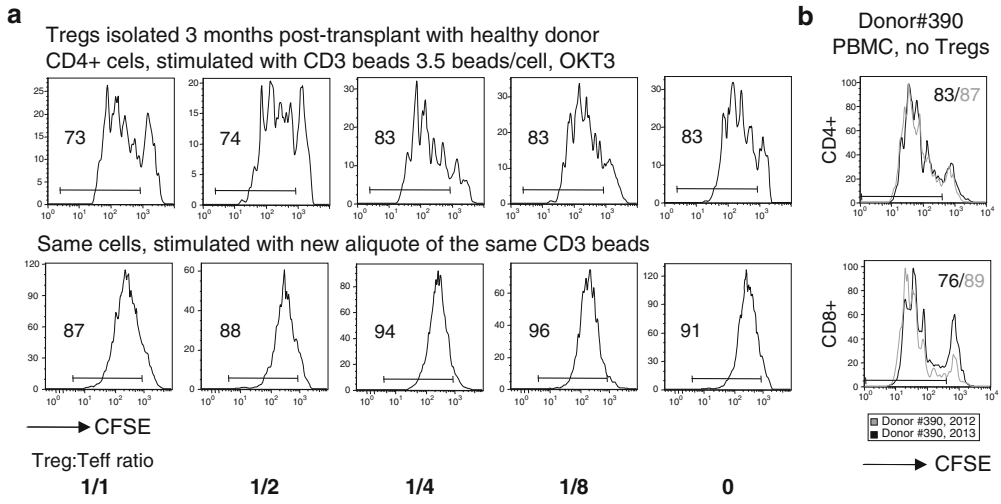
4.3.1 Choice of Healthy Donor and Detection of Optimal Stimulation

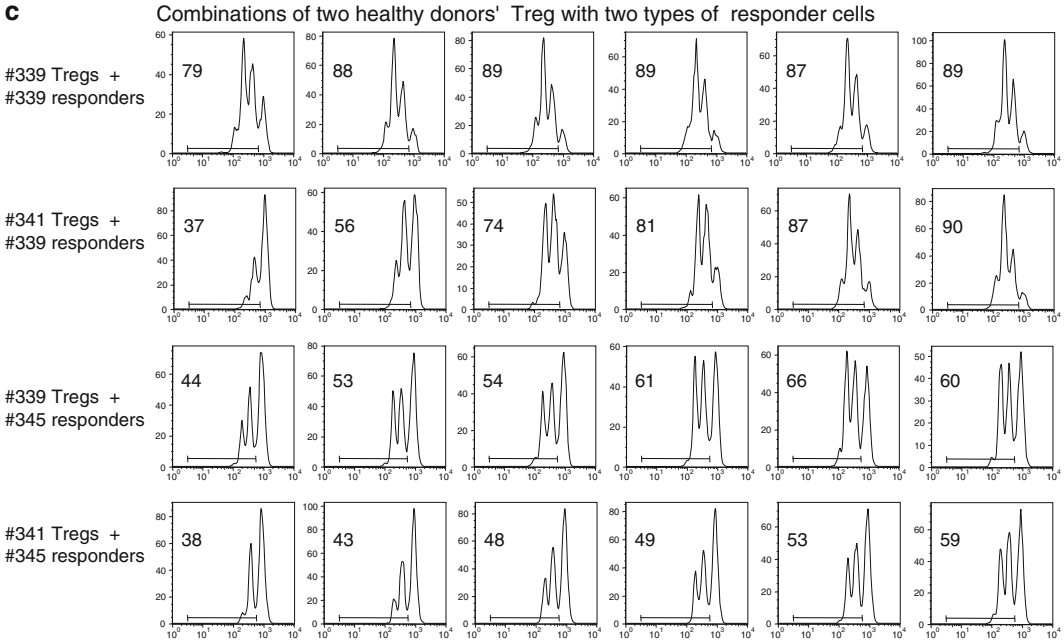
In contrast to the near-uniform proliferative potential of murine responder cells that, under standard conditions, are only affected by technical factors (cell isolation, cell media, CD3 antibody vendor lot), human PBMC responders do not provide such consistency. In our experience, studies involving human Treg suppression assays require serial preliminary tests determining PBMC responsiveness to stimulation, in order to detect the optimum range of stimulation for each donor. The most ideal stimulatory condition for a Treg suppression assay is located slightly below the level where responder PBMC reach a plateau of maximal division. Thus, if a given donor's PBMC divisions are 70 % at 2/1 CD3-bead/cell ratio, 80 % at 2.5/1 and 85 % at 3/1 and above, then 2.5/1 may be the optimal stimulation for a Treg suppression assay.

A second important factor for donor selection is the high consistency of his/her PBMC divisions, evaluated in different cryopreserved aliquots obtained from the same blood sample. Moreover, cryopreserved cells should not demonstrate any significant differences from the corresponding freshly isolated cells, which ensure appropriate cryopreservation-thawing techniques and therefore limit inter-assay variability in experimental conditions. In our current cryopreservation method (Materials and Methods), the majority of healthy donors satisfy these criteria. Comparisons of PBMC from 2 to 5 different donors, with a range of stimulatory conditions within the same experiment, will help to detect persons whose cells are mostly distinct from other donors in their stimulation/co-stimulation requirements. Usually, these donors are not an appropriate choice for use as standardized healthy donor responders as they often demonstrate significant variability after cryopreservation, impaired cell division, or enhanced resistance to Treg suppression. When the best donor and range of optimal stimulatory conditions are established, 2–3 suppression assays with autologous and allogeneic Tregs will help to adjust stimulatory requirements to ensure high division rate with low Treg ratio, and with suppression of division with high Treg ratios. In the following clinical study, division of cryopreserved aliquots of responder cells can be used to maintain standardized conditions for subsequent suppression assays. This is very useful when new aliquots of anti-CD3-coated beads need to be re-calibrated (Fig. 4a), or cell culture media or other reagents need to be replaced, etc.

4.3.2 Cryopreserved vs. Freshly Isolated Responder Cells

Taking into account the potential problems related to cryopreservation, the idea of using the same person as a source of freshly prepared standard PBMC responders for small trials (regarding that just 3 mL of blood is usually sufficient for this purpose) may be an attractive alternative. However, in our experience, healthy donor cells serially collected at different times may not be yield comparable results (Fig. 4b). Thus, well-cryopreserved aliquots of the same PBMC specimen remain the better alternative for standardized investigation of Treg function.


a
Tregs isolated 3 months post-transplant with healthy donor
CD4+ cels, stimulated with CD3 beads 3.5 beads/cell, OKT3
73
74
83
83
83
Same cells, stimulated with new aliquote of the same CD3 beads
87
88
94
96
91
CFSE
Treg:Teff ratio
1/1
1/2
1/4
1/8
0
b
Donor#390
PBMC, no Tregs
CD4+
83/87
CD8+
76/89
Donor #390, 2012
Donor #390, 2013
CFSE


c
Combinations of two healthy donors' Treg with two types of responder cells
#339 Tregs +
#339 responders
79
88
89
89
87
89
#341 Tregs +
#339 responders
37
56
74
81
87
90
#339 Tregs +
#345 responders
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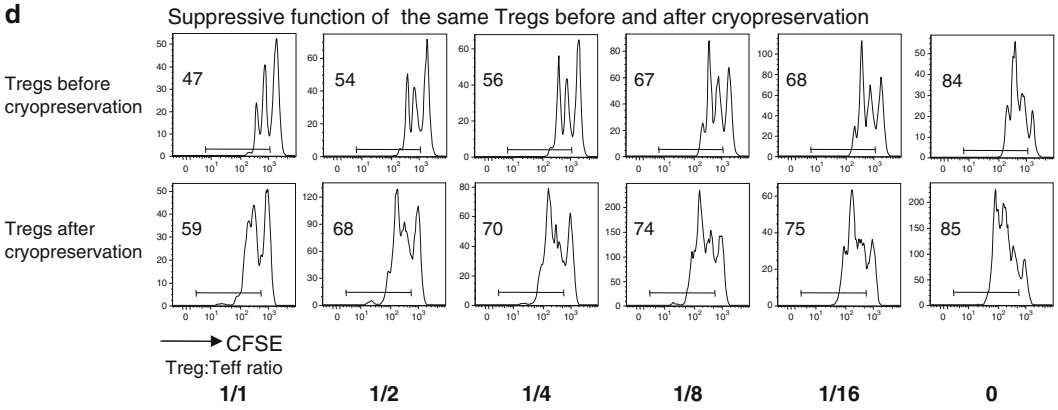
53
54
61
66
60
#341 Tregs +
#345 responders
38
43
48
49
53
59


d
Suppressive function of the same Tregs before and after cryopreservation
Tregs before
cryopreservation
47
54
56
67
68
84
Tregs after
cryopreservation
59
68
70
74
75
85
CFSE
Treg:Teff ratio
1/1
1/2
1/4
1/8
1/16
0

4.3.3 Sensitivity of Suppression Assay Depends of Responders

The optimal power to detect differences in Treg suppressive function may be obtained from responders that showed active divisions without Tregs, and are easily suppressed at high Treg ratios. In Fig. 4c, Tregs from two healthy donors were evaluated in combination with sets of two responder cells. With #339 responders, #341 Tregs demonstrate substantially stronger suppressive function, while #345 responders, due to a "narrow" interval within 1/1-0 Treg/Teffs ratios, cannot serve as a sensitive tool for Treg evaluation. Importantly, as mentioned above, the mutual relation of "stronger" vs. "weaker" Tregs (i.e., #341 and #339 Tregs, respectively, here) did not change when combinations of autologous or allogeneic, resistant or easily suppressed cells were used; therefore, any appropriate healthy donors cells can be used for this analysis, with results differing only in sensitivity.

4.3.4 Use of Cryopreserved Human Tregs

For clinical trials, when arrival of new samples for functional Treg tests is often unpredictable, and can occur after hours or interrupt ongoing experiments, use of cryopreserved PBMC for Treg isolation (or use of isolated cryopreserved Tregs) with later functional evaluation at a convenient time is a highly desirable option. Unfortunately, we have found that while murine Tregs sustain cryopreservation well and do not demonstrate substantial differences in their suppressive function in vitro and in vivo (in this paper [12], part of in vitro tests and all in vivo Tregs tests we performed using cryopreserved murine Tregs), most of freshly isolated human Tregs demonstrate impaired suppressive function after cryopreservation (Fig. 4d). As we were unable to stabilize the suppressive function of cryopreserved human Tregs, even those derived from healthy donors, we aimed to avoid introducing such variability into our clinical trials. However, in our experience, in vitro-expanded human Tregs sustain cryopreservation well, and their suppressive function, as well response to epigenetic compounds, were comparable between fresh and cryopreserved counterparts [13]. Indeterminate changes in functional activity of freshly isolated vs. cryopreserved human

Fig. 4 Factors affecting standardization of Treg suppression assays. (**a**) Tregs isolated at 3 months post-Tx from a liver allograft recipient demonstrated some suppression with healthy donor's responders when stimulated with current aliquot of anti-CD3 beads (*top*), but a new aliquot of anti-CD3 beads used in the same 3.5/1 ratio, clearly over-stimulated responder cells, which led to extinction of Treg suppressive function (*bottom*). (**b**) PBMC from the same healthy donor, collected with 1-year period, demonstrate differences in their divisions. Both PBMC samples were cryopreserved and used within the same experiment. (**c**) Selection of the best responder cells for human Treg suppression assay. While use of both #339 and #345 responders demonstrate that #341 Tregs have better suppressive function than #339 Tregs, responder cells with larger range between 1/1 and 0 Tregs ratios (*top two rows*, #339 responder) provide better sensitivity to discriminate Treg function. (**d**). Impaired function of human Treg function after cryopreservation. While cryopreserved human Tregs were viable and had no evident changes in their phenotype when evaluated by flow cytometry after thawing, they demonstrated impaired suppressive function

Tregs does not preclude their use in experiments where the same cells are using as a control and testing subsets, as for studying of compounds affecting Tregs, providing that fresh Treg cells demonstrate similar results in verification experiments.

4.3.5 Use of Decreased Cell Numbers in Suppression Assay

Importantly, the number of available Tregs is one of the most critical limiting factors not only in clinical trials, but also in some murine disease models. Therefore, we tested suppressive function using different starting Treg numbers, while Treg/Teffs ratios were preserved. In our conditions, murine cells stimulated with irradiated CD3-depleted splenocytes and soluble anti-CD3 antibodies, had markedly diminished proliferation at concentration of APC 30,000 cells/well or less, likely by progressive deficiency of co-stimulation. If the concentration of murine APC was maintained at 50,000 per well, Treg suppression assays showed generally comparable results in conditions from 50,000 to 20,000 cells/well at 1/1 ratios (Table 1). These results also suggest that the standard conditions of a murine Treg suppression assay, soluble factors produced by murine Tregs may operate locally in paracrine manner. However, they are unlikely to play an important role in modification of culture conditions affecting distant responder cells within the same well, since the shapes of Treg suppression curves did not differ when 50,000 or 20,000 Tregs were used in the same 200 μl total volumes.

Results of similar cellular titration with human Tregs do not provide such reproducible results due to high variability, but we

Table 1
Effects of different startingnumbers of cells in murine Treg suppression assays

Concentration, cells per well	Divisions of CD4+ Teffs at corresponding Treg/Teff ratio, %				
	1/1	1/2	1/4	1/8	0
50,000	29	45.4	67.2	77.4	93.7
40,000	27.8	**32.3**	58.1	75.9	94.8
30,000	**20.9**	**35.8**	63.1	80	96.4
25,000	30.8	41.6	**47.5**	73.1	97.3
20,000	26.8	42.5	60	79.8	95.7

Treg suppression assays were performed using 20, 25, 30, 40, and 50×10^3 of Tregs/Teffs per well in 1/1 ratios. Soluble anti-CD3 mAbs were added to final concentration of 1 μg/ml, and each well had 50×10^3 of irradiated CD3-depleted APC. The final volume of culture media in each well was equal to 200 μl, and the same U-bottom plate was used. Values that considerably differ from corresponding counterparts are bolded. This experiment was performed twice with similar results

have not observed significant differences in suppression function at 50,000 to 35,000 cells/well. At smaller numbers, 15,000–20,000 cells/well at 1/1 ratio in V-bottom plates, human Tregs tend to show an enhanced suppression in comparison with higher cellularity controls, but pipetting inaccuracies in serial dilutions, and therefore variability of results within duplicates, increase. As a practical approach, we routinely use 30,000–35,000 human Tregs at a 1/1 ratio, which allows to obtain more data from clinical samples with restricted blood volume or from post-transplant immunosuppression lymphopenic samples, but still provides reasonable accuracy in cell counting and pipetting.

4.3.6 Use of a Single Treg-Responder Cell Ratio if Treg Cell Numbers Are Limiting

Shortage of available Tregs in clinical studies often lead to attempts of researchers to save cells using just one given ratio in suppression assay for all samples. In our opinion, this approach has significant shortcomings. First, equal Treg function at one given ratio does not mean true equal function. Thus, in high Treg/Teffs ratios, when suppression is maximal and responders are not resistant, "weaker" Tregs can reach the same levels of feasible suppression as the "stronger" Tregs, and apparent differences in that case can be observed only when number of Teffs per one Treg increases (examples with raw data, Supplemental materials of [12]). Conversely, at lowest Treg/Teff ratios, suppression curves from Tregs with different suppression capabilities may merge together due to already equally diminished effects of suppression (Fig. 4, C 1/16 ratios at two top rows). Therefore, use of serial Tregs/Teffs dilutions increase the resolution of suppression assays, by increasing data for comparison and by leveling results of small pipetting errors with low cell numbers. Integrating curves of suppressive function over all measured ratios may additionally increase statistical power (see below).

4.4 Common Problems with Drug Testing

4.4.1 Compound Has no Specificity for Tregs

Since therapeutic manipulation of Tregs is an important area of interest, the effects of numerous compounds on Treg function have been tested. Unfortunately, the design of some studies precludes unambiguous interpretation of the results. One of the most frequent problems is the absence of any experiments showing the specificity of a given compound on Tregs vs. other cells, such as Teffs, especially for a compound that compromises Treg function. Figure 5a shows the results of Treg suppression assay with a chemically active compound isolated from Australian pigweed [14] and suggested to have a detrimental effect on Treg functionality. Murine Tregs, preincubated with as little as 400 nM of this compound, demonstrated impaired suppressive function in comparison with Tregs pre-incubated with inactive control. However, such results cannot be interpreted as a Treg-related or Treg-specific effect, as murine Teffs, pretreated with the same compound, also showed impaired division (Fig. 5b). Thus, despite the absence of evident cell toxicity at the tested concentrations of active

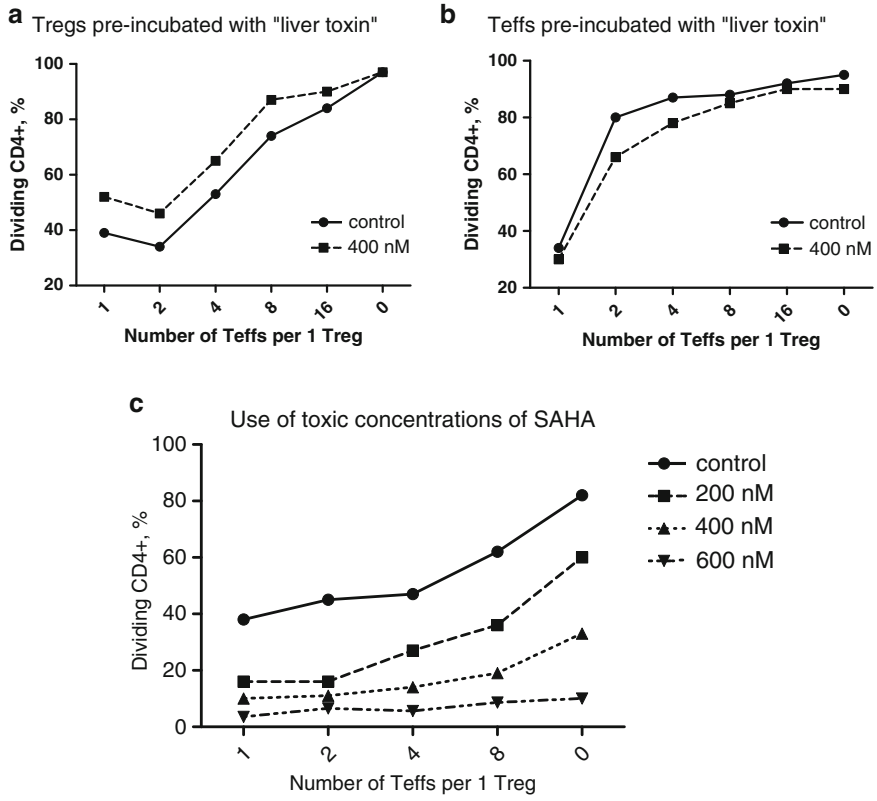


Fig. 5 Use of Treg suppression assay to evaluate compounds suggested to affect Treg function. Murine Tregs (**a**) or Teffs (**b**) were preincubated with a chemically active compound isolated from Australian pigweed plant, or with control inactive compound, for 2 h, washed twice and tested in a Treg suppression assay. In preliminary experiments, 400 nM of compound did not show any evident toxic effects during 3 days of culture of murine splenocytes. While data show impaired Treg suppression (**a**), additional data (**b**) demonstrated impaired division of pretreated CD4+CD25– Teffs, suggesting the effect of the compound is not specific for Tregs. Each experiment was performed twice with different concentrations of compounds and similar results. (**c**) Human Tregs, CD4+CD25– Teffs and CD3-depleted irradiated APC were incubated with different concentrations of SAHA for 4 days. As the concentrations of SAHA used directly inhibited Teff division, a finding of enhanced Treg function due to SAHA treatment cannot be distinguished from direct inhibitory effect of SAHA on responder cells, and therefore no reliable conclusions can be derived from such experiments

compound, a given compound can impair T cell division rate and the functional ability of T cells without compromising their viability, and therefore does not specify a Treg-mediated effect, at least during these preliminary in vitro experiments.

4.4.2 Compound Directly Affects Responder Cells

Another common problem in study design using compounds suggested to enhance Treg function is the use of reagents that directly suppress T cell divisions and/or disrupt APC co-stimulatory function in the suppression assay, when all three cellular participants (Tregs, Teffs, and APC) are treated together. In that case, effects on Tregs (stimulation of function and therefore decrease of

responder cell divisions) cannot be distinguished from effects on responders (direct inhibition of responder divisions). Figure 5c shows results of in vitro human suppression assay with increasing concentrations of the clinically approved HDACi, SAHA. A gradually decreased rate of Teff divisions corresponded with increased direct effect of SAHA on responder cells (clearly seen at zero-Treg ratios, Fig. 5c). Therefore, in this design, the data cannot be attributed to effects of SAHA on Treg function. For such experiments, it is reasonable to perform a series of preliminary tests to establish the concentration of compound that has no obvious effect on responder cell proliferation. For murine cells, these preliminary tests are usually not problematic since they usually yield a reasonably narrow range of optimal concentrations that maintain as long as animals, operator and assay conditions are unchanged. However, with human cells, we found very high individual variability in such concentrations (Table 2). To overcome this problem, in human Treg suppression assays we test each compound at 2–4 different concentrations, and choose the one that shows no effects on

Table 2
Results of preliminary tests to establish range of concentrations of epigenetic compounds that do not affect Teff division in the absence of Treg cells

	Decitabine (DNMTi)				Valproic acid (HDACi)				
	Donor and results: toxic effect, %				Donor and results: toxic effect, %				
	Donor1	Donor 3	Donor 5		Donor 1	Donor 2	Donor 4	Donor 5	Donor 6
15.6 nM	*n/t*	*n/t*	**0**	**15.6 µM**	*n/t*	*n/t*	*n/t*	**0**	*n/t*
31.25 nM	*n/t*	*n/t*	**7.6**	**31.25 µM**	*n/t*	**2.1**	*n/t*	**0**	**3.5**
62.5 nM	*n/t*	*n/t*	**5.8**	**62.5 µM**	*n/t*	**7.6**	*n/t*	**3**	**4**
0.125 µM	*n/t*	*n/t*	**9.34**	**0.125 mM**	*n/t*	**10.5**	*n/t*	**2.7**	**2.7**
0.25 µM	*n/t*	**14.7**	**13.75**	**0.25 mM**	*n/t*	**25.4**	**5.6**	**7.8**	*n/t*
0.5 µM	*n/t*	**17.3**	*n/t*	**0.5 mM**	**11.5**	*n/t*	**13**	*n/t*	*n/t*
1 µM	*n/t*	**19.7**	*n/t*	**1.0 mM**	**46.5**	*n/t*	*n/t*	*n/t*	*n/t*
3.42 µM	**8.4**	*n/t*	*n/t*						
6.84 µM	**14.7**	*n/t*	*n/t*						
13.69 µM	**10.4**	*n/t*	*n/t*						
27.38 µM	**15.5**	*n/t*	*n/t*						
54.75 µM	**17.4**	*n/t*	*n/t*						

Healthy donor's CD4+CD25– Teffs were CFSE labeled, incubated with irradiated autologous CD4-depleted APC and with listed concentrations of compounds. Differences between divisions rate in control (no compound) vs. tested concentrations are shown as "Toxic effect, %." "n/t"—not tested

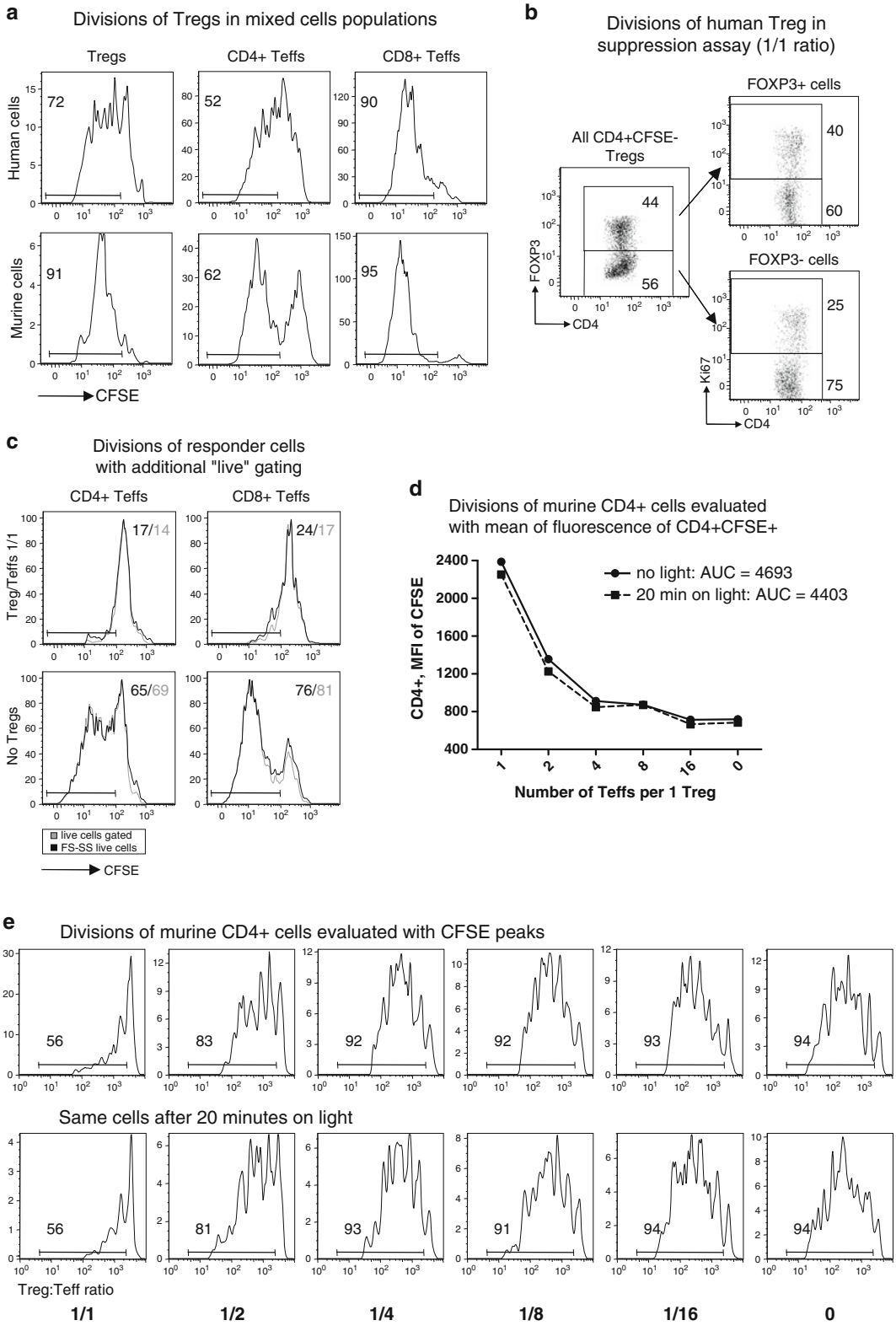
responders in the absence of Tregs. Even this design, carefully controlled for cell viability (toxic effects) and with elimination of results that are not related to Tregs (if direct effects on responders' division rates were observed), it is not ensured that effects are indeed Treg intrinsic. In that case, the validation experiments with Tregs, pretreated separately [13], and/or confirmatory in vivo experiments [12] with similar results, should be performed.

4.5 Readout of Suppression Assay

4.5.1 Thymidine Incorporation vs. CFSE Dilution

For decades, thymidine incorporation has been considered as a "gold standard" for MLR assays, and, subsequently, as a readout for Treg suppression assays. Multiple data showed an absence of Treg divisions upon anti-CD3 stimulation (or even with dual CD3/CD28 stimulation with soluble antibodies, plate-bound antibodies or with CD3/28 beads). These data led to assertions that all divisional activity, evaluated with thymidine incorporation, originated from Teff cells. However, this assumption of Tregs non-division is true only in the absence of Teffs in the suppression assay. In mixed cell populations, such as PBMC, lymph node cells or splenocytes (Fig. 6a), both murine and human Tregs divide well, and their division rate is usually even higher than in CD4+ Teffs, and sometimes approaches the division rate of CD8+ responders. The same is true for Treg suppression assays: use of two different fluorochromes allows for the evaluation of the division of responder and Treg cells in murine or human assays [15]. Similarly to murine cells, Ki-67 staining is also highly positive in human Tregs after suppression assays, especially for cells with stable FOXP3 expression that retain the Treg phenotype during the 4–5 days of assay (Fig. 6b). Therefore, when thymidine incorporation is employed in Treg suppression assays, data reflect division of both Tregs and Teffs, with additional confounding factors such as different Treg/Teffs ratios and level of suppression toward Teffs. These factors cannot be separated by this technique. Another important concern is that thymidine incorporation provides data of cellular division evaluated just within the last day of assay, while maximal suppression may occur within the first hours of Treg/Teff contact [16]. In contrast to thymidine-based assays, dilution of CFSE (or similar reagents) allows evaluation of cell division by responders and also, if needed, Tregs. An investigator can also perform co-staining with

Fig. 6 Different approaches to the analysis of Treg suppression assay data. (**a**) Human (*top*) and murine (*bottom*) Treg division in mixed cell populations consisting of PBMC (*top*) or splenocytes (*bottom*). Cells were stimulated with CD3, without addition of IL-2. Percent of dividing cells is shown. (**b**) Human Treg division in 4 day suppression assays, 1/1 ratio. Tregs with stable FOXP3+ phenotype upregulated Ki-67 better than Tregs that lost FOXP3. (**c**) Differences in cell division when live cells were gated according to FS-SS properties (*black histograms*), or when FS-SS gating was complemented with live/dead staining (*grey histograms*). Differences in CFSE mean fluorescence (**d**) or in percent of dividing cells (**e**) of the same cells after 20 min of light exposure of murine cells in Treg suppression assay


a
Divisions of Tregs in mixed cells populations
Tregs
CD4+ Teffs
CD8+ Teffs
Human cells
Murine cells
72
52
90
91
62
95
CFSE
b
Divisions of human Treg in suppression assay (1/1 ratio)
All CD4+CFSE- Tregs
FOXP3
CD4
44
56
FOXP3+ cells
40
60
FOXP3- cells
25
75
Ki67
CD4
c
Divisions of responder cells with additional "live" gating
CD4+ Teffs
CD8+ Teffs
Treg/Teffs 1/1
No Tregs
17/14
24/17
65/69
76/81
live cells gated
FS-SS live cells
CFSE
d
Divisions of murine CD4+ cells evaluated with mean of fluorescence of CD4+CFSE+
CD4+, MFI of CFSE
no light: AUC = 4693
20 min on light: AUC = 4403
Number of Teffs per 1 Treg
e
Divisions of murine CD4+ cells evaluated with CFSE peaks
56
83
92
92
93
94
Same cells after 20 minutes on light
56
81
93
91
94
94
Treg:Teff ratio
1/1
1/2
1/4
1/8
1/16
0

CD4 or CD8 antibodies, or study additional responder subsets such as naïve/memory populations, subsets of cells with upregulated activation markers, and Tregs after suppression assay for any markers of interest.

4.5.2 Methods to Enhance Quality of CFSE Peaks

Various concerns can also arise using CFSE-based methodology. While forward-scatter/side-scatter (FS-SS) gating after a suppression assay is usually sufficient to discern dead vs. live cells, less satisfactory results may be obtained when biosafety regulations (as for clinical samples) or intracellular co-staining require the use of fixed cells. In these cases, use of fixable live-dead markers prior to fixation (Material and Methods) gives significantly improved flow cytometry data. For unfixed cells, results of cell division, when FS-SS live gated cells or FS-SS + live/dead gating strategy are used, give similar data (Fig. 6c) although FS-SS gating tends to slightly flatten suppressive curves (providing more divisions at high Treg ratios and less divisions in low Treg ratios in comparison with live/dead gated subsets).

Use of pulse width gating to include only single cells for evaluation additionally improves the quality of CFSE peaks, and can be useful when tissue cells (splenocytes, lymph node cells) are used, or when toxic effects are expected. For human PBMC in standard suppression assay conditions, very few cells are not singlets. In our experience, the quality of CFSE peaks, making them still easily distinguishable by the end of an experiment, is usually not an issue in murine Treg suppression assays. However, human PBMC responders from some healthy donors and a considerable percent of patient PBMC do not divide synchronously, and therefore do not provide clearly distinguishable peaks. In that case, use of isolated CD4+ or CD8+ responders with irradiated, CD3-depleted APC, instead of CFSE-labeled PBMC, can improve the shape of CFSE peaks.

Due to difficulties with resolution of peaks, some researchers prefer to use the mean of CFSE fluorescence instead of identification of nondividing subsets. However, this approach suffers from a high level of variability: multiple factors (number of cells for labeling, protein content in cell media during labeling, temperature and time of labeling, age of CFSE aliquot stock solution, light exposure for CFSE and for labeled cells, cell viability and their time in assay) affect the brightness of CFSE signal, making it difficult to perform reliable inter-assays comparisons that are often critical in clinical studies. CFSE brightness may be so labile as to compromise results even in the same experiment, when multiple concentrations of testing compound are compared with controls that were run previously. Here we show comparison of CFSE mean fluorescence (Fig. 6d) vs. CFSE peaks (Fig. 6e) after 20 min of routine room light exposure. When CFSE mean of fluorescence was used for evaluation, nondividing and once divided cells with highest CFSE fluorescent means lost their brightness faster, leading to shrinkage

of suppression curves (and false decreases in Treg suppressive function) within as little as 20 min of light exposure.

For hampered CFSE-peak shapes, an additional co-staining with Ki-67 helps to accurately establish location of nondividing CFSE peak either for murine or human responders (Fig. 7a). A great advantage of this method is there is no need to routinely fix cells and co-stain them with Ki-67, and can be a result-saving strategy when exploratory flow cytometry shows hardly distinguishable CFSE peaks.

4.5.3 Effects of CD4+CD25– Gating on Responders

Since ideally most Teffs are CD4+CD25– and Tregs are CD4+CD25+, some researchers use CD25 co-staining and gate for the CD25– subset in CFSE+ Teffs to evaluate cell division. We consider this approach unsuitable since murine and human Teff cells easily upregulate CD25 after just overnight stimulation. Furthermore, with human T cells, CD25– gating will falsely increase Treg suppressive function since CD25+ Teffs have a greater division rate compared to those that retain the CD25– phenotype (Fig. 7b). The same is true for mouse C57BL/6 cells (Fig. 7c, left), but, interestingly Th2-biased BALB/c mice demonstrate the inverse; in these mice, CD25+ cells showed impaired cellular division during suppression assays, compared to CD25– T cells (Fig. 7c, right). Taken together, the use CD25+ or CD25– gating for responder T cells may provide additional readouts, but should not be used as a method to discern Teffs from Tregs.

4.5.4 Alternative Ways to Evaluate of CFSE Peaks: Division Index and Calculation of Progenitors

Use of division index instead of percent of dividing cells, as described [17], may be an excellent alternative providing higher sensitivity, but it is valid only in conditions where all CFSE peaks are easily visible and distinguishable from each other, which is often not the case with clinical samples. However, our AUC-based method to calculate Treg suppressive function as one number for all Treg/Teffs ratios may be applied for division index data as well as for percent of divisions (see below).

Another readout method in Treg suppression assays came from studies with cell lines and is based on the calculation of progenitors, as reviewed [17]. This method works on the assumption that each cell produces two daughter cells after division, and therefore the final number of cells in the last division peak is the result of this geometric progression with a denominator of 2. This method is appropriate for cell lines, where normally only a very small percent of cells stop division, and few die during an experiment under normal conditions. However, in our experience, primary cells always demonstrate noticeable levels of apoptosis, and also can stop after 1, 2, or 3 rounds of division due to multiple factors, just one of which is suppression by Tregs. The shape of typical CFSE peaks with murine or human primary T cells, with reduced numbers of dividing cells in the last division rounds, demonstrates this point.

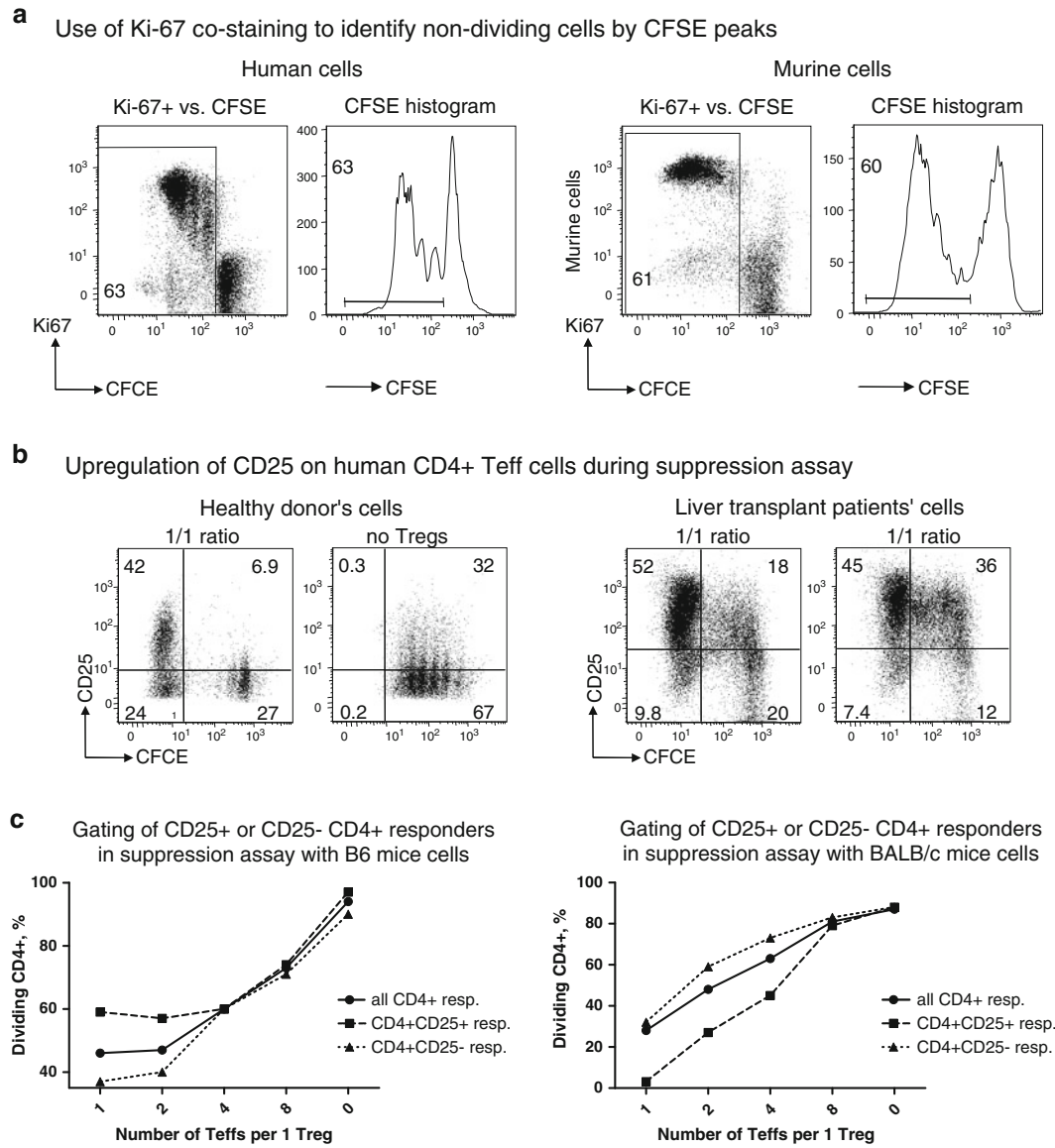


Fig. 7 Readout of CFSE-based suppression assay. (**a**) Co-staining with Ki-67 helps to locate the first CFSE peak of nondividing cells in human (*left*) or murine (*right*) suppression assays. (**b**) In human suppression assays, Teffs cells that upregulated CD25 are better dividers than CD25– cells. Healthy donor (*left*) or pediatric liver allograft patients (*middle* and *right*) Tregs were used in suppression assays with autologous CD4+CD25– Teffs (*left*) or with healthy donor PBMC (*middle* and *right*) as responders cells. 4 days later, cells were stained for CD4 and CD25, and CD25+ cells were plotted vs. CFSE in CD4+ gated cells. Tregs are located on left two quadrants of each dot plot. (**c**) In murine Treg suppression assays using cells from C57BL/6 mice, CD4+CD25+ responder cells demonstrate better divisions than CD25– Teffs (*left*), while using cells from BALB/c mice, cells with upregulated CD25 proliferated less than CD4+CD25– Teffs (*right*). Each murine experiment was performed twice with the same results

4.6 How to Calculate AUC for Treg Suppression Assay

4.6.1 Two Modifications of X-Axis Labeling to Increase Sensitivity of Assay

Area-under-curve (AUC) calculations are commonly used with glucose tolerance tests and in pharmacokinetic studies. Mathematically, it is a definite integral, but for practical purposes most statistical software use trapezoidal approximations for calculation. Any standard statistical package can be used to obtain results of Treg suppressive function as a standard calculation of AUC. For this method, we use an assumption that serial dilution of Tregs in Treg/Teffs ratios is the equivalent of a time function on the *x*-axis, where concentration of a given medication in patient's plasma gradually decreases. Therefore, Treg suppression at any given ratio from 1/1 to 1/16 (number of Teffs per Treg) or lower, if needed, is plotted on the *y*-axis against corresponding ratio. It should be noted that in this modification, AUC is sensitive to variability of results in low Treg/Teffs ratios and therefore good for Treg suppressive curves where the greatest differences occur at low ratios. Conversely, if responder cells are sensitive to suppression, and raw Treg curves demonstrate the highest differences in suppression in high Treg/Teffs ratios such as 1/1 and 1/2 (or even 2/1), it may be reasonable to replace "number of Teffs per 1 Treg" on *x*-axis to numbers equivalent to degrees of Treg dilutions. Therefore, in first scenario (AUC is sensitive to low ratios) the x-axis is labeled as 1, 2, 4, 8 and 16, while in second scenario the x-axis has numbers 0, 1, 2, 3, and 4. Both modifications will yield similar results in terms of discrimination of "weaker" vs. "stronger" Tregs, but the degree of these differences would differ according to the initial shape of the raw Treg curves.

4.6.2 AUCs for Drug Use vs. AUCs in Clinical Treg Studies

Two additional modifications of AUC calculations of suppressive function include experimental designs with internal controls vs. serial assessments of Treg function. Examples of the latter are evaluations of Treg suppressive functions in cross-sectional clinical studies [10], or calculations of Treg suppressive function in follow-up clinical studies, whereeach patient provides Treg samples at different times. For such studies, Tregs assays should be highly standardized within their conditions (see above) to ensure that resulting data will be intercomparable, and patient's Tregs would be the single main factor that differs between experiments. An example of AUC calculation for this type of studies is shown in Fig. 8. Here, raw data of four Treg suppression assays, evaluating Tregs from the same liver allograft recipient at different time points are shown. For these experiments, autologous CD4+CD25– responders were used, and 7 day post-transplant (post-Tx) data with maximal levels of immunosuppression clearly show impaired CD4+ division. Interestingly, in this patient, CD4+ T cells recovered their proliferative activity by 3 months post-Tx (Fig. 8a). Then, raw suppression data were re-calculated as standardized suppression using the formula $S = ((\text{divisions without Tregs} - \text{divisions}$

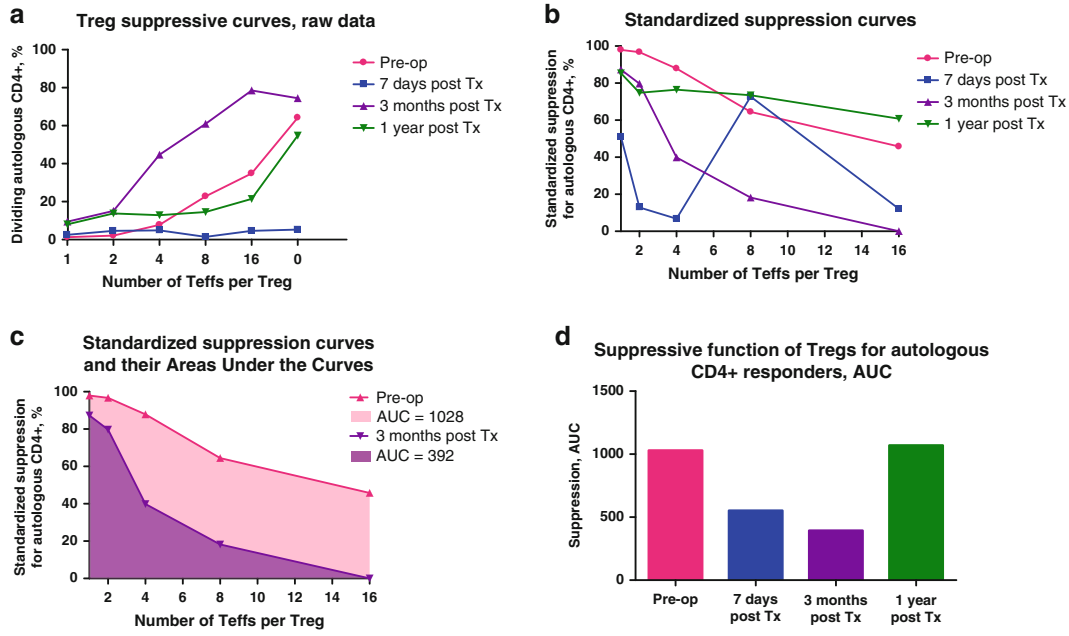


Fig. 8 Calculation of standardized suppression and AUC for Treg assays in clinical Treg studies. (**a**) Raw data showing CD4+ Teff proliferation in four autologous suppression assays, performed with samples from the same liver allograft recipient. Cells were isolated preoperative (Pre-op), and at 7 days, 3 months, and 1 year post-Tx. (**b**) Standardized suppression (*S*) was calculated using $S = ((\text{divisions without Tregs} - \text{divisions in current ratio})/\text{divisions without Tregs}) \times 100$. (**c**) Two areas under the standardized suppression curves, as in (**b**), are shaded for illustrative purposes. Corresponding AUC values are shown in graph legend on *right*. (**d**) Suppressive function of four Treg samples for autologous CD4+ responders, calculated as AUCs

in current ratio)/divisions without Tregs) × 100, where *S* is standardized suppression [18]. Due to significantly impaired division of autologous cells at 7 days post-Tx, standardized suppression is evidently compromised: the small variability in cell divisions occurred at 1/8 Treg/Teffs ratio turned out to produce a high suppression peak after re-calculation (Fig. 8b). This example illustrates the trial design issues discussed above, where autologous cells, if they have impaired division, cannot provide reliable Treg data but rather tend to lead to false-positive suppression findings, and, conversely, resistant autologous Teffs tend to lead to false negative assessment of Treg suppressive function. Figure 8c uses the same data as for Fig. 8b, but actual areas under two suppressive curves are shaded for illustrative purposes, and two corresponding AUCs values are provided in the legend. Clearly pre-Tx Treg suppressive function was better, although autologous cell results have to be validated with standardized conditions, i.e., with healthy donor responder cells. Finally, Fig. 8d shows results for all four suppressive curves calculated as AUCs.

Examples of experimental design with internal controls are tests with epigenetically active compounds [13], or WT vs. different KO mice [19], or control vs. disease groups of mice, i.e., experiments where Treg function is compared in experimental vs. control conditions. The experimental conditions for each independent experiment in that design may be not comparable with each other. Thus, different healthy donors Tregs with their own autologous responders may be used each time, mouse groups can differ in age, gender, duration of experiment, etc., but within a given experiment, all cells (except for Tregs) are the same. In that case, standardization of raw Tregs suppression curves may be calculated firstly as min-max normalization (*see* Fig. 9a and Table 3, Steps 1 and 2), and then transformed into standardized suppression using $\Upsilon_t = 100 - \Upsilon$ formula for standardization, where Υ_t is standardized transformed suppression (in %), and Υ is normalized suppression (*see* Fig. 9a–c and Table 3, Steps 2 and 3 for detailed example). Resulting AUCs (Fig. 9d) may be additionally calculated as a fold ratio to control Tregs' AUC (Fig. 9e), which provides an option to group results of different experiments into the final groups with p value calculations for statistical differences [13].

In summary, we have used this described AUC calculation method in murine and human studies for over 6 years, and found it to be convenient, sensitive and reproducible in any given Treg suppression assay design, as well as a viable method to standardize between different investigators.

5 Discussion

Assessment of Treg suppressive function remains one of the most reliable methods for identification of Tregs ex vivo, and also facilitates the evaluation of new Treg-associated markers, including surface molecules, cytoplasmic or nuclear factors, and epigenetic modifications. Within the past several years, many detailed and thoughtful protocols on the essential elements of Treg suppression assays have been published [11, 17, 18, 20–26]. Therefore, we focused this work on features of Treg suppression assays, especially with regard to clinical samples. We have described the importance of assay standardization, and provided a new method for reliably measuring and calculating Treg suppressive function.

There is a great variability in conditions of Treg suppression assays [17], and multiple factors may affect the results [11]. Historically, most Treg assays employed thymidine incorporation, which has exquisite sensitivity to detect differences using low cell numbers, and does not require access to a flow cytometer. Unfortunately, this method involves the false assumption that Treg divisions do not compromise the final results due to their

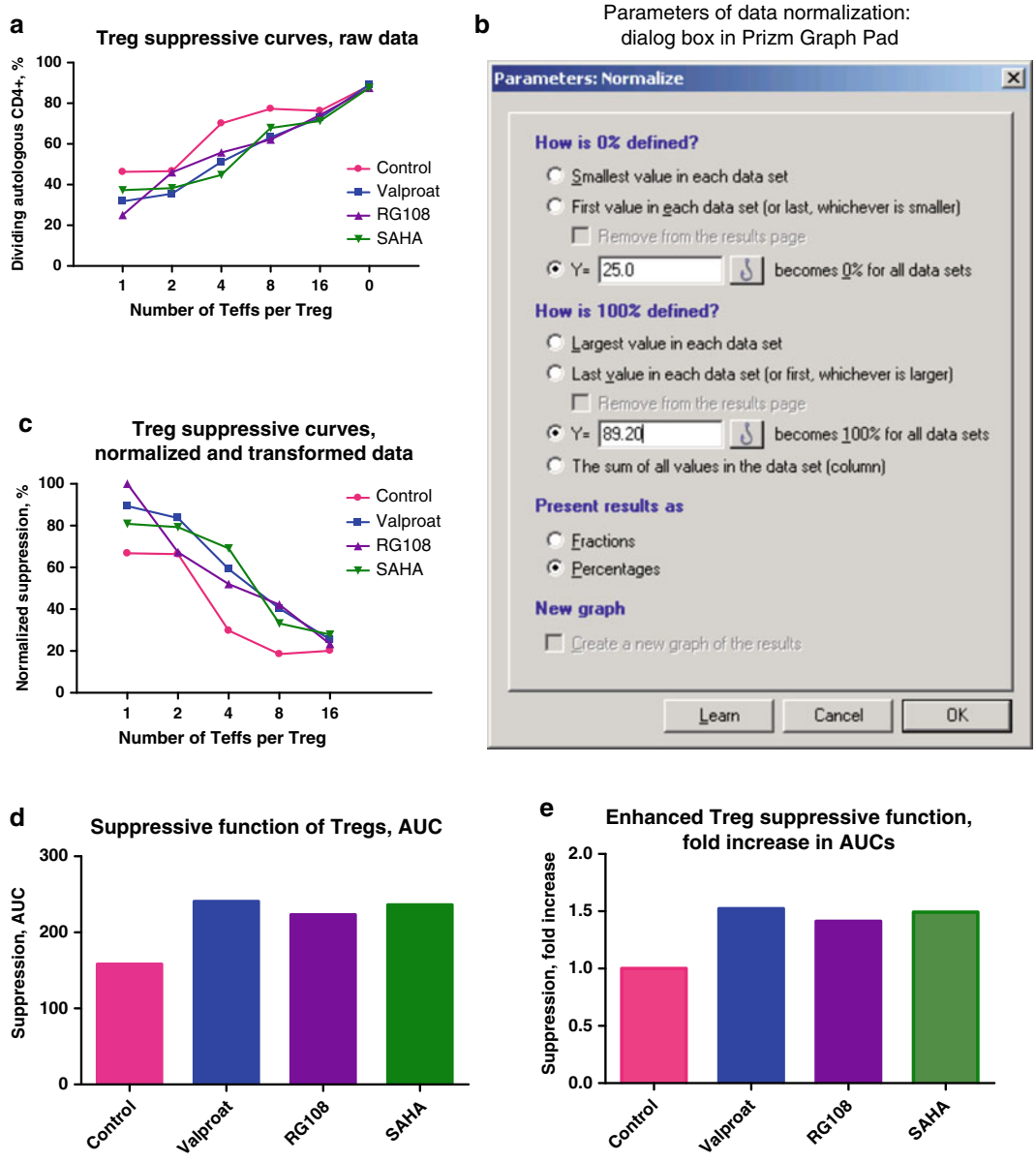


Fig. 9 Calculation of standardized suppression and AUC for Treg assays with epigenetic compounds. Healthy donor CD4+CD25+ Tregs, CD4+CD25– Teffs, and CD4-depleted irradiated APC were used in a suppression assay with different concentrations of valproic acid (HDACi), RG108 (DNMTi), and SAHA (HDACi). Then, one concentration of each compound, which demonstrated no direct inhibitory effect of given compound on Teff divisions, was chosen for following calculations. (**a**) Raw data show division of CD4+ Teffs at corresponding Treg/Teff ratios. Data are plotted as number of Teffs per (one) Treg vs. corresponding division of CD4+ Teffs. Table 3, Step 1 shows the same data as % of dividing CD4+ Teffs. (**b**) Within this experiment, the lowest and the highest divisions (bolded in Table 3, Steps 1 and 2) were used to define 0 and 100 % for percentage normalization. A screenshot of the dialog box from GraphPad Prism with corresponding data is shown. (**c**) Data from Table 3, Step 3, are plotted. Normalized suppression data were transformed using formula: $Y_t = 100 - Y$, where Y_t is transformed normalized suppression, and Y is normalized division. Y values show normalized and transformed suppression. All values in ratios without Tregs were excluded from further AUC calculations. (**d**) Treg suppressive function, calculated in AUCs units, is shown. All compounds clearly enhance Treg suppressive function. (**e**) The same data as (**d**) were re-calculated with control Treg suppressive function as denominator. As a result, data shown fold increase of Treg suppressive function for each compound vs. control (which is 1). These data may be further grouped with results of similar suppression assays with other Tregs and responders, to compare means of enhanced Treg function and p value for differences

Table 3
Calculation of enhanced Treg suppressive function in an experiment with epigenetic compounds

Step 1. Raw data: divisions of CD4+ cells				
Number of Teffs per Treg	*Divisions of CD4+, %*			
	Control	*Valproate*	*RG108*	*SAHA*
1	46.30	31.80	**25.0**	37.30
2	46.60	35.50	46.0	38.30
4	70.10	51.20	55.8	44.80
8	77.30	63.30	62.1	67.90
16	76.30	72.80	74.2	71.30
0	88.70	**89.20**	87.5	87.60
Step 2. 0–100 % Normalized data				
Number of Teffs per Treg	*Normalized divisions of CD4+, %*			
	Control	*Valproate*	*RG108*	*SAHA*
1	33.18	10.59	**0.00**	19.16
2	33.64	16.36	32.71	20.72
4	70.25	40.81	47.98	30.84
8	81.46	59.66	57.79	66.82
16	79.91	74.45	76.64	72.12
0	99.22	**100.00**	97.35	97.51
Step 3. Transformed data				
Number of Teffs per Treg	*Transformed normalized data, suppression, %*			
	Control	*Valproate*	*RG108*	*SAHA*
1	66.82	89.41	100.00	80.84
2	66.36	83.64	67.29	79.28
4	29.75	59.19	52.02	69.16
8	18.54	40.34	42.21	33.18
16	20.09	25.55	23.36	27.88
0	*0.78*	*0.00*	*2.65*	*2.49*

Healthy donor CD4+CD25+ Tregs, CD4+CD25− Teffs, and CD4-depleted irradiated APC were used in a suppression assay with different concentrations of valproic acid (HDACi), RG108 (DNMTi), and SAHA (HDACi). Then, one concentration of each compound, which demonstrated no direct inhibitory effect of given compound on Teff divisions, was chosen for following calculations. In Step 1, raw data show division of CD4+ Teffs at corresponding Treg/Teff ratios. Within this experiment, the lowest and the highest divisions (bolded) were used to define 0 and 100 % for percentage normalization in the next step. For Step 2, the smallest CD4+ division value in current experiment defined as 0 % and biggest one as 100 % (both are bolded). Corresponding dialog box in Prizm Graph Pad is shown at Fig. 9b. For Step 3, data transformed as $\Upsilon_t = 100 - \Upsilon$, where Υ_t is transformed normalized suppression, and Υ is normalized division. For further calculations, data of "0 Treg" ratios are excluded (shown in italic)

"anergic" status in vitro. In the current manuscript, and previously [15], we showed that murine and human Tregs indeed divide vigorously during Treg suppression assays, and the level of IL-2 produced by Teffs, even at a 1:1 Treg:Teffs ratio, is sufficient to overcome any "anergic" Treg state in vitro. Thus, only CFSE-based suppression assays [26] or analogs provide data on responder cell divisions that are free of contamination by Treg division. With regard to concerns of the sensitivity of CFSE-based assays,

we showed that cell numbers can be reduced to 20,000–25,000 of responders/Tregs in 1/1 ratios without any adverse effects, provided cell numbers are consistent between different experiments in the same study. Moreover, with relatively low cell numbers (40,000 and less), we never observed artifactual suppression due to the competition of highly activated CD25+ non-regulatory T cells, isolated as Tregs, with responder cells for growth factors or stimulation, as discussed [17]. Conversely, in our studies, highly activated human non-regulatory T cells stimulated healthy donor responder cells to divide, or had no effect. Use of flow-based assays for evaluation of division of responder cells can provide researchers with opportunities to obtain significant additional data. Cell culture media may be collected to evaluate soluble factors (IL-10, IL-2, IFN-γ, IL-17), cells may be fixed and co-stained with antibodies against cytokines and for transcriptional factors, and they may also be studied for expression of maturation and cell lineage markers. For example, staining cells at the end of the Treg suppression assay for Helios allowed us to study the effect of the presence or absence of Helios on Treg and Teff cell function, proliferation, and Teff susceptibility to Treg suppression [15].

As Tregs employ diverse mechanisms for their suppression, which may be regulated by cytokine milieu and other uncharacterized environmental factors [27], and depend on the type of responder cells and ongoing immune processes [28], Treg suppression assays can be modified accordingly, allowing a focus on defined conditions. Use of allergens for stimulation in order to study allergies [23], or use of donor-specific alloantigens to study Treg abilities to suppress anti-donor immune response in transplant studies [29, 30] are examples of such modifications. However, the results of these modifications should be interpreted with caution, as they may not reflect true in vivo situations. Thus, direct alloantigen recognition plays a major role only in early stage of alloantigen responses, and is then replaced by indirect recognition [31, 32], and alloantigen repertories may be significantly modified by post-ischemic transplant events and chronic graft deterioration. Outcomes may also vary based on use of a donor's native APC, or donor-antigen loaded APC isolated post-Tx. By contrast, use of standard polyclonal stimulation, which affects all Teff and Treg subsets, in comparison with healthy controls or pre-Tx cells, may provide more reliable evidence of alterations in Treg functionality, and should be performed at least as a control in any types of specifically modified Treg suppression assays.

To conclude, we propose that the utilization of standardized and carefully controlled conditions of Treg suppression assays, along with AUC calculations, may significantly improve the reproducibility of published results and therefore help to further improve our knowledge of Tregs and their suppressive mechanisms.

Acknowledgement

This work was supported by funding from the National Institutes of Health (DK087270, AI073489, UL1-RR026314 NCRR/NIH to WWH, K08DK092282 to MHL, and K08AI095353 to UHB). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

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Chapter 5

Characterization and Immunoregulatory Properties of Innate Pro-B-Cell Progenitors

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Abstract

Control of T-cell responses can be achieved by several subsets of B cells with immunoregulatory functions, mostly acting by provision of the anti-inflammatory cytokine IL-10 or exhibiting killing properties through Fas ligand (Fas-L) or granzyme B-induced cell death. We herein describe the characterization as well as the cellular and molecular mechanisms mediating the suppressive properties of bone marrow immature innate pro-B cell progenitors that emerge upon transient activation of Toll-like receptor 9. They are licensed by activated T-cell-derived IFN-γ to become suppressive by up-regulating their Fas-L expression and inducing effector $CD4^+$ T-cell apoptosis. They also up-regulate their own IFN-γ production which dramatically reduces T-cell production of a major pathogenic cytokine, IL-21. A single adoptive transfer of as little as 60,000 of them efficiently prevents the onset of spontaneous type 1 diabetes in recipient nonobese diabetes (NOD) mice, highlighting the remarkable regulatory potency of these so-called CpG-proB cell progenitors compared to regulatory cells of diverse lineages so far described. The CpG-proB cell activity is prolonged in vivo by their differentiation after migration in the pancreas and the spleen into B-cell progeny with high Fas-L expression that can keep up inducing apoptosis of effector T cells in the long term.

Key words Regulatory B cells, B-cell progenitors, Toll-like receptors, IFN-γ, Killer B lymphocytes, Fas-L, IL-21, Type 1 diabetes, Cell therapy, Tolerance

1 Introduction

The participation of B cells in various immunopathologic settings, particularly in autoimmune diseases, has been amply documented. Today, it is well known that their function is not limited exclusively to the secretion of antibodies since they have been shown to display influence on T-cell-driven diseases either acting as antigen-presenting cells or through their ability to produce various cytokines. These properties have led to the implementation of various B-cell-targeted depletion strategies as a mean for potential treatment. Paradoxically, in autoimmune encephalomyelitis, an experimental model of multiple sclerosis, an autoimmune disease targeting the myelin components and leading to demyelination and subsequent paralysis, B-cell depletion could trigger different

Maria Cristina Cuturi and Ignacio Anegon (eds.), *Suppression and Regulation of Immune Responses: Methods and Protocols, Volume II*, Methods in Molecular Biology, vol. 1371, DOI 10.1007/978-1-4939-3139-2_5,

effects depending on the time of depletion. Matsushita et al. showed that when B-cell depletion, using antibodies targeted against CD20, was accomplished at the peak of the disease or afterwards at the recovery phase, it was associated with reduced disease scores, whereas worsening of disease scores were reported when depletion took place at the immunization phase, thereby suggesting that B cells at the initiation phase of the disease exhibited regulatory properties in vivo. Additionally, the same group provided evidence also for the protective B-cell type identifying a new rare subset expressing the $B220^{+}CD5^{+}CD1d^{hi}$ phenotype, exerting immune regulation through the production of the anti-inflammatory cytokine IL-10 (thereafter named B10 cells) [1, 2].

Different subsets of B cells have since been reported to display immunoregulatory properties (see for review [3–9]), including the above cited $CD19^{+}CD5^{+}CD1d^{hi}$ cells [2], transitional type 2 marginal zone precursor B cells (T2-MZP-Bs) in mice with arthritis [10], follicular B cells and marginal zone B cells and, more recently, $CD138^{+}$ plasma cells [11]. These various B-cell subpopulations at diverse maturation stages can acquire regulatory properties provided they receive several activation signals including both innate and adaptive signals. These signals include either stimulation through Toll-like receptor (TLR) associated with B-cell receptor signaling, cognate interactions with T cells through CD40, as well as provision of several cytokines [11–16]. Even though these diverse signals are effectively provided, only a small percentage of B cells gain regulatory properties, for instance the capacity to secrete the anti-inflammatory cytokine IL-10, questioning the efficiency of this process. Although today it is believed that regulatory B cells do not form a separate lineage, at variance with regulatory T cells, whether a particular stage of differentiation in the B-cell lineage is more prone to acquire regulatory properties and whether immature B-cell progenitors can exhibit tolerogenic properties and differentiate into mature regulatory B cells has not been investigated so far.

Interestingly, it has been shown that TLRs are expressed even on highly immature cells such as hematopoietic stem cells and progenitors that upon stimulation by innate or danger signals do not remain confined within the bone marrow. They can traffic throughout the body, even within tissues, towards infectious and inflammatory signals [17, 18]. It was demonstrated that common lymphoid progenitors (CLP) stimulated via their TLR receptors are diverged from the B-cell lineage and instead differentiate into myeloid dendritic cells that actively take part in the anti-infectious defense [19]. Based on previous demonstrations that mobilized [20, 21] or activated bone marrow progenitors, in tumoral and inflammatory settings [22] as well as in parasite infection [23] can acquire tolerogenic properties, we hypothesized that TLR signals could instead promote the emergence of hematopoietic progenitor

cell populations with tolerogenic properties that could be valuable for cell therapy for the control of unwanted T-cell responses and particularly autoimmune diseases.

2 Characterization of TLR-Activated B-Cell Progenitors with Tolerogenic Properties

To test this hypothesis, we performed total bone marrow cell culture with TLR agonists and investigated whether new c-kit$^+$ progenitor populations with immunoregulatory properties could emerge. Indeed, we showed that within CpG-activated bone marrow cells emerged a new population of tolerating progenitor cells able to provide protection against nonobese diabetic (NOD) mice, an experimental model for type 1 diabetes upon their adoptive transfer.

The following conditions permitted the emergence and isolation of this protective population: incubation of total bone marrow cells for 18 h at 37 °C in 5 % CO_2 in RPMI 1640 medium supplemented with 10 % fetal calf serum and antibiotics, in the presence of 10 μM CpG-B (CpG 1668). We then performed a positive selection of c-kit$^+$ cells using automated magnetic selection (Robosep, StemCell Technologies) and thereof electronically cell-sorted c-kit$^+$ Sca-1$^+$ B220$^+$ IgM$^-$ cells of small size with a FACS Aria cell sorter (Becton Dickinson). As the phenotype of these progenitors—CD127$^+$ CD24$^+$ but also CD43$^+$, CD1d$^+$ and Sca-1$^+$—appeared close to that of a pro-B-cell stage of differentiation although with some differences likely to result from CpG-stimulation, and based on preliminary evidence of their differentiation into the B-cell lineage, we named them CpG-proB cells as a population which emerges upon activation with CpG [24].

An adoptive transfer of only about 60,000 cells by i.v. injection at 6 weeks of age in NOD mice was able to provide significant and dose-dependent protection against T1D whereas transfer of only 12,500 of the same population of cells had no similar protective effect. Furthermore, these cells still protected 50 % of mice from T1D onset even if injected in 16-week-old recipients, just before disease onset. Therefore, these CpG-proB cell progenitors are far more active at a per cell basis than any other previously reported immunoregulatory cell subset and their regulatory potential thus appears inversely correlated to their frequency. In the NOD mice, in our hands, approximately 50,000 CpG-proB cells can be obtained from a single donor mouse when using bone marrow recovered from tibiae and femurs. Interestingly, injection of non-stimulated control pro-B cells had no protective effect against T1D suggesting that TLR-9 signaling may confer tolerating inprinting.

To investigate whether cell populations with the phenotype of CpG-proBs can also transiently emerge after stimulation with other

known TLR-agonists, we performed the same bone marrow cell cultures in the presence of a large array of TLR ligands. Only MyD88-dependent TLR agonists efficiently promoted the emergence of a CpG-proB like cell subset and additionally, CpG-B was unable to trigger CpG-proB cell accumulation in the bone marrow derived from MyD88-deficient NOD mice. CpG likewise prompted the emergence of the same B-cell progenitors within the bone marrow 18 h post-injection (i.p.) in NOD mice, suggesting that such highly potent regulatory population might play a role in vivo as well.

3 Migration and Differentiation Properties

In order to assess the life-span, migration and differentiation properties of the adoptively transferred CpG-proBs in NOD mice that develop spontaneous type 1 autoimmune diabetes, we used CD45.2 congenic donor mice and traced the adoptively transferred progenitors into $CD45.1^+$recipient NOD mice. We observed that the injected progenitor cells migrated within the first 5 days to the pancreas, which is the target tissue of the autoimmune response in NOD mice. A more detailed follow-up showed that they can be recovered also within the pancreatic draining lymph nodes at approximately 3 times less cell counts than in the pancreas, staying in these organs for about 15 days. Later on and only 20 days after injection, they finally accumulated in the spleen where they persisted at least 1 month after injection. The total number of recovered $CD45.2^+$ cells found in the recipient mice corresponded approximately to the number of injected cells, an observation which, taking into account the inevitable cell loss, suggested that some proliferation had occurred.

Along with their migration, differentiation of the progenitors occurred, exclusively into more mature cells of the B-cell lineage but not into any other hematopoietic lineage. While the progeny that initially migrated to the pancreas still expressed c-kit, as did the injected progenitors, when reaching the pancreatic lymph nodes they gradually lost c-kit expression and in the spleen developed into different stages of B-cell maturation including transitional type 2 marginal zone precursor B cells as well as follicular and marginal zone B cells.

4 Cellular and Molecular Mechanisms of Action: CpG-proBs Trigger Effector T-Cell Apoptosis and Dramatically Reduce Their Production of a Major Pathogenic Cytokine in T1D, IL-21

In order to clarify the protective mechanism of CpG-proBs, we investigated whether these cells had a direct effect on either the pathogenic $CD4^+$ T cells or on regulatory T cells. Isolated

CpG-proBs had no effect on the proliferation of anti-CD3 + anti-CD28-activated $CD4^+CD25^+$ (all $Foxp3^+$) regulatory T cells in a cell culture in vitro. Conversely, CpG-proBs were efficient in inhibiting the proliferation of activated $CD4^+CD25^-$ cells at a 1:1 and 1:2 T-cell:CpG-proB ratio. This inhibition of proliferation was correlated with enhanced apoptosis of co-cultured T cells. Although CpG-proB cells expressed a number of death-inducing molecules including TRAIL, PDL1, and PDL2 as well as FasL, their capacity to trigger apoptosis of the co-cultured $CD4^+$ T cells was Fas-FasL dependent, being only inhibited by co-incubation with a neutralizing anti-FasL antibody but none of the other corresponding antibodies.

Moreover, qPCR array analysis of T cells that had escaped apoptosis in the co-culture assay with CpG-proBs demonstrated that they had up-regulated by 55-fold their expression of Fas-ligand, in keeping with their propensity to apoptosis, and additionally had considerably enhanced their pro-Th1 and cytotoxic profile at the expense of a Th2-profile with increased expression of IFN-γ, t-Bet and decreased levels of IL-13 and Gata3. They also concomitantly up-regulated eomesodermin and downregulated ICOS levels, together resulting in the dramatic 77-fold reduction of IL-21 transcript levels.

These molecular pathways were likewise modulated in vivo in T cells from CpG-proB NOD recipients. The $CD4^+$ T-cell pancreatic infiltrates showed a dramatic reduction of $CD44^{hi}CD62L^-CD4^+$ effector memory T cells correlated with reduced IL-21 production capacity contrasting with enhanced IFN-γ intracytoplasmic production. Pancreatic homogenates of CpG-proB recipients likewise displayed significantly reduced IL-21 protein levels relative to control mice with T1D. Therefore, CpG-proBs target pathogenic cellular and molecular mechanisms in T1D, by killing effector T cells and controlling their production of IL-21, a major pathogenic cytokine in T1D [25–27] with *IL-21* representing a susceptibility gene for type 1 diabetes in both mice and humans [28, 29].

5 An IFN-γ Driven Interplay but No Role for IL-10 in the Suppressive Effect of CpG-proBs on Their Target T Cells

One of the most common functional properties of so far described B regulatory cells is their capacity to secrete the anti-inflammatory cytokine IL-10. However, CpG-proBs did not produce IL-10 and neither $CD4^+$ T nor $CD19^+$B cells in CpG-proB recipient mice were displaying enhanced production of IL-10.

Instead, CpG-proBs, constitutively after their isolation, massively produced IFN-γ, proposing a crucial role for this cytokine in their protective mechanism of action. The important role of IFN-γ produced either by T cells or the CpG-proBs was shown with the use of mice deficient for IFN-γ. None of the previously

described effects, induction of apoptosis or reduction of the pathogenic cytokine IL-21 occurred in the absence of IFN-γ in either T cells or CpG-proB cells. When co-cultured with $CD4^{+}CD25^{-}$ T cells isolated from IFN-γ-deficient donors, INF-γ-competent CpG-proB cells (isolated from wild-type (WT) NOD donors) were unable to inhibit the T-cell proliferation or to trigger T-cell apoptosis and to up-regulate their own expression of Fas-L, suggesting that the provision of IFN-γ by activated T cells was the signal responsible for conferring suppressive properties onto CpG-proBs. Moreover, during the co-culture with WT T cells, CpG-proBs up-regulated their own IFN-γ production. In turn, IFN-γ deficient CpG-proB cells did not enhance IFN-γ production by T cells and did not reduce their IL-21 production. This mechanism of action points out the regulatory role of IFN-γ—either direct or indirect through IFN-γ dependent gene-encoded molecules, yet to be identified—since its enhanced production by both T and CpG-proB cells was necessary for achieving a complete suppressive effect *in vitro*. In addition, its regulatory role in vivo as well was demonstrated by the incapacity of CpG-proB cells isolated from the bone marrow of IFN-γ-deficient donor NOD mice to prevent T1D onset in vivo after their adoptive transfer in NOD mice (Fig. 1).

The production of IFN-γ by B cells has been described in various settings. Constitutive production of low levels IFN-γ by immature bone marrow B cells [30] and IFN-γ production by TLR-activated follicular and marginal zone B cells have been previously reported [31, 32], the latter with functional outcomes such as regulation of the Th1 response to *Salmonella enterica* infection [33]. Mature follicular B cells from *Toxoplasma gondii*-infected mice were shown to produce IFN-γ upon ex vivo restimulation with pathogen extracts [34]. Bao et al. [35] recently reported that innate B cells with a $CD11a^{hi}$ $CD16/32^{hi}$ phenotype emerged from follicular B cells 3 days post-infection with bacterial and viral agents, even in IFN-γR deficient mice. Upon ex vivo restimulation with anti-CD40, these innate B cells produced as much IFN-γ as NK cells. Their provision of IFN-γ was required for protection against *L. monocytogenes* infection and depended on activation of the Bruton's tyrosine kinase (*btk*)—but not the*t-Bet*-dependent pathway-, as *btk*-deficient mice lacked these $CD11a^{hi}$ $CD16/32^{hi}$B cells and did not clear *L. monocytogenes* infection. Therefore, IFN-γ production by CpG-proBs as well as other described B cells has functional role and may link innate and adaptive immune responses.

6 CpG-proB Cell Progenitors Mature into FasL-Expressing Immunoregulatory Progeny

The limited life-span of hematopoietic progenitors contrasted with the long-lasting protection against spontaneous T1D provided by a single injection of only 60,000 CpG-proBs and suggested that

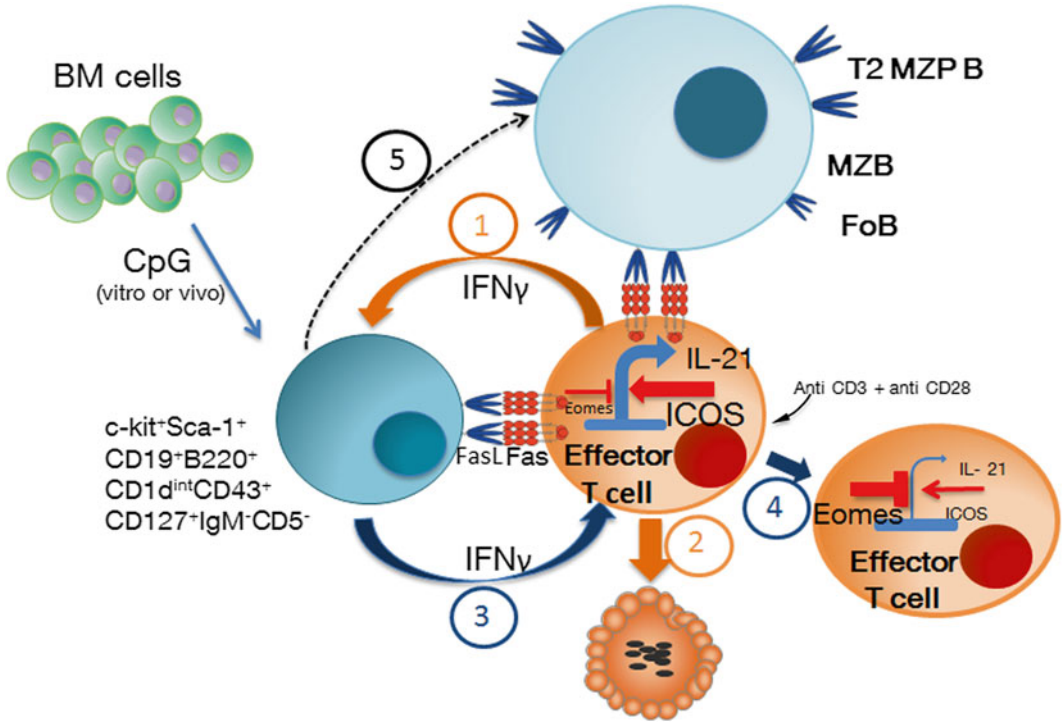

Fig. 1 Cellular and molecular mechanisms underlying the protective effects of adoptively transferred CpG-proB cells against type 1 diabetes in NOD mice. (1) IFN-γ derived from TCR-activated T cells promotes expression of Fas-L on CpG-proB cells. (2) FasL-expressing CpG-proB cells trigger $CD4^+$ T-cell apoptosis. (3) IFN-γ derived from activated T cells also enhances IFN-γ levels produced by CpG-proB cells, which (4) enhances within spared $CD4^+$ T-cells eomesodermin and ICOS levels, resulting in the dramatic reduction within T-cells of the production of IL-21, a cytokine playing a major role in T1D pathogenesis. (5) CpG-proB cells migrate to the pancreas, the pancreatic lymph nodes and the spleen sequentially where they differentiate into various B-cell mature subsets (T2-MZP-Bs, MZ, and FO B cells) all expressing higher FasL levels than the recipient's B cells in the corresponding tissues and able to trigger apoptosis of T cells in the long term

the influence of the B-cell progenitors was somehow prolonged over time. Indeed, we showed that their more mature B-cell progeny could contribute to the protection. These mature B cells, recovered either from the pancreas or the spleen of $CD45.1^+$ CpG-proB recipients, maintained a remarkably high FasL expression—higher than that of the host $CD45.2^+$B cells in the same compartments—and remained able to trigger the apoptosis of $CD4^+$ T cells, even if recovered as far as 1 month after the adoptive transfer of CpG-proBs. Therefore, a long-term control of effector diabetogenic T cells which was initially operated by the acquired FasL expression in CpG-proB cells in contact with activated T cells is further maintained in vivo through the differentiation of CpG-proBs into $FasL^{hi}$ mature B-cell progeny in both pancreas and spleen (Fig. 1). CpG-proB cells and their mature B-cell progeny thereby share the cytotoxic properties originally described in PMA + ionomycin-activated spleen B cells [30], in LPS-treated NOD mice based on FasL expression of mature B cells [36] and in

Shistosoma infection by splenic CD5⁺ B1a cells expressing high levels of FasL that triggered CD4⁺ T-cell apoptosis [37, 38]. In humans, regulatory B cells with cytotoxic activity have been reported. Leukemic, myeloma cells and EBV- and HIV-infected cells were shown to express FasL allowing them to escape immune surveillance [39, 40]. B cells producing instead granzyme B, under the influence of IL-21 production by T cells, were likewise shown to inhibit tumor defense [41].

The capacity of TLR-activated B-cell progenitors to migrate into the autoreactive inflammatory target site, develop into an immunoregulatory mature B-cell progeny that ensures long-term targeting of effector T cells represent remarkable properties than might be harnessed in cell therapy for controlling unwanted T-cell immune responses and establishing long-lasting immune tolerance.

Acknowledgements

We are indebted to Christophe Gras for technical assistance and thank Jérôme Mégret for expert cell-sorting. This work was supported by institutional grants from Centre National de la Recherche Scientifique and Institut National de la Recherche Médicale, and by grants from Humanis, IKY (State Scholarship Foundation, Greece), Juvenile Diabetes Research Foundation, Fondation de la Recherche Médicale Prix Mescle and Association Française pour la Recherche sur la Sclérose en Plaques (ARSEP). R.M. was recipient of a doctoral grant from the Ministry of Education and Research, France, and subsequently from Ligue Nationale Française contre le Cancer. S.K. was supported by a grant from Domaine d'Intérêt Majeur StemPôle, Région Ile de France and subsequently from Association Française pour la Recherche sur la Sclérose en Plaques (ARSEP).

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Chapter 6

Generation and Characterization of Mouse Regulatory Macrophages

Laura Carretero-Iglesia, Marcelo Hill, and Maria Cristina Cuturi

Abstract

In the last years, cell therapy has become a promising approach to therapeutically manipulate immune responses in autoimmunity, cancer, and transplantation. Several types of lymphoid and myeloid cells origin have been generated in vitro and tested in animal models. Their efficacy to decrease pharmacological treatment has successfully been established. Macrophages play an important role in physiological and pathological processes. They represent an interesting cell population due to their high plasticity in vivo and in vitro. Here, we describe a protocol to differentiate murine regulatory macrophages in vitro from bone marrow precursors. We also describe several methods to assess macrophage classical functions, as their bacterial killing capacity and antigen endocytosis and degradation. Importantly, regulatory macrophages also display suppressive characteristics, which are addressed by the study of their hypostimulatory T lymphocyte capacity and polyclonal T lymphocyte activation suppression.

Key words Regulatory macrophages (Mreg), Cell therapy, In vitro differentiation, Bacterial killing assay (BKA), Suppression

1 Introduction

Pharmacological and biological compounds are widely used to treat immunological disorders that lead to an excessive response of the immune system. However, those agents unselectively target important cellular and molecular pathways, leading to undesired side effects. Therefore, cell therapy arises as a good strategy to modulate immune responses by induction of specific peripheral tolerance [1].

Macrophages constitute a highly heterogeneous cell subset, comprising cells derived from different embryonic and adult precursors. Macrophages are involved in both physiological and pathological processes [2]. In steady state, macrophages are responsible of maintaining homeostasis in a variety of peripheral tissues (dermis, bone, lungs, spleen, adipose tissue …) by clearance of apoptotic cells, development and metabolic regulation. They also play fundamental roles as immune sentinels. After pathogen

Maria Cristina Cuturi and Ignacio Anegon (eds.), *Suppression and Regulation of Immune Responses: Methods and Protocols, Volume II*, Methods in Molecular Biology, vol. 1371, DOI 10.1007/978-1-4939-3139-2_6,

encounter, macrophages get activated and are responsible of the initiation of an inflammatory microenvironment which triggers the recruitment of other immune cell types, which perpetuate inflammation. Meanwhile, macrophages are also involved in the resolution of inflammatory processes, through phagocytosis of cellular debris and tissue reconstitution [3].

Those antagonistic functions displayed by macrophages in vivo give a hint of the plasticity of this population. Therefore, in vitro differentiation of macrophages from precursor cells or ex vivo modification of isolated macrophages can be a good strategy to polarize macrophages towards a regulatory profile for their use in clinical practise.

Regulatory macrophages (Mreg) have already been generated in mouse [4] and humans [5]. In the setting of transplantation, those Mreg have successfully been able to prolong cardiac graft survival in a fully mismatched mouse transplant model. A clinical trial including two patients has also shown their capacity to maintain graft functionality with only tacrolimus monotherapy after Mreg administration [6].

In this chapter, we describe a methodology to generate in vitro another subset of Mreg using only low doses of M-CSF. Mreg conserve the functional characteristics of classical macrophages while being able to suppress polyclonal T lymphocytes activation and to be hypostimulatory in in vitro allogeneic co-cultures.

2 Materials

2.1 General Materials

- Complete medium: 1× DMEM medium (Gibco) supplemented with 10 % FCS (Lonza), 0.05 mM β-ME (Sigma-Aldrich), 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 1× MEM NEAA, 100 U/ml penicillin, 0.1 mg/ml streptomycin (all from Gibco).
- Sterile PBS 1× (Phosphate saline buffer, pH 7.2–7.4).
- PFE: sterile PBS 1× supplemented with 2 % FCS and 2 mM EDTA.
- Vacuum-driven filtration system, 0.22 μm (Millipore).
- 15 and 50 ml centrifuge tubes (Greiner bio-one).
- 24-Well flat-bottom plates and 96-well round-bottom plates (BD Falcon).

2.2 Material for In Vitro Mregs Generation

- Complete medium.
- Mouse recombinant M-CSF (mrM-CSF) (Peprotech).
- 6- to 8-Week-old C57BL/6 mice (Janvier, France).
- 2.5 ml syringe and 26G needle.

- RBCL (Red Blood Cell Lysis) solution: 0.15 M NH_4Cl, 0.01 M $KHCO_3$ and 100 μM Na_2EDTA, filtered and adjusted to pH 7.4.
- 100 μm nylon filters (BD Falcon).
- Non-culture-treated 10 cm Petri dishes (VWR).

2.3 Material for Bacterial Killing Assay

- Antibiotic-free complete medium: complete medium without penicillin and streptomycin.
- FCS (Lonza).
- *E. coli* bacteria (Agilent Technologies).
- Liquid LB médium (Sigma-Aldrich).
- LB-agar plates (Sigma-Aldrich).
- Gentamicin (Sigma-Aldrich).
- 0.5 % Sodium Deoxycholate (Sigma-Aldrich).

2.4 Material for Endocytosis and Antigen Degradation

- OVA-Alexa Fluor647 (Invitrogen).
- DQ-OVA (Invitrogen).
- PFA 2 % (Paraformaldehyde 2 %).

2.5 Material for Allogeneic Co-culture and Suppression Test

- 6- to 8-Week-old C57BL/6 or Balb/C mice (Janvier, France).
- 100 μm nylon filters (BD Falcon).
- CD90.2 or Pan T Isolation Kit II, mouse (Miltenyi Biotech).
- AutoMACS separator or columns and magnets (Miltenyi Biotech).
- CFDA-SE (CarboxyFluorescein DiAcetate Succinimidyl Ester) (Invitrogen).
- DynaBeads mouse T-Activator CD3/28 (Gibco): Anti-CD3 and anti-CD28-coated microbeads.
- BD™IMagnet (BD Biosciences Pharmigen).

3 Methods

3.1 Differentiation of Mregs from Bone Marrow Precursors

In order to generate Mreg in vitro, several culture conditions were tested: harvesting time points, serum amounts and lots, type of plastic recipient for cell culture, and mrM-CSF concentrations. Macrophages that displayed the desired phenotype and function were obtained after 15 days of culture in complete medium supplemented with 10 % of FCS and cultured in untreated Petri dishes with 0.2 ng/ml of mrM-CSF. Cell yields obtained using the different conditions are shown in Table 1. The following protocol considers the chosen culture conditions:

Table 1
Yield of adherent cells recovery under different culture conditions

Initial concentration (cells/ml)	Volume (ml/dish)	rmM-CSF (ng/ml)	Culture time (days)	
			7	15
10^6	10	0.2	0.3	2.3
		0.4	0.4	2.7
		1	1.2	2
2×10^6	10	0.2	0.8	2
		0.4	0.8	2.8
		1	0.8	2.9

Two initial cell concentrations (10^6 or 2×10^6 cells/ml) were cultured for the indicated times (7 or 15 days) in presence of different doses of rmM-CSF (from 0.2 to 1 ng/ml). Numbers show the yield of recovery of adherent cells ($\times 10^6$). Around 95 % of adherent cells displayed $CD11b^+F4/80^+$ phenotype

1. After sacrifice, remove tibias and femurs from 6- to 8-week-old C57BL/6 mice (*see* **Note 1**) and flush bone marrow using a 2.5 ml syringe and a 26G needle.
2. Recovered cells into a 50 ml tube and centrifuge them for 10 min at $500 \times g$. Discard supernatant and resuspended cell pellet in 5 ml of RBCL solution for 5 min in order to eliminate erythrocytes.
3. Add 45 ml of PFE to stop the lysis and centrifuge for 10 min at $500 \times g$. Discard supernatant.
4. Repeat **step 3**.
5. Resuspend cell pellet in 10 ml of PFE and filter cells using a 100 μm filter.
6. Determine cell number and resuspend cells into pre-warmed complete medium to a final concentration of 10^6 cells/ml. Add 0.2 ng/ml of mrM-CSF.
7. Dispatch 10 ml of the suspension into each Petri dish. Incubate cells at 37 °C and 5 % CO_2.
8. At day 3 of the culture, add 10 ml of preheated complete medium supplemented with 0.2 ng/ml of mrM-CSF to each Petri dish.
9. At day 7, replace 10 ml of medium from each plate. Centrifuge cell suspension, discard supernatant, and resuspend pellets in the same volume to the initial of preheated complete medium supplemented with 0.2 ng/ml of M-CSF.
10. At day 15, discard medium containing non-adherent cells. Add 10 ml of cold PFE are to each Petri dish. Adherent cells are harvested by pipetting up and down. Mregs are collected into

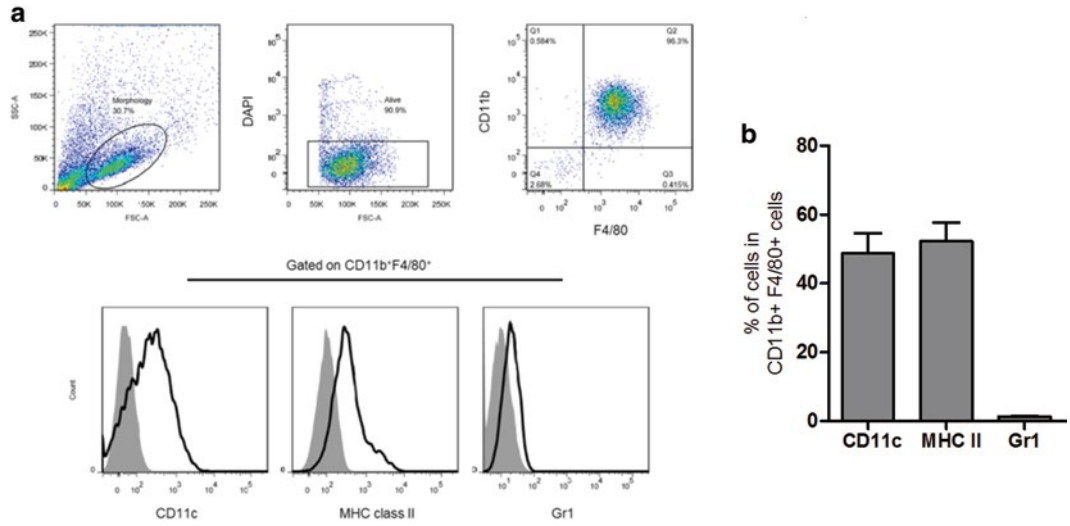

Fig. 1 Mreg phenotype at day 15. Adherent cells were harvested at the end of the differentiation period. Phenotype was assessed by flow cytometry. (**a**) Representative plots of Mreg phenotype. (**b**) Mreg surface markers expression in $CD11b^+$ $F4/80^+$ cells (mean 93.6 ± 2.1). Data are represented as mean ± SEM of four independent experiments

50 ml tubes and centrifuged. Supernatant is discarded and pellets are resuspended in PFE and pooled into a final volume of 10 ml of PFE.

11. Phenotypical characterization of recovered macrophages can be performed by flow cytometry. Typically, virtually all recovered Mregs are CD11b/F4/80 positive, whereas half of the population expresses CD11c and MHC class II markers and Gr1 marker is absent (Fig. 1).

3.2 Study of Macrophages Function

In this assay, we evaluate the capacity of macrophages to kill internalized bacteria. The readout is the quantification of live bacteria at different times after phagocytosis. The following protocol has been modified from Sokolovska et al. [7].

3.2.1 Bacterial Killing Assay

1. Culture and harvest macrophages as previously described. Wash cells with PFE. Count cell numbers and resuspend cell pellet in antibiotic-free complete medium to a concentration of 10^6 cells/ml. Dispose 1 ml per well in a 24-well flat-bottom culture plate. Each well represents a condition or a time point. It is recommended to perform three replicates per point. Allow macrophages to adhere to the bottom of the culture plate for 1 h at 37 °C.
2. Prepare live *E. coli* (*see* **Note 2**):
   (a) Day 2: Spread *E. coli* into LB-agar plates and incubate ON at 37 °C.

(b) Day 1: Grow one separate colony into liquid LB ON at 37 °C.

(c) Day 0:

- Make serial dilutions of bacteria into pre-warmed liquid LB.
- Incubate different dilutions at 37 °C for 1–2 h.
- Determine the optical density (OD) of the suspension at 600 nm (*see* **Note 3**) and calculate bacterial concentration (x) as follows:

$$x = \frac{\text{measured O.D.} \times 5 \times 10^8 \,\text{CFU} / \text{ml}}{1\,\text{O.D.}} \left(\text{CFU} / \text{ml}\right)$$

(d) Resuspend bacteria into antibiotic-free complete medium to a concentration of 10^7 bacteria/ml (*see* **Note 4**).

3. Infect macrophages by adding 10 µl of the bacterial preparation to each well and centrifuge the plate for 4 min at 500 × *g*.
4. To allow macrophages phagocyte *E. coli*, incubate plates at 37 °C 5 % CO_2 for 30 min.
5. Wash wells twice with warm PBS. Add 500 µl of complete medium supplemented with 50 µg/ml of gentamicin. Incubate for 1 h at 37 °C. This step allows killing of remaining non-phagocyted bacteria.
6. Wash wells twice with warm PBS. Add 500 µl of complete medium with 5 % FCS containing 5 µg/ml of gentamicin. This is time point 0 (*see* **Note 5**). Incubate for the desired time points.
7. Harvest macrophages by washing wells twice with warm PBS and scraping.
8. To assess intracellular bacteria, centrifuge cell suspension, discard supernatant and lyse macrophages by adding 100 µl of 0.5 % sodium deoxycholate (*see* **Note 6**). Pipet up and down and vortex vigorously to release intracellular bacteria (*see* **Note 7**).
9. Make serial dilutions of the lysate and plate bacteria onto LB-plates. Incubate ON at 37 °C and count CFU. Results can be expressed as remaining "alive bacteria per initial macrophage number" (*see* **Note 8**). Titration of CFU counts can be performed in order to choose the best MOI to perform further (Fig. 2).

3.2.2 Endocytosis and Antigen Degradation

To perform this protocol, two ovalbumin (OVA) modified proteins are used. OVA protein conjugated with Alexa Fluor 647 fluorochrome (OVA-AF647) is used to evaluate endocytosis. AF647 fluorochrome displays invariable fluorescence despite differences in environmental factors. To evaluate antigen degradation DQ-OVA

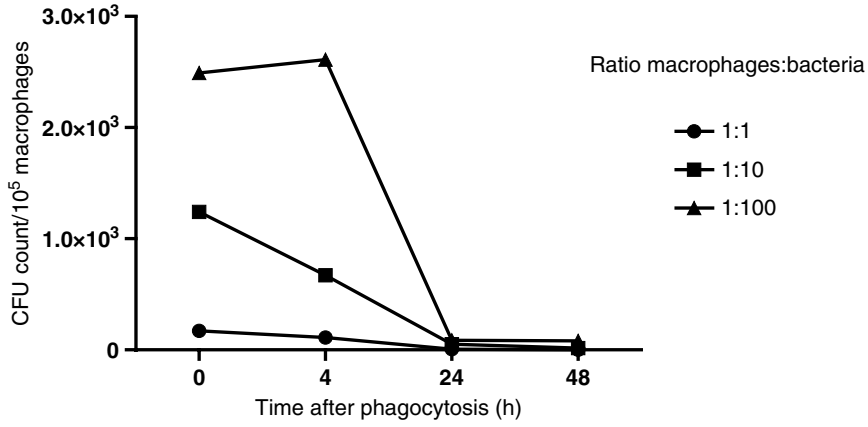

Fig. 2 Macrophage bacterial killing kinetics. Evaluation of *E. coli* killing capacity was assessed as previously described. A representative experiment evaluating different time points and different MOIs (ratio 1:1 (*circles*); 1:10 (*squares*), 1:100 (*triangles*)) is shown

is used. DQ-OVA is a self-quenched conjugate that only emits fluorescence upon proteolytic digestion. DQ-OVA fluorescence excitation and emission are close to fluorescein (FITC) but contrary to FITC-labeled proteins, DQ-OVA is labelled with a photostable dye in a pH range of 3–9, which makes it suitable for phagosomal antigen degradation studies.

1. Culture and harvest macrophages as previously described. Wash cells with PFE. Count cell numbers and resuspend cell pellet in complete medium to a concentration of 10^6 cells/ml.
2. Place 1 ml of the cell suspension into as many tubes as conditions to test. At least two 15 ml tubes are needed, one for the sample and one for the negative control.
3. Place one tube containing cells at 37 °C and another on ice (negative control) for at least 30 min.
4. Add both fluorescent OVA proteins into macrophage cell suspension at a final concentration of 1 μg/ml each.
5. Incubate tubes for the desired time points either at 37 °C or on ice.
6. Resuspend each tube and recover 100 μl of each cell suspension. Stop endocytosis and degradation by placing them rapidly into ice and wash with cold PFE. From this point on, cells must be kept on ice (*see* **Note 9**).
7. Cells can directly be analyzed by flow cytometry or fixed with PFA 2 % for 20 min, then washed with PFE, and stored at 4 °C (*see* **Note 10**).
8. Determine the percentage and the mean fluorescence intensity in the APC channel (for endocytosis) and FITC channel

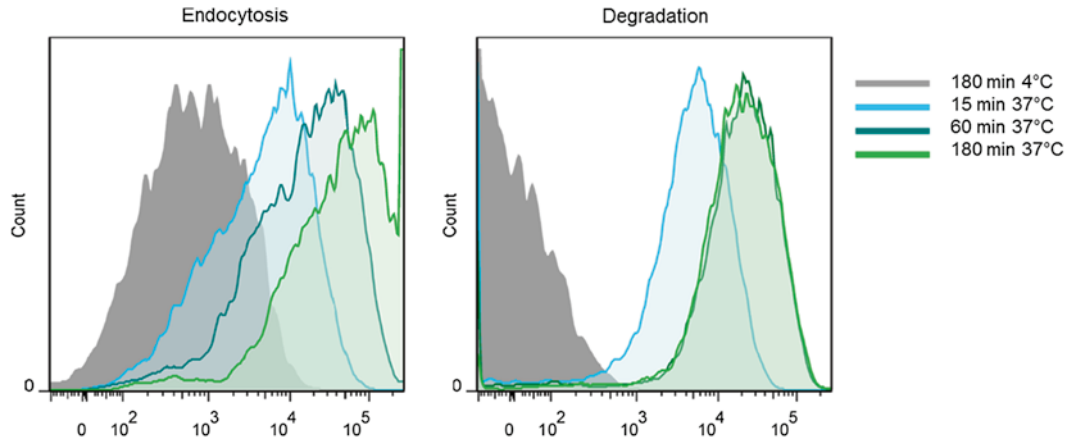

Fig. 3 Endocytosis and antigen degradation kinetics. Evaluation of endocytosis and antigen degradation were assessed as previously described. A representative histogram displaying different time points is shown. 4 °C control is shown filled in *grey*

(for antigen degradation). Figure 3 shows an example of the endocytic and degradative capacity of Mreg at different time points, whereas Mregs continuously endocyte OVA particles, and degradation rises a maximal kinetics at 60 min.

3.3 Assessment of Mregs Immunoregulatory Properties

In order to assess the ability of Mreg to induce hypoproliferation of allogeneic T lymphocytes, co-cultures between complete mismatched cells are performed. Proliferation of responder cells from Balb/C mice are used as the readout of the assay.

3.3.1 Allogeneic Co-culture

1. Prepare macrophage dilutions:
   (a) Culture and harvest macrophages as previously described. Wash cells with PFE. Count cell numbers and resuspend the pellet in complete medium to a concentration of 5×10^6 cells/ml.
   (b) Perform serial dilutions. Ratios can range from 1:2 to 1:128 (Mregs:T lymphocytes).
2. Prepare allogeneic T lymphocytes from spleen or lymph nodes (LN) from Balb/C mice:
   (a) Crush spleen or LN on a 100 μm nylon strainer with a syringe.
   (b) Rinse the strainer with PFE and recover cells in a 50 ml tube. Centrifuge the cell suspension.
   (c) Discard supernatant and resuspend cellular pellet in 5 ml of RBCL solution for 5 min in order to eliminate erythrocytes.
   (d) Add 45 ml of PFE to stop the reaction and centrifuge for 10 min at 500 × *g*. Discard supernatant.

(e) Repeat **step d**.

(f) Resuspend cell pellet in 10 ml of PFE and filter them using a 100 μm filter.

(g) Determine cell number and proceed to magnetic separation of T lymphocytes, following the manufacturer's instructions.

(h) After purification, label T lymphocytes with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE). Wash to remove the excess of dye.

(i) Determine lymphocyte numbers and resuspend them in complete medium at a density of 10^6 cells/ml.

3. Pour the following into round-bottom 96-well plates (*see* **Note 11**).
   - 100 μl of Mreg suspension (of each dilution) or 100 μl of complete medium as a basal T lymphocyte proliferation control.
   - 100 μl of Balb/C CFDA-SE labelled T lymphocytes.
4. Culture plates at 37 °C and 5 % of CO_2 for 4 days.
5. Assess T lymphocyte's proliferation by CFDA-SE dilution using flow cytometry. An example of the hypostimulatory capacity of Mregs is shown in Fig. 4.

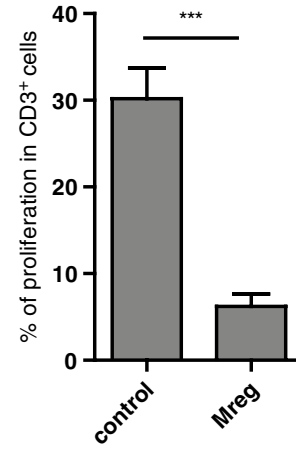

Fig. 4 Mregs display hypostimulatory capacity in allogeneic co-cultures. Mreg capacity to stimulate allogeneic T lymphocytes was evaluated in direct 4d co-cultures as previously described. The figure shows a 1:8 ratio (myeloid cell:T lymphocyte). Control cells were generated by culturing bone marrow precursors with 40 ng/ml of GM-CSF for 8 days. Data are represented as mean ± SEM of three independent experiments. Statistical analyses were performed using Mann-Whitney test, two-tailed, ***$p < 0.0001$

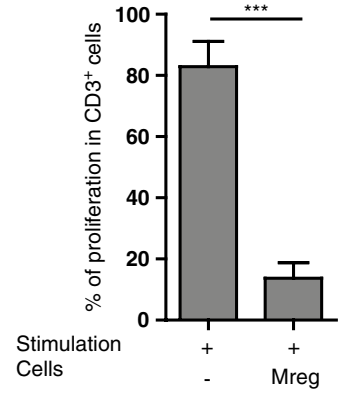

Fig. 5 Mregs suppress polyclonal T cell activation. Mreg capacity to stimulate suppress a CD3/aCD28-stimulated T lymphocytes proliferation was evaluated as previously described. The figure shows a 1:2/1/5 ration (myeloid cell:T lymphocytes:microbeads). Data are represented as mean ± SEM of three independent experiments. Statistical analyses were performed using Mann-Whitney test, two-tailed, ***$p < 0.0001$

3.3.2 Suppression Test

In this protocol, T lymphocytes are polyclonally stimulated with αCD3/αCD28-coated microbeads (*see* **Note 12**). Syngeneic Mregs are added to the culture to test their suppressive capacity.

1. Culture and harvest macrophages as previously described. Wash cells with PFE. Count cell numbers and resuspend the pellet in complete medium to a concentration of 10^6 cells/ml.
2. Prepare syngeneic T lymphocytes from C57BL/6 mice as already explained in "allogeneic co-culture" (**step 2**). Resuspend CFDA-SE-labeled T lymphocytes to concentration of 10^6 cells/ml.
3. Prepare polyclonal stimulation: wash αCD3/αCD28 coated microbeads with PBS using the BD™IMagnet and resuspend them in complete medium to a final concentration of 2×10^5 beads/ml.
4. Pour the following into round-bottom 96-well plates:
   - 100 μl of C57BL/6 CFDA-SE-labeled T lymphocytes.
   - 50 μl of αCD3/αCD28-coated beads.
5. Incubate for 30 min at 37 °C and 5 % of CO_2 (*see* **Note 13**).
6. Add 50 μl of Mreg suspension or 50 μl of complete medium as a proliferation control.
7. Culture plates at 37 °C and 5 % of CO_2 for 4 days.
8. Assess T lymphocyte's proliferation by CFDA-SE dilution using flow cytometry. Mreg are able to efficiently suppress polyclonal T lymphocyte proliferation (Fig. 5).

4 Notes

1. BM derived Mreg can be generated from any mouse strain. However, functional differences may be observed depending on the mouse strain they derive from [8].
2. Other bacterial strains can be used. In that case, it is important to adapt reagents used for bacterial growth and spread to the bacterial strain used.
3. Bacteria should be used when they are at the exponential phase of the culture, obtained when $OD_{600nm} = 0.4–0.6$.
4. The initial bacterial concentration depends on the MOI chosen for the experiments. It is recommended to try different MOIs before starting experiments.
5. Phagocytic capacity of macrophages can also be analysed by counting internalized bacteria at time point 0, expressing values as "number of CFU/initial bacteria numbers."
6. Alternatively, macrophages can be lysed by resuspension in 0.2 % Triton-X 100.
7. At that point, cell lysates can be conserved at 4 °C for some days.
8. In some cases, it may be important to count living macrophages after harvesting (**step 8**). Values can then be expressed as "number of CFU/10^3 live macrophages."
9. The same tube is used for each time point. When needed, take 100 μl of the cellular dilution for endocytosis/antigen degradation analysis.
10. Cells can be stained for a viability dye before flow cytometry analysis. It must be essential when treating cells with new drugs which may affect cell viability.
11. It is recommended to perform triplicates of each ratio.
12. Alternatively, αCD3-coated plates and soluble αCD28 antibody stimulation can be used.
13. This step is to facilitate the recognition between antibodies and their target surface molecules before addition of Mregs. If coated plates and soluble antibodies are used, this step can be skipped.

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Chapter 7

Generation and Expansion of T Helper 17 Lymphocytes Ex Vivo

Darya Alizadeh and Nicolas Larmonier

Abstract

CD4+ T helper (Th) lymphocytes are essential elements of the complex cellular networks regulating the initiation, development, and termination of adaptive immune responses. Different independent and specialized subsets of Th cells can be distinguished based on their dedicated transcription factor and cytokine expression profiles. Th17 lymphocytes have been described about a decade ago as CD4+ Th cells producing high quantity of IL-17A as a signature cytokine. Since their initial discovery, Th17 have drawn intense scrutiny for their dominant role in the pathogenesis of multiple autoimmune, infectious diseases and allergy. The influence of Th17 lymphocytes in cancer remains however ambiguous. The plethoric functions of Th17 may rely on the remarkable plasticity of these cells, endowed with the ability to transdifferentiate into other Th subpopulations depending on the environmental cytokine context. The possibility to generate Th17 ex vivo has facilitated the elucidation of the signals and transcription factors required for their differentiation and functions and has allowed for the evaluation of their functions following adoptive transfer in vivo. Several protocols have been developed to produce Th17 in vitro. The intent of this chapter is to provide examples of procedures for generating and expanding Th17 ex vivo.

Key words Th17 lymphocytes, Mouse, Human, Ex vivo generation

1 Introduction

CD4+ T helper (Th) lymphocytes critically contribute to the development and coordination of immune responses. They provide essential support to CD8+ cytotoxic T lymphocytes (CTLs) [1, 2], B lymphocytes [3], natural killer cells (NK) [4] and significantly participate to the recruitment and activation of innate cells such as macrophages and dendritic cells (DC) [5–7]. The activation of naïve CD4+ T lymphocytes requires the recognition of antigenic peptides in the context of MHC Class II by their specific receptor (TCR) together with co-stimulatory signals delivered by antigen-presenting cells. Different subtypes of activated antigen-experienced CD4+ T cells exhibiting distinct phenotypic and functional properties can be produced depending on the nature of the cytokines present in the microenvironment [8–10]. A growing number

Maria Cristina Cuturi and Ignacio Anegon (eds.), *Suppression and Regulation of Immune Responses: Methods and Protocols, Volume II*, Methods in Molecular Biology, vol. 1371, DOI 10.1007/978-1-4939-3139-2_7,

of Th lymphocyte subsets have been characterized based on their cytokine secretion profile and their dedicated differentiation programs controlled by specific transcription factors [11–13]. These Th lineages include well-characterized Th1 and Th2 cells as well as regulatory (immunosuppressive) T lymphocytes (Treg) and more recently identified Th9, Th22, T follicular, and Th17 [7, 12, 14, 15].

Cardinal features inherent to Th17 include the secretion of substantial amounts of interleukin 17A (IL-17A) and the expression of the transcription factor retinoic acid receptor-related orphan receptor gamma t (RORγt) [16, 17]. In addition, Th17 cells produce IL-21 and IL-22 [18] and, depending on the differentiation/environmental conditions, secrete variable amounts of TNFα, IFNγ, and/or GM-CSF [19–23]. Th17 represent a dominant pro-inflammatory lymphocyte subpopulation involved in the elimination of pathogens that are not cleared by Th1 or Th2 responses. These cells are also potent inducers of tissue inflammation and contribute to the pathogenesis of multiple autoimmune diseases in animals and humans [12, 21, 24]. Of note, the role of Th17 in the development of cancer remains debatable [25]. Adoptive transfer experiments have supported the antitumoral effects of Th17, highlighting their potential therapeutic interest [23, 26–29], but the significance of the presence of endogenous Th17 that develop in a context of progressing cancer is unclear [18, 30, 31]. This unstable balance between the pro- versus antitumoral function of Th17 likely stems from the high plasticity of these cells, capable of trans-differentiating into either pro-inflammatory effector cells such as Th1 [32] or immunosuppressive FoxP3$^+$Treg [33] depending on the environmental conditions [25]. In this context, the concentration of TGFβ1 present in the milieu is an important factor determining the pro- or anti- inflammatory characteristics of polarized Th17.

Many different protocols have been used to generate Th17 in vitro from naïve CD4$^+$ T lymphocytes using specific cytokine cocktails. In mice, TGFβ1, IL-6, and IL-23, in the presence of TCR and CD28 signals (antigen-presenting cells, plate-bound anti-CD3 plus anti-CD28 or anti-CD3/anti-CD28-coated microbeads) are required and sufficient to induce the differentiation of naïve CD4$^+$CD25$^-$ T cells into Th17 [25, 34]. IL-6, by inhibiting TGFβ-induced FoxP3 expression, blocks Treg differentiation, resulting in the polarization of naive T cells towards IL-17-producing RORγt$^+$ lymphocytes. However, some studies have demonstrated that pathogenic Th17, responsible for the development of experimental autoimmune encephalomyelitis (EAE), can be generated in IL-6$^{-/-}$ mice [20, 35]. These results highlight the possibility that Th17 can also develop in absence of IL-6. IL-21 has been identified as an alternative factor capable of inhibiting TGFβ-induced FoxP3 expression in the absence of IL-6 [20, 36]. IL-21 is produced in large quantities by Th17 and fosters an autocrine amplification feed-back loop increasing Th17 generation in

the absence of IL-6 [37]. IL-23 also plays an essential role in Th17 development. The IL-23 receptor (IL-23R) is composed of IL23R and IL-12Rβ2 [38]. The level of expression of IL-23R by naïve $CD4^+$ T cells is very low, while Th17 highly express this receptor. In agreement with these considerations, IL-23 does not seem required for Th17 initial lineage commitment, but this cytokine enhances Th17 expansion and promotes their survival, stabilization and pro-inflammatory properties [39]. Consistently, the number of Th17 is significantly reduced in IL-23p19-deficient mice compared to their wild-type counterparts [40, 41]. Additionally, IL-23 substantially contributes to the pathogenic functions of Th17 [40, 42]. Finally, IL-23 is a critical factor required for the generation of prolonged in vitro Th17 cultures [39]. IL-1β, by inducing interferon regulatory factor 4 (IRF4) which is a critical regulator of IL-21, has also been reported as an additional factor involved in the differentiation of Th17 cells in pro-inflammatory environments [43, 44]. In humans, the precise parameters required for Th17 differentiation remain unclear. Although some reports have argued that TGFβ1 may not be needed for human Th17 generation [45–47], others have reported that this cytokine is essential for the development of these cells [36, 48]. The possibility of producing Th17 with TGFβ plus IL-21 but not IL-6 has been proposed [36] and IL-1β alone or in combination with TGFβ has also been used to generate human Th17 [49]. Importantly and similar to the results obtained in mice, the presence of IL-23 is required for Th17 stabilization and optimal proliferation [50].

As Th17 cells have gained increasing interest in the field of autoimmunity, infectious diseases and cancer, the development of protocols allowing for the production of large number of these cells ex vivo is essential to further study their function, regulation, lineage stability and impact in different pathologies. For instance, the developmental fate of Th17 adoptively transferred to cancer-bearing hosts and the influence of the tumor environment on their lineage commitment and pro-inflammatory properties remain to be clearly elucidated. The possibility to harness the therapeutic potential of these cells against infectious agents or cancer represents another application of ex vivo-generated Th17. In this chapter, we describe examples of procedures suitable for in vitro generation of mouseand human Th17 lymphocytes from naïve $CD4^+CD25^-$ T cells.

2 Materials

2.1 Murine Th17 Generation

1. Six- to eight-week-old mice.
2. Complete medium for murine Th17 generation: RPMI 1640 supplemented with 10 % FBS, 100 U/ml penicillin, 100 μg/ml

streptomycin sulfate, 0.5× MEM nonessential amino acids, and 1 mM sodium pyruvate.

3. Naive $CD4^+CD62L^+$ T Cell Isolation Kit II, mouse (Miltenyi Biotech, catalogue # 130-093-227).
4. AutoMACS separator or LS Columns and magnets (MidiMACS or QuadroMACS (Miltenyi).
5. Anti-CD3/anti-CD28-coated (expansion/activation) microbeads (Invitrogen).
6. Magnet (DynaMag™-15 Magnet, Invitrogen) to recover washed microbeads.
7. Cytokines: mouse IL-12, IL-2, IL-7, IL-6, TGFβ (Peprotech) and IL-23 (R&D Systems).
8. Blocking antibodies: anti-mouse IFNγ and anti-mouse IL-4 antibodies (Affymetrix eBioscience).

2.2 Human Th17 Generation

1. Human PBMC.
2. Complete Medium (CM): AIM-V medium containing glutamine, nonessential amino acids, 10 mM Hepes buffer, and 1 mM sodium pyruvate.
3. Ficoll-Paque™ PLUS (GE Healthcare Bio-Sciences AB).
4. Miltenyi Human Naïve T Cell Isolation Kit II (Cat#: 130-094-131).
5. AutoMACS separator or LS Columns and magnets (MidiMACS or QuadroMACS (Miltenyi).
6. Human Anti-CD3/anti-CD28-coated microbeads (Invitrogen).
7. Magnet (DynaMag™-15 Magnet, Invitrogen) to recover washed microbeads.
8. Cytokines: Human IL-6, TGFβ1, IL-1β, IL-2 and IL-23 (Miltenyi).

2.3 Real-Time PCR

1. NanoDrop ND1000 spectrophotometer (NanoDrop).
2. Primers specific for RORγT and T-bet (ABI; Applied Biosystems), RNA extraction kit (Qiagen RNAeasy Mini kit), Reverse transcription (iScript cDNA synthesis kit), and Real-Time PCR reagent (IQ Supermix) (Bio-Rad).
3. Bio-Rad iCycler iQ real-time PCR detection system (Bio-Rad).

2.4 Flow Cytometry

1. Flow cytometry buffer (1× PBS, 1 % BSA).
2. Human FcγR (Fc-gamma receptor)-binding inhibitor (Affymetrix Ebioscience).
3. 1× Fixation/Permeabilization buffer (Affymetrix Ebioscience).

4. Anti-human/mouse RORγt-PE (clone AFKJS-9), anti-human/mouse Tbet-PE (clone eBio4B10), mouse IgG1K Isotype Control-PE (clone P3.6.2.8.1), rat IgG2a K Isotype Control-PE (Clone eBR2a) (Affymetrix Ebioscience).
5. LSRII-Fortessa flow cytometer (BD bioscience).
6. Flowjo software (Vx, Tree Star, Inc).

2.5 ELISAs

1. Human IL-17A ELISA Ready-SET-Go!® (Affymetrix ebioscience).
2. Human IFN-gamma ELISA Ready-SET-Go!® (Affymetrix ebioscience).

2.6 General Materials

1. Sterile PBS.
2. Red blood cell lysis buffer (BD biosciences).
3. Cell strainer.
4. Centrifuge tubes.
5. Hemocytometer.
6. 0.4 % Trypan blue.
7. MACS sorting buffer (0.5 % BSA, 2 mM EDTA).
8. 24-Well plates and Petri dishes.

3 Methods

3.1 Generation of Murine Th17 Lymphocytes Ex Vivo

1. Euthanize mice by carbon dioxide asphyxiation or other approved techniques and harvest spleens. Cut the spleens in small pieces with scissors and dissociate the tissues in a 40 μm cell strainer with a 5 ml syringe plunger in a Petri dish containing complete medium to obtain a single-cell suspension.
2. Collect the cell suspension in a 50 ml tube and centrifuge at 300 × *g* for 5 min at 4 °C. Remove the supernatant.
3. Resuspend the pellet in red blood cell lysis buffer and incubate for 1 min.
4. Add complete culture medium and centrifuge (400 × *g* for 5 min at 4 °C). Remove the supernatant.
5. Resuspend cells in MACS sorting buffer and determine the number of viable cells using trypan blue and a hemocytometer.
6. Isolate naive $CD4^+CD25^-CD62L^+$ T lymphocytes using a $CD4^+CD62L^+$ T cell isolation kit II strictly following the manufacturer's instructions. The isolation requires two steps: (1) Depletion of non-$CD4^+$ T cells using a biotin-antibody cocktail II and anti-biotin microbeads. Cells are separated using an AutoMACS device (program "Depletes") or LS columns and a

MidiMACS or QuadroMACS magnet and the negative fraction enriched in $CD4^+$ cells (flow-through) is collected (outlet port neg1 of the AutoMACS). (2) Positive selection of $CD4^+CD62L^+$ T cells using CD62L microbeads. Cells are separated using an AutoMACS device (program "Possel") or MS columns, and the positive fraction is collected (outlet port pos1 of the AutoMACS). Cell labeling is performed in MACS sorting buffer and incubation times are as described by the provider.

7. Centrifuge cells (400 × *g* for 5 min at 4 °C). Remove the supernatant.
8. Resuspend purified naïve $CD4^+$ T cells at a concentration of 10^6 cells/ml in complete culture medium.
9. Wash the appropriate amount of anti-mouse CD3/anti-mouse CD28-coated expansion/activation beads (*see* **step 10**) in a 15 ml tube with PBS and EDTA and recover the beads using a DynaMag™-15 Magnet as instructed by the provider.
10. Add washed expansion/activation beads at a ratio of 2 beads to 1 naïve $CD4^+$ T lymphocyte.
11. Add cytokines and blocking antibodies: 40 ng/ml IL-6, 0.5 ng/ml TGFβ1, 5 μg/ml blocking anti-IFNγ, and 5 μg/ml blocking anti-IL-4 antibodies.
12. Plate cells in 24-well plates (1 ml, 10^6 cells/well).
13. After 3 days, collect cells, remove expansion/activation beads using a DynaMag™-15 Magnet and repeat **steps 7–9**.
14. Add 40 ng/ml IL-23 and plate cells in 24-well plates (1 ml, 10^6 cells/well).
15. On day 6, cells expressing the transcription factor RORγt and producing IL-17 can be obtained (Fig. 1). The generated cells can be characterized by real-time PCR, flow cytometry or ELISA for expression of specific markers and transcription factors and cytokine production. These polarized cells can also be used for further analyses in vitro or to evaluate their therapeutic potential in vivo [26].

3.2 Generation of Human Th17 Lymphocytes Ex Vivo

1. Collect blood from healthy volunteers or patients as desired (100–300 ml).
2. Dilute blood in 1× PBS (approximately 1 to 2 dilution).
3. Pipet 15 ml of Ficoll-Paque™ PLUS in a 50 ml tube.
4. Gently layer 30 ml of diluted blood onto the Ficoll.
5. Centrifuge the samples at 930 × *g* for 20 min at room temperature with no brake.
6. Collect the peripheral blood mononuclear cells (PBMC) layer from the Ficoll gradient and transfer the cells in a 50 ml tube.

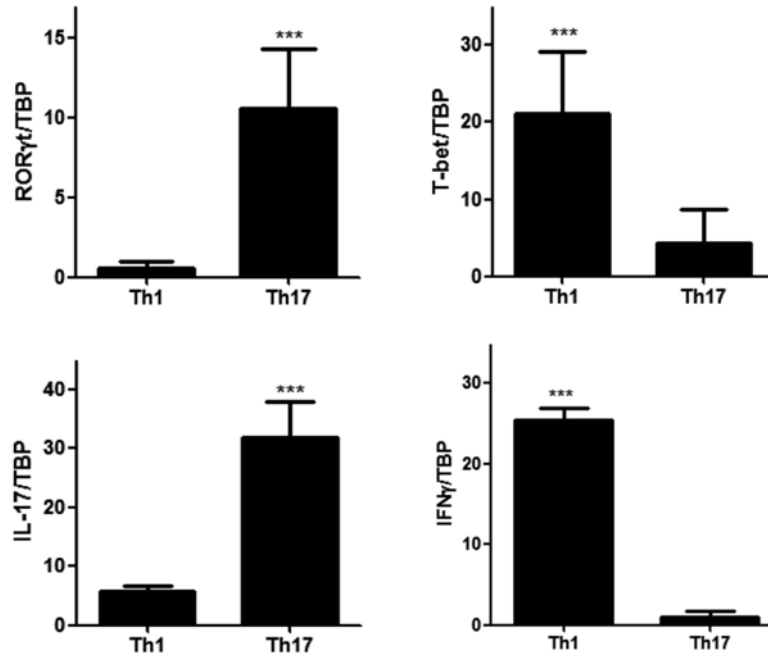

Fig. 1 Example of mouse Th17 lymphocyte generation in vitro. Mouse naïve CD4+ T cells were cultured in Th17-polarizing conditions and analyzed for RORγt and IL-17 expression. Cells were collected at the end of the differentiation period and RORγt, Tbet, IL-17, and IFNγ mRNA expression was determined by RT-PCR. Th1 cells, generated as reported [26], were used as comparison. Data are represented as mean ± SEM of three different experiments (***, $p < 0.001$)

7. Centrifuge cells (300 × *g*, 5 min, room temperature) and wash twice in PBS.
8. Resuspend the cells in MACS sorting buffer and determine cell number.
9. Perform isolation of naïve T lymphocytes using Miltenyi Human Naïve T Cell Isolation Kit II strictly following the provider's instructions. Untouched naïve CD4+ T cells are isolated by depletion of non-CD4+ T lymphocytes and of memory CD4+ T cells that are labeled with a cocktail of biotin-conjugated antibodies and anti-biotin microbeads. Cells are separated using an AutoMACS device (program "Depletes") or LS columns and a MidiMACS or QuadroMACS magnet and the negative fraction enriched in naïve CD4+ cells (flow-through) is collected (outlet port neg1 of the AutoMACS).
10. Centrifuge cells (300 × *g*, 5 min, room temperature) and determine cell number.
11. Resuspend naïve CD4+ T cells in CM (~1 million cells/ml).
12. Wash the appropriate amount of anti-human CD3/anti-human CD28-coated expansion/activation beads (*see* **step 13**)

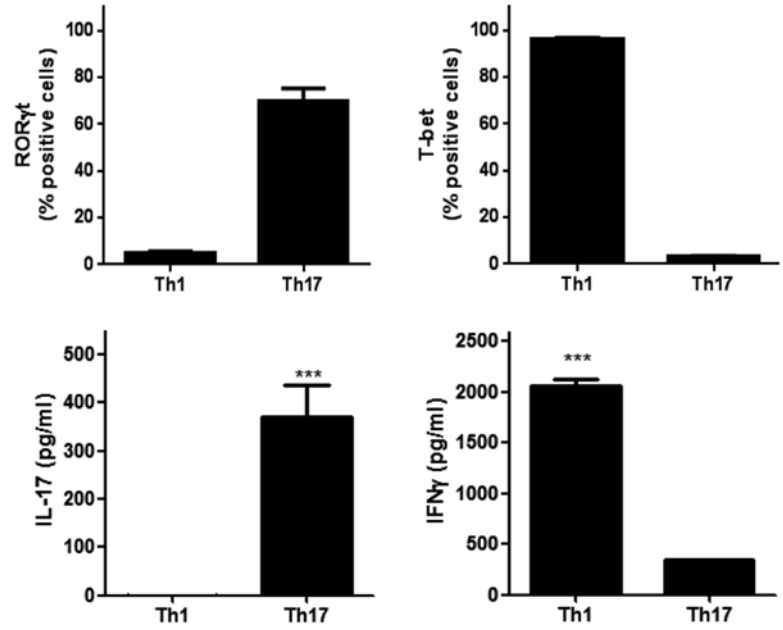

Fig. 2 Example of human Th17 lymphocyte generation in vitro.Human CD4+ T cells were cultured in Th17-polarizing conditions and analyzed for RORγT expression and IL-17 production. Cells were collected at the end of the differentiation period and the percentage of RORγt + and Tbet + cells was determined by flow cytometry. The concentration of IL-17 and IFNγ was detected by ELISA in 48 h culture supernatants (10^6 cells/ml). Th1 lymphocytes were used as comparison. Th1 were generated following the same procedures as for Th17 cells, except that (**a**) human IL-2 (20 UI/ml), human IL-12 (10 ng/ml), human IL-7 (20 ng/ml) were used, (**b**) the beads to T cell ratio was 1 to 1, and (**c**) cells were cultured for a total of 3 days (***, $p < 0.001$)

in a 15 ml tube with PBS and EDTA and recover the beads using a DynaMag™-15 Magnet strictly following the provider's instructions.

13. Add washed beads at a ratio of 2 beads to 1 naïve CD4+T lymphocyte.
14. Add cytokines: 20 ng/ml human IL-6, 5 ng/ml TGFβ1, 50 ng/ml IL-1β, 5 ng/ml IL-2.
15. Plate cells in 24-well plates (1 ml, 10^6 cells/well).
16. After 3 days, add 50 ng/ml human IL-23 in 1 ml of fresh CM per well.
17. Split cell by half on day 5 and add fresh CM to each well.
18. CD4+ T cells expressing RORγt and producing IL-17 (Fig. 2) can be collected on day 6–7 for further analyses. The generated cells can be characterized by real time PCR, flow cytometry or ELISA for expression of specific markers and transcription factors and cytokine production.

3.3 Characterization of Ex Vivo-Generated Th17 Cells

3.3.1 Real-Time PCR

1. Extract total mRNA from the collected cells using Qiagen RNAeasy Mini kit strictly following the manufacturer's protocol.
2. Measure RNA concentration using NanoDrop ND1000 spectrophotometer.
3. Run reverse transcription reaction using 250 ng of total RNA and iScript cDNA synthesis kit, following the manufacturer's instructions.
4. Real-time PCR reaction: 20 μl of the PCR reactions are set up in 96-well plates containing 10 μl 2× IQ Supermix, 1 μl TaqMan® primer/probe set, 2 μl of the cDNA synthesis reaction, and 7 μl of nuclease-free water. Reactions are run and analyzed on a Bio-Rad iCycler iQ real-time PCR detection system.
5. Analysis: Cycling parameters are determined and resulting data are analyzed by using the comparative C_t method as means of relative quantification, normalized to an endogenous reference (TATA Box Bonding Protein, TBP) and relative to a calibrator (normalized C_t value obtained from control cells), and expressed as $2^{-\Delta\Delta}C^t$ (Applied Biosystems User Bulletin #2: Rev B "Relative Quantification of Gene Expression").

3.3.2 Flow Cytometry

1. One million cells are washed in flow cytometry buffer and incubated for 15 min with human FcγR (Fc-gamma receptor)-binding inhibitor.
2. Cells are incubated with 1× fixation/permeabilization buffer at 4 °C for 30–45 min.
3. Cells are washed twice with 1× permeabilization buffer.
4. Cells are incubated with anti-human/mouse RORγt-PE, anti-human/mouse Tbet-PE or Mouse IgG1K Isotype Control-PE) or Rat IgG2a K Isotype Control-PE at 4 °C for 30 min.
5. Samples are washed twice with 1× permeabilization buffer, then once with 1× PBS.
6. Samples are resuspended in 1× PBS and analyzed using a LSRII-Fortessa flow cytometer.
7. Data are analyzed using Flowjo software.

3.3.3 ELISA

1. Generated human Th cells are cultured for 48 h (10^6 cells in 1 ml CM per well of a 24-well plate) in the presence of anti-human CD3/anti-human CD28-coated expansion/activation beads (2 beads to 1T lymphocyte) and the culture supernatant is collected and centrifuged (300×*g*, 5 min, room temperature).
2. IL-17 and IFN-γ are detected in cell-free culture supernatants by enzyme-linked immunosorbent assays using human IL-17A

ELISA Ready-SET-Go!® or Human IFN-gamma ELISA Ready-SET-Go!® reagent sets strictly following the manufacturer's instructions.

4 Notes

1. To obtained optimal cell purity, start from single-cell suspension and strictly follow the provider's instructions regarding cell number and amount of beads (Miltenyi Biotech).
2. Keep cells on ice or at 4 °C during the isolation procedures until plating. Use pre-chilled MACS buffer (Miltenyi Biotech).
3. When purifying PBMC on Ficoll monolayers, ensure that the blood does not mix with the Ficoll before centrifugation. When collecting the PBMC ring, do not mix with or collect Ficoll (Miltenyi Biotech).
4. Cell viability of purified naïve T lymphocytes before initiation of the cultures should be 90–100 % (Miltenyi Biotech).
5. Activation beads should be washed in a minimum of 1–2 ml of buffer by extensive mixing (Miltenyi Biotech).
6. Examine cell cultures every day and split cultures if necessary depending on growth rate. Cell clusters should be observed. Do not let cells overgrow (Miltenyi Biotech).
7. Cytokine reconstitution and storage should be performed according to the provider's instructions. Avoid using products that are close to their expiration date. Avoid freeze/thaw cycles (Miltenyi Biotech).

Acknowledgments

The authors thank Martin Asimis for technical assistance with the characterization of human Th17 cells and Claire Larmonier for technical assistance with real-time PCR.

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Part II

Animal Models of Immunoregulation

Chapter 8

Autoimmune Diabetes: An Overview of Experimental Models and Novel Therapeutics

Sylvaine You and Lucienne Chatenoud

Abstract

Type 1 diabetes (T1D) results from a chronic and selective destruction of insulin-secreting β-cells within the islets of Langerhans of the pancreas by autoreactive $CD4^+$ and $CD8^+$ T lymphocytes. The use of animal models of T1D was instrumental for deciphering the steps of the autoimmune process leading to T1D. The non-obese diabetic (NOD) mouse and the bio-breeding (BB) rat spontaneously develop the disease similar to the human pathology in terms of the immune responses triggering autoimmune diabetes and of the genetic and environmental factors influencing disease susceptibility. The generation of genetically modified models allowed refining our understanding of the etiology and the pathogenesis of the disease. In the present review, we provide an overview of the experimental models generated and used to gain knowledge on the molecular and cellular mechanisms underlying the breakdown of self-tolerance in T1D and the progression of the autoimmune response. Immunotherapeutic interventions designed in these animal models and translated into the clinical arena in T1D patients will also be discussed.

Key words Autoimmunity, Type 1 diabetes, Pancreatic beta-cells, Autoantigens, Experimental models, T lymphocytes, NOD mice, Immunotherapies

1 Introduction

Type 1 diabetes (T1D), also termed insulin-dependent diabetes, is an organ-specific autoimmune disease that mostly affects children and young adults and whose incidence steadily increases in industrialized countries [1, 2]. Autoimmune diabetes results from the selective destruction of pancreatic insulin-secreting β-cells by an immune-mediated inflammation, involving autoreactive $CD4^+$ and $CD8^+$T lymphocytes, which progressively invade the islets (insulitis) [3]. Diabetic symptoms are hyperglycemia, glucosuria, polyuria, and polydipsia. Current conventional treatment is substitutive and consists in chronic administration of exogenous insulin which, in spite of significant advances, is still associated with major constraints (multiple daily injections, risks of hypoglycemia) and lack of effectiveness over long term in preventing severe

Maria Cristina Cuturi and Ignacio Anegon (eds.), *Suppression and Regulation of Immune Responses: Methods and Protocols, Volume II*, Methods in Molecular Biology, vol. 1371, DOI 10.1007/978-1-4939-3139-2_8,

degenerative complications (retinopathy, nephropathy, neuropathy). For patients presenting poor diabetes control (including hypoglycemia unawareness and severe hypoglycemic events) or kidney failure as complication of their autoimmune disease, only transplantation of whole pancreas or of isolated islets of Langerhans, to reconstitute the β-cell mass, may lead to insulin independence and restore glycemic control associated with beneficial effects on secondary complications. However, in addition to the difficulties related to the organ availability and the transplant procedure, these grafts are subjected to the risks of rejection. Therefore, better understanding the pathological process leading to T1D and developing new strategies to halt the aberrant autoreactivity toward pancreatic β-cellautoantigens remains of great interest and a major challenge.

Studies on the etiology of T1D benefited from the availability of several rodent animal models allowing an in-depth analysis of the disease. Depending on the model, the animals present a spontaneous or an induced form of the disease which provides important clues for a deeper understanding of the various genetic, immune, and pathophysiological mediators involved in the breakdown of self-tolerance to pancreatic β-cells. Our aim here is to describe the different experimental models that have been established in an autoimmune prone or a normal genetic background (Table 1) to gain knowledge on T1D and to define clinically applicable therapeutic strategies.

2 Experimental Models

2.1 Spontaneous Diabetes

2.1.1 The Non-Obese Diabetic Mice

The non-obese diabetic (NOD) mice is the most widely used animal model for the study of T1D [4]. It has been developed more than 30 years ago and was originally spontaneously derived from the inbred cataract Shionogi (CTS) mice characterized by cataracts, themselves derived from the outbred Imperial Cancer Research (ICR) mice [5]. As in humans, T1D in NOD mice results from the chronic destruction of β-cells in the pancreas by the immune system leading to insulin deficiency, hyperglycemia, and glycosuria.

Under specific pathogen-free conditions, diabetes incidence reaches 80–95 % of female mice and 20–40 % of males by 40 weeks of age. This variability in disease incidence is in part related to the pathogen environment as the higher incidence is observed in "very clean" specific and opportunistic-free conditions (SOPF) and the sex bias is abrogated under germ-free conditions where NOD males develop T1D at a similar rate than females [1, 6]. In conventional non-SPF environment, frequency of diabetes can drop to levels as low as 10 % [7]. Animals decontaminated by embryo transfer and housed in isolator show again a 90 % disease incidence

Table 1
Experimental models of T1D

Spontaneous models	Mouse model	The non-obese diabetic (NOD) mouse		[5]
	Rat model	The bio-breeding (BB) rat		[59]
Genetically modified models	In normal non-autoimmune prone mouse strains	Rat insulin promoter (RIP)-driven expression	RIP-LCMV GP/NP	[77–79]
			RIP-HA	[80]
			RIP-HEL	[81]
			RIP-OVA	[82]
			RIP-IFNγ	[85, 90, 91]
			RIP-IL-2	[87]
			RIP-TNF α	[86]
			RIP-B7.1	[88, 89]
			RIP-I-A or I-E	[85]
	In the NOD autoimmune prone strain	T CR transgenes cloned from islet-infiltrating T cells	BDC2.5	[26, 94]
			8.3	[92]
			4.1	[93]
		Rat insulin promoter (RIP)-driven expression	RIP-LCMV GP/NP	[97]
			RIP-B7.1	[99]
			RIP-I-A or I-E	[101, 102]
			RIP-IL-4	[104]
			RIP-IL-10	[100]
			RIP-TGF-β	[105]
			RIP-FasL	[103]
		Invalidation of candidate genes	Ins2$^{-/-}$	[106]
			IFNγ$^{-/-}$	[107]
			IL-12$^{-/-}$	[108]
			IL-4$^{-/-}$	[109]
			IL-10$^{-/-}$	[110]
			CD28$^{-/-}$	[111]
			B7.1/B7.2$^{-/-}$	[54]
		Expression of human molecules (humanized models)	HLA-DR4	[112]
			HLA-A*0201	[114]
			HLA-A*1101	[118]
			HLA-B*0702	[118]
			huCD3ε	[119] [120]

as early as the first generation. In addition to T1D, NOD mice are prone to thyroiditis, sialitis, autoimmune hemolytic anemia, and lupus-like syndrome following particular treatments [8–12].

Autoimmune diabetes is a multigenic disease and, as in diabetic patients, extensive studies have been performed on the genetics of NOD mice addressing the presence of polymorphic loci positively or negatively associated with diabetes development. Chromosomal regions containing *Idd* (insulin-dependent diabetes) susceptibility loci have been identified [13, 14]. Variations in the major histocompatibility (MHC) genes, encoded in *Idd1*, are the major genetic

component controlling diabetes onset in NOD mice as well as in T1D patients. In addition to the common class I alleles *Kd* and *Db*, NOD mice express a unique MHC class II molecule, *I-Ag7* and do not express an *I-E* region [15]. Development of diabetes is dependent on the homozygosity for this particular MHC region. The I-A^{g7} molecule is characterized by a substitution of the aspartic acid residue by a serine at position 57 on the β chain [16]. Similarly, aspartic acid at position 57 of the human DQ8 β chain provides protection from T1D and substitution of this residue increases susceptibility to diabetes [17]. Non-MHC-linked candidate genes or regions have also been identified in human and mouse genome-wide association studies (GWAS) [18, 19]. Among them, *Idd3* and *Idd5* encode critical genes involved in immune responses, i.e. *Il-2* or *Ctla4*, *Cd28*, *Icos*, and *Bcl2*, respectively [20, 21]. *Idd9* carries molecular variants of *Cd30*, *Tnfr2*, and *Cd137* and may also regulate the development and function of $CD4^+CD25^+Foxp3^+$regulatory T cells [22, 23]. *Idd7* contains the insulin 2 (*Ins2*) locus, the mouse ortholog of *IDDM2* in humans [24]. *Idd18* includes the *Vav3* locus involved in B- and T-cell signaling [25].

Type 1 diabetes is a slowly progressing disease and clinical manifestations are preceded by a long-lasting asymptomatic phase that starts at 3–4 weeks of age with the first wave of immune cells infiltrating pancreatic islets (insulitis) [26]. The infiltrate progresses and becomes more prominent but remains under the form of a peripheral non-aggressive insulitis until 8–10 weeks of age. During this period, antibodies arise against β-cell autoantigens such as insulin/proinsulin, GAD (glutamic acid decarboxylase), IA2 (β cell-specific protein phosphatase), the p277 peptide of hsp60, the islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), the zinc transporter ZnT8 [27]. By 10–12 weeks of age, insulitis becomes invasive and aggressive, β-cells are destroyed and overt hyperglycemia occurs when 70–75 % destruction of the functional β-cell mass has been achieved.

Development of T1D is mediated by T cells recognizing the various autoantigens mentioned above [28–34]. Importantly, in polyclonal NOD mice, both $CD4^+$ and $CD8^+$ populations are required as adoptive transfer of either populations isolated from the spleen of diabetic NOD mice into immunoincompetent NOD hosts (neonates, irradiated adults, NOD-*SCID*, NOD-$RAG^{-/-}$), does not provokepancreatic β-cell destruction and hyperglycemia [35–37]. It has been shown that autoreactive T-cell pools, notably the clonotypes recognizing their MHC/peptide ligand with a high avidity/affinity, progressively expand in the periphery and are recruited in the target tissue [38, 39]. These T cells get activated by antigen-presenting cells (APC) mostly in the pancreatic lymph nodes (PLN) as illustrated by the fact that removal of PLN in young pre-diabetic NOD mice prevent diabetes development [40]. Based on the observation that there is a physiological β-cell apoptosis

peaking at 2–3 weeks of age during the pancreas organogenesis in mice and that macrophages of NOD mice exhibit deficient phagocytosis, it has been suggested that the initiation of the autoimmune involved the uptake of β-cell autoantigens by APC (mainly dendritic cells) which then migrate to the PLN where they present autoantigenic peptides to autoreactive T cells [41, 42]. In addition, release of death-associated molecular pattern molecules (DAMP) by apoptotic/necrotic β-cells may also stimulate APC and contributes to induction of inflammation through innate receptors such as Toll-like receptors (TLR) [43]. Lastly, Diana et al. showed that when physiological β-cell death occurs, the innate immune cell dialog engaged between plasmacytoid dendritic cells (pDC), neutrophils, and B-1a cells is critical for T1D development [44].

Adoptive transfer of B cells or injection of serum from overtly diabetic NOD mice, in contrast to T-cell transfer, in immunoincompetent NOD mice did not induce diabetes revealing the lack of intrinsic pathogenicity of B lymphocytes or of autoantibodies. However, B cells are mandatory for diabetes development as B-cell-less NOD mice are completely protected from disease. These results argue for a key role of B cells in antigenic presentation [45, 46].

In parallel to autoreactive effector T cells, numerous studies reported the presence in NOD mice of T cells endowed with suppressive capacities. Initial studies focused on the L-selectin (CD62L) as a marker to discriminate pathogenic from regulatory T cells (Treg). Indeed, $CD4^+CD62L^+$ thymocytes or splenocytes protect from diabetes transfer in vivo [47–49]. In contrast, the $CD25^-CD62L^-$ T-cell fraction includes potent diabetogenic effectors already present in very young pre-diabetic (6-week-old) NOD mice and capable of transferring diabetes in NOD-*SCID* recipients as efficiently as spleen cells from diabetic animals [50]. Following the seminal work of the group of S. Sakaguchi describing natural, thymus-derived $CD4^+CD25^+$ Tregs, compelling evidence was accumulated showing the predominant role of such Tregs expressing the lineage marker forkhead/winged helix (FoxP3) transcription factor in the control of diabetes [51, 52]. Selective deprivation of natural Tregs, as induced by thymectomy at 3 weeks of age or by administration of depleting CD25 antibodies to young animals or by invalidation of the *CD28* gene (CD28-deficient NOD mice), promotes disease acceleration and exacerbated Th1 responses [53–55]. $CD4^+CD25^+Foxp3^+$ T cells isolated from the thymus or the spleen and lymph nodes of young pre-diabetic NOD mice can prevent from diabetes induced by diabetogenic effector T cells in NOD-*SCID* recipients [50]. This control is carried out in a TGFβ-dependent manner [50, 56]. Interestingly, progression to overt diabetes in NOD mice is associated with a decline in the functional capacity of Tregs, particularly within the pancreas where both the inflammatory milieu and an insufficient availability of IL-2 affects

Treg homeostasis [50, 57]. In addition, data, corroborated in other models of autoimmunity, pointed to key age-dependent differences in pathogenic T cells that are not only quantitative but also qualitative and render them progressively insensitive to Treg- and TGFβ-mediated immune regulation [50, 58]

2.1.2 The Bio-Breeding Rat

The second model of spontaneous T1D is the bio-breeding diabetes prone (DD-DP) rat whose first description was published by Nakhooda et al. in 1977 [59]. As in the NOD mice, the polygenic imprint of T1D was also evidenced in this model showing predisposing genes (*Iddm*) encoded in MHC and non-MHC susceptibility loci. In particular, BB-DP rat express the $RT1^u$ haplotype which encodes the D^u/B^u MHC class II alleles (*Iddm1*), mandatory for the development of autoimmune diabetes [60, 61]. The GTPase of the immune-associated nucleotide-binding protein 5 (*Gimap5*, *Iddm2*) also contributes to diabetogenesis. This protein exerts pro-survival functions by regulating calcium influx [62]. Homozygote mutations of *GIMAP5* induce spontaneous apoptosis of mature T lymphocytes, leading to a state of lymphopenia as shown in the BB-DP rat [63]. Additional susceptibility loci have been identified showing significant linkage to the development of T1D, revealing the presence of genes associated with the human disease such as *Ins*, *Ptpn22*, *Il-2*, and *Il-21* [64].

In contrast to the NOD mouse model, both male and female BB-DP rats develop autoimmune diabetes reaching 90 % of the colony by 4 months of age under specific pathogen-free conditions. Disease onset is preceded by an asymptomatic phase characterized by progressive infiltration of pancreatic islets by immune cells, notably $CD4^+$ and $CD8^+$ T cells that will destroy the β-cells [65].

T cells endowed with regulatory properties (Tregs) have also been identified in the BB-DP rats. They were first defined by the expression of the antigen RT6, now named ART2, which is an adenosine diphosphate (ADP)-ribosyltransferase expressed at the cell membrane. BB-DP rats are lymphopenic for this subset due to an abnormal intra-thymic T-cell maturation and an elevated rate of apoptosis of recent thymic emigrants in the liver [66, 67]. In vitro, thymocytes from BB-DP rats failed to differentiate into $ART2^+$ T cells in fetal thymic organ cultures [68]. The importance of these $ART2^+$ T cells in the control of T1D was illustrated by studies using the bio-breeding diabetes-resistant rats (BB-DR) which express the same MHC molecules but are protected from spontaneous T1D [69]. These rats are not lymphopenic and present normal numbers of $ART2^+$ T cells that can be generated in vitro from fetal thymus. Administration of a depleting anti-ATR2 (RT6) antibody induces diabetes in BB-DR rats [70] and adoptive transfer of BB-DR $CD4^+ART2^+$ T cells into BB-DP recipients prevents diabetes development [71, 72]. Expression of ART2 is mainly confined

to the naturally occurring $CD4^+CD25^+Foxp3^+$Treg cell subset which is also deficient in the BB-DP rats [73]. $CD4^+CD25^+Foxp3^+$ Tregs exhibit suppressive capacities in vitro and in vivo and can control diabetogenic effectors. Finally, in the rat, a $CD4^+$ Treg population was also identified by the absence or low expression of CD45RC, as exemplified in a model of T1D induced by thymectomy and sublethal irradiation [74]. $CD4^+CD45RC^{-/low}$ T cells, isolated from BB-DR rats, express low levels of Foxp3 which correlates with the fact that about 20 % of them express CD25. Interestingly, in vivo, both $CD4^+CD45RC^-CD25^+$ and $CD4^+CD45RC^-CD25^-$ T cells can protect from diabetes onset when infused into BB-DP rats or when co-transferred with diabetogenic T cells in immunoincompetent hosts [73].

In addition to the BB-DP strain, the LEW.1AR1/Zmt-iddm rat is another good experimental model of human T1D, expressing the MHC class II genes encoded in the $RT1^u$ haplotype [75]. Spontaneous diabetes develops in association with an invasive insulitis including $CD4^+$ and $CD8^+$ T cells, B lymphocytes, macrophages, and natural killer cells. In contrast to BB-DP rats, lymphopenia is not observed in this model and differentiation of $RT6^+$ T cells is normal.

2.2 Diabetes Induced by Genetic Manipulation

2.2.1 In Normal Non-Autoimmune Prone Mouse Strains

To better understand the contribution of defined cellular and molecular mediators in the pathogenic autoimmune responses, transgenic models of T1D were generated in normal strains, i.e. genetically resistant to spontaneous autoimmunity. Most of these models rely on a rat insulin promoter (RIP)-driven expression of neo-antigens in pancreatic β-cells, originally described by the group of D. Hanahan [76]. Such neo-antigens, considered as self-antigens, include lymphocytic choriomeningitis virus (LCMV) nucleoprotein (NP) or glycoprotein (GP) [77–79], influenza hemagglutinin (HA) [80], hen egg lysozyme (HEL) [81], and ovalbumin (OVA) [82]. Pancreatic β-cell expression of these antigens does not per se induce diabetes. The disease develops upon immunization with the cognate antigens, viral infections, or transfer of antigen-specific T cells. For example, infection with LCMV triggers rapid and severe disease in RIP-LCMV GP mice (within 14 days) [77, 79]. Interestingly RIP-LCMV NP mice, that express NP both in β-cells and thymus, develop slow-onset diabetes (several months) after inoculation of LCMV. In this latter model, high-affinity LCMV-specific $CD8^+$ T cells are deleted in the thymus due to negative selection and only the low-affinity $CD8^+$ T cells survive and migrate to the periphery [79]. In the RIP-LCMV GP model, high-affinity LCMV-specific $CD8^+$ T cells do not undergo negative selection and are exported at the periphery where they get activated and rapidly destroy β-cells.

Another possibility to induce T1D in these transgenic models is to breed the mice expressing neo-antigens in β-cells with mice

expressing neo-antigen-specific T-cell receptor (TCR) transgenes. However, quite interestingly, these double transgenic models do not all develop the disease. Mice expressing HA in β-cells and HA-specific TCR transgene in T cells developed T1D immediately after birth and died within 10 days [83]. Contrasting with these results, crossing RIP-LCMV GP mice with LCMV-specific TCR transgenic mice did not promote diabetes [77]. Clonal deletion or anergy were not causing this immune tolerance as transgenic TCR T cells were detected in the thymus and the periphery and in vitro, they were able to kill target cells expressing LCMV GP. These data led to the concept of "immunological indifference" due in part, to an inappropriate co-stimulation of effector T cells [84]. In vivo, development of diabetes was triggered following infection of the double TCR-GP transgenic mice with LCMV, which may have provided a danger signal and adequate antigen presentation thus inducing full activation of LCMV-specific T cells and destruction of LCMV GP expressing β-cells [77].

To further examine the factors triggering T1D, mice expressing various inflammatory cytokines, co-stimulatory molecules or MHC class II in β-cells have been produced. Expression of IFNγ, IFNα, or IL-2 under the RIP promoter induced progressive inflammation of the islets and destruction of β-cells [85–87]. Overexpression of co-stimulatory B7.1 molecules is not sufficient to provoke an autoimmune response but combination with TNFα or IL-2 resulted in autoimmunity [88, 89]. Mice transgenic for MHC class II molecules (I-A or I-E) have been produced as aberrant expression could be a triggering factor of diabetes development [90, 91]. Neither insulitis nor diabetes was observed in these models.

2.2.2 *In the NOD Autoimmune Prone Strain*

Studies in genetically modified NOD mice have generated valuable knowledge on diabetogenesis. In particular, the availability of NOD mice expressing TCR transgenes cloned from islet-infiltrating T cells has provided important information on the development, the activation, the migration, and the recruitment of autoreactive T cells to the target tissue. NOD mice transgenic for the TCR recognizing the insulin B chain 9–23 peptide (4.1), the IGRP 206–214 peptide (8.3), or chromogranin A-derived vasostatin-1 peptide 29–42 (BDC2.5) have been widely used to monitor the important checkpoints controlling the diabetogenic process [26, 92–94]. BDC2.5 $CD4^+$, 4.1 $CD4^+$, and 8.3 $CD8^+$ T-cell clones can induce diabetes on their own when transferred into immunoincompetent NOD mice. In addition, severe diabetes was observed and occurred within few weeks in 4.1-NOD-$RAG^{-/-}$ or BDC2.5-NOD-$RAG^{-/-}$ mice which cannot rearrange endogenous TCR genes [49, 93]. Contrasting with these models, 8.3-NOD-$RAG^{-/-}$ develop less aggressive disease as compared to 8.3-NOD-$RAG^{+/+}$ mice due to their requirement of a $CD4^+$ T-cell-derived signal to migrate

efficiently to pancreatic islets [92]. Expression of the BDC2.5 or the 4.1 TCR transgene in the normal NOD background induced insulitis but not overt diabetes although the TCR transgenic T cells are produced in large amount in the thymus, are not deleted through negative selection and are exported to the periphery where they constitute the majority of the T-cell repertoire [92]. Some studies suggested that this low diabetes incidence resulted from the control of diabetogenic transgenic cells by Tregs absent in the NOD-RAG$^{-/-}$ background [95, 96].

NOD mice expressing the LCMV nucleoprotein under the control of RIP have been generated and, as shown in non-autoimmune prone mice, they develop severe diabetes after infection with LCMV [97]. This virally induced transgenic NOD mouse model has been used to demonstrate the existence of a cross-talk between iNKT cells, pDCs, and Tregs in the pancreatic islets that contribute to disease prevention [98] and allows to study the impact of viruses as environmental factors on the development of autoimmune diabetes.

NOD mice expressing cytokines, MHC class II or co-stimulatory molecules in their β-cells have also been very informative. Acceleration of diabetes development was observed following overexpression of B7.1 or IL-10 [99, 100]. In contrast, transgenic expression of protective MHC class II molecules (I-A or I-E), immunomodulatory cytokines (IL-4, TGFβ), or Fas-ligand prevented diabetes [101–105].

Finally, the generation and the study of numerous NOD mice deficient for candidate molecules implicated in diabetes development or protection contributed to our understanding of T1D pathophysiology. Proinsulin 2$^{-/-}$ (*Ins2*$^{-/-}$) NOD mice exhibit an accelerated insulitis and T1D related to the lack of thymic expression of ins2 [106]. Anti-Ins2 autoantibodies were increased in these mice and their T cells showed an enhanced diabetes transfer ability. Surprisingly, NOD mice invalidated for inflammatory pro-Th1 cytokines (IFNγ, IL-12) or modulatory pro-Th2 cytokines (IL-4, IL-10) did not exhibit significantly reduced or exacerbated T1D incidence [107–110]. These data suggest that cytokines may have redundant roles in the progression or the protection from T1D. Lastly, deficiency in co-stimulatory molecules affected diabetes development which was unexpectedly accelerated in CD28$^{-/-}$ or double B7.1/B7.2$^{-/-}$ NOD mice [54, 111]. This effect was related to the profound depletion in CD4^{+}CD25^{+}Foxp3^{+}Tregs observed in these mice as Tregs require B7/CD28 signaling for their thymic generation.

2.2.3 Humanized NOD Mice

Human leukocyte antigen (HLA) class I or class II molecules have been introduced in NOD mice to further analyze their pro- or anti-diabetogenic properties and to identify T-cell epitopes relevant to the human disease. HLA transgenic mice expressing

HLA-DR4 have been instrumental for the characterization of class II-restricted insulin epitopes [112]. In T1D patients, numerous pancreatic β-cell peptides are recognized in the context of HLA-A*0201 [113]. The generation of HLA-A*0201-transgenic NOD mouse (NOD.HHD) allowed the identification of autoreactive islet-infiltrating $CD8^+$ T cells specific for insulin or IGRP epitopes which were also recognized by some human $CD8^+$ T-cell clones derived from diabetic patients [114–117]. Finally, the insulin C-peptide recognized by $CD8^+$ T cells was identified in NOD mice expressing human β2-microglobulin and HLA-A*1101 or HLA-B*0702, which are members of the A3 and B7 HLA haplotypes, respectively [118].

Additional humanized NOD mouse models have been generated with the aim of testing the therapeutic efficacy of antibodies recognizing human molecules. In particular, CD3 antibodies being strictly species-specific, transgenic NOD mice expressing the epsilon chain of the human CD3 complex (huCD3ε), that is the target of therapeutic antibodies, have been produced [119]. The immune system of NOD-huCD3ε mice develops normally in terms of $CD4^+$, $CD8^+$, and Treg cells. All T cells express on their surface "hybrid" TCR/CD3 complexes including both human and murine epsilon chains at a 1/1 ratio. In vitro, these T lymphocytes respond adequately to stimulation with anti-human antibodies in terms of proliferation or cytokine production. Importantly, in vivo, NOD-huCD3ε mice exhibit an incidence of autoimmune diabetes that is comparable to that observed in conventional NOD mice (80–85 % in females at 40 weeks of age). Thus these mice represent a valid preclinical model to screen for the therapeutic efficacy of human CD3 monoclonal antibodies.

Similarly, transgenic NOD mice expressing the human CD20 molecules in B cells (hCD20/NOD) were generated to assess the effect of B-cell depletion using anti-human CD20 on the occurrence and the treatment of T1D [120]. Expression of the transgene did not alter the development of the immune system, and the induction of efficient humoral responses. Incidence of diabetes was very similar to wild-type NOD mice in both males and females. Thus, hCD20/NOD mice were used to investigate the efficacy and mode of action of anti-human CD20 antibodies in T1D setting.

3 Immunotherapy in Type 1 Diabetes

The experimental models described above were instrumental for the design of immunotherapeutic strategies aimed at restoring self-tolerance to pancreatic β-cells and therefore preventing or reversing autoimmune diabetes. Many lessons drawn from these studies were transferred to the clinic in the attempt to find a real cure for human T1D [121].

3.1 Autoantigen Therapy

Autoantigen-based therapy has generated considerable interest since numerous studies in experimental models demonstrated the therapeutic efficacy of β-cell autoantigens administration in preventing autoimmune diabetes through the inhibition of pathogenic T-cell responses and the induction of immunoregulatory mechanisms. The success of such therapy depends on the nature of the antigen (whole protein or selected peptides) and the adjuvant, the dose injected, the administration routes and the timing of injections in relation to disease progression. Immunization of young pre-diabetic NOD mice (3–6 weeks of age) with insulin, GAD or the p277 peptide of the hsp60 protein, using different routes of administration (intravenous, subcutaneous, oral, and nasal) conferred significant protection from disease [122–127]. Depending on the model, restoration of self-tolerance was associated with the induction of Th2 (IL-4), Th3 (TFGβ), or Tr1 (IL-10) responses [123, 124, 128, 129]. Interestingly, tolerance induced toward the injected antigen/peptide could spread to other organ-related autoantigens through by stander suppression, initially described in the experimental autoimmune encephalomyelitis (EAE) model [130]. Administration of autoantigens in older NOD mice, already showing invasive insulitis, was much less efficient in preventing T1D, except in few cases [131, 132].

Translation of this strategy to the clinical arena was disappointing, as most of the results were negative or showed limited effect. Intervention trials using insulin or GAD in pre-diabetic patients (presenting at least two autoantibodies to β-cell antigens) or in patients with recent-onset T1D showed no significant difference in terms of disease incidence or exogenous insulin needs, whatever the route of administration tested [133–136]; {Harrison, 2004 #7022; Nanto-Salonen, 2008 #7054; Wherrett, 2011 #7089; Ludvigsson, 2012 #7042}. In contrast, encouraging results were obtained in phase II trials using subcutaneous injections of the Hsp60 p277 peptide (Diapep277) in terms of preservation of β-cell function [137, 138]. These positive findings were confirmed in a recent phase III trial enrolling 457 newly diagnosed patients having received DiaPep277 or placebo quarterly for 2 years [139]. C-peptide secretion was preserved in DiaPep277-treated patients as compared to controls and this effect was accompanied by a decrease in hypoglycemic events reflecting a better glycemic control.

Interesting strategies using autoantigen-coupled apoptotic cells or autoantigen-coupled biodegradable nanoparticules were developed by the group of S. Miller. Indeed, intravenous administration of insulin or insulin peptide B_{9-23} chemically cross-linked through ethylene carbodiimide to syngeneic splenocytes (Ins-ECDI-SP or $InsB_{9-23}$-ECDI-SP) was very effective at protecting NOD mice from diabetes [140]. Interestingly, a 50 % disease remission was obtained in new onset diabetic NOD treated with Ins-ECDI-SP [141]. Similarly, adoptive transfer of diabetes induced by

transgenic BDC2.5 T cells was abrogated after treatment with p31-ECDI-SP, p31 (1040-p31) being a mimotope of chromogranin $A_{29–43}$ peptide [141]. Contrasting with these data, the use of GAD or IGRP peptides coupled with syngeneic splenocytes did not afford diabetes protection suggesting that only few epitopes may play critical role in modulating the disease [140, 141]. The translation of the Ag-ECDI-SP strategy to the clinic was supported by data in a humanized NOD mouse model in which human HLA-A*0201 molecules are expressed instead of murine MHC Class I (NOD.β2mnull.HHD). Administration of a combination of $CD8^+$ T-cell epitopes $IGRP_{228–236}$/$IGRP_{265–273}$ coupled to ECDI-SP prevent from diabetes development in pre-diabetic NOD.β2mnull.HHD [142]. Recently, to foster clinical application of this methodology, polystyrene beads (PSB) or biodegradable poly(lactic-co-glycolic acid) (PLGA) nanoparticles have been proposed as an alternative to the use of syngeneic cells as cellular vehicle. Following promising results in EAE, the mouse model of multiple sclerosis, preliminary experiments have been conducted in T1D.Treatment of recipient NOD-*SCID* mice with p31-ECDI-PLGA nanoparticles inhibited diabetes induction by activated BDC2.5 transgenic T cells [143]. Mechanistic studies suggested that the i.v. injected Ag-ECDI-SP or Ag-ECDI-PLGA nanoparticles were uptaken by splenic or liver APCs through scavanger receptors such as MARCO, leading to an upregulation of PD-L1/2 inhibitory receptors (but not co-stimulatory molecules) and production of immunomodulatory cytokines such as IL-10 and TGFβ. These tolerogenic APCs induce (1) anergy or apoptosis of activated antigen-specific T cells, (2) anergy of naïve antigen-specific T cells, and (3) generation and expansion of induced Treg capable of controlling the pathogenic process [143].

Lastly, an elegant recent study by Kasagi et al. described a therapeutic approach allowing the in vivo generation of autoantigen-specificTregs capable of preventing and treating autoimmunity [144, 145]. This strategy relies on the ability of phagocytes to release massive amounts of TGFβ after engulfment of apoptotic cells [146]. NOD mice received whole body γ irradiation to induce apoptosis of immune cells (including T and B cells and macrophages) followed by administration of GAD65 peptides and macrophages. This apoptosis-antigen combined therapy efficiently prevented T1D and also halted disease progression in recently hyperglycemic NOD mice. TGFβ was mandatory to establish tolerance and, in the presence of GAD65 autoantigenic peptides, directed the conversion of naïve $CD4^+$ T cells into GAD65-specific $Foxp3^+$Tregs that suppressed the autoimmune responses in the long term. The apoptosis-antigen therapy was applied to EAE with the same efficacy [144]. Importantly, replacing the whole body γ irradiation by injection of anti-CD20 and anti-CD8 antibodies induced similar results thus providing clues for considering clinical translation of this approach.

3.2 Anti-inflammatory Therapy

This therapeutic strategy targets innate immune cells, notably macrophages and neutrophils that accumulate within the islets of Langerhans in early phases of diabetes development, at the time of physiological β-cell apoptosis. These innate cells, activated by β-cell destruction, produce inflammatory cytokines such as IL-1, IL-6, and TNFα that promote the activation of pathogenic autoreactive effectors in addition to being toxic for pancreatic β-cells thus negatively impacting on their survival.

In NOD mice, blocking the IL-1/IL-1 receptor pathway using monoclonal antibodies or IL-1Ra antagonist partially reduced diabetes development but did not revert established disease [147, 148]. In the clinic, a first non-randomized phase I trial showed reduced insulin requirements in 15 recent-onset children with type 1 diabetes treated with anakinra, a recombinant non-glycosylated form of the human IL-Ra [149]. A Phase II placebo-controlled trials using anakinra or canakinumab (anti-IL-1β antibody) was launched but no therapeutic efficacy was observed [150].

A randomized, placebo-controlled pilot study was also initiated in 18 pediatric T1D patients who received anti-TNFα (ethanercept) or placebo [151]. Results showed an increased endogenous insulin secretionsuggesting a preservation of β-cell mass.

3.3 Therapies Using Immunomodulatory Drugs

Experimental models have been extensively used to test the efficacy of therapies targeting the immune players responsible for the aberrant destruction of pancreatic β-cells, i.e. T cells, B cells, and APCs.

3.3.1 Monoclonal Antibodies

Monoclonal antibodies recognizing TCR/CD3 complex, CD4 and CD8 co-receptors, MHC II molecules, co-stimulatory molecules (CD40L), adhesion molecules (LFA-1 and ICAM-1), or CD20 showed efficacy at preventing diabetes when applied in young (3–6-week-old) pre-diabetic NOD mice [120, 152–155]. In contrast, administration of CTLA-4Ig fusion protein did not afford T1D protection [54]. CTLA-4Ig (abatacept) was tested in T1D patients [156]. The decline of C-peptide level was delayed in treated patients but this did not translate into a decrease in exogenous insulin needs over the 24-month follow-up.

Very few therapies were able to reverse established diabetes, i.e. to cure the disease. Among them, monoclonal antibodies to CD3 epsilon chain (CD3 Ab) have proven efficacy in treating diabetic NOD mice, reaching more than 80 % remission [157]. This remission is long-lasting and associated with restoration of self-tolerance toward pancreatic β-cells as exemplified by the survival of syngeneic islet grafts in CD3 Ab-treated NOD mice [158]. In contrast, these mice were able to reject skin allografts with a kinetic comparable to untreated NOD mice, demonstrating that the treatment did not induce a profound and sustain T-cell depletion. Indeed, the depleting effect of CD3 Abs is partial and transient (25–50 % depending on the use of the non-mitogenic $F(ab')_2$

fragments or the mitogenic whole antibody). Importantly, although CD3 Abs target virtually all T cells, activated effector T cells are the most sensitive to their depleting effect [159, 160]. In contrast, $CD4^+CD25^+Foxp3^+$Tregs are relatively spared by CD3 Ab-induced apoptosis and their proportion increases in the early post-treatment period. This explains, at least in part, why CD3 Ab exert their tolerogenic effects only in the context of an ongoing immune reaction, a finding corroborated in experimental models of transplantation [160, 161] and underlying the importance of the therapeutic window. T cells, not depleted by CD3 Ab therapy, undergo a process of antigenic modulation characterized by the internalization of the TCR/CD3 complexes following ligation of the CD3 antibodies, rendering T cells blind to antigenic stimulation [162]. This situation induced a clearance of insulitis, leaving the pancreatic islet free of inflammation and the 25–30 % remaining β-cells regain functions and insulin-producing capacities. A key element in CD3 Ab-induced tolerance is the role of macrophages and immature DCs engulfing CD3-Ab-induced apoptotic T cells and releasing massive amounts of TGFβ in the microenvironment [146]. TGFβ can modulate the autoreactive T-cell responses and also promote Treg expansion and/or suppressive capacities, either directly or indirectly via APC, notably dendritic cells that acquire tolerogenic properties (decrease of MHC and co-stimulatory molecules, upregulation of PD-L1, ICOSL) allowing an efficient control of pathogenic effectors over the long term [163, 164]. In this anti-inflammatory environment, not only Tregs regain efficient suppressive functions (that are TGFβ-dependent), but also pathogenic T cells regain their sensitivity to regulation afforded by Tregs and TGF-β [165].

All these data encouraged clinical translation of CD3 Ab therapy in T1D patients. Two humanized CD3 Abs, mutated in their Fc portion to inhibit binding to Fc receptors and thus massive T-cell activation and inflammatory cytokine release, have been used: otelixizumab and teplizumab. Using a protocol very similar to that applied in NOD mice (1–2 week CD3 Ab treatment), two phase II trials enrolling recently diagnosed T1D patients showed very promising results [166, 167]. The treatment efficiently preserved β-cell function for several months, maintaining significantly higher levels of C-peptide as compared to placebo-treated patients and thus decreasing exogenous insulin needs. In the otelixizumab trial, insulin requirements were still reduced in the intent-to-treat population as compared to the placebo group at 4 years of follow-up [168]. Although side effects were overall minor, transient reactivation of Epstein–Barr virus (EBV) in some patients raised safety concerns [169]. However, efficient EBV-specific humoral and cellular immune response developed in all CD3 Ab-treated patients demonstrating that CD3 Abs did not induced generalized immunosuppression: T1D patients are able to mount adequate immune responses to exogenous unwanted antigens.

These positive results gave rise to phase III clinical trials using both antibodies. The *Defend-1* study (Tolerx & GSK) using otelixizumab failed in achieving the primary end-points and the study was therefore stopped. The reason evoked for these disappointed results is the dose of antibody used, i.e. 3.1 mg (cumulative dose), arbitrarily chosen with the aim of limiting side-effect and notably EBV reactivation, which corresponds to a 15 time reduction as compared to the 48 mg provided in the phase II trial. The second phase III trial, the *Protégé* study, using different cumulative doses of teplizumab (launched by Macrogenics & Eli Lilly) did not also meet the primary end-points at 1 year (glycated hemoglobin $A_{1c} < 6.5$ % and insulin dose < 0.5 U/kg/day) [170]. However, as these primary efficacy outcomes were distinct from the one used in previous phase II trials, *Protégé's* data were reanalyzed using C-peptide and insulin requirement as end-points as used in other trials. Results showed a significant therapeutic effect that was dose-dependent as only patients who received the highest dose of teplizumab (34 mg) had significantly decreased insulin needs compared with placebo [170]. In addition, the best response was observed in children (8–11 years old) as well as in patients diagnosed for T1D less than 6 weeks.

The availability of transgenic NOD mice expressing the human CD3epsilon chain (NOD-huCD3ε) shall provide critical insights for optimizing safety and efficacy of CD3 antibody therapy. Indeed, a short low-dose treatment of overtly diabetic NOD-huCD3ε mice with otelixizumab induces the same durable remission of spontaneous diabetes we observed in conventional NOD mice treated with anti-murine CD3-specific antibodies [119]. Immune mechanisms involved in this remission were identical to those described in wild-type mice with notably a key role of $CD4^+CD25^+Foxp3^+$Tregs and TGFβ in inducing and sustaining tolerance.

Monoclonal antibodies to CD20 (rituximab) induced diabetes remission in 30 % of cases in transgenic NOD mice expressing the human CD20 molecules in B cells (hCD20/NOD) [120]. The treatment induced a major B-cell depletion and induction of Tregs as well as B cells endowed with regulatory capacities. Based on these results, a clinical trial was launched in T1D [171]. Rituximab therapy showed a significant effect on β-cell preservation during the induced B-cell depletion, as revealed by a higher C-peptide level as compared to the placebo arm. However, the effect was transient. In addition, the reduced B-cell number and IgM levels observed for several months following treatment raised safety concerns on the ability of the patients to mount efficient antibody responses.

To further improve therapeutic efficacy and reduce side effects of treatment, and knowing that both T and B cells are essential for T1D pathogenesis, a combined therapy targeting both compartments, using intravenous anti-CD20 and oral anti-CD3 antibodies, was tested in hCD20/NOD mice [172]. A synergistic effect

was observed as the combined treatment prevented from diabetes development and disease reversal was observed in more than 60 % of new onset diabetic mice. The therapeutic effect was associated with increased proportion and suppressive capacities of $CD4^+Foxp3^+$Tregs in the spleen and pancreatic lymph nodes of treated mice as well as the induction of IL-10-producing $CD4^+$ T cells in the small intestine.

3.3.2 *Interleukin-2*

More recently, another strategy based on the administration of low dose of interleukin-2 has been investigated in experimental T1D models and in the clinic. The rationale of this approach is to boost immunoregulatorypathways,inparticular$CD4^+CD25^+Foxp3^+$Tregs to afford a better control of autoreactive pathogenic T cells. All T cells require IL-2 for their survival and growth but this is even more the case for Tregs whichconstitutively express the high-affinityIL-2 receptor.

Using the NOD mouse model, it has been demonstrated that progression to overt diabetes is associated with a decline in the functional capacity of Tregs, particularly within the pancreas, where both the inflammatory milieu and an insufficient availability of IL-2 affect Treg survival and homeostasis [50, 57]. Interestingly, a continuous treatment (from 5 to 20 weeks of age) with low-dose IL-2 or IL-2/anti-IL-2 complexes significantly protected from diabetes development [57]. This strategy was also efficient at reversing established diabetes as a 5-day treatment-induced T1D remission in 60 % of the mice [173]. The therapeutic effect was associated with an increased proportion of $CD4^+Foxp3^+$Treg in pancreatic islets (not observed in the spleen or pancreatic lymph nodes). Phenotypic and transcriptomic analysis suggested that intra-islet Treg cell function and survival were upregulated after IL-2 treatment [57, 173].

Based on a previous report showing synergistic effect between IL-2 and rapamycin in preventing autoimmune diabetes in NOD mice [174], a phase I clinical trial was launched in T1D patients [175]. Treg proportions increased in the treated patients but returned to pre-treatment values after the 1-month IL-2 administration. In parallel, increased peripheral blood NK cells and eosinophils and decreased serum TGFβ were observed. More importantly, the IL-2/Rapa therapy worsened pancreatic β-cell functions as revealed by a decline in C-peptide levels, showing the limitation of this approach.

Recently, a randomized, double-blind, phase I/II clinical study has been performed in T1D patients to identify a safe and efficient dose of IL-2 capable of expanding Tregs without inducing serious adverse effect [176]. A 5-day course of IL-2 (Aldesleukin) promoted a dose-dependent rise in Tregs proportion over the study period. Glucose metabolism was not altered but mild to moderate side effects and NK cell expansion were more frequently observed

in patients treated with the highest dose of IL-2 (3 MIU/day). A phase II trial in children with recently diagnosed type 1 diabetes is currently ongoing to further define the therapeutic dose and to optimize the benefit/risk ratio of this treatment in T1D.

3.3.3 Vitamin D

Vitamin D3, and more precisely its 1,25-Dihydroxyvitamin D3 (1,25(OH)2D3, calcitriol) activated form, is a potent immunomodulatory agent due its capacity to prevent dendritic cell maturation [177]. In NOD mice, vitamin D deficiency during embryogenesis and the first 3 months of life exacerbated T1D development [178]. In contrast, repeated administrations of 1,25(OH)2D3 or its analog to young NOD mice were very effective at protecting from diabetes development [179, 180]. Vitamin D3 therapy inhibits IL-12 production and pathogenic Th1 responses while increasing the proportion of Treg in pancreatic lymph nodes [180, 181].

In the clinic, a case–control study reported that vitamin D supplementation in early childhood was associated with a decreased risk of developing T1D [182]. However, administration of calcitriol in recent onset diabetic patients did not impact on disease progression as C-peptide levels and insulin requirement were similar between the treated and the placebo groups [183, 184].

3.4 Treg Cell Therapy

Therapeutic approaches were developed in the last few years, using Tregs as a cell therapy product for the treatment of autoimmune diabetes. The group of JA Bluestone reported that sorted $CD4^+CD25^+CD62L^+$ Tregs from NOD mice expanded very efficiently in response to anti-CD3 and anti-CD28 coated beads in presence of recombinant IL-2 [185, 186]. These cells maintained a Treg phenotype as revealed by high levels of CD25, CD62L, PD-1, CTLA-4 and Foxp3 and they inhibited proliferation and cytokine production (IFNγ, IL-2) by effector T cells. However, in vivo, in an adoptive transfer model into lymphopenic hosts, these polyclonal expanded Tregs were not able to significantly prevent diabetes development induced by spleen and LN cells from diabetic NOD mice. In contrast, administration of low numbers of antigen-specific BDC2.5 Tregs, previously expanded in vitro using the same methodology as polyclonal Tregs, effectively inhibited diabetes induced by polyclonal or transgenic BDC2.5 effector T cells [185]. Furthermore, infusion of BDC2.5 expanded Tregs reversed established diabetes in 60 % of new onset diabetic NOD mice. This later result suggests that Treg therapy may hold promise in reversing T1D and restoring self-tolerance.

In the clinic, the feasibility of Treg cell therapy was supported by the establishment of a protocol allowing robust expansion of human Tregs from T1D patients stimulated with anti-CD3/anti-CD28 beads in the presence of IL-2 and rapamycin [187]. These Tregs were isolated on the basis of their $CD4^+CD127^{low/-}$ or

$CD4^+CD127^{low/-}CD25^+$ phenotype. Both populations showed similar expansion fold. Expanded Tregs retain Foxp3 expression although a decline was noticed and exhibited comparable in vitro suppressive capacities. A phase I trial is currently ongoing in T1D to assess the safety of infusing escalating numbers of polyclonal $CD4^+CD127^{low/-}CD25^+$ Tregs in new onset diabetic patients.

3.5 Autologous Bone Marrow Transplantation

Autologous hematopoietic stem cell transplantation (AHSC) is a radical approach allowing a de novo generation of the immune system. The therapeutic effect of such replacement therapy is attributed, at least in part, to granulocyte colony-stimulating factor (G-CSF)-based protocols used for stem cell mobilization which may induce hematopoietic progenitors capable of inhibiting T-cell proliferation and favoring Treg expansion [188, 189]. Thus, in several severe cases of autoimmunity, transplantation of mobilized stem cells did not lead to recurrence of disease as we could have expected but rather a cure of disease. A phase I/II trial was launched in newly diagnosed diabetic patients. Peripheral AHSC were mobilized with cyclophosphamide and G-CSF, harvested by leukapheresis, cryopreserved and reinfused in T1D patients who had received a conditioning regimen (cyclophosphamide and rabbit antithymocyte globulin) [190, 191]. Interesting results were obtained as most of the patients showed increased C-peptide levels and became insulin-independent for 1–35 months. However, in all patients the disease eventually relapsed. In addition, all patients experienced transplantation-related complications of high-dose immunosuppression and two patients developed bilateral nosocomial pneumonia. In addition, three patients presented late endocrine dysfunction, and male patients showed impaired testicular functions characterized by low sperm motility and morphological alterations [192]. Therefore, due to the complexity of the procedure and the potential life-threatening complications, the beneficial effect of AHSC transplantation for T1D patients remains controversial.

4 Conclusions

The availability of spontaneous or genetically modified models of T1D allowed a very productive research on the genetic and pathophysiological mechanisms responsible for breakdown of self-tolerance toward pancreatic β-cells, revealing the complexity of the disease. These models were instrumental for monitoring the therapeutic effect of therapies targeting the various components of the autoimmune responses, providing the basis for active clinical translation. Halting disease progression or reversing established T1D requires reprogramming of the immune system in an antigen-specific manner. Combination therapies using immunomodulating

drugs, antigen-specific approaches, and/or anti-inflammatory protocols may be the solution for inducing robust tolerance that is sustained in the long term.

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Chapter 9

Recent Advances in the Treatment of Immune-Mediated Inflammatory Diseases

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Abstract

The treatment of immune-mediated inflammatory diseases (IMIDs) has dramatically improved over the last two decades by the development of a series of targeted biological therapies. This paper focuses on new developments in the treatment of IMIDs. In particular, we discuss how different ways of targeting the same mediators can lead to different efficacy and safety profiles, using B cell targeting as example. In addition, we discuss the emerging field of 'small molecules' that target specifically intracellular processes related to cytokine signaling, cell activation, cell migration, and other processes relevant to tissue inflammation.

Key words Immune-mediated inflammatory diseases, Treatment, B cells, Signal transduction

1 Introduction

Immune-mediated inflammatory diseases (IMIDs) encompasses disorders where tissue and organ inflammation is primarily driven by aberrant immune responses. In contrast to the 'secondary' involvement of the immune system in infectious diseases and oncology, the trigger of IMIDs is the immune system itself. Importantly, recent advances in our understanding of immunity and inflammation revealed that IMIDs can be driven not only by autoimmunity, defined here as abnormal responses of T and/or B lymphocytes against self-antigens, but also by auto-inflammation, this is self-directed tissue inflammation driven by aberrant or uncontrolled innate immune response triggered by local factors at tissues sites predisposed to disease. The former group encompasses diseases such rheumatoid arthritis (RA), type I diabetes, and systemic lupus erythematosis, whereas gout and sarcoidosis are examples of autoinflammatory diseases.

The treatment of IMIDs has dramatically improved over the last two decades by the development of a series of targeted biological therapies. Indeed, combined fundamental and translational immunology research has revealed that specific inflammatory

Maria Cristina Cuturi and Ignacio Anegon (eds.), *Suppression and Regulation of Immune Responses: Methods and Protocols, Volume II*, Methods in Molecular Biology, vol. 1371, DOI 10.1007/978-1-4939-3139-2_9,

mediators (in particular cytokines) and cells were 'master switches' in specific IMIDs and that targeting these cellular and molecular players with antibodies or soluble receptors potently down-modulated chronic inflammation. The first and major success story is TNF blockade, which is very effective to treat a variety of IMIDs including RA, spondyloarthritis (SpA), psoriasis, and inflammatory bowel disease. Other major anti-cytokine therapies are directed towards IL-1 and IL-6 and, more recently, the IL-23/IL-17 pathway. Besides targeting cytokines, a second very successful approach was to target pathogenic cell subsets, with as prime example B cell depletion with the anti-CD20 antibody rituximab. Originally developed to treat lymphomas, this compound turned out to be also very effective in the treatment of RA and other autoimmune diseases. Thirdly and finally, pathogenic cell can not only be depleted but one can inhibit their interaction with other pathogenic cells (such as in the case of co-stimulation blockade by CTLA-4-Ig or abatacept) or with molecules directing their migration into target tissues (such as the anti-alpha4 integrin antibody natalizumab).

Existing and emerging therapies targeting cytokines, cells, and cellular interactions have been extensively described in the literature and are not reviewed in detail here. This chapter rather focuses on two specific new developments in the treatment of IMIDs. Firstly, we discuss how different ways of targeting the same mediators can lead to different efficacy and safety profiles, using B cell targeting as example. We discuss novel drugs beyond rituximab that target other B cell surface molecules, other B cell subsets, and B cell growth factors. Secondly, we discuss the emerging field of "small molecules" that target specifically intracellular processes related to cytokine signaling, cell activation, cell migration, and other processes relevant to tissue inflammation.

2 Targeting B Cells

2.1 Targeting B Cells with Anti-CD20 Monoclonal Antibodies

B cells contribute to chronic inflammatory disease by secreting cytokines, providing co-stimulatory signals to T cells, presenting antigen in the context of antibody production, and producing auto-antibodies. Therefore, selective depletion of these cells alters the immune response and reduces inflammation. Antibody-mediated depletion of B cells can be achieved via different mechanisms of which antibody-dependent cellular cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) are most widely used. The validity of this approach had been demonstrated by the use of the anti-CD20 antibody rituximab in RA [1] as well as ANCA-associated vasculitis [2], modest effects in SLE [3, 4], systemic sclerosis (SSc) [5], Sjogren's syndrome (SS) [6], and multiple sclerosis (MS) [7]. Rituximab is currently tested in pemphigus, AIHA, and ITP [8].

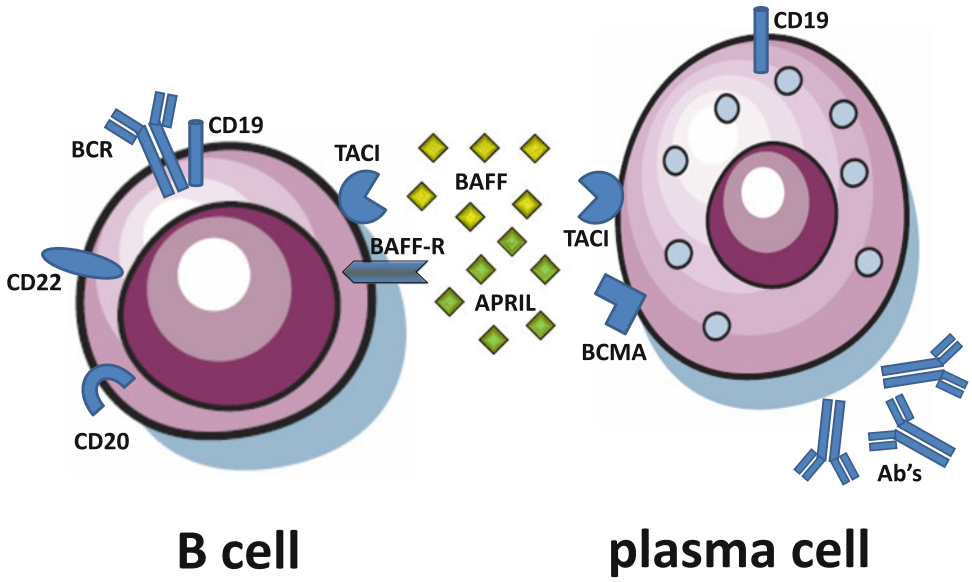

Fig. 1 Surface molecules of B cells and plasma cells of soluble factors targeted in IMIDs. Schematic overview of B cell and plasma cell surface receptors or other molecules. The survival factors B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) bind to their respective receptors on B cells (BAFF-receptor, BAFF-R) (=TNFRSF13C), and transmembrane activator and calcium-modulating ligand interactor, TACI (=TNFRSF13B) and plasma cells (B cell maturation antigen, BCMA (TNFRSF17), and TACI. Targeting APRIL and BAFF affects both B cells and plasma cells. B cells can be targeted specifically via the B cell restricted antigens CD20 and CD22. Targeting CD19 affects both B cells, plasmablasts, and a subset of plasma cells. *Ab's* antibodies, *BCR* B cell receptor

Based on the efficacy and relatively good safety profile of rituximab (a rare but very severe complication of rituximab treatment is progressive multifocal leukoencephalopathy, a devastating demyelinating disease caused by reactivation of the JC virus [9]), other antibodies targeting CD20 are currently in development with the aim to improve the efficacy and safety profile (Fig. 1).

Ofatumumab is a fully humanized IgG1 mAb which binds a CD20 epitope distinct from the binding site of rituximab. Ofatumumab has enhanced CDC activity compared to the other anti-CD20 mAbs [10]. In RA data from initial phase 1/2 and 3 studies point towards favorable effects on disease activity [11, 12]. In a phase 2 dose-finding study in MS ofatumumab treatment resulted in a substantial reduction in new and total lesions [13].

Ocrelizumab is a humanized anti-CD20 mAb that binds a different but overlapping epitope from rituximab. It has similar CDC, but 2–5-fold increased ADCC [14]. Overall, it appears that fewer anti-drug antibody responses are elicited during ocrelizumab treatment. In RA patients ocrelizumab treatment resulted in reduced disease activity and a reduction in joint damage, however this was accompanied by an increased risk of infections which led to termination of development for RA [15, 16]. In SLE nephritis, overall renal response rates with ocrelizumab were numerically but not

statistically significantly superior to those with placebo, while ocrelizumab treatment was associated with a higher rate of serious infections in the subgroup receiving background MMF [17]. Nevertheless, in MS after initial favorable results [18], additional clinical trials in different forms of this disease are still ongoing.

Veltuzumab is a humanized IgG1 anti-CD20 mAb with both structural and functional differences from rituximab. It has shown promising clinical activity in relapsing ITP [19] and is also being evaluated for RA [20], but no results have been disclosed yet.

2.2 Targeting Other B Cell Surface Molecules

CD19 is a B cell-restricted antigen that regulates the threshold for B cell activation and, in contrast to CD20, is maintained on plasmablasts and subsets of plasma cells (Fig. 1) [21]. Therefore, targeting CD19 is expected to have a more profound effect than anti-CD20 therapy [22]. MEDI-551 is a humanized IgG1 afucosylated mAb targeting CD19 with enhanced ADCC effector function [23]. It is currently under evaluation in clinical trials for systemic sclerosis (SSc) (Clinical Trials.gov: NCT00946699) and MS [24].

CD22 is considered to be a B cell antigen (expressed on the majority of IgM^+IgD^+ B cells, but less so on germinal center B cells and plasma cells), which can also be detected on basophils and dendritic cells (Fig. 1) [25]. However, CD22 has been demonstrated to play an important role in the control of B cell activation, B cell survival, and cell-cycle progression following activation [26]. Epratuzumab is a humanized IgG1 mAb directed against CD22 with modest ADCC, but no CDC activity (most likely due to rapid internalization of CD22 after Ab binding) [27]. In an open-label phase 1/2 study in Sjogren's syndrome (SS) epratuzumab treatment was well-tolerated and resulted in a moderate clinical responses [28]. A phase 2 study in SLE patients also demonstrated favorable clinical effects [29, 30]. Phase 3 trials in SLE are currently ongoing.

2.3 Targeting B Cell Survival Factors

Besides targeting B cell themselves, a novel strategy consists of targeting B cell growth and survivial factors. Indeed, B cell function and survival depends on various factors of which the TNF family members B-cell activating factor (BAFF or BlyS) and a proliferation induced ligand (APRIL) are probably most important in the context of autoimmune diseases. Interestingly, BAFF and APRIL also support plasma cell survival (Fig. 1) [31].

Belimumab is a fully human IgG1 mAb that selectively inhibits BAFF, which results in B cell apoptosis [32]. It is effective in SLE in patients with active, autoantibody positive disease [33] and was approved by the EMA and FDA for this indication in 2011. Belimumab was not very successful in RA [34], however its efficacy is currently under investigation for ITP, Waldenstrom's macroglobulinemia, idiopathic membranous glomerulonephropathy, Sjogrens syndrome (SS), prevention of kidney transplant rejection, and myasthenia gravis (reviewed in [35]).

Tabalumab is a humanized IgG4 antibody that binds and neutralizes both soluble and membrane-bound BAFF [36]. A phase 2 dose-ranging study of subcutaneous tabalumab for the treatment of active RA patients with an inadequate response to methotrexate was successful [37]. Clinical trials in SLE [38] and MS [39] are ongoing, but results have not been published yet.

Atacicept is a fusion protein soluble receptor construct of Transmembrane Activator and Calcium-modulating ligand Interactor (TACI) and the Fc part of human IgG1 (TACI-Ig) [40]. TACI is a receptor that is normally expressed both on B cells and on plasma cells and binds both BAFF and APRIL [41]. It has been tested in SLE [42] and RA [43, 44], but in general was not successful. In MS atacicept even worsened disease activity [45]. One explanation for this may be that atacicept also targets survival factors for regulatory B cells without full depletion of pathogenic B cells [46]. This example as well as the other emerging biological drugs discussed above in the context of B cell targeting illustrate well that different ways of approaching a therapeutic target can result in strongly different efficacy and safety profiles.

3 Targeting Intracellular Signaling Pathways

Besides novel approaches to target extracellular molecules (including cytokines, growth factors, surface markers, co-stimulatory molecules, and adhesion molecules), intense efforts have been made in the last years in identifying intracellular targets, since all inflammatory responses are initiated by activation of intracellular signal transduction pathways. Examples of key molecules in these intracellular pathways are mitogen-activated protein kinases (MAPKs), nuclear factor-kappaB (NF-κB) activating kinases, Janus kinase (JAK), spleen tyrosine kinase (Syk), and phosphoinositide 3′kinase. Here we discuss the advances in targeting MAPKs, NF-κB, and JAKs as examples.

3.1 Targeting MAPK

The family of mitogen-activated protein kinases (MAPKs) play a central role in the regulation of various biological processes that are involved in immune responses, such as proliferation, differentiation, pro-inflammatory gene expression, and survival. MAPKs are activated in response to environmental stress factors, such as TLR ligands, cytokines, growth factors, and radiation. Subsequently, MAPKs induce signaling by phosphorylating specific target proteins. MAPKs consist of three main groups that all have specific roles in the regulation of cell function: p38 MAPKs, extracellular signal-regulated protein kinases (ERKs), and c-jun NH_2 terminal kinases (JNKs). Recently, several additional atypical MAPKs such as ERK5, ERK3/4, ERK7/8, and Nemo-like kinase have been described [47], but these are less well studied and are not discussed here.

3.1.1 p38 Inhibitors

p38 has four isoforms (α, β, γ, and δ), of which p38α and p38β are ubiquitously expressed. Activation and phosphorylation of p38 is regulated by the upstream MAPK kinases (MKK)3 and MKK6 that are phosphorylated by multiple MKK kinases (MAP3Ks). Particularly p38α is a signaling molecule that regulates pro-inflammatory cytokine production (such as TNFα, IL-1β, and IL-6), which makes it an attractive target for many IMIDs including RA. Consequently, intense efforts have been made to develop small molecule p38 inhibitors. However, despite being effective in preclinical models of arthritis, to date clinical trials in RA have all failed due to poor efficacy or toxicity, including hepatotoxicity (reviewed in [48]). Yet, in inflammatory bowel disease (IBD) initial clinical trials with the p38 inhibitor Semapimod (CNI-1493) appeared promising [49] and follow-up studies have established a mild beneficial effect in a limited number of patients [50]. A potential explanation for these rather disappointing results may lie in the fact that p38 also has anti-inflammatory effects or that blocking one kinase may lead to compensatory effects in other kinases that regulate the same genes. Therefore, an alternative more effective strategy may be to block upstream kinases such as MKK3/6 [48].

3.1.2 ERK Inhibitors

The ERK family consists of two conventional MAPK, namely ERK 1 and ERK2, that are activated by the MAPKKs MEK1 and MEK2 in response to growth factors, including platelet-derived growth factor (PDGF) and epidermal growth factor (EGF). ERK1 and ERK2 are important for cell proliferation and differentiation [47]. FR180204, an ERK inhibitor, has been shown to be effective against mouse collagen-induced arthritis, a representative animal model of RA. The MEK1/2 inhibitors PD98059 and U0126 are not competitive with respect to ATP, but appear to physically interact with MEK1/2 thereby preventing phosphorylation and/or conformational transition that generates the activated enzyme. More recently, additional noncompetitive inhibitors of MEK1/2 with greater bioavailability (PD184352 and PD0325901) have been developed and entered clinical trials as potential anticancer agents (reviewed in [47]). However, no clinical trials in IMIDs have been performed so far.

3.1.3 JNK Inhibitors

The three JNK isoforms (JNK1, JNK2, and JNK3) are involved in many processes that contribute to chronic inflammation such as matrix metalloproteinase (MMP) and cytokine production, cell migration, and angiogenesis [51, 52]. JNK1 and JNK2 are widely expressed, and therefore most attention of pharmaceutical companies has gone out to target these isoforms [51]. SP600125, a direct inhibitor of JNK activity, decreased paw swelling in rat adjuvant-induced arthritis, which was accompanied by a near-complete inhibition of radiographic damage [53]. However, this inhibitor lacked specificity and was replaced by more selective inhibitors.

At present, several companies have JNK inhibitors that are in different stages of development, but no data of clinical trials in IMIDs have been reported.

3.2 NF-κB Inhibitors

The Nuclear Factor-kappaB (NF-κB) family of transcription factors is crucially involved in the regulation of immune responses in IMIDs (reviewed in [54]). NF-κB can be activated via two distinct pathways: the canonical pathway and the alternative or noncanonical pathway. The canonical pathway is most extensively studied and can be activated by stimulation of a variety of cell membrane receptors including tumor necrosis factor receptor (TNF-R), IL-1 receptor, and Toll-like receptors, in response to their respective pro-inflammatory ligands, as well as via triggering of classic immunoreceptors like the T-cell receptor (TCR) or the B-cell receptor (BCR). In this pathway, inhibitor of κB (IκB) kinase (IKK)β is required for NF-κB activation, whereas IKKα is redundant (reviewed in [55]). The canonical NF-κB pathway is essential both in acute inflammatory responses and in chronic inflammatory diseases such as RA and inflammatory bowel disease [56]. In RA IKKβ is a key regulator of synovial inflammation and the importance of the canonical NF-κB pathway in arthritis is underlined by the beneficial effects of specific IKKβ inhibition in preclinical models of arthritis [57, 58]. Fuelled by these results and beneficial effects of NF-κB inhibition in preclinical models of other inflammatory diseases, more than 700 compounds with inhibitory effects on NF-κB signaling have been reported [59]. However, clinical trials are hitherto lacking, presumably by fear of toxicity associated with global NF-κB inhibition or off-target effects. This could potentially be solved by selective targeting of the NF-κB inhibitor to a specific cell type, for instance using a multimodular recombinant protein that specifically binds to cytokine-activated endothelium, which has been demonstrated to work very elegantly under inflammatory conditions in vivo [60].

The noncanonical NF-κB pathway can be triggered by the activation of members of the TNF-receptor superfamily including the lymphotoxin β receptor (LTβ-R), CD40, B cell activating factor belonging to the TNF family (BAFF) receptor, and receptor activator of NF-κB (RANK). Of note, these receptors not only trigger the noncanonical NF-κB pathway, but simultaneously also the canonical pathway. The noncanonical NF-κB pathway is strictly dependent on NF-κB inducing kinase (NIK) and IKKα homodimers, but does not involve IKKβ or IKKγ. Overall, this pathway is involved in lymphoid organ development and adaptive immune responses [61]. Recently, we established that noncanonical NF-κB signaling in endothelial cells stimulates pathological angiogenesis in chronic inflammation [62]. Consequently, NIK inhibition using specific small molecule inhibitors could perhaps be an effective new treatment option for chronic inflammatory diseases [63].

3.3 Janus Kinase (JAK) Inhibitors

The Janus kinase (JAK) family consists of four members: JAK1, JAK2, JAK3, and tyrosine kinase 2 (TYK2). JAKs associate with different cytokine receptors and via phosphorylation of tyrosine residues create docking sites for one or more signal transducer and activators of transcription (STAT) molecules. JAK1, JAK2, and TYK2 are ubiquitously expressed, whereas JAK3 is primarily expressed in hematopoietic cells. JAK1 and JAK3 convey signals from cytokine receptors that contain the IL-2 receptor common γ chain and mediate signaling by IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, cytokines that are essential for the development and maturation of T cells. JAK2 is associated with hematopoietic growth factor receptors and cytokine receptors for IL-1, IL-6, and IL-17 that are critically involved in various aspects of immune cell function (reviewed in [64]). Consequently, inhibiting JAKs blocks multiple aspects of cytokine signaling, which makes them attractive targets for many IMIDs. Of all protein kinase inhibitors, JAK inhibitors have entered the clinic first. Tofacitinib (also known as CP-690550) is a potent JAK1 and JAK3 blocker, that also inhibits JAK2 to a certain extent. It was effective in preclinical models of arthritis and transplantation [65, 66]. Tofacitinib successively entered clinical trials, which demonstrated efficacy in RA [67, 68], IBD [69], and psoriasis [70]. In 2012 tofacitinib was approved for the treatment of RA in the USA, Japan and Russia. However, the European Medicines Agency (EMA) did not approve tofacitinib for RA, mainly due to concerns about the risk of serious infections. Nevertheless, EULAR included tofacitinib in their recommendations for the treatment of RA as a therapeutic option after biological treatment has failed [71].

4 Conclusion

The treatment of IMIDs continues to improve as we develop a better understanding of the pathogenesis of these diseases and the pathways that are suitable for targeting. Importantly, however, the clinical exploration of novel targeted therapies also contributes directly to our understanding of the function and role of specific pathways in vivo. This interaction between fundamental immunobiology and translational research has been key to many novel developments in the field of IMIDs.

These developments are not only related to an ongoing expansion of 'classical' target pathways (cytokines, growth factors, surface molecules, co-stimulation, adhesion) but also to fine-tuning of the way to approach these targets, as discussed for B cells. The key message here is that a single pathogenic pathway may operate in completely different ways depending on the exact immunological and tissue context. As we discussed recently for another key inflammatory pathways, the IL-23/IL-17 axis, studying the context of

inflammation is as important as understanding the pathway to determine how, when and where this pathway should be optimally targeted [72, 73].

This may be further improved by new developments in recombinant antibody technology allows for the generation of bispecific antibodies that have the ability to bind to two different epitopes on the same or different antigens. This may have significant advantages over targeting one epitope, especially in complex multifaceted diseases [74], since with a single therapeutic entity two targets can be blocked or engaged. This approach has been rapidly adopted by the oncology and hematology field, and attempts are also made in the field of clinical immunology and rheumatology. An example of this is a bispecific hexavalent antibody comprising epratuzumab and veltuzumab (anti-CD22/CD20), which may lead to improved treatment of SLE and other IMIDs, but has not been formally tested yet [75].

Finally, new horizons are opening with completely novel targets such as the intracellular signaling pathways. This review discussed a few examples in order to highlight the enormous progresses and promises ahead of us, but is obviously far from complete. For example, there is crucial emerging knowledge in the role of epigenetic modifications in the initiation and maintenance of tissue inflammation and, accordingly, small molecules modifying for example DNA methylation and histone modifications are in (pre)clinical development [76]. To date, one clinical trial with a histone deacetylase (HDAC) inhibitor has been performed in systemic juvenile inflammatory arthritis. Oral administration of the nonselective HDACi givinostat (ITF2357) resulted in significant therapeutic benefit after 12 weeks, particularly with respect to arthritis activity, with a relatively good safety profile [77]. These and other new developments will continue to revolutionize the treatment of IMIDs and contribute to the ongoing evolution from nonspecific immune suppression to targeted immunomodulation and, ultimately, genuine disease modification and cure.

Acknowledgements

SWT is supported by a VENI grant and a Clinical Fellowship from the Netherlands Organization for Scientific Research (NWO/ZonMw), and grants from the Dutch Arthritis Foundation. DLB is supported by a VICI grant from the Netherlands Organization for Scientific Research (NWO), and grants from the Dutch Arthritis Foundation.

Disclosure of conflicts of interest: The authors declare no competing financial interests.

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Chapter 10

Application of Humanized Mice in Immunological Research

Wenwei Tu and Jian Zheng

Abstract

During the past decade, the development of humanized mouse models and their general applications in biomedical research greatly accelerated the translation of outcomes obtained from basic research into potential diagnostic and therapeutic strategies in clinic. In this chapter, we firstly present an overview on the history and current progress of diverse humanized mouse models and then focus on those equipped with reconstituted human immune system. The update advancement in the establishment of humanized immune system mice and their applications in the studies of the development of human immune system and the pathogenesis of multiple human immune-related diseases are intensively reviewed here, while the shortcoming and perspective of these potent tools are discussed as well. As a valuable bridge across the gap between bench work and clinical trial, progressive humanized mouse models will undoubtedly continue to play an indispensable role in the wide area of biomedical research.

Key words Humanized mice, Immunology, Immune regulation

1 Introduction

During the past century, the application of rodent animal models, especially diverse gene-engineered mouse models, provided indispensable platforms and numerous valuable information for the advances in experimental medicine and biological research. However, the gap between species is still the most challenging obstacle for translation of results from rodents to humans. With the great advancement of technology in molecular biology and gene modification, the attempt to establish "humanized" mouse models has made a leap since 1990s [1–3]. Nowadays, a wide variety of humanized mouse models have been generated and applied in nearly all fields of biomedical research [4]. In this chapter, we briefly review the history and classification of humanized mouse models and then summarize the current situation and recent advancement of their application in biomedical research, especially in the research of immune-related diseases.

Maria Cristina Cuturi and Ignacio Anegon (eds.), *Suppression and Regulation of Immune Responses: Methods and Protocols, Volume II*, Methods in Molecular Biology, vol. 1371, DOI 10.1007/978-1-4939-3139-2_10,

1.1 History and Classification of Humanized Mice

In general, the "humanized mice" are composed of three main classes: human gene-expressed transgenic mice (*human gene-transgenic mice*), which are modified by gene knock-in or replacement technology to express one or more human specific genes; humanized mice carrying human tissue, such as the liver (*humanized liver mice*) in which murine hepatocytes are completely or partly replaced by infused human-original hepatocytes; humanized mice equipped with functional human immune system (*humanized immune system mice*), which are established on immunodeficient mice by transplanting human immune organs or cells to reconstitute human immune system in mice and thus referred to special "humanized mice." In the following section, we briefly review the history and current advance of human gene-transgenic mice and humanized liver mice, and then focus on humanized immune system mice.

1.1.1 Human Gene-Transgenic Mice

Human gene-transgenic mice are closer to gene engineered mice rather than "humanized mice." Although the expressions of human gene or protein in transgenic mice provides the platform for studying in vivo role of specific human gene or molecule, the value of these data is limited in translational medicine due to the lack of human microenvironment and signal networks in these mice.

The most widely used human gene-transgenic mice are human leukocyte antigen (HLA)-transgenic mice [5]. These HLA-expressed transgenic mice represent for a useful tool in studying in vivo TCR-restricted immune responses and thus were adopted in the studies of immune-related diseases during the first 10 years of this century. For example, HLA-A0201-transgenic mice were used in inducing $CD8^+$ T cell-restricted type I diabetes (T1D) [6] and experimental autoimmune encephalitis (EAE) [7], while HLA-DRB1-transgenic mice were applied in establishing $CD4^+$ T cell-mediated EAE [8], system lupus erythematosus (SLE) [9] and rheumatoid arthritis (RA) models [10]. More recently, the respective role of HLA-DR2 and HLA-DQ8 in EAE [11] and autoimmune diabetes [12] was also studied through transgenic mice. Meanwhile, transgenic mice with distinct HLA subtype expression favor the study of HLA-related susceptibility on specific diseases, such as EAE [13], experimental autoimmune uveitis [14], arthritis [15–17], allergic bronchopulmonary aspergillosis-like pulmonary responses [18], and celiac disease [19]. Although the application of HLA-transgenic mice has been reduced due to the simplification of diseases into specialized immune responses, the combination of HLA-transgenic technology and reconstitution of human immune system in immunodeficient mice has re-assigned them vitality in biomedical research, which will be discussed in the next section.

Other transgenic mice used in immune-related studies included humanized α1KI mice [20], humanized θ-defensins mice [21], humanized toll-like receptor (TLR) 4/MD2 mice [22],

humanized type I interferon (IFN) mice [23], and humanized tumor necrosis factor (TNF) mice [24]. Similar to HLA-transgenic mice, their combination with humanized-immune system in mice will certainly strengthen their translational capability in the future.

1.1.2 Humanized Liver Mice

Humanized liver mice were established as early as 2001 and mainly applied in the study of drug metabolism, excretion, toxicity [25, 26], and the in vivo activity of enzymes such as human cytochrome P450 [27]. Moreover, the establishment of chimeric mice with humanized liver accelerated the progress of the studies in hepatitis virus B [28, 29], C [30, 31], D [32], and human cytomegalovirus infection [33], which had all been blocked by the lack of optimal animal models in "pre-humanized mice time." On the other side, Chen et al. tried to stabilize the function of cryopreserved human hepatocytes in immune competent mice through a novel system called "human ectopic artificial livers (HEALs)," which involved juxtacrine and paracrine signal in polymeric scaffolds. They claimed that mice transplanted with HEALs exhibited persistent normal liver function for weeks and thus provided a window for drug-related investigation [34]. However, the efficacy and value of humanized liver mice in the development of drug are still on debate due to the proposed side effects such as ongoing liver injury caused by transgenic and the influences on "normal metabolism" mediated by exogenous treatment [35, 36]. Apart from these, the potential application of humanized liver mice in immune-related research also deserves further exploration because liver also represents for a critical component of human immune system.

1.1.3 Humanized Immune System Mice

The development of humanized immune system mice could be divided into three phases corresponding to the establishment of *Prkdc*^{scid} (protein kinase, DNA activated, catalytic polypeptide; severe combined immunodeficiency) mutation in CB17 mice, the development of NOD (non-obese diabetic)-SCID mice, and the generation of immunodeficient mice homozygous for mutation at IL (interleukin)-2 receptor γ chain locus [2, 37]. Each breakthrough mentioned previously significantly improved the engraftment of human immune cells or pluripotent stem cells and stood as milestone on the way to "real humanized mice." The engraftment of multiple human immune components in these mice surpassed conventional human-gene knock-in in breaking the limited viewpoint of studying specific molecules under isolated environment. This unique advantage of humanized immune system mice favors their general application in immune-related studies, and opens a window for researchers to observe the interaction among human immune cells in vivo. In the following content, we focus on the characteristics and application of these mouse models and simply refer them as "humanized mice" if not otherwise specified.

Currently, IL2γc$^{-/-}$ mice established on NOD/scid and recombination activating gene 2 (Rag2)$^{-/-}$ Balb/C background were most widely used strains for the reconstitution of human immune system in vivo [38–40]. Recently, by using bone marrow, liver, thymus (BLT) co-transplantation, Lavender et al. engrafted high levels of multi-lineage hematopoiesis and organized lymphoid tissues in C57BL/6-Rag2$^{-/-}$γc$^{-/-}$CD47$^{-/-}$ triple-knockout mice. These humanized mice sustained human cell and tissue engraftment as long as 29 weeks post-transplantation without the development of chronic graft-versus-host diseases (GVHD), and thus represented for a new advancement in establishment of humanized mice [41].

1.2 Reconstitution of Immune System in Humanized Mice

The reconstitution of functional immune system is the key to evaluate the successful establishment of humanized mice. The graft used for reconstituting human immune system includes stem cells [42], BLT [41], and peripheral blood cells [43] according to specific objectives. Generally, stem cell and BLT transplantation exhibit advantage in establishing stable multi-lineage hematopoietic cells but might need additional treatment for improving development of specific cell subpopulations. On the contrary, humanized mice established by peripheral blood cells provide a ready platform for studying the functions of mature immune cells but the length of window appropriate for research is still limited by chronic GVHD and ongoing reduced engraftment. To maximize the potential of humanized mouse model, some progresses have been made recently. Firstly, pretreatment or gene-engineering of pluripotent stem cell exhibited satisfactory effects on improving engraftment of immune cells [38, 42, 44]. Secondly, human growth factors, cytokines [44, 45] or signal regulatory protein alpha (SIRPa)-expressed [46] immunodeficient mice demonstrated superior engraftment for specific immune cell subpopulations as well. In the following paragraphs, we briefly review current status of the reconstitution of specific immune cell subpopulations in humanized mice.

Lymphocytes are most important components of immune system and thus draw a major attention. Although human peripheral blood mononuclear cells (PBMC) transplantation led to rapid reconstitution of human lymphocytes in humanized mice, it was found that after initial activation and induction of antibody production, human T cell lymphocytes enter an unresponsiveness status due to loss of human professional antigen-presenting cells (APC), which could be reversed by adoptive transfer of human APC [47] or activating organ-resident myeloid dendritic cells (DC) through poly(I:C) treating [48]. Meanwhile, stem cell-transplanted humanized mice displayed diversified T cell repertoire, but the gap between HLA and murine major histocompatibility complex (MHC) molecules prohibited the induction of efficient T cell-mediated primary immune responses in vivo [49, 50].

To overcome these problems, HLA-expressed immunodeficient mice were generated and their efficacy has been confirmed [51]. Another concern origins from Th1 and Th17 immunocompetence in humanized mice [52], which supports the utility of their application as surrogate model in transplantation rejection and autoimmunity but might cause some unwanted immune responses against murine tissue antigen as well.

Distinct from their T cell companion, reconstitution of functional B lymphocytes is generally poor in humanized mice and needed to improve in the future although their primary repertoire were principally unaltered by the differences between mouse and human stromal environments [53] and their ability to produce antigen-specific antibody was partly developed [54].

As described previously, the reconstitution of myeloid cells not only guarantees immune system intact, but also determines the development and function of both adaptive and innate lymphocytes [47, 48, 55]. Unfortunately, monocytes and other myeloid cells usually exhibit immature phenotype and impaired function in humanized mice [56], which could be partly rescued by human colony stimulating factor (CSF)-1 [57]. However, the improvements in their survival, differentiation and even migration and residence [58] are still urgently required. Besides leukocytes, other blood components also play important roles during immune response and regulation. Recently, Hu et al. established the full reconstitution of human platelets in humanized mice after depletion of murine macrophage [59], which represents for an interesting attempt in constructing a more "humanized" circulation in mice.

In summary, the optimization of humanized mouse model is still on the way and the advances in molecular biology, cellular biology, and system biology will definitely bring new era to the development of this useful tool.

2 Applications of Humanized Mice

The applications of humanized mice cover nearly all fields of biomedical research and here we concentrate on immune-related studies, especially those aiming at the mechanisms and translational potentials of immune regulation and suppression. We also briefly summarize the benefits brought by these potent models in tumor, infectious diseases, and vaccine studies.

2.1 Development of Immune System

2.1.1 Lymphocytes

2.1.1.1 T Cells

Benefiting from humanized mouse model established by BLT or $CD34^+$ stem cell transplantation, research on the development of human T cells made a great progress in the past 5 years. In 2011, Choi et al. induced human $CD4^+CD8^+$ double-positive (DP) T cells, $CD4^+$ and $CD8^+$ single-positive (SP) T cells, $CD34^+CD38^{lo}CD1a^-$ (thymus setting-progenitors, TSP),

$CD34^+CD38^{lo}CD1a^-$ (early T lineage progenitors, ETP), and $CD34^+CD38^+CD1a^+$ pre-T cells in liver of humanized mice by intrahepatic injection of $CD34^+$ stem cells, establishing a wonderful platform for investigating human T cell development [60]. However, Joo et al. found that human T cells educated by murine MHC in mice without a human thymus differ from normal human T cells marked as higher expression of CD45RO and promyelocytic leukemia zinc figure protein (PLZF) regardless of similar development stages [61]. Correspondingly, Danner et al. generated HLA-DR4-expressed NOD-$Rag1^{-/-}\gamma c^{-/-}$ mice and demonstrated the critical role of HLA class II molecule for development of functional T cells by infusion with HLA-DR-matched human hematopoietic stem cells [62]. Meanwhile, the roles of IL-12 [63] and Notch [64] signals during the development of human $CD4^+$ and $CD8^+$ T cells were evaluated by human hematopoietic stem cell-transplanted mice. Moreover, using a human stem cell factor, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3-expressed NOD/scid-$\gamma c^{-/-}$ mice, Billerbeck et al. found the increased accumulation of human $CD4^+Foxp3^+$ T cells in blood, spleen, bone marrow and liver. Most importantly, these $CD4^+Foxp3^+$ T cells exhibited potent suppressive capability on T cell proliferation, which made a significant contribution to study of human regulatory T cells (Treg) development in vivo [65].

2.1.1.2 B Cells

As described previously, the development of human B cells in humanized mice is relatively weak compared to T cells. In 2011, Choi et al. evaluated the efficacy of Busulfan, a chemotherapeutic agent, and claimed that it could efficiently improve the reconstitution of human specific antibody-producing B cells, T cells, macrophage, and even DC from $CD34^+$ cord blood cells with less toxic effects [66]. On the other hand, Kim et al. found that co-transplantation of fetal bone tissue with fetal thymus could facilitate the development and reconstitution of human B cells from fetal liver-derived $CD34^+$ cells together with T cells [67].

2.1.1.3 Other Lymphocytes

Besides adaptive lymphocytes like T and B cells, innate lymphocytes development-related factors were also illustrated in humanized mouse model. As early as in 2008, Huntington and Di Santo made a periodic review on the application of humanized mice in the research of NK cell development [68]. In 2011, Pek et al. further confirmed the crucial role of IL-15 in NK cell development in bone marrow and liver with humanized mouse model [69]. We believe that more studies in the development of other innate lymphocytes such as NKT, γδ-T cells and innate-like T cells (ILT) will be reported in the near future.

2.1.2 Myeloid Cells and Other Cells

Myeloid cells are generally regarded as more fragile and difficult to survive in "strange environment", which made it attractive and subtle to improve reconstitution of these sensitive cells. The

addition of human original cytokines such as GM-CSF and IL-4 was generally accepted as an efficient way to improve DC maturation [70]. Similarly, the effects of macrophage colony-stimulating factor (M-CSF) and Fms-related tyrosine kinase (FLT)-3 ligand on promoting the development of macrophage [71], and $CD141^+$ and $CD1c^+$ DC [72] have also been confirmed in humanized mouse model respectively. Moreover, the development of megakaryocytes was replicated and used as index of dengue virus-infection in humanized mouse model recently [73], which also supported the multi-lineage hematopoietic cell development in humanized mice. Finally, transplantation of human stem cells from bone marrow of patients with bone marrow failure syndrome into humanized mice provided invaluable tools for evaluating novel gene-targeted therapy before clinical trial [74].

In summary, the reconstitution of diverse human immune cell populations from their pluripotent progenitors in immunodeficient mice has become a potent platform for investigating the development of human immune system while the next question is how to create a more "humanized" environment in mice for human cells [75].

2.2 Autoimmune Diseases

The advances in the study of autoimmune diseases in humanized mice, especially those T cell-mediated diseases, are always correlated with development of HLA-transgenic technology. In 1999, Bachmaier et al. generated a CD4–CD8– double-knockout mice transgenic for human CD4 and HLA-DQ6 to specifically reconstitute the human HLA-DQ6/CD4 arm in mice and established a dilated cardiomyopathy model [76], which was one of the earliest attempt for applying humanized mouse model in the study of autoimmune diseases. Using similar strategy, Eming et al. established a RA model in a HLA-DR4/human CD4/TCR combined transgenic mice with the stimulation of a RA-related human autogenic protein HCgp-39 in 2002 [77]. However, the lack of human immune system reconstitution in these models constrained their representative for the whole map occurring during autoimmune diseases. On the other side, Shultz et al. established T1D model in NOD/scid-$\gamma c^{-/-}$ mice by co-transplanting with human stem cell and islet cells [78, 79]. Importantly, this group pointed out the potential of HLA-transgenic immunodeficient mice in optimization of these models and provided some interesting preliminary data [78]. Soon after, inflammatory arthritis and type 2 diabetes models were established in HLA-transgenic humanized mice by David [80] and Schultz groups [81] respectively. As we mentioned previously, T cell-mediated immune responses were generally incomplete in humanized mice established on conventional immunodeficient mice, which usually led to insignificant clinical symptoms [82] and thus limited the application of these models. The involvement of HLA not only improves the efficacy of immune responses, but also provides a platform for study of the

relationship between HLA subtypes and specific diseases susceptibility. Nevertheless, the complexity and individuality of HLA phenotypes in healthy donors or patients still remain as the biggest challenge in rebuild of physiopathology process in relatively limited HLA-expressed humanized mice.

Due to relatively weak reconstitution of human B cells in humanized mice, the establishment of B cell or antibody-mediated autoimmune diseases seems to be more difficult than those T cell-mediated autoimmune diseases. Kerekov et al. rebuilt the clinical pathogenesis in humanized mice with cells transferred from SLE patients and evaluated the potential of B cell-targeted therapy with a chimeric molecule containing a monoclonal antibody against human inhibitory complement receptor type I coupled to a decapeptide that mimic DNA antigenicity [83]. In 2012, another group led by Duffield recapitulated systemic vasculitis in humanized mice by treating them with anti-proteinase-3 IgG isolated from patients [84]. With the improvement in reconstitution of multiple components of human immune system in humanized mice, it is predictable that the induction of diverse human B cell-mediated autoimmune diseases in vivo will be accessible soon.

2.3 Transplantation-Related Diseases

Application of humanized mice models in transplantation-related diseases arises as early as the birth of humanized mice but the process is so tortuous till now due to chronic exogenous rejection and ongoing decrease of immune cells [85]. In 2001, Coates established an allogeneic skin rejection model in humanized NOD/scid chimeric mice and examined the therapeutic effects of human myeloid DC transduced with an adenoviral IL-10 gene [86]. In 2006, Marcheix et al. rebuilt a human chronic vascular rejection model in humanized SCID/beige mice with human mesenteric arterial grafts [87]. In 2012, Yi et al. determined the suppressive capacity of in vitro-expanded human $CD4^+$ Treg on porcine islet xenograft rejection in humanized mouse model and found the crucial role of IL-10 in Treg-mediated protection [88]. In above three studies, investigators planted solid grafts into immunodeficient mice before reconstitution of human immune system and induced rejection by infusion of mature human cells. However, the long-term outcome of these models is still not clear.

In order to further mimic clinical situation, human $CD34^+$ stem cells were applied in establishing humanized mice. Using this strategy, three independent groups reported allogeneic islet transplantation [89], xenogeneic islet rejection [90], and xenogeneic skin rejection [91] models during 2010–2012. Unfortunately, insufficient development of immune cell populations in these humanized mice still stayed as an obstacle and even led to the failure of rejection [89]. To solve this problem, some other groups tried to develop a more “mature” human immune system in humanized mice by transplanting human peripheral blood cells.

In 2013, our group reported a novel human allogenic GVHD model established on humanized mice reconstituted with human PBMC [92]. This model reproduced typical clinical process of acute GVHD occurring during allogeneic bone marrow transplantation without apparent interruption of exogenous reactivity. Using this model, we evaluated the protective effects of human $CD8^+$ Treg induced ex vivo by allogeneic CD40-activated B cells and found that human $CD8^+$ Treg could inhibit GVHD and induce long-term tolerance without compromising general immunity and graft-versus-tumor (GVT) activity [92]. The potent regulatory activity of the $CD8^+$ Treg was mainly mediated by the expression of cytotoxic lymphocyte antigen (CTLA)-4 on cell surface, while their alloantigen-specificity and the ability to induce the long-term tolerance favor their clinical application. More importantly, this strategy might reduce clinical dependence on limited HLA-match donors and largely improve the survival chance of millions of patients who are waiting for bone marrow transplantation.

Humanized mouse model undoubtedly brings new hope for transplantation research, but we also need to keep in mind that a lot of questions are still waiting to be answered on this way. As emphasized by Brehm and Shultz, keys to successful humanized mouse model included available immunodeficient mouse strains, the choice of tissue to transplant and the specific human immune cell population that can be grafted [85].

2.4 Other Inflammatory Disease

Besides autoimmune diseases and transplantation-related diseases, humanized mice models are also useful to study some other inflammatory diseases.

In 2002, Hammad et al. compared the Th2 allergic inflammation in the lung of humanized mice reconstituted with PBMC. To induce inflammatory reaction, DCs from home dust mite (HDM)-allergic patients or healthy donors were injected intratracheally and mice were then repeated exposed to aerosol of HDM. In contrast to IFN-γ secretion induced in mice receiving normal DCs, those injected with DCs from patients induced IL-4 and IL-5 production accompanied with the increase of IgE production, which represents characteristics of Th2 response [93]. In 2003, Firouzi et al. used a humanized SCID mouse model confirmed the crucial role of T cells during multiple sclerosis-associated retrovirus particle-caused brain hemorrhage [94], while Sheu et al. found that circulating IgM played the main pathogenic role in skeletal muscle ischemia-reperfusion injury based on their research on hPBL-SCID mice in 2009 [95]. In the meantime, Unsinger et al. established a sepsis model in humanized mice elevated human pro- and anti-inflammatory cytokines as well as a dramatic increase in human T and B cell apoptosis, which was generally found in patients with sepsis [96]. More recently, Vudattu et al. determined the adverse effects of anti-CTLA-4 antibody (ipilimumab) including hepatitis,

lymphadenopathy, and other inflammatory sequelae in humanized mouse model [97].

In addition to immunopathology study, humanized mouse model was also applied in studying the underlying mechanisms of injury repair. By plating retroviral vector-modified human skin on nude mice and adding human keratinocyte growth factor (KGF) to artificial wound in the skin, the re-epithelialization was significantly accelerated [98]. Although this model could not be described as "real" humanized mice because no human immune system was involved in it, this attempt initiated an innovative application of humanized mice.

Compared to satisfactory reconstitution of circulating blood cells, the successful reconstitution of mucosa immunity in humanized mice is still absent till now. Mucosa, especially respiratory and digestive tract surface, plays indispensable role in protection and immune regulation. However, the residence and exchange of immune components in the locus are still difficult to rebuild in animal models because the physiological dynamics remains largely unknown [99]. Another reason is due to their complex gnotobiotic microenvironment. To meet this requirement, Gordon's group firstly established a humanized gnotobiotic mice by transplanting fresh or frozen adult human fecal microbial communities into germ-free C57BL/6J mice and then investigated the effect of diet on human gut microbiome [100]. Similarly, Kashyap et al. determined the relationship among diet, gastrointestinal transit and gut microbiota using the same model [101], while Macrobal et al. further compared the difference between gnotobiotic humanized mice and conventional mice urine and fecal metabolomics profiles [102]. Recently, another exciting breakthrough in immune reconstitution of the gastrointestinal tract was reported by Nochi's group. They developed human gut-associated lymphoid tissue (GALT) in mouse cryptopatches and succeeded in generating functional intestinal immunity marked by human IgA secretion in a BLT-NOD/scid mice model [103]. The combination of this humanized mouse model and gnotobiotics transfer will greatly improve our understanding on intestinal physiology and immune regulation.

2.5 Other Applications

2.5.1 Tumor

The occurrence of humanized mouse model provided a perfect platform for evaluating immunotherapy against tumor. The earliest attempts of inducing antitumor immune responses in humanized mice focused on the generation of specific antibody but the outcome varied due to unstable humanization of models [104, 105]. In the new century, researchers started to pay more attention on developing complete tumorigenicity, especially metastasis process and its relationship with stromal cells, in immunocompetent humanized mice, and made some significant advances in multiple fields like human prostate cancer [106], mixed-lineage leukemia

(MLL) [107], human primary squamous cell carcinoma [108], and human T-cell leukemia virus (HTLV)-induced T cell leukemia [109]. Based on these progresses, some novel immunotherapy strategies were evaluated on humanized mouse models, such as inhibitory receptor Ig-like transcript (ILT)-3 depletion or blockade in melanoma [110] and IL-15-enhanced NK cell-mediated cytotoxicity against human breast cancer [111]. Recently, our group reported a novel application of pamidronate, a phosphoantigen generally used to treat osteoporosis, in treating Epstein-Barr virus (EBV)-induced B cell lymphoproliferative disease in humanized mouse model reconstituted with human PBMC [112]. This "new application of an old drug" was mediated by expanding and activating human Vγ9Vδ2-T cells, a small cell population of human lymphocytes, which might inspire further exploration of currently available resources. More importantly, the established of donor- and tissue-specific humanized mouse tumor models will undoubtedly play an indispensable role during the development of individual therapies in the future [113].

2.5.2 Infectious Disease

2.5.2.1 HIV

The development of humanized mice represents a milestone in the history of human immunodeficiency virus (HIV) study. The new generation of humanized mice not only improved our understanding on transmission, latency, and pathogenesis of HIV [114–119], but also provided unprecedented platform for antiviral study. Besides further exploration of efficient virus-specific neutralization antibodies [120–125] and conventional antiretroviral or antimicrobial therapies [126–128] in these models, the efficacy of vectored immunoprophylaxis [129] and CCR5-targetd treatment [130–132] in preventing HIV transmission were evaluated as well. Meanwhile, the crucial roles of HIV-specific $CD8^+$ T cells [133, 134] and plasmacytoid DC (pDC) [135, 136] in the replication of virus and activation of immune responses, and their potentials in targeted therapy were also investigated. Other novel immunotherapy assays performed in humanized mouse model included blockade of programmed cell death (PD)-1 receptor [137, 138], engineering HIV-resistant T cells from short-hairpin RNA (shRNA)-expressing hematopoietic stem/progenitor cells [139], and inhibition of HIV replication by a chimera containing an RNA aptamer with high binding affinity to the HIV envelop protein gp120 and virus neutralization properties and a small interfering RNA (siRNA) triggering sequence-specific degradation of HIV RNAs [140]. Moreover, a preliminary study on mechanisms underlying viral controlling in HLA-B*57 elite controller or suppressor (ES) was completed in humanized BLT mice and demonstrated that elite suppressors are capable of controlling HIV-1 due to the possession of unique host factors rather than infection with defective virus in vivo [141]. Nowadays, we could even make in-depth study on the cell dynamics in HIV-infected humanized mice model

with the help of intravital microscopy [142]. Therefore, it is countable that the future molecular biology will bring more surprise to the efforts of gene therapy against HIV [143].

2.5.2.2 Other Infectious Disease

Except for application in HIV-related studies, humanized mouse model also brought span-new opportunities for other human infectious diseases [144], especially those blood-borne pathogen-caused diseases such as dengue virus infection [145–149], EBV infection [150–155], HCMV infection [156], HTLV infection [157], and malaria parasite infection [158]. On the other hand, humanized mouse models for Leishmaniasis [159], Salmonella Typhi infection [160, 161], herpesvirus infection [162, 163], Mycobacteria infection [164, 165], and group B Streptococcus (GBS) infection [166] have been established. These efforts fill in the lacks of suitable animal models for those human-specific pathogen-caused diseases and push forward the correlating investigations on development of prevention and treatment, although some technological obstacles like the replication of natural infection and transmission routes are still needed to resolved.

In 2011, our group used PBMC-transplanted humanized mouse model to evaluate a novel therapeutic strategy by targeting the host rather than the virus for treating influenza virus infection. We demonstrated that aminobisphosphonate can control influenza disease through boosting human Vγ9Vδ2-T cell immunity and this beneficial effect is active against viruses of varying subtypes and virulence [43]. Nevertheless, differences in the characteristics of molecules, tissues, and organs between human and mice might impair efficiency of pathogen infection and initiation of specific immune responses [167]. In 2005, Lassning et al. increased the susceptibility of mice on human coronavirus by crossing aminopeptidase N (APN), the receptor for human coronavirus (hCoV)-229E, and transgenic mice into signal transducer and activator of transcription (Stat)-1 null mice [168]. This work, together with HLA- and human cytokines/growth factor-transgenic technology [169], provided successful examples for future studying human infectious agents in humanized mice. In the next stage, improvement of versatility and variability of human immune system in humanized mouse model and application of gene-modified pathogens [170] will definitely enhance translational efficiency of these models.

2.5.3 Vaccine

The usage of humanized mice in the development of vaccines targeting human diseases including EBV, HIV-1, dengue virus, influenza virus, severe acute respiratory syndrome (SARS) corona virus, and carcino-embryonic antigen (CEA) has obtained outstanding achievements during the past decade, while the introduction of HLA transgenic immunodeficient mice further accelerated the advancement in this field [171–173]. With the improvement of immune cell population reconstitution, more and more novel vaccination protocol will be carried out in humanized mice.

3 Perspective

Compared to conventional mice and non-human primate model, humanized mice exhibit great advantage in translational potential, reproductive capacity and data repeatability, economical and ethical concerns. The increasing applications of diverse humanized mice models in biomedical research during the past two decades significantly improved our understanding on human physiological and pathological, especially immunological process at systemic, cellular, and molecular levels. This further accelerated the development of current translational medicine significantly. Nevertheless, there are several major caveats on their development remain to be dealt with, including complete replacement of murine MHC with diversified HLA molecules and efficient methodology to express corresponding growth factors and cytokines at specific time and organs [174]; how to prolong the maintenance of human engraftment, promote the development of myeloid cells and increase relatively weak quantity and quality of immune cells [175]; and the limited development of lymph nodes, inter-organ traffic of immune cells, and the reconstitution of red blood cells and granulocytes [176]. In another word, the most important issue is to find the convenient and cost-effective ways to construct appropriate human-like microenvironment including physical structure, intercellular contact and molecular signals transfer in humanized mice. It is foreseeable that knowledge exchange in the age of big data will bring an even more bright future to this advancing tool than ever.

Acknowledgment

This work was supported in part by the Area of Excellence program supported by the University Grants Committee of the Hong Kong SAR, China (AoE/M-12/06 and AoE/M-06/08).

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Chapter 11

Humanized Mice as Preclinical Models in Transplantation

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Abstract

Animal models have been instrumental in our understanding of the mechanisms of rejection and the testing of novel treatment options in the context of transplantation. We have now entered an exciting era with research on humanized mice driving advances in translational studies and in our understanding of the function of human cells in response to pathogens and cancer as well as the recognition of human allogeneic tissues in vivo. In this chapter we provide a historical overview of humanized mouse models of transplantation to date, outlining the distinct strains and share our experiences in the study of human transplantation immunology.

Key words Humanized mouse model, Transplantation, Immunology

1 Introduction

Transplantation remains the treatment of choice for patients with end stage organ disease. Despite improvements in short-term outcome, the obligatory protracted use of powerful nonspecific immunosuppressants has led to an accelerated rise in morbidity and mortality as a result of chronic rejection and associated toxicity.

There is, therefore, enormous interest for other therapeutic alternatives in the current struggle to improve long-term outcomes, thus negating the use of conventional immunosuppressive drugs.

Translational research has relied heavily on studies conducted in animal models, offering a powerful tool in the fundamental understanding of therapeutics and biology behind diseases. However, the significant use of traditional murine models has exposed the caveats of species-specific differences in the immune systems between humans and mice and, in some instances,

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Maria Cristina Cuturi and Ignacio Anegon (eds.), *Suppression and Regulation of Immune Responses: Methods and Protocols, Volume II*, Methods in Molecular Biology, vol. 1371, DOI 10.1007/978-1-4939-3139-2_11,

hampering the translation of novel protocols to the clinic having resulted in serious harm to research volunteers. An example being the adverse effects of anti-CD28 monoclonal antibody (mAb) in patients despite the lack of effects in mice [1].

Recent years have seen the evolution of humanized mouse models in which human hematolymphoid cells and tissues are engrafted in immunodeficient mice, providing a unique opportunity to study human biology in an animal surrogate. These models have proven to be particularly useful in the transplant setting, lending knowledge to the basic immunological mechanisms underlying allogeneic transplant rejection. More recently considerable effort has been placed on optimizing the various humanized mouse models to ensure better engraftment of the human immune system, as well as broadening the scope of various models of transplantation, to now include skin, islets, and artery.

These mice have proved to be invaluable preclinical models for the evaluation of human specific therapeutics. This chapter details the different strains of mice available, the materials used for reconstitution and the transfer options together with a review of the most well-known humanized mouse models in the setting of graft-versus-host disease (GvHD) and allograft rejection.

2 Strain Development for Humanized Mice and Immune Cell Reconstitution

The concept of humanized mouse models is centered on the reconstitution of immunodeficient mice with viable human cells/tissues with the rationale of emulating an in vivo setting in line with human physiology. Mice in an immunodeficient state are more receptive to xenografts, allowing for the assembly of various human systems without the complication of graft rejection [2]. Of importance, to promote the successful engraftment of human cells and tissues in these mice, both the murine innate and adaptive immune systems need to be suppressed.

The discovery of the severe combined immunodeficiency (*Prkdcscid*), SCID mutation, in 1983 was the turning point in the development of humanized mouse models. The catalytic subunit of *Prkdc* is implicated in the rearrangement of B and T cell receptors, and as such mice homozygous for this mutation display impaired development of T and B lymphocytes [3–5].

Initial studies using the CB17-*scid* mice described the successful engraftment of human peripheral blood mononuclear cells (PBMC) [6], haematopoietic stem cells (HSC) [7] and fetal tissues [8]. Despite this, the overall level of engraftment with human cells reported was low, in part due to the rejection of the cells by the host's active innate immune response, most notably natural killer (NK) cells, left unaffected by the mutation. In addition, the CB17-scid mice also showed leakiness of murine T and B cells, whereby

mature murine T and B cells developed in these aging mice, due to the spontaneous rearrangement of T- and B-cell receptors.

Attempts to improve this model saw the preconditioning of SCID mice with γ-radiation which, despite emptying the mouse of almost all of the host stem and haematopoietic cells, led to radiation sensitivity, as a result of the underlying defect in DNA repair [9, 10]. Further efforts to improve engraftment led to the proposal of a second injection of human cells and chemical macrophage depletion [11–13]. Nonetheless, the search for improved engraftment in these mice and the persistence of the host innate immune system was the driving incentive in the quest for a universal humanized mouse model.

Since SCID mice were highly sensitive to radiation, an alternative was the use of mice deficient in the expression of either recombination-activating gene (Rag)-1 (*Rag1null*) or Rag-2 (*Rag2null*), required for the generation of mature T and B cells [14, 15]. In this regard, Rag-1 and Rag-2 knockout mice established a new strain with restrained leakiness of murine T and B cells and radiation sensitivity previously ascribed to earlier strains, but still retained high levels of host NK cells.

In view of the inconvenience of a persistent host innate immunity, and in the search of better mouse strains that supported human cell engraftment, the *Prkdcscid* mutation was crossed onto different strain backgrounds. As a result, the later derivation of the non-obese diabetic (NOD)-*scid* mice allowed for higher levels of human cell engraftment as compared to the CB17-*scid* mice. With the aforementioned effects of the SCID mutation on the host's adaptive immune system, the combination of the NOD background synergistically dampens the host's immune system via several defects in the innate immunity including: defective macrophage function, reduced NK function and numbers, impaired dendritic cell (DC) maturation, lack of C5 complement component, and a polymorphism in the inhibitory receptor signal regulatory protein-alpha (SIRPα), described in detail below. It is important, however, to highlight that the spontaneous mutation in the *c5* gene, precluding macrophages to secrete C5, does not seem to be responsible for the better engraftment of human cells on NOD background strains [16]. Moreover, since NOD diabetes is T-cell mediated, the incorporation of SCID mutation resulted in diabetes-free mice. While these NOD-*scid* mice are considered the "gold standard" in xeno-transplantation studies, leakiness alongside a predisposition to thymic lymphomas in the majority of the mice, severely decreases their life-span [17, 18], making this model still far from ideal.

Introduction of a Rag1 or Rag2 mutation in NOD mice resulted in reduced leakiness of murine immune cells, although the engraftment levels in these mice remained low [19–21] and the problems with the development of lymphomas still persisted [18, 22].

2.1 Persistence of Murine Innate Immune System and Targeted Disruption of IL2-Rγ^c Gene

In the following years, research was focused on developing mice both free from murine B and T cells and devoid of an innate immune system, particularly with regards to NK cell function. Since several groups were working to solve the problem, many different mouse backgrounds were used. In addition to the scid mutation on the CB17 mice, the disruption of the lysosomal trafficking (*Lyst*) gene by the beige mutation (*Lystbg*) resulted in impaired NK cell function. These mutations were crossed onto BALB/c [23] and C57BL/6 [24] backgrounds. Although SCID/Beige strains of mice still required high numbers of human cells, it was a very useful model for skin engraftment since it accepted human skin grafts with minimal infiltration of murine immune cells [25].

In later years, protocols to increase levels of PBMC engraftment were published [26] and a year later protocols to attain a higher level of engrafted human cells from transplanted cord blood HSC [27].

Further attempts to improve the model saw the targeted mutations in the β2 microglobulin (*β2m*) and perforin (*Prf1*) genes. Mice lacking *β2m* (*β2mnull*) had a drastic reduction in major histocompatibility complex class I (MHC I) molecules leading to impaired NK development [28]. In line with this, a knock-out of *β2m* in NOD-*scid* mice (NOD-*scid*-$\beta2m^{-/-}$) showed improved engraftment rates as compared to the NOD-*scid* mice, due to the abolishment of NK cell activity secondary to homozygosity for the $\beta2m^{-/-}$ allele [28]. However, this model still proved to be problematic in view of the eventuality of thymic lymphomas and mice with $\beta2m^{-/-}$ also prone to developing hemochromatosis [29].

A major breakthrough in this area was the targeted disruption of the murine IL-2 receptor common gamma chain (*IL2-Rγc*) gene, a gene encoding the common cytokine chain and essential signaling component for the action of cytokines: IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 [2, 30].

Strains with the IL2-Rγ^c mutation (γ^c), when selectively bred together with NOD-*scid*-, NOD-*Rag1*-, or BALB/c-Rag2-deficient mice, creating NOD-*scid* gamma (NSG), NOD-*Rag1* gamma (NRG) and BALB/c-*Rag2* gamma (BRG), yielded a variety of more severely immunocompromised recipient mice, achieving far superior levels of human cell engraftment [31]. It was shown that the absence of functional receptors for IL-7, IL-15, and other cytokines might prevent the development of NK cells and early lymphoma cells, resulting in better engraftment of the transferred cells in turn prolonging the life expectancy of these mice (for a comprehensive mouse strain review *see* ref. 2).

Although throughout this book chapter we use simplified abbreviations of mouse strains, the general consensus when detailing primary papers requires, that the full genotype be mentioned, since gross abridgements of mice strains can result in exclusion of vital information, for example, both NOD.Cg-*PrkdcscidIl2rγtm1Wjl*/SzJ and NODShi.Cg-*PrkdcscidIl2rγtm1Sug*/Jic are NSG strains,

but the former has a complete deletion of the IL-2Rγ chain while the latter has a truncation and can still bind cytokines. For the purpose of this chapter, to differentiate these strains they are referred to as NSG and NOG mice, respectively. All the strains detailed above are in current use with their applicative selection dependent on the associated relative merits and drawbacks to each model.

As the years progressed and science advanced, it became increasingly more apparent that the specific strain background was integral in the development of successful humanized mouse models. The NOD mouse background was ordinarily considered superlative in this respect, offering a number of genetic advantages that promoted the engraftment of human immune systems [17]. In agreement, a direct comparison of immunodeficient IL2-Rγ$^{c-/-}$ mice on either a NOD background (NSG, NOG) or BRG revealed that the NOD background supported significantly higher levels of human cell engraftment following the injection of human HSCs [26, 32, 33].

The strength accredited to the NOD mouse model was found to be attributed to a polymorphism of the *SIRPα* gene. SIRPα expressed on murine host macrophages interacts with CD47, expressed on the surface of human cells, providing an inhibitory signal to the murine macrophage, referred to as the "don't eat me signal," preventing phagocytosis. Whilst polymorphisms in the NOD mice increase the receptive activity of SIRPα, distinct polymorphisms in BALB/c mice blunt its response leaving human cells open to host phagocytosis [34].

In order to validate the importance of SIRPα in models of humanized mice, BRG mice were genetically manipulated to introduce the human SIRPα so as to regenerate the "don't eat me signal" resulting in significantly improved levels of HSC engraftment. Bearing in mind the flaws of the NOD background with regard to radiation sensitivity and their short life-span precluding any complex genetic manipulation, the success of BRG-hSIRPα mouse strains with engraftment, on par with NSG models, has opened the gates to experimental genetics on a more robust mouse strain [35].

2.2 Engraftment of Human Immune Cells; the Role of Cytokines, HLA-Transgenic (Tg) Mice, CD47 Gene Inactivation

Research around the world on humanized mouse models initially concentrated on the success of effective human cell engraftment; however as the field matured in both technology and understanding, the focus shifted to challenging the fundamental integrity of the engrafted human immune system. In the early 1990s Lapidot et al. used several cytokines/growth factors to enhance the humanization of immunodeficient mice with human HSCs [7]. Following this, several research groups have adopted the rationale put forward by Lapidot, now looking at the administration of human factors/cytokines, in isolation or as produced from non-hematopoietic cells, with low or undetectable cross-reactivity with their mouse counterpart (reviewed in [36, 37]).

In this fashion, exogenous IL-7 and IL-15 have been reported to boost the development of human T and NK cells in BRG mice [38–40]. However, the systemic injection of these cytokines was found to pollute the desired physiological environment whilst also bringing into question issues of cost-effectiveness. In answer to this predicament lentiviral expression and hydrodynamic injections of DNA were trialed to avert from the concerns of physiological adulteration [38, 41, 42]. However, these transgenic mice overexpressing cytokines under the CMV promoter have the potential to undesirably transform the development of the engrafting immune system expanding/depleting or favoring some cell population/functions over others [43].

By far the most reliable approach is the knock-in replacement of mouse genes with their human counterparts. This method ensures the physiological and site-specific regulation of gene expression; however, it does come with its own shortcomings of cost, time and labour. Recently, Flavell's group demonstrated the feasibility of this endeavor by knocking in human cytokines/factors into corresponding BRG mouse loci [37] investigating the effects of the single cytokine/factor CSF-1 [44], IL-3/GM-CSF [45], and thrombopoietin [46] on the development and function of an engrafted human immune system. Following this success, they pushed yet further, interbreeding their transgenic mice resulting in the creation of the mouse strains known as MITRG and the MISTRG, which also incorporated the human SIRPα [47]. This strain outperformed NSG mice, showing the maturation of a functional human immune system including, myeloid cells, monocytes, basophils, eosinophils, DC and NK cells, as well as haematopoietic stem cell survivor. However, the significant number of reconstituted human cells, required in the MISTRG strain, over-populates the mice, limiting the development of essential murine cells, giving rise to anaemia.

Other than the provocation of cytokines and growth factors in these immunodeficient mice, different pre- and post-myeloablative regimens have also been proposed to facilitate engraftment. Therefore, pretreatment regimens now include radiation, chemotherapy, or a combination. Other studies have highlighted additional pretreatment options, targeting the remaining murine cells. Examples include the depletion of macrophages with clodronate-containing liposomes [48] or anti-CD122 mAB for NK cell depletion [7, 19]. In addition, a review by Nevozhay et al. provides an outline of the irradiation protocols and effective doses in various different mouse models [49].

While the total B cell repertoire seems to be accounted for, differentiation and function of these cells is suboptimal in humanized mice [50, 51]. This is in due in part to the lack/limited $CD4^+$ T cell help.

It was demonstrated that thymopoiesis is normal in humanized mice, but the muffled "cross talk" between murine thymic stroma

and human haematopoietic precursors may prove to be suboptimal [52]. Furthermore, the lack of proper education by human MHC class I and II (HLAs) and expression of mouse MHC on the thymus is in part responsible for the poor T cell education. Hence, several HLA-transgenic (Tg) mice were crossed on immunodeficient backgrounds to generate humanized mice with HLAs. This HLA-Tg humanized mice showed improved T cell responses with detectable tetramers + T cells after vaccination protocols [53, 54]. Recently, Garcia et al. generated a murine MHC deficient, HLA-Tg Rag/γ^c bearing the human SIRPα gene, which improved reconstitution after injection of human HSC and HLA-restricted T cell responses [55].

Reports of several new strains tackling different problems have just been published, amongst them is the B6-Rag2/γ^c with a CD47 gene inactivation (CD47$^{-/-}$) which achieved human immune system reconstitution in the bone marrow, liver, and thymus (BLT) humanized mouse model [56, 57] and the Kit mutants in NSG and BRG backgrounds that allowed human HSC reconstitution and sustained multilineage throughout several serial transplantations, without the need for preconditioning the mice [58].

Whilst the incorporation of the γ^c deficiency in several mouse strains has significantly advanced humanized mouse models, these mice rarely develop lymph nodes (with exception of the mesenteric) and show extremely low levels of human cell reconstitution in the gut in the BLT model [59, 60].

Such studies merely highlight the plastic designs of current humanized mouse models on the path to defining the "perfect" model. However, for now the decision as to which strain of mice is to be used relies on the underlying question and hypothesis that needs to be tested in the field of interest. The diversity of protocols and approaches extends far wider than the choice of mouse strain alone, also calling into question the specific human immune cell population that can be engrafted, pretreatment options, the route of transfer as well as the choice of tissue to transplant.

2.3 Route of Administration and Post-treatment Options

Figure 1 depicts the various routes of administration of immune cells in mice to date [6]. Transfer options include injection of human PBMCs into the peritoneal cavity, intravenous injection into the tail and organs (e.g. spleen) of adult mice (reviewed in [61]). It has been reported that when SCID mice were injected in the peritoneal cavity with human PBMCs, cells were detected in that area for 3 weeks as compared to the injection in other organs, where cells were detected up to 5 months [62]. On the other hand, administration of human HSCs has been reported in newborns and adult mice. The injection of cells into the bone marrow cavity (i.e. femur) has been trialed, but it is only used on rare occasions. More commonly is the intracardiac and intrahepatic injection of human HSCs in newborn mice harboring the IL2-R$\gamma^{c-/-}$ and

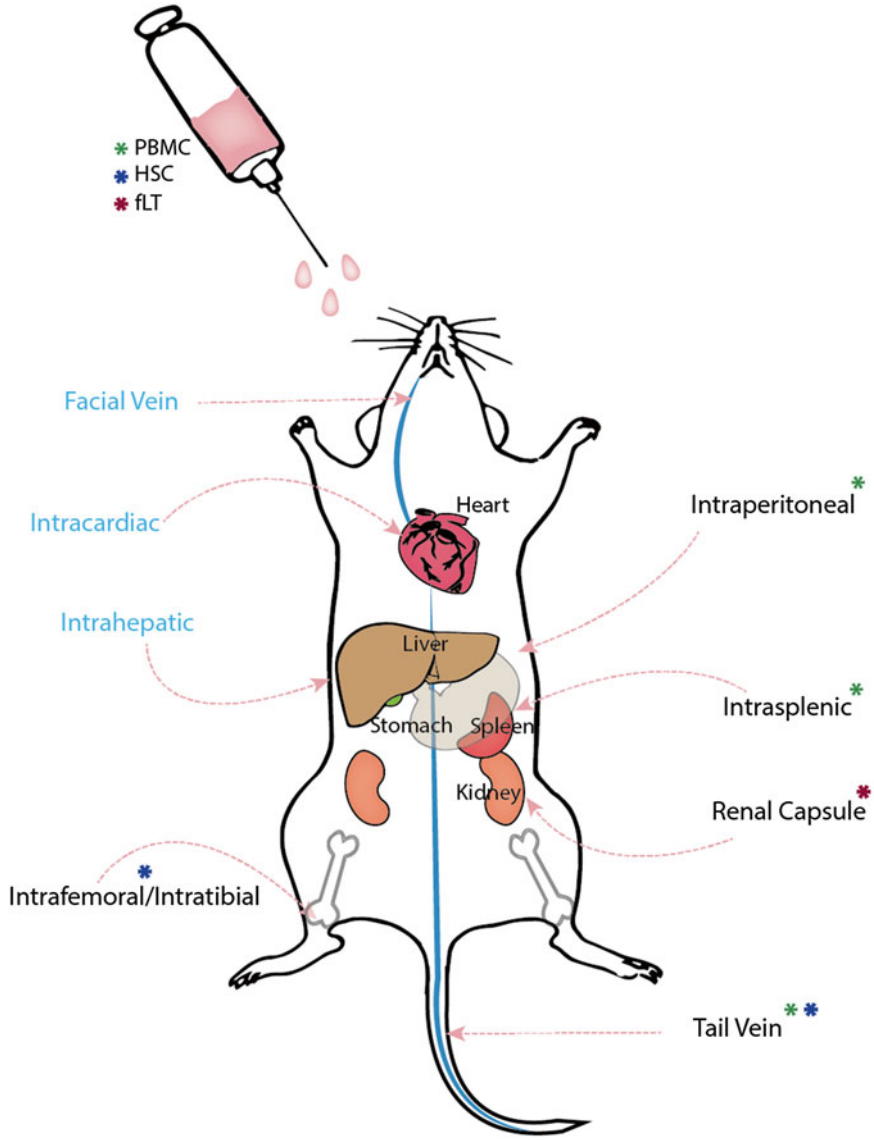

Fig. 1 Routes of human cell administration in immunodeficient mice. Transfer options include the injection of human cells intravenously via the tail vein, into the peritoneal cavity, intraosseously via the femur, intrahepatically, intrasplenically, and beneath the renal capsule. Routes written in *blue* (intravenous, via the facial vein, intracardiac, and intrahepatic) all denote routes of administration applied in newborn mice, PBMCs are routinely delivered to adult mice through intravenous injections into the tail vein or intrasplenic or intraperitoneal injections. Human stem cells are injected into adult mice intravenously via the tail vein or intrasplenically. Fetal liver and thymus fragments are delivered to adult mice under the renal capsule. Abbreviations: *fLT* fetal liver thymus fragments,*HSC* human stem cells, *PBMC* peripheral blood mononuclear cells. Figure adapted from Schultz et al. 2007

the intravenous injection of human HSCs into adult mice, where injection of newborn mice showed more efficient engraftment than adult mice [32].

More complex models include the transplantation of human fetal thymus and liver under the kidney capsule, follow by irradiation and administration of human HSC [61]. This model, known as the bone marrow (although they are in fact from fetal origin), liver and thymus (BLT) model, has high levels of human cell engraftment, adequate T cell education and when performed in NOD-*scid* mice, colonization of human cells in the gut [59, 60].

Overall, these studies highlight the various factors that have to be borne in mind in the efficient engraftment of human cells in the humanized mouse models.

3 Application of Humanized Mouse Models of Transplantation

3.1 Graft-Versus-Host Disease

GvHD is a fatal complication that may develop in patients receiving allogeneic bone marrow transplants (BMT) for the treatment of various haematological conditions such as acute/chronic leukemia, aplastic anaemia or congenital immunodeficiency.

Over 20 years ago, Mosier et al. first demonstrated that the induction of xenogenic GvHD was possible in the CB17-*scid*, immunodeficient mice, following transplantation of human PBMCs [6]. In recent years many other humanized mouse models have been used in the GvHD research, namely: NOG [63], NOD-scid [63], NOD-*scid-B2m*$^{-/-}$ [64], NSG [65], and the BRG mice [48]. Several limitations have hindered the outright translation of these models in the setting of GvHD primarily when considering the relative levels of engraftment, in the CB17-*scid* and the NOD-scid mice in particular, where the host's innate immune system interferes with complete engraftment. Furthermore, a relatively large number of human PBMCs have to be administered, intraperitoneally, to induce disease [66], which conflicts with the usual route of administration in humans, where cells are infused intravenously. Van Rijen et al. proposed a solution to this by using BRG mice which permitted the pathological induction of GvHD following intravenous injection of human PBMCs [48]. However, drawbacks to this model necessitated a large number of cells to be infused and the requirement of total body irradiation, prior to injection, which resulted in considerable variation in disease onset. In the search for the optimal humanized mouse model for the study of GvHD, research to date has suggested that the use of the NOG or NSG mice provides the most stable platform. In these models, smaller numbers of donor cells have been shown to be sufficient where intravenous injection/total body irradiation have not been necessary [63, 65].

In agreement, we have recently compared the engraftment of human lymphocyte populations in both NSG and BRG mouse strains [67]. In this study we concluded that NSG mice are associated with faster rates of engraftment and development of GvHD,

with phenotypic analysis of the engrafted cells demonstrating the prevalence of tissue homing with a T-effector memory phenotype. This finding correlates with the response reported in human GvHD, whereby a strong anti-host effector cell reactivity and cutaneous tissue infiltration has been well documented, thus suggesting the strengths of this model as an informative preclinical tool.

Despite these successes, it must, however, be noted that the symptoms induced in humanized mice of GvHD correlate with the levels of human cell engraftment [19] with the suggestion that this may not necessarily correlate with the human GvHD phenotype. It is, therefore, difficult to translate the induced GvHD response to the acute and chronic equivalent of the human GvHD. Studies have proposed that the differentiation between acute and chronic GvHD in mice can be based on the predominant T cell subset: Th1 in acute-, Th2 in chronic- GvHD or cytokine production: TGF-α/IL-1 in acute-, TGF-β in chronic- GvHD and/or the presence of autoantibody production or systemic fibrosis, classified within the category of chronic GvHD [68]. In this regard, the use of humanized mice to inform translational research in the study GvHD is also reliant on careful histological and cytokine/infiltrating cell analysis.

3.2 Skin Allografts

For many years human skin allografts have been considered ideal in the study of allogeneic transplantation immunology in humanized mouse models in view of the considerable immunogenicity of skin allografts and the relative abundance of the organ [69]. Additionally, the rationale for their use is not only based on the ease of harvest of the source of tissue specimens from healthy individuals, but also the possibility of concurrently recovering autologous PBMC from the skin donor. Of significance, once engrafted on the immunodeficient mice, the transplanted tissue preserves its original architecture.

Early attempts to generate humanized mouse models of skin transplantation utilized the CB17-*scid* host [70, 71]. In this regard, Kawamura et al. transplanted human skin allografts on CB17-*scid* mice engrafted with either PBMCs from a donor that had or had not been previously sensitized to alloantigens [72]. They showed that PBMCs from the sensitized donor were unable to reject skin allografts, proposing that unless pre-existing alloreactive memory cells were present the PBMCs injected into these mice were not functional.

Attempts to improve the model and levels of PBMC engraftment, saw higher numbers (3×10^8) of human PBMCs injected in these mice, following pretreatment with the anti-asialo GM1 polyclonal antibody, which depleted murine NK cells [71]. A second strategy also included the irradiation of the CB17-*scid* mice prior to injection of human splenocytes [70], resulting in higher levels of human cell engraftment as compared to the human PBMC. This approach also accelerated the study of rejection of human skin

allografts, whereby within 3 weeks of human skin transplantation, 75 % of the engrafted splenocytes had rejected the human skin.

The evolution of the humanized mouse model saw groups using the SCID/Beige mice, allowing the engraftment of human PBMCs without the need to deplete the NK cells [23, 73]. Moreover, these mice readily accepted the human skin grafts, with minimal injury or infiltration with murine immune cells [74]. This mouse model has since proven useful in characterizing allograft injury mediated by human T cells [75, 76]. Despite the merits of this model, the poor reconstruction of the immune system, whereby the survival of innate immune cells and B cells was incomplete in comparison to the overpowering persistence of T cells, warranted continued efforts directed at developing a model with a more comprehensive human immune system. In this regard, adult SCID/Beige mice were irradiated and injected with human $CD34^+$ HSC derived from the peripheral blood of granulocyte colony-stimulating factor-mobilized individuals [74]. Despite the promising concept the results were disappointing, still falling short of developing a replica human immune system in mice.

Recent years have seen the use of NSG mice as potential models to study skin allograft rejection mediated by human PBMC. However, human skin grafts on unmanipulated NSG mice showed extensive perivascular infiltration of murine immune cells that resulted in graft injury [77]. Injection of human PBMCs into GR1-depleted NSG mice bearing human skin allografts resulted in allograft rejection, complete loss of human vasculature and destruction of the epidermal and dermal layers, with infiltration of human CD45 cells [77]. The high engraftment of human cells and consistent rejection of human skin allografts in NSG mice have also been the impetus for the use of this model by our group and others to study the potential of regulatory T cell (Treg) therapy in preventing transplant rejection [78–80]. In agreement, we have shown the efficacy of human graft-specific Tregs, enriched based on co-expression of activation markers CD69 and CD71, in preventing alloimmune dermal tissue injury in NSG (and BRG) mice, transplanted with human skin. In our model, mice were reconstituted with allogeneic PBMCs (5×10^6), depleted of $CD25^+$ cells, at 4–6 weeks after transplantation in order to induce allograft damage, with or without in vitro-expanded Tregs [80]. We further showed that higher doses of PBMCs (10×10^6 to 20×10^6 per mouse) routinely resulted in engraftment of both CD4 and CD8 T cells, whereas lower doses resulted in the predominant engraftment of CD4 T cells, with no detectable antigen presenting cell (APC) compartments. In this study, successful human cell engraftment was established whereby $CD45^+$ cells constituted >0.5 % of total splenic lymphocytes. In this regard, the model proved valuable in supporting the importance of allospecific Tregs in the setting of transplantation.

A subsequent study by our group provided further supporting evidence to these findings [79]. Of note, in this study only BRG mice were used, necessitating weekly intraperitoneal injection of anti-mouse Gr1. However, no difference between the two different mouse models was noted in our studies [80].

3.3 Islet allografts

Type I diabetes is a chronic autoimmune condition, characterised by the destruction of insulin-secreting pancreatic β cells, resulting in the dysregulation of blood glucose levels. Available treatment options include human islet cell transplantation, with the promise of restoring normal glycemic control [81]. However, this treatment modality is fraught by late-stage allograft rejection and the need to be maintained on lifelong immunosuppression [82]. As a result, humanized mouse models have been invaluable in the investigation into the mechanisms of rejection alongside the trial of novel therapeutic strategies of immunomodulation in order to suppress T-cell-mediated alloimmune responses [83–85].

As in the case of the humanized mouse model of skin transplantation, initial experiments, using humanized mice in islet transplantation were carried out using CB17-*scid* mice [11, 86]. It was demonstrated that the transplantation of human islets under the renal capsule restored normoglycemia in these mice that had been rendered diabetic by a single intraperitoneal injection of streptozotocin (STZ) [86]. In this study, the injection of allogeneic splenocytes resulted in rejection of the transplanted islets, characterized by infiltration of human CD8 T cells at the graft site.

Subsequent developments in the model of islet cell transplantation saw the use of the NSG mice, after reports of high engraftment levels of human PBMCs, allowing for the careful study of human T cell function, following their injection. As such, the injection of allogeneic PBMCs into STZ-treated diabetic NSG mice, successfully transplanted with human islet allografts, resulted in hyperglycemia and a loss of detectable human C peptide [87]. The rejection was confirmed histologically by the absence of insulin-positive cells at the graft site and the presence of a marked cellular infiltrate.

Further studies used BRG mice in the generation of the humanized mouse model of islet cell transplantation, highlighting the validity of this model, as well, to test novel cell therapy intervention in providing protection against human islet allograft rejection [88].

In the studies outlined above, however, mice were reconstituted with human PBMCs with the limitation of a predominant engraftment of T cells. In this regard, our group has shown the utility of HSC reconstituted NSG mice in studying innate immune responses to human islet allografts [85]. Here, NSG mice at 4–6 weeks of age were irradiated with 240 cGy and within 24 h received intravenous injection of 2×10^5 $CD34^+$ stem cells, enriched by

positive selection from cord blood (NHS Cord Blood Bank, London, UK). The mice generated in this study were approximately 30 % chimeric with human $CD45^+$ cells, encompassing T and B cells, as well as Tregs ($CD4^+CD25^+FOXP3^+$), monocytes/macrophages, $CD11c^+$ DCs, and NK cells, promoting the reconstitution of human lymphohematopoiesis. However, despite the faithful presence of B cells in their entirety, in support of published work [89–91], associated responses were impaired in the mice. This may have arisen secondary to a defective interaction between the human B and T cells, apparent from a lack of HLA expression in the mouse thymus [91, 92]. This notion was validated by studies showing that the injection of human $CD34^+$ cells into immunodeficient transgenic mice, expressing HLA molecules improved antigen-specific T-cell interactions and antibody responses [91, 93].

In our study, 12–16 weeks post-injection of human $CD34^+$ cells and continual assessment of human engraftment, mice were given a single intraperitoneal injection of STZ and 2–3 days post-injection were transplanted with human islet allograft under the kidney capsule. In this model, we were also able to study the biology of Tregs in protection against allograft rejection where, a total of 6×10^6 ex vivo expanded human Tregs, expressing the same HLA-DR as the engrafted $CD34^+$ stem cells were injected intravenously into recipient mice immediately after islet transplantation.

The presence of complement protein, C3d, deposition and infiltration of macrophages ($CD11b^+$) and neutrophils ($CD66b^+$) into rejecting islet allografts in these mice supported the advantage of this model in detailing the intricacies of the innate immune responses. In addition, one of the assets of this model was the absence of GvHD [8], as compared to PBMC reconstituted NSG mice which die of GvHD within the first 30 days, making this model ideal in the study of interventional transplantation therapies [87]. Based on the merits posed by the use of this model, we were able to show a delay in islet rejection post adoptive transfer of Tregs. Moreover, histological analysis demonstrated that there were significantly less infiltrating macrophages, neutrophils, and $CD4^+$ T cells, with the preservation of the islet structure, in the grafts from the Treg-treated animals. This model not only provided supportive data for the development of clinical strategies to use Treg cell therapy in the control of human islet rejection, but also provided a mechanistic insight into the biology of Treg function in vivo.

In agreement with previously published work we provided data that the suppressive properties of Tregs were not limited to the effects on T cell response, but also the inhibition of the pathology mediated by the cells of the innate immune system [94, 95]. Thus, we suggest that the HSC reconstituted NSG mice are currently a favored model to study human islet rejection.

3.4 Artery Allografts

In view that the endothelial cell lining of blood vessels is one of the main targets of acute and chronic rejection [96] several investigators sought to develop a humanized mouse model of the blood vessels to study the immunology behind this process and test novel treatment options. In this regard, Wood et al. developed a model, using the BRG mice, reconstituted with human PBMCs, and incorporating a human aortic graft to study human Treg function in vivo [97]. Their results showed that grafts from mice reconstituted with PBMCs alone exhibited extensive areas of vascular intimal hyperplasia and a decrease in luminal diameter, whereas the co-transfer of expanded Tregs prevented this vasculopathy [97]. Such a study highlights the important use of this model as a preclinical platform for the investigation of novel therapeutic interventions. In line with this, a subsequent study by the same group reported the use of the same mouse strain, showing the inhibition of transplant arteriosclerosis with the combined regimen of low dose rapamycin and Tregs [98]. This study highlights the importance of humanized mouse models as influential preclinical platforms, supporting the concept that rapamycin can be incorporated as an adjunctive therapy to improve the efficacy of Treg based cell therapy in clinical practice.

It must, however, be noted that one of the first models of xeno-vessels transplantation was in SCID/Beige mice with pig epicardial coronary arteries inserted into the murine infrarenal aorta [99]. Other studies progressed onto the transplantation of human arteries [100], with subsequent injection of allogeneic PBMCs, evoking an immune response against the transplanted arterial graft, resembling post transplant human graft arteriosclerosis [101].

Moreover, the injection of human HSC in SCID/Beige mice has also been used to study artery graft rejection [74]. In this model, human artery allografts were rapidly infiltrated with human macrophages and extensively calcified following transplant on HSC-engrafted SCID/Beige mice.

Despite these studies, when comparing the BRG mice as compared to the SCID/Beige for vascular allograft rejection, it has been proposed that the former is more advantageous in view of both the engraftment of human artery and human PBMCs. As a result, this model has been used extensively in recent studies of human artery allografts [97, 102, 103].

4 Conclusions and Future Perspective

The true diversity of humanized mouse models of transplantation have allowed for their extensive use in several different settings. These models now form an integral part of translational biomedical research providing insight into the complexities of transplantation immunology and the power of therapeutic interventions.

While the field is ever-growing in science and knowledge, the search for the perfect humanized mouse model of transplantation is far from over. This warrants the necessity to not only optimize the currently available models, but also develop new models of transplantation to study other organs of interest such as liver, lung, and gut. Additionally, following recent advances in the area of regenerative medicine, the future may see the use of artificial human organs, HSCs developed from embryonic stem cells or inducible pluripotent stem cells in this setting. Although these techniques have not as yet found their way to the field of humanized mice, it is only a matter of time before new models will arise from the transplantation of artificial human organs and HSCs.

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Chapter 12

Dextran Sulfate Sodium (DSS)-Induced Acute Colitis in the Rat

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Abstract

Inflammatory bowel diseases (IBDs) are complex multifactorial disease thought to result from inappropriate immune responses to the gut microbiota, in genetically susceptible individuals, under the influence of environmental factors. Among the different animal models developed to help in understanding IBDs pathophysiological mechanisms as well as to achieve pharmacological preclinical studies, the dextran sulfate sodium (DSS)-induced colitis model is the most widely used because of its simplicity, cost-effectiveness, and similarity with human IBDs. This section provides with a detailed protocol that we validated in our laboratory to perform DSS-induced acute colitis in the Sprague-Dawley (SPD) rat.

Key words Colitis, Animal models, Rats, Dextran sodium sulfate, Inflammatory bowel disease, Crohn's disease, Ulcerative colitis

1 Introduction

Crohn's disease (CD) and ulcerative colitis (UC), the two major clinically defined forms of inflammatory bowel diseases (IBDs), are chronic remittent or progressive disorders of the gastrointestinal tract, with an estimated prevalence of 250 cases per 100,000 individuals in Western countries [1, 2]. Both CD and UC are characterized by intestinal inflammation and epithelial injury and are mediated by shared and distinct inflammatory pathways [3]. Etiology of IBDs is still not fully understood but it is widely acknowledged that they result from inappropriate and/or deregulated immune responses to the commensal gut microbiota, in genetically predisposed individuals and under the influence of environmental lifestyle factors [3]. Understanding these complex interactions is of importance in order to develop new targeted therapies of clinical interest that are still deeply of need. To achieve this, animal modelsof IBDs represent contributive tools as they allow manipulations and interventions that are not possible with human studies [4]. Among them, the dextran sulfate sodium

Maria Cristina Cuturi and Ignacio Anegon (eds.), *Suppression and Regulation of Immune Responses: Methods and Protocols, Volume II*, Methods in Molecular Biology, vol. 1371, DOI 10.1007/978-1-4939-3139-2_12,

(DSS)-induced colitis model is the most widely used one as beyond its shared characteristics with human IBDs etiology, pathogenesis, and therapeutic response, it is relatively simple, rapid to set up, and cost effective.

DSS is a sulfated polysaccharide whose colitogenic properties were first reported in hamsters [5] and extrapolated a few years later to mice [6] and rats [7, 8]. The exact mechanisms through which DSS induces intestinal inflammation are unclear but may be the result of direct damage of the monolayer of epithelial cells in the colon, leading to the crossing of intestinal contents (e.g., commensal bacteria and their products) into underlying tissue and therefore induction of inflammation [9]. Clinical manifestations of the colitis usually include watery diarrhea, occult blood in stools, and weight loss. In the acute model, rectal bleeding and diarrhea may occur as early as 2–3 days following DSS administration and inflammation is fully installed within 6–7 days. Animals then recover if DSS administration is stopped, allowing the study of gut epithelium healing. Weight loss starts 4–5 days following the initiation of DSS-treatment and continues over the next few days, including 4–5 days after DSS removal. Importantly, the DSS model is not dependent on adaptive immunity and is thus useful to analyze the contribution of the innate immune system to the installation of intestinal inflammation [10]. Inflammation is normally limited to the colon with some species-dependent variations [9]. Histopathological findings closely resemble that of human IBDs, especially UC [6], but the inflammatory environment created includes features of both CD and UC [11]. Finally, the DSS-induced colitis has been validated as a relevant model for testing of therapeutic compounds of interest to treat human disease [12].

In this chapter we describe the procedure we have validated in our laboratory to perform DSS-induced colitis in SPD rats.

2 Materials

1. Sprague-Dawley male rats (Centre d'Elevage Janvier, Le Genest-St Isle, France) 7–9 weeks old (weighing 170–200 g) (*see* **Note 1**).
2. 4.5–6.0 % w/v dextran sulfate sodium (DSS) salt (molecular weight 35,000–55,000 g/mol; TdB Consultancy AB, Uppsala, Sweden) in autoclaved drinking water.
3. Animal balance, accurate to 0.1 g.
4. Rat cages fitted with a water bottle.
5. Anesthesia induction chamber (4.0 % isoflurane, airflow 1 L/min).
6. Euthanasia induction chamber (CO_2, airflow 3 L/min).
7. 70 % Ethanol.

8. Sterile forceps.
9. 5 % PBS-buffered formalin.
10. Permanent ink marker for rat identification.
11. Scalpel.
12. PBS.
13. Small scissors.
14. Petri dishes.
15. Ice.

3 Methods

3.1 Prepare Rats for DSS-Induced Colitis

1. On the first day of DSS administration, house SPD rats at no more than four per cage. Rats should have ad libitum access to food and water.
2. Label each rat on the tail using the permanent ink marker (or any other convenient method if preferred). The DSS model is highly variable and labeling allows to follow-up each individual rat susceptibility to colitis.
3. Weigh all the rats to determine baseline weight. Try to equilibrate average weights to avoid significant differences between each group to be tested (e.g., water controls and DSS-treated rats).

3.2 Prepare the DSS Solution to Be Administered

1. Dissolve 5.5 % (w/v) DSS powder in the drinking autoclaved water of experimental rats. Stir at room temperature with a magnetic bar until a limpid solution is achieved. As DSS stability is better in a dry form, only prepare the volume required for the experiment (consider 40 mL/rat/day) (*see* **Note 2**).
2. Fill the cage water bottle with 400 mL of the DSS solution or water depending on the groups. Unused DSS solution can be stored in the fridge for up to 7 days.
3. Observe DSS solution or water intake to make sure that equal amounts are consumed in each groups. Refill cage water bottle with adapted drinking solution every 2–3 days (*see* **Note 3**).

3.3 Daily Monitor Rats for Disease Severity

(a) Measure body weight each day and calculate the % of weight loss as compared to baseline weight using the following formula (*see* **Note 4**):

(b) $[(\text{weight day } X - \text{baseline weight}) / \text{baseline weight}] \times 100$.

(c) Place each rat in an individual empty cage to collect and score feces for consistency and blood (Table 1).

3.4 Sacrifice Rats and Collect Organs

1. On the day of sacrifice, remove the food from cages 4 h before euthanasia. Rats should first be anesthetized in an induction chamber containing 4.0 % isoflurane (airflow 1 L/min).

Table 1
Clinical scoring of the DSS-induced acute colitis

Feces consistency	Score	Blood in feces	Score	Total
Normal	0	No blood	0	Consistency + blood scores
Wet	1	Bloody stools and/or blood around the anus	1	
Soft	2	Severe bleeding	2	
Water diarrhea	3	–	–	

Once unconscious, rats are transferred in a chamber saturated with CO_2 (airflow 3 L/min), according to approved institutional animal ethical protocols (*see* **Note 5**).

2. Collect blood for serum analyses.
3. Once sacrificed, spray 70 % ethanol onto the ventral face of the rat and carefully perform a midline incision to open the peritoneal cavity.
4. Remove and weigh the spleen (*see* **Note 6**).
5. If needed, remove mesenteric lymph nodes for cellular analyses and bacterial translocation assessment.
6. Carefully remove the colon by cutting just after the ileocecal junction and at the terminal end of the rectum.
7. Take a representative picture of colons from rats belonging to each different group.
8. Measure colon length (*see* **Note 7**).
9. Gently remove fecal content by manual displacement with forceps and by flushing with ice-cold PBS using a blunt needle attached to a syringe.
10. Cut colon pieces depending on need (*see* **Note 8**).
11. For histopathological analysis, cut a colon fragment of about 1 cm and carefully open it on the mesenteric face using small scissors. Remove diet content, feces, and blood by gently shaking the fragment in a petri dish filled with ice-cold PBS. Place the fragment in 5 % neutral buffered formalin for at least 24 h before treating it (*see* **Note 9**).
12. For RT-qPCR or WB analyses snap freeze tissues in liquid azote and store them –80 °C until use (*see* **Note 10**).
13. Colons can be cultured ex vivo to measure inflammatory mediators secretion. Prepare colon sections as in 11 but wash them in HBSS with 1.0 % penicillin and streptomycin. Measure

sample weight using a balance accurate to 0.1 mg. Cut the colon section in 1–2 mm fragments and transfer them in a 24-well plate containing 1.0 mL of serum-free RPMI 1640 medium with 1.0 % penicillin and streptomycin. Incubate 24 h a 37 °C, 5.0 % CO_2 incubator. Collect supernatants and centrifuge 10 min at 20,000 × *g*, 4 °C. Store at −80 °C until analysis (*see* **Note 11**).

4 Notes

1. In our experience DSS-colitis develops more efficiently and reproducibly in young rats that in older ones, probably due to food and water requirement considerations. Even if colitis can develop in both male and female rats, more robust colitis are obtained with males.
2. Severity of the colitis is highly variable, depending on rats strains used as well as vendors and housing facilities [8, 13]. Variability is also dependent on the DSS providers and manufacturing lots. It is thus recommended to purchase DSS in bulk from one given lot and to perform preliminary experiments to determine the optimal dose of DSS to be administered for inducing a robust colitis over a period of 7 days. Be careful that DSS MW is comprised between 35,000 and 55,000 g/mol, as it is also a critical parameter for colitis induction. For SPD rats, 4.5–6 % DSS doses should be tested to obtain a slow and steady onset of colitis.
3. Usually DSS is administered for 7 days to induce an acute colitis and then replaced by regular water to allow healing to occur. A complete recovery can be observed by day 20.
4. A body weight loss >20 % reflects a highly severe disease and rats should be sacrificed.
5. For analyzing the peak of acute colitis, sacrifice should be performed at days 6–7. After replacing the DSS solution by regular water, rats will continue to lose weight until days 10–12 but the healing process would have begun. To better analyze the healing phase, sacrifice rats between days 14 and 20.
6. Increased spleen weight can reflect the severity of inflammation. Culturing spleen lysates on LB agar is also helpful to appreciate bacterial translocations. Similarly, liver lysates can also be cultured.
7. In our experience, colon length from 9 weeks old rats in the water group is usually around 20 cm at sacrifice whereas it is around 14–16 cm in the DSS-treated group.
8. In SPD rats, DSS-induced colitis is more prominent in the distal part of the colon. When comparing different groups of

Table 2
Histopathological scoring of DSS-induced colitis

Aspect	Score
Normal	0
Slight increase in cellularity	1
Increased cellularity including neutrophils, mild edema	2
Focal erosions, ulcerations of the mucosa	3
Large and or multifocal mucosal ulcerations	4
Loss of mucosal architecture	5

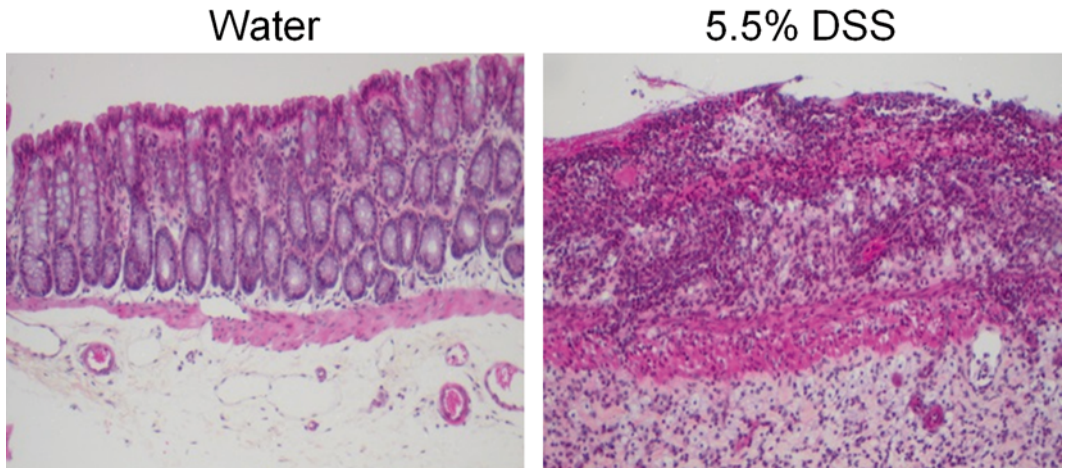

Fig. 1 Representative H&E staining of colon sections from water controls (*left panel*) or 5.5 % DSS-treated (*right panel*) rats during 7 days

animals, make sure that analyzed samples are coming from the same colon region.

9. Determination of histopathological severity should be performed in a blinded fashion by a trained pathologist on hematoxylin and eosin-stained sections. Histological scoring can be performed as indicated in Table 2. Figure 1 illustrates H&E sections of distal colon in water and DSS-treated animals.
10. DSS has been reported to inhibit the activity of both reverse transcriptase and Taq polymerase enzymes, epically when colons are removed before switching the DSS solution to regular water [14]. It is thus recommended to extract RNA with Qiagen RNeasy Mini Kit (Qiagen, Germantown, Maryland) or to remove DSS as described elsewhere [14].
11. Measuring sample weight is important here to report cytokine production by mg of tissue.

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Chapter 13

Corneal Immunosuppressive Mechanisms, Anterior Chamber-Associated Immune Deviation (ACAID) and Their Role in Allograft Rejection

Oliver Treacy, Gerry Fahy, Thomas Ritter, and Lisa O'Flynn

Abstract

Corneal transplantation is the most frequently performed transplant procedure in humans. Human leukocyte antigen matching, while imperative for other types of organ transplants, is usually not performed before cornea transplantation. With the use of topical steroid immunosuppressants, which are subsequently tailed off to almost zero, most corneal transplants will not be rejected in recipients with low risk of graft rejection. This phenomenon has been described as immune privilege by Medawar many years ago. However, this immune privilege is relative and can be easily eroded, e.g. by postoperative nonspecific inflammation or other causes of corneal or ocular inflammation. Interestingly, corneas that are at high risk of rejection have a higher failure rate than other organs. Considerable progress has been made in recent years to provide a better understanding of corneal immune privilege. This chapter will review current knowledge on ocular immunosuppressive mechanisms including anterior chamber-associated immune deviation and discuss their role(s) in corneal allograft rejection. Ultimately, this evolving information will be of benefit in developing therapeutic strategies to prevent corneal transplant rejection.

Key words Cornea, Transplantation, ACAID, Immunosuppression, Tregs

1 Introduction

The cornea is one of only a select few tissues in the body that enjoy immune-privileged status by maintaining immunological ignorance (others include the brain, testes, the pregnant uterus, and the anterior chamber (AC) of the eye). Any corneal inflammatory events, if allowed to proceed unabated, may break down immunological barriers. The most serious consequence for the cornea is immunological destruction of corneal endothelial cells, which have no capacity to regenerate. The result of this rejection process is corneal edema, corneal opacification, and blindness. The concept of relative immune privilege, as applied to the cornea, is supported by observations of high survival rates of corneal allografts without routine human leukocyte antigen (HLA)

Maria Cristina Cuturi and Ignacio Anegon (eds.), *Suppression and Regulation of Immune Responses: Methods and Protocols, Volume II*, Methods in Molecular Biology, vol. 1371, DOI 10.1007/978-1-4939-3139-2_13,

matching between donor and host tissues and the use of only locally applied topical corticosteroids, as opposed to using systemic immunosuppressants which would be standard for other organ transplants. Corneal allograft-associated immune privilege, however, cannot be ascribed to one single mediator or mechanism and is likely to be the result of multiple anatomical, physiological, and immunomodulatory properties inherent to the allograft itself and also the host graft bed [1].

2 Physiological and Anatomical Properties of the Immune-Privileged Cornea

Physiologically, the normal, healthy cornea is both avascular and devoid of lymphatic vessels, thereby shielding itself from immune-mediated attack by denying potentially harmful infiltrating immune cells getting access to the graft and preventing transport of antigens and antigen-presenting cells (APCs) to secondary lymphoid tissues, such as the draining lymph nodes (DLN) [2]. It has been shown that maintenance of corneal avascularity is due to constitutive expression of soluble vascular endothelial growth factor receptor-1 (VEGFR-1) by epithelial cells [3]; with another study demonstrating that administration of VEGF receptor-3 (VEGFR-3) can also suppress hemangiogenesis [4]. Another naturally occurring angiogenesis inhibitor is endostatin. Endostatin can inhibit endothelial cell functions by several means, including attenuation of VEGF receptor signaling and its subsequent binding to $\alpha_5\beta_1$ integrins [5]. Furthermore, Tan and colleagues could show that both syngeneic and allogeneic corneal grafts produce endostatin and while levels remained high in syngeneic grafts, they began to decrease in allografts 10 days post-transplantation. This correlated with early recruitment of allo-specific T cells into grafts, which led to the destruction of endostatin-producing cells and ultimately allograft rejection in 75 % of cases [6]. The authors also found that local administration of exogenous endostatin treatment could attenuate allograft rejection.

Lymphangiogenesis, on the other hand, is suppressed by secretion of soluble VEGFR-2 (which inhibits VEGF-C activity) by keratocytes and corneal epithelial cells but, interestingly, does not affect hemangiogenesis [7]. In this study, the authors showed that soluble VEGFR-2 could inhibit lymphatic vessel infiltration into "high-risk" corneal grafts, that is, graft beds in which intrastromal sutures had been placed 2 weeks prior to transplantation to induce lymphangiogenesis and hemangiogenesis. Their results also showed that a single intracorneal injection of soluble VEGFR-2 was sufficient to significantly prolong corneal allograft survival compared to untreated recipients [7].

Another important factor with regard to corneal immune privilege is the weak or absence of expression of MHC class I

and II antigens, respectively, by corneal epithelial, stromal, and endothelial cells. This has the effect of limiting immunogenicity to foreign antigens as the capacity for antigen uptake is attenuated [8].

3 Soluble and Cell Membrane-Bound Mediators of Corneal Immunosuppression

In addition to the physiological and anatomical properties outlined above, the cornea also expresses numerous cell membrane-bound immunomodulatory molecules and molecules capable of inducing apoptosis of effector immune cells. These include Fas ligand (FasL, CD95L), complement regulatory proteins (CRPs), tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), programmed death-ligand 1 (PD-L1), and MHC-Ib. FasL is an apoptosis-inducing molecule expressed by a number of ocular cells, including corneal epithelial and endothelial cells and can induce apoptosis of infiltrating Fas receptor (CD95)-expressing effector T cells and neutrophils [9, 10], thereby protecting the corneal graft from immune-mediated rejection. The importance of FasL was highlighted by studies in which murine corneal allografts with defective FasL expression, when transplanted, displayed a much higher incidence of immune-mediated rejection when compared to wild-type controls [11, 12]. Similar to FasL, corneal cells also express PD-L1 which, following engagement with its receptor programmed death (PD)-1 on T cells, leads to inhibition of T-cell proliferation and induction of apoptosis as well as reduced interferon (IFN)-γ secretion, thereby promoting corneal allograft survival [1, 13–15]. Further evidence supporting the key role of PD-L1 in determining the fate of corneal allografts comes by way of a study by Shen and co-workers where they reported that murine C57BL/6 PD-L1$^{-/-}$ allografts were more susceptible to rejection than wild-type allografts when placed into Balb/c hosts [14]. Furthermore, we have shown recently that lentivirus-mediated overexpression of PD-L1 on donor corneas leads to >80 % allograft survival compared to just 20 % in untreated allogeneic control grafts underlining the importance of this molecule in protecting corneal tissues from immune response [16]. TRAIL is another pro-apoptotic molecule that, similar to FasL and PD-L1, can induce apoptosis of inflammatory cells. However, although corneal cells do express TRAIL [17, 18] and evidence suggests adenovirus-mediated overexpression of TRAIL can prolong murine corneal allograft survival [19], no reports have yet been published establishing a link between TRAIL expression and preservation of corneal immune privilege specifically. CRPs play a vital role in protecting cells from complement-mediated damage and are expressed predominantly by the corneal epithelium [20]. Soluble CRPs are also present in physiologically relevant quantities in the aqueous humor (AH) that bathes the corneal endothelium and function to protect

this pivotal nonregenerative cell layer from complement-mediated lysis [20, 21]. Additionally, some CRPs, such as decay accelerating factor (DAF, also known as CD55), can disrupt APC:T cell interactions and contribute to corneal allograft immune privilege in this way [22] . Indeed, Esposito and colleagues [22] demonstrated in a murine corneal transplant model that when either the donor cornea or the recipient graft bed is deficient in DAF, rapid rejection ensues.

The AH contains a large amount of immunosuppressive molecules that contribute significantly to maintaining immunological ignorance. These include anti-inflammatory cytokines (e.g. tissue growth factor (TGF)-β2), complement inhibitors [21], neuropeptides, alpha-melanocyte stimulating hormone, vasoactive intestinal peptide, and calcitonin gene-related peptide [23–26]. A soluble version of FasL is also found in the AH and is an important endogenous immunosuppressant as it can suppress neutrophil recruitment and activation [27, 28]. As mentioned earlier, corneal cells only weakly express MHC class I molecules, if at all and this is an important characteristic in maintaining immune privilege. However, the corneal cells may become a target for natural killer (NK) cells, as these cells are programmed to target and kill any cell that does not express MHC class I molecules [29]. The cells at highest risk of attack are those comprising the corneal endothelium and it has been shown that rats undergoing corneal allograft rejection have NK cells present in their AH, which bathes the endothelium [30]. To counteract this possible NK cell-mediated cytolysis, however, the AH contains physiologically relevant levels of TGF-β and macrophage migration inhibitory factor, both of which are capable of neutralizing the effects of NK cells [31–34].

4 Anterior Chamber-Associated Immune Deviation and Corneal Allograft Fate

As described previously, key mechanisms which characterize ocular immune privilege are the unique anatomical and cellular barriers of the eye and the expression of key immunomodulatory molecules including but not limited to interleukin (IL)-10, TGF-β, FasL, and PD-L1 [9, 12, 14, 16, 20, 35–37]. In addition to maintaining a local immunosuppressive environment, the eye is also capable of orchestrating systemic immunoregulatory responses against intraocular antigens. This mechanism of ocular immunosuppression has been given the term anterior chamber-associated immune deviation (ACAID) [38–40]. In corneal transplantation, the donor allografts are in direct contact with the AC and it is this location that correlates closely with the allograft's capacity to survive and induce ACAID to donor alloantigens [41–43].

ACAID, an atypical systemic response to alloantigens is not, as once believed, due to immunological ignorance but rather an active process that induces unique cellular mechanisms which can suppress destructive cellular responses such as delayed type

hypersensitivity (DTH) and cytotoxic T lymphocytes (CTLs) [44]. Studies have illustrated AC injection of donor cells prior to orthotopic corneal transplantation results in a significant increase in the acceptance of corneal allografts in rats and mice [41, 45]. However, it is the introduction of alloantigens during and after routine keratoplasty that is believed to contribute to the ACAID role in corneal transplantation survival [41].

The AC contains AH consisting of biologically relevant concentrations of various immunomodulatory factors as previously described [20, 35–37]. Compelling evidence demonstrates that the F4/80-positive APCs in the eye, maintained in an immature state due to the presence of constitutively expressed TGF-β2, capture intraocular antigens [46]. These antigen-bearing APCs subsequently migrate through the blood stream to the marginal zone of the spleen where they induce the formation of ACAID-regulatory T cells (Tregs) [39, 41, 43, 47]. Both cell-associated and soluble antigen injected into the eye has been detected in the DLN at 6 h and in the spleen after 16–24 h post injection [48, 49]. Once ocular-derived APCs enter the spleen, a series of complex cellular interactions which are not yet fully understood involving CD4 + T cells, natural killer (NK) T cells, B cells, and γδT cells culminate in the generation of Tregs that suppress DTH responses in an antigen-specific manner [42, 50, 51].

Eventually, antigen-specific Tregs that mediate ACAID emerge from these cell clusters in the spleen [39, 44]. It is these antigen-specific CD4+ and CD8+ Tregs that contribute to ocular immune privilege by down-regulating immune responses and protecting a graft from immune rejection after transplantation [52]. The first "afferent" set of cells made up of $CD4^{+}$Tregs prevent the activation and differentiation of antigen-specific effector T helper cells (Th1). Following this, a second set of "efferent" cells consisting of the $CD8^{+}$Tregs are associated with the inhibition of DTH [40]. Interestingly, it is these different forms of immune tolerance which have been demonstrated to be involved in the induction of ACAID and play a role in the promotion of corneal allograft survival [53, 54]. These findings suggest that ACAID may be required for long-term survival of corneal allografts and indicates that immune privilege in the eye is sustained through the cooperation of various cells from organs other than the eye itself.

The concept of ACAID, in which antigen-bearing APCs migrate from the eye, is not yet proven in humans and animal studies have demonstrated that cells do not need to leave the ocular microenvironment for antigen to induce a reduced DTH [55, 56]. The nature of the APC which promotes ACAID-induced tolerance is unclear, but mice deficient in cells expressing the macrophage surface marker F4/80 fail to generate tolerance after injection following donor antigen challenge [57]. Others have suggested that ocular fluids containing material such as soluble proteins from incoming inflammatory cells enter the blood circulation and arrive

at the spleen where further amplification of the NKT cell/F4/80 spleen cell-mediated process of T-cell apoptosis occurs [58]. Winton et al. also suggest antigen may travel from the eye to the spleen, lymph nodes of the head and neck, and mesenteric lymph nodes in a soluble form through blood and lymph [56].

The CD4+ T cells which recognize alloantigen via the indirect pathway are the cells which are believed to be required for induction of corneal allograft rejection [59]. It also has been described that IFN-γ is not necessary for the rejection of MHC-mismatched corneal grafts. However, IFN-γ and Th1 immune mechanisms have been demonstrated to be necessary for the rejection of MHC-matched but minor histocompatibility mismatched corneal allografts [60]. Interestingly, it has been recently demonstrated that IFN-γ is needed for alloantigen-specific ACAID CD8+ Tregs to execute their suppressive function but not required for the establishment of ACAID CD8+ Tregs [51]. Paunicka et al. provide evidence that the Tregs induced by AC injection of alloantigens (i.e. ACAID) are different from the Tregs induced by corneal allografts [51]. For example, in vivo administration of anti-CD8 antibody abolishes ACAID but has no effect on the immune privilege of corneal allografts. The authors suggest that an additional role of IFN-γ in exerting suppression during ACAID may be its ability to enhance the susceptibility of CD4+ effector cells to be suppressed by CD8+ Tregs [51].

Several cell-based therapies have been explored for their capacity to modulate the immune system of the corneal transplant recipient [61–63]. As described, APCs are key players in determining the induction of ACAID or tolerance. APCs are the cells with the capacity to transmit antigen-specific signals and direct adaptive immune responses. In one study by Khan et al. corneal allograft survival was prolonged by intravenously administering CTLA4-KDEL-expressing dendritic cells (DCs), however, this was only when the DCs were capable of indirect presentation of alloantigen [62]. Using donor-derived tolerogenic DCs, Hattori et al. demonstrated that corneal allograft recipients significantly suppress the indirect pathway of allorecognition and that this led to the inhibition of CD4+IFN-γ T cell frequencies. This DC cell therapy was also associated with an increase in Foxp3 expression in the Treg cell compartment [61]. We have recently shown that intravenous injection of donor bone marrow-derived DCs (BMDCs) or donor BMDCs treated with dexamethasone significantly prolongs corneal allograft survival without the need for additional immunosuppression. With both cell therapies, a significant reduction in the level of allograft cellular infiltration and a significant increase in the ratio of intragraft FoxP3 expressing regulatory cells in both the allograft and the DLNs were observed [63].

As well as examining APC-derived cell therapies, much interest has also been focused on the development of mesenchymal stromal cell (MSC)-based therapies to promote corneal allograft survival

[64, 65]. We recently demonstrated that allogeneic MSC treatment prolongs corneal allograft survival by suppressing peripheral immune responses and promoting an intragraft immunoregulatory milieu. This response was associated with a higher proportion of splenic CD4+Foxp3+ Treg cells [64]. Interestingly, Zhang et al. illustrated that systemic administration of human umbilical cord-derived MSCs (hUC-MSC) could potentiate the antigen-specific immune-suppressive responses induced by ACAID. The authors also demonstrated how administration of hUC-MSC was associated with increased cytokine production and Treg cell expansion within the spleen, capable of promoting and maintaining ACAID [66].

Aside from Treg expansion in the spleen, the possibility that Tregs can be induced locally within the eye has been a topic of much debate [55]. However, there is some evidence to suggest that naïve T cells that gain access to the ocular microenvironment may be skewed toward a Treg phenotype in situ [55]. Tregs recruited into the eye from the periphery, therefore, may be critical to tip the balance and together with local conversion help induce an immunosuppressive microenvironment and bring about resolution of potential transplantation rejection [55]. It must be noted that ACAID is not maintained when DTH is present under normal conditions and ACAID may be disturbed/abolished due to surgery-induced trauma, viral infection or chronic inflammation, all of which may contribute to corneal allograft rejection.

5 Conclusions

Relative ocular immune privilege is a fascinating area of immunology research. It is not the result of a single immunosuppressive mechanism, but rather is a combination of both local and systemic immunomodulation involving soluble factors as well as regulatory cells. Although corneal transplants benefit from relative ocular immune privilege, this privilege can be lost with subsequent failure of the transplant and blindness. Transplant immunology and eye research is proving beneficial at identifying factors and processes that protect cells and tissues from immune-mediated destruction or rejection. Future research will further elucidate the mechanisms of ocular immune privilege and open new areas of immunomodulation, particularly with respect to patients at high risk of corneal transplant rejection, that may also benefit other transplant models or immune-mediated diseases.

Acknowledgements

This work was supported by the Science Foundation Ireland Investigator Award to TR (12/IA/1624) and by the European Union Regional Development Fund.

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Chapter 14

Food Allergies: Novel Mechanisms and Therapeutic Perspectives

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Abstract

Childhood food allergy (FA) rates have rapidly increased with significant direct medical costs for the health care system and even larger costs for the families with a food-allergic child. The possible causes of food allergy become the target of intense scrutiny in recent years. Increasing evidence underline the importance in early life of gut microbiome in the development of allergic diseases. There are a range of factors in the modern environment that may be associated with changes to both the gut microbiome and risk of FA, such as mode of delivery, antibiotic exposure, infant feeding practices, farming environment, and country of origin. Knowledge of the relationship between early life gut microbiome and allergic diseases may facilitate development of novel preventive and treatment strategies. Based on our current knowledge, there are no currently available approved therapies for food allergy. More studies are needed to evaluate the safety and efficacy of allergen-specific and allergen-nonspecific approaches, as well as combination approaches.

Key words Immunotherapy, Probiotics, Intestinal microflora, Immune system, Tolerance acquisition

1 Introduction

Food allergy (FA) is a major health issue in Western countries with a substantial effect on quality of life of both patients and their relatives. On the basis of numerous studies, food allergy likely affects nearly 5 % of adults and 8 % of children, with growing evidence of an increase in prevalence [1]. Although any food can provoke a reaction, relatively few foods are responsible for the vast majority of significant food induced allergic reactions: cow's milk (2.2 %), peanuts (1.8 %), and tree nuts (1.7 %) are the most common allergens in children, and shellfish (1.9 %), fruits (1.6 %), and vegetables (1.3 %) are the most common allergens in adults. Recent publications focusing on peanut allergy indicated increases with a doubling (UK) or tripling (USA) in diagnoses [2, 3]. In general, childhood FA to milk, egg, wheat, or soy typically resolves during childhood, whereas allergies to peanut, tree nuts, fish, and shellfish

Maria Cristina Cuturi and Ignacio Anegon (eds.), *Suppression and Regulation of Immune Responses: Methods and Protocols, Volume II*, Methods in Molecular Biology, vol. 1371, DOI 10.1007/978-1-4939-3139-2_14,

are persistent. However during the last decade, a changing pattern in FA was observed with an increased prevalence, severity of clinical manifestations, and risk of persistence until later ages in Western countries [4]. Over the last 20 years rates of potentially life-threatening reactions to food (anaphylaxis) have steadily risen in the developed world [5]. For these reasons, there is a strong need for an expansion of pre clinical research to improve understanding of the mechanisms and to develop novel effective strategies to prevent and treat FA.

2 New Insights into the Pathogenesis of Food Allergy

There is a complex interplay of environmental influence and genetics that underlie the immunopathogenesis of food allergy and the manifestations of various food-induced allergic disorders. The gut microbiota is emerging as a crucial "internal" environmental exposure [6]. We briefly summarize what are the evidences that demonstrate an association between microbial exposure in early life and the development of food allergy.

Microbial gut colonization begins after birth and this process is affected by the newborn infant's gestational age, mode of delivery and first feeding strategies. The colonizing bacteria originate mainly from the mother's gut and vaginal tract [7]. After delivery, breast feeding continues to enhance the original inoculum by the introduction of specific lactic acid bacteria, Bifidobacteria, and other bacteria from the mother's skin. These bacteria set the basis for gut microbiota development and modulation. An imbalance in the compositional configuration of the gut microbiota, dysbiosis, alters the host-microbiota homeostasis, which is a requisite for the development and function of immune cells in the gut associated lymphoid tissue. The importance of this reciprocal regulation of the microbiota and immune system culminates in early infancy, when the balance between homeostasis and inflammation programs later disease risk. In particular, early exposure to commensal bacteria plays a crucial role in Th1/Th2 polarization and proper immune regulatory mechanisms. Germ free animals do not develop oral tolerance and maintained a Th2 type immune response to orally administered ovalbumin. This could be corrected by the reconstitution of the microbiota at the neonatal stages, but not any reconstitution implemented at a later ages [8]. These findings documented a decisive role of the gut microbiota for the acquisition of food oral tolerance in early life. Exposure to a normal intestinal microflora in early life allows for a change in the lymphocyte Th1/lymphocyte Th2 balance, favoring a Th1 cell response [9], while an imbalance in the compositional configuration of the gut microbiota, dysbiosis, alters the host-microbiota homeostasis, producing a shift of the Th1/Th2 cytokine balance toward a Th2

response and a consequent activation of Th2 cytokines with an increased production of immunoglobulin E [10]. Imbalance in intestinal microbiota composition has been documented in patients with food allergy [11]. It was recently found that clostridia strains promote the development of regulatory T cells in the intestine, and when a mix of human clostridia strains were administered to mice, they could suppress the development of food allergy [12, 13]. Recently, a new link between dysbiosis and food allergy development has been provided. Maternal use of antibiotics before and during pregnancy was associated with an increased risk of cow's milk allergy in the offspring and the risk of cow's milk allergy increased with increasing number of child's antibiotics used from birth to diagnosis [14]. In a recent review Marrs et al. conducted a systematic review to test the hypothesis that microbial exposure can modulate the risk of developing FA and concluded that factors influencing microbial exposure, such as mode of delivery, rural animal exposure, diet, childhood infections, immunizations, and antibiotic use, may be partly responsible for rising FA burden, but further prospective studies using double-blind placebo controlled food challenges as an outcome are required [6].

3 Allergen-Specific and Non-allergen-specific Therapies

Based on our current knowledge of the immune basis of food allergy, therapeutic strategies have focused on reducing levels of allergen-specific IgE, enhancing levels of allergen-specific IgG or IgA, suppressing Th2 effector cells, or enhancing regulatory T cells through a variety of allergen-specific and allergen non-specific strategies [15].

3.1 Allergen-Specific Therapies

In the past 10 years allergen immunotherapy by the oral, sublingual, or epicutaneous routes has been the subject of intense research focus. Results are promising when desensitization, defined as protection from food-induced reactions while receiving therapy, is used as a primary outcome [16–18]. However, there is a lack of clarity about safety and long-term efficacy of the treatment. Adverse reactions to oral immunotherapy are not uncommon, and a significant number of subjects experience adverse reactions of sufficient severity or persistence to prevent continuation of immunotherapy. The most successful trials report that at least half of patients who begin immunotherapy do not achieve successful long term tolerance [19, 20]. Preclinical research on food allergy immunotherapy safety has primarily focused on modifications to allergen structure to reduce IgE binding. Allergens can be modified through heating, which denatures the proteins and destroys conformational epitopes; digestion, which forms peptides that are too short to cross-link IgE but maintain T cell epitopes would have the capacity to generate

T cell-mediated immunomodulation; and chemical modification, for example glycosylation of allergens can significantly modify their immunogenicity and allergenicity [15].

3.2 Modified Allergens and Adjuvants for Allergen Immunotherapy

Some allergens can be modified simply through heating. Heating denatures the proteins and destroys conformational epitopes, and there are also matrix effects that influence digestion and absorption of the allergens. Milk- or egg-allergic children enrolled in intervention studies in which they incorporated extensively heated milk or egg into the diet outgrew their unheated egg or milk allergy more quickly than a control group that received standard of care [21, 22], and this inclusion of milk or egg was associated with changes in immune parameters consistent with an immunotherapeutic response (elevation in IgG4, decreases in allergen-specific IgE). Heating to reduce allergenicity is applicable to egg or milk, but not to antigens such as peanut where high heat increases allergenicity rather than reducing it. Allergens can be also modified through digestion, which forms peptides that are too short to cross-link IgE but maintain T cell epitopes would have the capacity to generate T cell-mediated immunomodulation. Immuno-dominant peptides in the peanut allergens Ara h 1 [23] and Ara h 2 [24] have recently been identified with the goal of developing peptide immunotherapy. In addition to digestion and heating, allergens can be modified by chemical modification. Glycosylation of allergens can significantly modify their immunogenicity and allergenicity. Carbohydrate structures can both promote and suppress allergenicity. There is evidence that exposure of some allergens to high heat can enhance allergenicity through glycation, which allows for recognition of the allergens by pattern recognition receptors on antigen-presenting cells [25, 26]. But glycosylation can also result in enhanced immune tolerance. At preclinical level, research on improvements in efficacy of food allergy immunotherapy is focused on adjuvant optimization. Adjuvants that amplify either a Th1 response or a regulatory response may be necessary to sufficiently suppress the Th2-skewed immunity that drives the allergic response to foods. Many of these adjuvants are of microbial origin and range from whole heat-killed bacteria to co-administered purified microbial products to fusion proteins incorporating allergen and adjuvant in one. By binding to innate pattern recognition receptors on antigen-presenting cells, these adjuvants are thought to drive the T cell response away from a Th2 response. Adjuvants not only modify the nature of the immune response, but amplify the response such that significantly lower doses of allergen may be sufficient for an immunotherapeutic effect. The immune basis of tolerance induced by allergen immunotherapy for food allergy is still the subject of intensive research; immunotherapy is associated with elevations in allergen-specific IgG4 and IgA, and reductions in diversity of epitopes recognized by allergen-specific IgE, skin prick test wheal

size, allergen-induced basophil activation, and allergen-induced Th2 cytokine production [27–29]. These parameters are associated with immunotherapy, but so far there have been no biomarkers described that successfully predict tolerance versus desensitization in response to immunotherapy.

3.3 Allergen-Nonspecific Therapies

Therapies that are not allergen specific are especially attractive because many patients have multiple food allergies and allergen immunotherapy with specialized allergen adjuvant constructs may be of limited value in these patients. In this field, there is a significant interest in probiotics and the possibility of manipulating the microbiome for therapeutic purposes. Recently, we demonstrated that treatment of cow's milk allergy (CMA) infants with an extensively hydrolyzed casein formula (eHCF) supplemented with the probiotic *Lactobacillus rhamnosus* GG (LGG) accelerates oral tolerance acquisition to cow's milk [30, 31]. Subsequently, we tested the hypothesis that eHCF plus LGG induced effect on oral tolerance thanks to an influence of this dietary intervention on the composition of the gut microbiota (Fig. 1). High-throughput sequencing technology (16S rRNA-based sequence analysis) was used to compare fecal samples from newly diagnosed CMA infants, collected before and after treatment with eHCF plus LGG, to

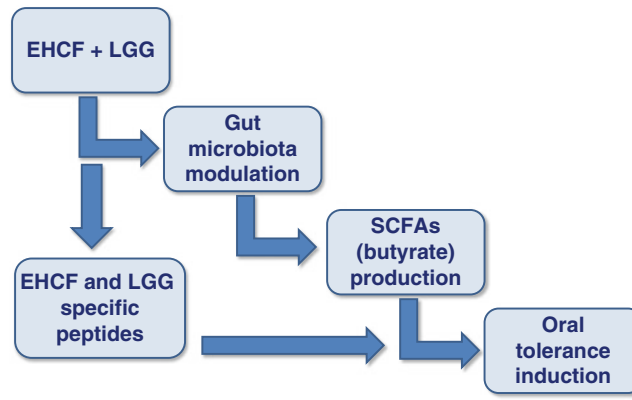

Fig. 1 A schematic representation of the potential mechanisms of action of eHCF plus LGG in children with cow's milk allergy. Treatment with EHCF plus LGG expanded gut microbiota populations associated with immunoregulatory effects and increased butyrate production at intestinal level. Gut microbiota is a crucial factor for food oral tolerance and it regulates an appropriate balance between immune effectors and regulatory pathways. Short chain fatty acids (SCFAs) such as propionate, acetate and butyrate are gut microbiota-derived bacterial fermentation products that selectively expand Tregs in the large intestine. These SCFAs stimulate the expansion and immune-suppressive properties of Tregs, such as the production of IL-10. Moreover, the specific immunomodulatory effect of eHCF plus LGG may be due to small specific peptides, which are absent in other formulas

those obtained from controls. Treatment with eHCF plus LGG expanded gut microbiota populations associated with immunoregulatory effects. Otherwise healthy infants with CMA were given eHCFs ($n=55$), eHCF with LGG ($n=71$), hydrolyzed rice formula ($n=46$), soy formula ($n=55$), or amino acid-based formula ($n=33$), and oral food challenges were performed after 12 months to assess acquisition of tolerance. The rate of tolerance after 12 months was significantly higher ($p<0.05$) in the groups receiving eHCF (43.6 %) or eHCF plus LGG (78.9 %) compared with the other groups: hydrolyzed rice formula (32.6 %), soy formula (23.6 %), and amino acid-based formula (18.2 %). Our in vitro and in vivo data suggest that eHCF containing LGG promotes oral tolerance through a combination of different mechanisms. These findings suggest a potential innovative therapeutic approach for children affected by FA.

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Part III

Immunomonitoring and Biomarkers

Chapter 15

Standardized Multi-Color Flow Cytometry and Computational Biomarker Discovery

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Abstract

Multi-color flow cytometry has become a valuable and highly informative tool for diagnosis and therapeutic monitoring of patients with immune deficiencies or inflammatory disorders. However, the method complexity and error-prone conventional manual data analysis often result in a high variability between different analysts and research laboratories. Here, we provide strategies and guidelines aiming at a more standardized multi-color flow cytometric staining and unsupervised data analysis for whole blood patient samples.

Key words Flow cytometry, Immune monitoring, Standardization, Data analysis

1 Introduction

Within the last 15–20 years, biologics aiming at influencing particular pathological aspects of inflammatory diseases such as solid organ transplantation and different autoimmune diseases have been introduced into clinical practice. With the inflammatory diseases being very divers and knowing that individual patients will respond differently to these biologics, monitoring the changes in composition and functionality of immune cells has and will even more become a crucial part of clinical diagnostics [1]. In addition, it has now become more and more evident that composition and function of leukocyte subsets are not static but may vary between individuals, are dependent on gender and age; and rather change during life [2–7]. To be able to compare the effectiveness of biologics and cellular therapeutics, standardization of the immune monitoring assays especially between different performing centers and over time is critical. Because the complexity of the immune system requires the measurement of multiple parameters in parallel and the characterization of many cell subsets, flow cytometry has become a very powerful tool for immune diagnostics [1]. However, the assay complexity in combination with pre-analytical factors such

Maria Cristina Cuturi and Ignacio Anegon (eds.), *Suppression and Regulation of Immune Responses: Methods and Protocols, Volume II*, Methods in Molecular Biology, vol. 1371, DOI 10.1007/978-1-4939-3139-2_15,

as staining procedures, compensation, leukocyte subset definition, and analysis also increases the variability, particularly when comparing results obtained within different laboratories [8–11]. Thus, in order to introduce flow cytometry into routine diagnostics procedure, standardization including an unsupervised approach for leukocyte subset identification at all steps is of outmost importance. Here we will describe our strategies/procedures for whole blood flow cytometry, which has been proven to result in reproducible results across different laboratories [1].

In the first section of this chapter, protocols that have been validated and successfully used in clinical trials for comprehensive multi-parameter immune phenotyping will be provided. The second part aims at giving a brief overview of computational methods for flow cytometric biomarker discovery with emphasis on accessible and easy to learn tools for clinical scientists and researchers.

2 Materials

2.1 Flow Cytometric Antibody Panels

1. Prepare an antibody pre-mix of each flow panel for the required numbers of samples according to the titrated amount of antibody as exemplified by Table 1.

2.2 Whole Blood Antibody Staining

1. *PBS/FSC-Buffer:*
   IsoFlow (Beckman Coulter) + 0.1 % NaN_3 + 2 % FCS. Pour 490 ml IsoFlow into 500 ml glass bottle. Add 0.5 g NaN_3 and 10 ml FCS to the bottle and mix well. Store buffer at 4 °C.
2. *Versa-Lyse-Fix-Solution:*
   Mix 8 ml Versa-Lyse and 200 µl IOTest 3 Fixative solution (10×, Beckman Coulter) in a 15 ml Falcon tube.
3. *1× Lysis-Buffer:*
   Mix 1.5 ml Red Blood Cell Lysing Solution (Miltenyi Biotec) and 13.5 ml Ampuwa.
4. *PBS-Fix-Buffer:*
   Mix 1 ml IsoFlow with 25 µl IOTest 3 Fixative Solution (10×).

2.3 Flow Cytometer Set-Up

1. Flow-Set Pro beads (Beckman Coulter, *see* **Note 1**).

2.4 Bioinformatics Tools/Programs

1. *Software for manual gating.*
   From the wealthy catalog of commercially available software products for conventional flow cytometry data analysis, i.e., by manual gating, only the most popular third-party packages shall be mentioned here. FlowJo™ (TreeStar), Kaluza® (Beckman Coulter), as well as FCS Express™ (DeNovo Software) are "state of the art" and feature intuitive user interfaces, flexibility and speed in data analysis and visualization as

Table 1
Example of antibody panels for complex multi-parametric whole flow cytometry as performed within the "ONE Study"

	FITC	PE	ECD	PC7	APC	APC-A700	APC-A750	PacBlue	KrOrange
1. Immune phenotyping	CD160 Clone 3GB	CD560 Clone N901	CD19 Clone J3-190	CD14 Clone RMO 52	CD 4 Clone 1388.2	CD 8 Clone 89.11	CD3 Clone UCHT 1	CD64 Clone 22	CD45 Clone J.33
2. T-cell subsets	TCRγδ Clone IMMU510	TCRαβ Clone IP26A	CD45RO Clone UCHL1		CD4 Clone 1388.2	CD8 Clone 89.11	CD3 Clone UCHT 1		CD45 Clone J.33
3. T-cell activation	CD57 Clone NC1	CD28 Clone J.33	HLA-DR Clone IMMU357	CD27 Clone 1A 4CD27	CD4 Clone 1388.2	CD8 Clone 89.11	CD3 Clone UCHT 1	CD45RA Clone 2H4LDH11LO89	CD45 Clone J.33
4. $T_{eff}/T_{mem} + T_{reg}$	CD127 Clone R34.34	CCR7 Clone 150503	CD62L Clone DREG56	CD25 Clone 81.49.9	CD4 Clone 1388.2	CD8 Clone 89.11	CD3 Clone UCHT 1	CD45RA Clone 2H4LDH11LD89	CD45 Clone J.33
5. B-cell phenotyping	IgD Clone IAD60	CD21 Clone BL13	CD19 Clone J3-119	CD27 Clone 1A 4CD27	CD24 Clone ALB 6		CD38 Clone LS198-4-3	IgM Clone SA-DA-4	CD45 Clone J.33
6. Dendritic cells	BDCA3 Clone 501733	LIN	CD123 Clone SSDCLY107D2	CD11c Clone BU15	BDCA20 Clone AC144		CD16 Clone 3GB	HLA-DR Clone IMMU357	CD45 Clone J.33

well as sophisticated reporting of the results. To date, no commercially available software provides a complete solution for automated data processing and gating, unsupervised population identification algorithms as well as statistical analysis for feature extraction and biomarker discovery.

2. *Open-source software for automated gating and unsupervised approaches.*
   The R Project for Statistical Computing/BioConductor platform constitutes a major and freely available resource for advanced flow cytometric data analysis [12–14]. Within the modular R framework the *flowCore* package provides the basis for handling cytometric data sets usually stored in list mode data (FCS) files with a multitude of adjoining packages for quality control, normalization, advanced tools for autogating and population identification based on a large number of different clustering algorithms as well as exploratory pipelines (*see* Table 2) for robust identification of clinical correlates (*see* Table 3).

3 Methods

3.1 Flow Cytometer Setup

The environment of the cytometer has a great impact on the quality of the measurements. Best is an installation of the cytometer in an air-conditioned room with protection of direct sun radiation. Variations in temperature affect the stability of the measurements. We describe here the setup procedure for a ten-color, three-laser Navios™ flow cytometer (Beckman Coulter). Similar procedures apply to other ten-color flow cytometers.

The standardization of the Navios™ cytometer performance can easily be done by (1) defining target channels for all fluorochromes using calibration beads (e.g., Flow-Set Pro beads, Beckman Coulter) and (2) generating Flow-Set bead lot-specific protocol files (*see* **Note 1**).

3.2 Staining of Whole Blood Samples

3.2.1 Staining Protocol 1 for Whole Blood Samples for Leukocyte Subsets Except B Cells (e.g., Panel 1 to 4 and 6 of Table 1)

1. Sample preparation: Use EDTA-whole blood stored within refrigerator (*see* **Note 2**), mix well prior to use. Label six 5 ml flow cytometry tubes with panel 1, 2, 3, 4, 2×6 and the corresponding sample ID. Add 100 µl EDTA-blood per tube.
2. Cell staining: Add pre-made antibody mixes according to tube label. Vortex samples for 10 s. Incubate samples for 15 min at room temperature in the dark. Vortex samples for 10 s. Add 1.5 ml 1× Versa-Lyse-Fix-Solution to all tubes. Vortex briefly. Incubate samples for 15 min at room temperature in the dark. Vortex tubes for 5 s. Add 2.5 ml cold PBS/FCS-Buffer to all tubes. Centrifuge samples at 300×*g* for 5 min at 4 °C. Decant the supernatants. Vortex tubes for 5 s. Unify both

Table 2
Selection of R packages for advanced flow cytometry data analysis

R package	Description	Ref.
flowCore	Framework for reading, writing, and processing of FCS files	[37]
flowStats	Data transformation, normalization, binning, and statistical methods	[32]
flowViz	Plotting of flow data for conventional visual inspection, i.e., by dot plots	[38]
flowQ	Quality control of raw data	[39]
QUALIFIER	Quality control of gated data	[40]
flowFP	Fingerprinting by probability binning for quality control and classification	[41, 42]
flowDensity	Automated sequential gating based on 1D kernel density estimation	[43]
flowMeans	*k*-Means clustering with adjacent cluster merging	[44]
Modalclust	*Hierarchical modal clustering*	[45]
Mclust	*n* Mixture model-based clustering	[46]
flowClust	*t* Mixture model-based clustering with the Box–Cox transformation	[47]
flowMerge	Model-based clustering with entropy-based merging	[48]
flowPeaks	*k*-Means clustering with *n* mixture peak merging	[49]
SamSPECTRAL	Spectral clustering after density-based down-sampling	[27]
SPADE	Density-normalized clustering and minimum spanning tree construction	[31]
flowMatch	Cell population matching between samples and meta-clustering	[50]
flowWorkspace	Import of hierarchical gating template from FlowJo™	[40]
openCyto	Scalable robust framework for automation of provided gating template	[51]
flowType	Automated phenotyping by 1D partitioning and significance assessment	[21]
RchyOptimyx	Extraction of population hierarchies correlated with outcome of interest	[33, 52]

panel 6 samples in one tube. Add 3 ml PBS/FCS to all tubes. Centrifuge samples at 300 × *g* for 5 min at 4 °C. Decant supernatant. Vortex samples for 5 s. Add 150 µl PBS/FCS-Buffer resulting in a final volume of about 250 µl (*see* **Note 3**).

*3.2.2 Staining Protocol 2 for Whole Blood Sample B-Cell Subsets (e.g., Panel 5 of Table 1, See **Note 4**)*

1. Sample preparation: Use EDTA-whole blood stored within refrigerator (*see* **Note 2**), mix well prior to use. Label two 5 ml flow cytometry tubes with panel 5 and the corresponding sample ID. Label one 50 ml Falcon™ tube with sample ID. Add 15 ml 1× Lysis-buffer into the 50 ml Falcon™ tube. Add 300 µl of blood and vortex. Incubate for 12 min at room temperature on test tube rotator. Add 10 ml cold IsoFlow. Centrifuge at 300 × *g* for 10 min at 4 °C. Decant supernatant and resuspend pellet (*see* **Note 5**). Add 300 µl cold IsoFlow and transfer the suspension into one of the 5 ml flow cytometry tubes. Add 4 ml cold IsoFlow. Centrifuge at 300 × *g* for 5 min at 4 °C. Decant supernatant and resuspend pellet in a maximum of 200 µl IsoFlow. Transfer 100 µl cell suspension into second labeled flow cytometry tube.
2. Cell staining: Add pre-made antibody mixes according to tube label. Vortex samples for 10 s. Incubate samples for 20 min at room temperature in the dark. Vortex samples for 10 s. Add 1 ml 1× PBS-Fix buffer. Vortex briefly. Incubate samples for 10 min at room temperature in the dark. Vortex tubes for 5 s. Add 2 ml cold PBS/FCS-Buffer and centrifuge samples at 300 × *g* for 5 min at 4 °C. Decant the supernatants. Vortex tubes for 5 s. Unify both samples in one tube. Add 150 µl PBS/FCS-Buffer resulting in a final volume of about 250 µl (*see* **Note 3**).

3.3 Sample Acquisition and Compensation

1. Acquire samples on the Navios™ using the generated protocol files for staining protocol 1 and 2.
2. When using multiple fluorochromes in an experiment correction of spillover of fluorochromes into another channel is required by the process called compensation, i.e., multiplying by the inverse of the spillover matrix. This is usually achieved by single-color control-stained cells or capture beads. An advanced alternative is called "fluorescence-minus-one" FMO controls, in which always one of the used fluorochromes is removed within the staining procedure. The spillover into this channel can be then compensated. However, for repeated measurements on, e.g., patient samples of several panels this can be time- and cost-intensive. Thus especially in longitudinal projects compensation has to be up-to-date and ideally performed by well-experienced analysts adjusting the photomultiplier tube PMT voltages in order to align the median fluorescence intensity (*see* **Note 6**) of the positive and negative populations in the neighboring channel (*see* Fig. 1).

3.4 Analysis of Acquired Flow Cytometric Data

1. *Manual hierarchical gating.*
   Conventional analysis of flow data relies on sequential manual gating, i.e., by setting cutoff values in univariate histograms or drawing regions (polygons, ellipses) around point clouds that

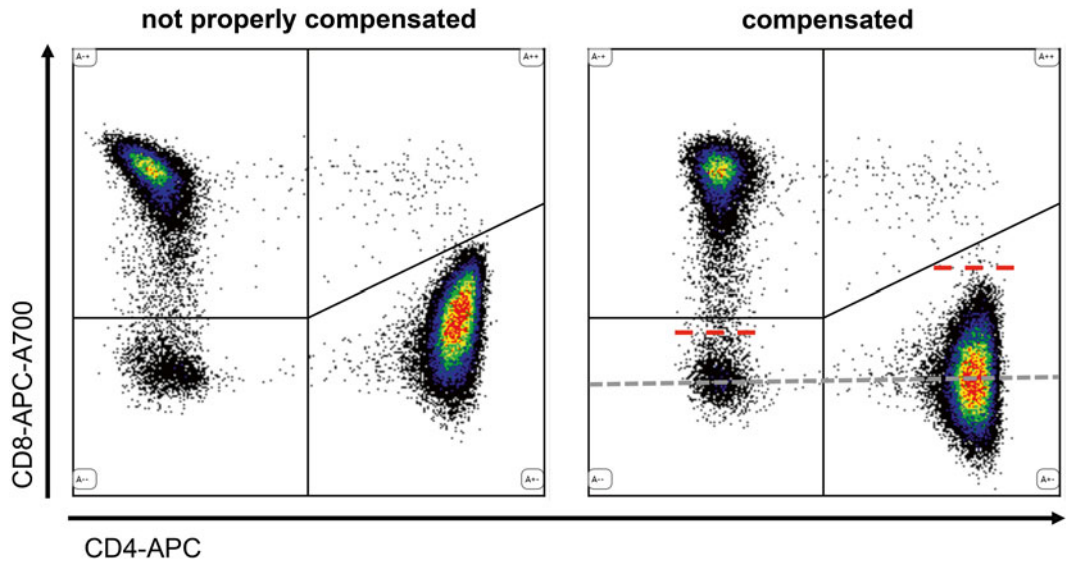

Fig. 1 Example of a not properly compensated and correctly compensated whole blood sample stained for CD8-APC-A700 and CD4-APC. Spillover of APC fluorescence into APC-A700 as visible in the *left* part of the figure was compensated until CD8-negative, CD4-negative (*bottom left*), and CD4-positive (*bottom right*) populations show the same median fluorescence intensity (*grey dashed line* in the *right* part) for the APC-A700 channel. Also indicated are the thresholds for APC-A700 autofluorescence for the CD4-negative and CD4-positive populations (*red dashed line*), which is higher for the CD4-positive population due to data spread after compensation

are positive or negative for a particular parameter, i.e., expression marker, in bivariate dot plots or histograms. Gating is the process to extend a cellular phenotype to more than two markers, by filtering the observed cells based on a region set in a parental dot plot and identifying target populations based on different marker(s) in a nested bivariate dot plot, thereby inherently imposing a gating hierarchy (or strategy) for a desired phenotype. Appropriate negative staining controls such as "fluorescence minus one" (FMO) [11] provide a basis for objectivity in identifying marker-positive cell subsets. However, scaling of the data within a plot, i.e., by appropriate transformations, as well as the exact location of an applied gate may vary considerably between individually skilled operators.

2. *Automated gating and unsupervised approaches.*
One of the main drawbacks in flow cytometric data analysis has been the subjective nature of traditional methods to identify the cellular subsets of interest. Strong analyst bias and technical variability have hampered inter-laboratory comparisons. Recent efforts to harmonize and standardize flow cytometry for immune monitoring in multi-centric clinical trials have been successful [9, 15, 16], paving also the way for computational methods to facilitate cytometric data analysis. A large number of flow cytometry-related bioinformatics tools are not

only readily available but also peer-reviewed and have been tested for performance [17].

3. *Web-based analysis.*
In an effort to also bring the computational methods to the broad community of scientists and cytometrists without programmatic skills, online solutions have been made available. The GenePattern Flow Cytometry Suite allows to build analytical pipelines from 34 open-source modules that leverage from the functionality developed in R/BioConductor [18]. A straightforward approach has been taken by CytoBank, a web-based application for basic and advanced, collaborative analysis, and storage of flow cytometric data. Particularly the advent of mass cytometry enabling simultaneous acquisition of 40 or more parameters has created a need among researchers for easy to use tools, such as the CytoBank built-in *SPADE* (spanning-tree progression analysis of density-normalized events) or *viSNE* (t-distributed stochastic neighbor embedding) algorithms (also available as MATLAB implementations) [19, 20]. These do not only allow an objective, unsupervised identification of cell populations but also their visualization by mapping relationships within the high-dimensional space into two-dimensional graphs (Fig. 2).

4. *Computational biomarker discovery.*
Until recently, comprehensive multi-parameter immune phenotyping has not been accessible to exploratory and predictive analytics, as a fully exhaustive manual gating is not feasible. The superiority of above-mentioned, unsupervised population identification algorithms to explore experimental or clinical high-dimensional flow cytometric data has been demonstrated by critical assessment [21–23]. Approaches to cellular biomarker discovery comprise following steps (for full details of the methodology the interested reader may refer to [17, 21–30] and references in Table 2, which may also serve as a typical analysis pipeline template):

   (a) Acquire a large enough cohort of samples to cover most of the biological heterogeneity and to have sufficient statistical power. Balance for possible confounders.

   (b) Reduce technical noise by using standardized procedures (see above).

   (c) Compensate fluorescence channels and scale data by appropriate transformations (i.e., hyperbolic arcsine, biexponential, or logicle transformation). Further routines to normalize the data are worth considering (*see* **Note 7**).

   (d) Preprocess cytometric data to exclude debris, dead cells, and doublets. Perform quality control by initial analysis of your data, i.e., to detect and exclude outliers (*see* **Note 8**).

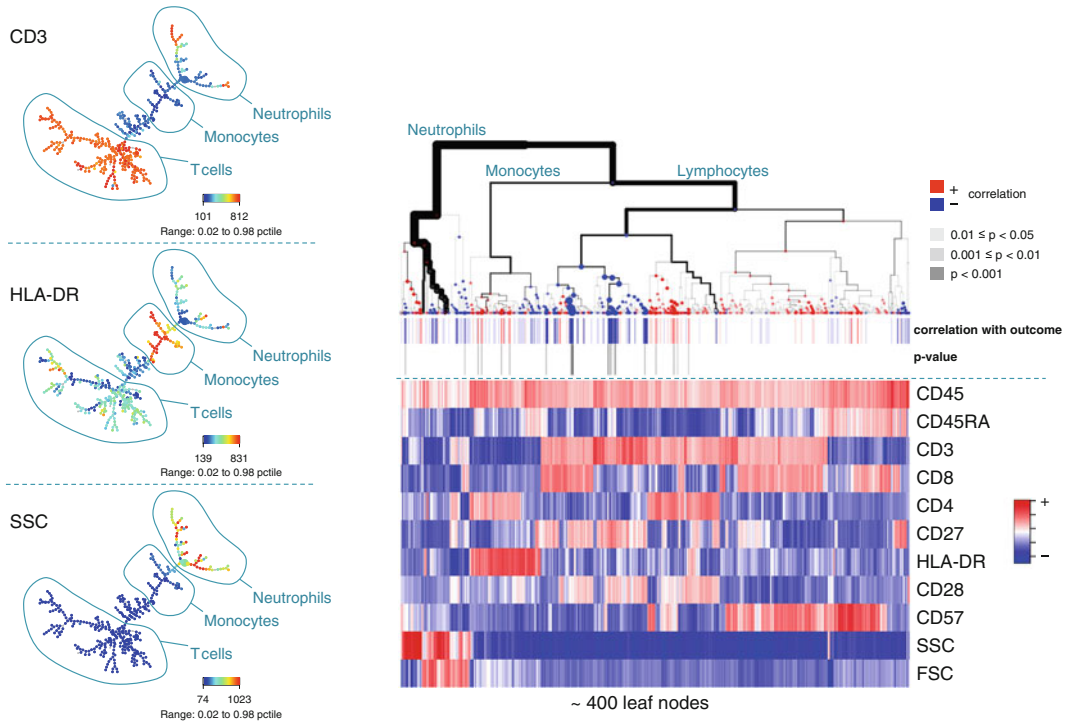

Fig. 2 Unsupervised population identification by density-normalized agglomerative clustering. Whole blood samples from 52 healthy donors were stained for T-cell activation markers and analyzed on a Navios™ (Beckman Coulter) according to standard operating procedures. All data files have been preprocessed by applying compensations, transformations, gating out doublets and debris and subsetting to CD45+ leucocytes. The *left panel* shows the minimum spanning-tree (MST) constructed by *SPADE* (R/Cytoscape plugin) after "meta"-clustering of all measured markers across all 52 samples. According to tree coloring for median fluorescence intensities of CD3 and HLA-DR expression, and side scatter (SSC) characteristics the MST was annotated manually for Neutrophils, Monocytes, and T cells. The size of each individual node (of approx. 400) reflects the contained cell count or frequency for a given sample. To the right, the *SPADE* result can be visualized in a heatmap showing all clustered parameters at once in blue-white-red coloring to accentuate negative and positive marker expression. Similarities between leaf (i.e., *SPADE*) nodes are represented in a dendrogram generated by hierarchical clustering (as opposed to a MST). Hierarchical nodes within the dendrogram are accessible to further analysis such as calculating cell frequencies of parental nodes, indicated by the increased edge thickness (branches) and statistical tests for, e.g., correlation with external variables. Point size of nodes in the dendrogram increases with "significance" of correlation. For leaf nodes sign and *p*-values, e.g., of Pearson's correlation metric (adjusted for multiple comparisons) are indicated by side bar colors

(e) Apply appropriate algorithm that exhaustively identifies cellular subsets for specified parameters/markers across all samples of a training set (*see* **Note 9**).

(f) *Variation 1.* Clustering on individual samples. Identified populations are unlabeled (randomly assigned numbers) and have to be matched between samples by a secondary clustering of individual cluster results (e.g., by *flowMatch*).

(g) *Variation 2.* Meta-clustering after merging of reduced data [27, 31] (e.g., by *SPADE* after density-dependent down-sampling) into a single file.

(h) *Variation 3.* As an alternative approach, use single-parameter partitioning (i.e., into positive and negative expression of a marker) and derive cell populations by combining partitions to all possible phenotypes (*flowType/RchyOptimyx* pipeline).

(i) Visualize and carefully interpret the results in terms of systematic variance and biological relevance (*see* **Note 10**).

(j) Identify candidate features that stratify clinical outcome, i.e., those populations that significantly discriminate between groups (input classes in the training set) or correlate with outcome of interest. Adjust p-values in case of multiple hypothesis testing (*see* **Note 11**).

(k) Generate classification models based on one ore more selected populations or phenotypes (usually, this would be multivariate classifiers, e.g., a logistic regression model, linear discriminant function, or self-learning algorithm, such as a support vector machine [26]; several advanced packages are available in R). Determine robustness of classifier in terms of sensitivity to variations within the training set by internal cross-validation or bootstrapping procedures to get confidence intervals for p-values and effect sizes.

(l) Externally validate the model or trained classifier (new hypothesis!) by testing its predictive performance on a new, independent set of samples taken from the patient population. Ideally, this is prospectively performed in a new randomized blinded clinical trial. Do not underpower the study (calculate necessary sample size, *see* **Note 12**).

4 Notes

1. Different batches of calibration beads such as Flow-Set Pro beads do vary in their mean fluorescence intensity (MFI) values, thus for consistent results within one project work with the same batch of beads. Otherwise, cytometer settings have to be translated between batches to new protocol files.
2. Stain samples within at least 8 h of blood collection, extended time between blood collection and staining results in high-performance variability.
3. Store at 4 °C in the dark until measurements, low variability is achieved when measured within 4 h after staining.

4. Whole blood staining of B cell subsets requires pre-washing of serum antibodies with PBS or lysis solution, which would otherwise interfere with surface IgM and IgG staining.
5. If cell pellet stays red repeat lysis. Pay attention blood samples are drawn at correct volumes. Performance of lysis solutions may also vary according to different batches. Report back to company if performance is unsatisfactory. Unsatisfactory lysis is also influenced by blood volume within collection tube.
6. Use median fluorescence intensity (grey dashed line in Fig. 2) as the threshold for autofluorescence in channel two may be higher for the positive population (red dashed line in Fig. 2), and thus adjusting for the mean fluorescence intensity could result in over-compensation.
7. Normalize systematic between-sample variation (as introduced by instrumentation differences and technical variation during sample acquisition) by aligning prominent features (landmarks) in the raw data on a per-channel basis [32].
8. Beware of piling of events (data points) on the margins of the data range, as, e.g., introduced by too high/low PMT values or insufficient transformation of compensated data to a logarithmic scale. These "boundary events" would contaminate any clustering and should be removed in advance.
9. Clustering algorithms, such as *SPADE*, are ideally suited to detect rare populations (leaf nodes) that are phenotypically different (i.e., in terms of the expression of specific markers). However, if a cohort of samples is rather uniform in cellular phenotypes, relevant, i.e., predictive differences arise from cell distributions (i.e., compositions) in complex phenotypes that are difficult to be interpreted and re-identified by a (hierarchical) gating strategy. The *flowType/RchyOptimyx* pipeline addresses this shortcoming, enabling the annotation and condensation of a large number of identified phenotypes into a single, most important hierarchy [33] Table 3.
10. Perform visual inspection of clusters/populations in conventional dot plots and assess the validity of the result ("does the

Table 3
Cellular phenotypes as clinical biomarkers

Type of biomarker	Clinical application
Diagnostic	To define presence, activity, or absence of a disease
Prognostic/predictive	To predict outcome of a disease or therapy
Monitoring	To asses treatment efficacy and safety
Intrinsic risk profile	To stratify/to personalize therapy

cluster make sense?", i.e., do mutually exclusive cell subsets fall in one cluster). You might have to tune input parameters of the algorithm (e.g., provide optimal *k*-number of clusters to resolve all possible cell populations, add some noise to avoid singularity issues, etc.) and rerun the clustering.

11. Test as appropriate. Discriminative properties of identified subsets might also be scored by evaluating the area under the receiver operating characteristics curve (AUC) [34]. If a multivariate classifier is to be constructed, consider regularization techniques to prevent overfitting of the model and improve predictive performance [35, 36].
12. Consult a statistician for trial design and sample size calculation.

Acknowledgement

This work was supported by the EU FP7 grant "The ONE Study" and the EU COST initiative "A-FACCT" provided to Birgit Sawitzki.

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Chapter 16

The Aryl Hydrocarbon Receptor in Immunity: Tools and Potential

Charlotte Esser

Abstract

The signaling pathway of the evolutionary old transcription factor AhR is inducible by a number of small molecular weight chemicals, including toxicants such as polycyclic aromatic hydrocarbons, bacterial toxic pigments, and physiological compounds such as tryptophan derivatives or dietary indoles. AhR activation is of immunological importance, but at the same time mediates toxicity of environmental pollutants, such as immunosuppression by dioxins. Measuring AhR activity and identification of ligands is thus of great interest for a variety of research fields. In this chapter, I briefly introduce the AhR signaling pathway, its role in immunology, and the tools and assays needed to analyze AhR signaling. Both are also needed when therapeutic applications are envisioned.

Key words Aryl hydrocarbon receptor, PAS-bHLH proteins, T cells, Innate lymphoid cells, AhR ligand binding and activation assays, TCDD, Immunotoxicity, Dioxin

1 Introduction

Interaction with the environment and building meaningful physiological responses is pivotal for organisms. In fact, much in the science of biology is about the study of cellular differentiation and interaction. Signaling and induction of gene expression are at the core of differentiation and response to external triggers. Signaling is mediated via signaling molecules and their receptors. Major signaling pathways are known. Surface receptor (e.g. cytokine receptors, G proteins) mediated transmission eventually amplify a signal via a cascade of downstream events (such as MAP kinase signaling). In contrast, nuclear receptors (e.g. steroid receptor or thyroid receptors) are transcription factors themselves. The aryl hydrocarbon receptor (AhR) is a nuclear receptor which can sense and respond to certain chemicals. AhR has been studied for a long time by toxicologists because it binds to and mediates toxicity to the environmental pollutant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and other, often anthropogenic, polycyclic aromatic

Maria Cristina Cuturi and Ignacio Anegon (eds.), *Suppression and Regulation of Immune Responses: Methods and Protocols, Volume II*, Methods in Molecular Biology, vol. 1371, DOI 10.1007/978-1-4939-3139-2_16,

hydrocarbons (PAH). However, it has always been considered unlikely that AhR has evolved to recognize a modern environmental pollutant. In recent years, the physiological functions of AhR signaling have been studied increasingly and revealed numerous pivotal functions of AhR for cell differentiation, proliferation, and function of immune cells [1–3]. AhR might also be a pattern recognition factor for bacterial pathogens [4]. AhR research is highly interdisciplinary. This chapter provides a discussion on the role for AhR in immunity and major tools in measuring and assessing AhR activation. Both are needed when therapeutic applications are envisioned.

2 The Family of PAS-bHLH Proteins

The AhR belongs to the family of Per-ARNT-Sim-basic-helix-loop-helix (PAS-bHLH) proteins [5, 6], one of the three main families of bHLH proteins. PAS-bHLH proteins are transcriptional regulators controlling essential gene expression in adaptive responses. The acronym PAS indicates a domain, which was first identified in the drosophila proteins PER and SIM, and in ARNT. The PAS domain is common for proteins which can sense environmental clues, such as oxygen in the case of the PAS-bHLH member HIF-1α, or polycyclic aromatic hydrocarbons in the case of the AhR [7]. Also bacterial PAS-containing proteins are known, e.g. redox sensor DOS from *Escherichia coli* [8]. Some plants use PAS domain containing proteins for photoreception [9]. The PAS domain is approximately 300 amino acids long and some PAS-bHLH proteins have two degenerate repeats, PAS-A and PAS-B. Characteristically, PAS-bHLH proteins form functional homo- or heterodimers via their PAS domains. Recently, the murine PAS-A domain has been crystallized [10], and the authors could detail the heterodimerization of the AhR with its partner molecule AhR-nuclear translocator (ARNT) via PAS. Also, the PAS domain includes the ligand binding domain (LBD). The LBD appears spatially conserved. AhR binds its ligand in the PAS-B domain [11, 12]. PAS-bHLH proteins are evolutionary old, members exist in both vertebrates and invertebrates, e.g. in the nematode *Caenorhabditis elegans*. A crystal structure of the full AhR has not been reported, although it would be of enormous interest. Figure 1 shows the basic sections of AhR protein. Table 1 lists members of PAS-bHLH proteins.

3 Biochemistry of AhR Signaling

The biochemistry of so-called canonical signaling via AhR is well-known (reviewed by [13]). AhR resides in the cytoplasm in a multi-protein complex, which consists of two heatshock protein

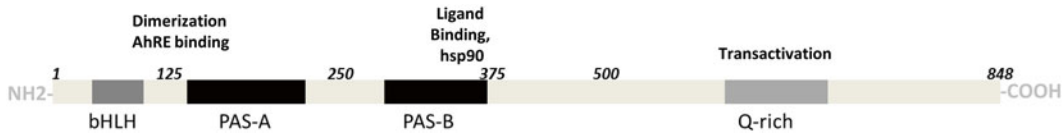

Fig. 1 Graphic scheme of domains in AhR. In humans, AhR is 848 amino acids long. A basic amino acid stretch is placed near the N-terminus, followed by a domain with two alpha-helices connected by a loop. This region binds to DNA and is important for nuclear translocation as well. At around amino acid 120 the first PAS domain begins. PAS domains are about 100 amino acids long. Dimerization with ARNT, ligand binding (at PAS-B), and also attachment with chaperoning proteins such as AIP occur in this region. Finally, toward the C-terminus a transactivation domain is found, necessary for the transcription factor activity of AhR

Table 1
Some members of PAS-bHLH protein family

"Sensor" Class I (class α)	Major function	Mouse model(s) available[a]	"Partner" Class II (class β)	Mouse model(s) available[a]
AhR	Chemical sensing/ immunity, metabolism	Yes	ARNT (=HIF1β)	Yes
AhRR	Suppression of AhR	Yes	ARNT2	Yes
HIF1α	Hypoxia sensing	Yes	BMAL1 (ARNTL1; MOP3)	Yes
HIF2α		Yes	BMAL2 (ANRTL2)	
HIF3α (IPAS)		–		
Sim1	Embryogenesis			
Sim2		Yes		
Clock	Circadian rhythm			
Per (lacks bHLH)		Yes		
NPAS2				
NPAS1	Neurogenesis			
NPAS3	Suppression of HIF			
NPAS4	Memory	Yes		

[a]To date

(hsp)-90 molecules, co-chaperon p23, and the immunophilin-related AhR-interacting protein (AIP, formerly known as XAP2 or ARA9). Hsp90 molecules prevent proteolytic degradation, while AIP prevents binding of the molecule importin [14], and thus premature nuclear import. The AhR complex disintegrates once a ligand binds into the AhR ligand binding pocket, and at the same time a nuclear translocation site (NLS) is exposed, and AhR is imported into the nucleus [15, 16]. Ligands of AhR can be either present in the cells, or must cross the cell membrane (and therefore are likely to be very lipophilic). There is no known AhR ligand transporter system, but import apparently requires dephosphorylation in

the NLS region [16]. Due to the omnipresence of ligands, a constitutive activity of AhR must be assumed. Within the nucleus, AhR dimerizes with ARNT molecules and binds to a short DNA sequence with the substitution intolerant core sequence (5′-GCGTG-3′). This sequence is called "dioxin-responsive element" (DRE), "xenobiotic response element" (XRE), or AhR-responsive element (AhRE). To initiate transcription, co-activators (such as SP1, NCOA1, p300) are recruited and chromatin structure changes. Eventually specific and general transcription factors enable that RNA polymerase II starts transcribing the gene [15, 17–20]. AhR-induced gene transcription is highly cell- and tissue-specific [21–23]. Many genes contain putative AhREs, but only few are actually targeted by AhR. The specificity is controlled by factors such as DNA accessibility, AhRE sequence and placement within promoter and other promoter elements, or availability of cofactors. Nonetheless, much of this is not understood and requires further research.

In addition to the canonical gene induction by AhR:ARNT, interactions of AhR with proteins from other signaling pathways have been described in recent years, most notably in cells of the immune system. AhR can associate with a number of proteins, including retinoblastoma protein, NFκB, STATs, c-maf, or β-catenin [24–31]. Apparently, this ability to tie into other signaling pathways allows for great flexibility and builds a network of cellular responses in cell proliferation, development, or inflammation. The interactions are highly cell- and situation-specific. Finally, purely cytoplasmic events can be initiated by dissociation of the AhR complex. A rapid increase in Ca^{2+} concentration or phosphorylation of the EGF receptor by c-src has been described in this context [32, 33].

4 Immunosuppression by TCDD and PAHs

PAHs are persistent in the environment and continue to be a regulatory challenge and ecological concern [34]. Measures have been taken in the last decades to remove PAHs from the environment but much remains to be done. New sources of pollution, such as electronic waste reclamation in developing countries have emerged, which lead to intolerably high intakes of toxic equivalents for workers and people living in the respective areas [35]. These new pollution scenarios must be dealt with by national governments in accordance with international conventions.[1] Toxicological assessment looks at various endpoints. One of them is immunotoxicity. Immunosuppression is caused by very low doses of TCDD in laboratory animals [36–38]. TCDD has a broad range of effects,

[1] http://chm.pops.int/Convention/ConventionText/tabid/2232/Default.aspx (accessed June 8, 2015).

causing atrophy of secondary lymphoid organs (thymus, lymph node, spleen) and functional impairment of many immune cells [39–42]. As a result, TCDD is immunosuppressive on the systemic level. Epidemiological evidence of TCDD immunotoxicty in humans was gathered after major poisoning incidents, such as the release of TCDD into the environment in 1976 after an explosion in a plant in Seveso, Italy. However, results were sometimes conflicting, and correlation with exposure is not always available. In general though, the data support the view that the human immune system is a target of dioxin-like substances [43]. Similarly, in vitro studies with human cells have shown that immune functions are adversely affected by dioxins, albeit a robust human biomarker for "immune competence impairment" is still lacking. This remains a major challenge for immunotoxicologists.

5 AhR and Immunity

Studies of AhR-deficient mice have highlighted its role for differentiation and function of immune cells. AhR is expressed highly in several hematopoietic cells from both the innate and adaptive immune system. Microarrays and studies using cell sorting combined with real-time PCR and Western blotting have identified Lin-Sca$^+$ and Sca$^-$ progenitor cells in bone marrow (BM), double-negative (CD4$^-$CD8$^-$) DN4 cells in thymus, CD4$^+$Th17 cells, innate lymphoid cells type 3 (ILC3), BM-derived dendritic cells (DC), γδ T cells, and Langerhans cells (LC) as subpopulations with high AhR levels [44–51]. Also keratinocytes, mast cells, and immune cells of the skin express AhR [52]. Dendritic cells (DC) and γδ T cells have simultaneously a constitutively high expression of AhR repressor (AhRR). The significance of this is not known.

5.1 T Cells

T cells are part of the adaptive immune system. Naïve T cells differentiate upon recognition of their cognate antigen and co-stimulatory signals provided by antigen-presenting cells (APC). Cytotoxic CD8+ T cells are capable of killing infected cells or cancer cells. T helper (Th) cells, on the other hand, orchestrate specific and innate immune responses by secretion of cytokines; for instance they help B cells to differentiate and undergo immunoglobulin class switching, and provide pro-inflammatory or immunosuppressive cytokines for other immune cells. Differentiation from naïve CD4+ T cells into T helper (Th) 1, Th2, or Th17 cells is driven by combinations of cytokines in the micromilieu, which are also provided by APC. AhR expression is not equal among T-cell subsets, or indeed other immune cells. It remains debated whether high AhR expression levels are indicative of physiological importance. However, AhR expression levels can be inducible in T cells and other cells [53–55].

One T-cell subset, Th17 cells, expresses higher constitutive amounts than other T helper cells [47]. Under in vitro Th17 differentiating culture conditions, AhR ligands promote the generation of Th17 [56]. It was further shown that AhR is needed for the expansion of this subset and secretion of IL-22 by Th17 cells [47, 57]. As IL-22 is very important for fighting bacterial infections, lack of AhR can lead to high susceptibility to certain infection [51]. However, Th17 cells and their cytokines are also known for their contribution to tissue destruction in autoimmunity. Unexpectedly, in experimentally induced autoimmunity models exposure to persistent and easily degradable AhR ligands (TCDD, ITE, or FICZ) ameliorated the disease, rather than exacerbating it [57–60]. Disease amelioration was explained by a shift toward generation of regulatory T-cell subsets (Treg). Likely, this is not a direct AhR effect. First, because evidence for induction of FoxP3 by AhR is inconclusive, and second, because mice with a constitutively active AhR in T cells have no increase in Treg [57, 60, 61]. It could be a question of a tolerogenic cytokine micromilieu generated by AhR effects on DC [62, 63]. Clearly, the in vivo situation is more complex, i.e. AhR ligands may induce Th17 directly, but Treg indirectly via DC, and this is balanced out by other parameters such as onset, route of exposure, immune status, and so on.

Regulatory T cells (Treg) secrete IL-10, an immunosuppressive cytokine, which also helps prevent tissue damage. Inducible Treg, natural Treg, and regulatory Tr1 cells were shown to be increased in mice injected with AhR ligands [29, 47, 57, 58, 64]. AhR cooperates with c-Maf in Tr1 cells to induce IL-10 transcription [29, 65]. Kynurenines, tryptophan metabolites, and high-affinity endogenous AhR ligands have been reported to promote Treg formation [57, 63, 66]. Thus, AhR is involved in the balance between Treg and Th17. However, there are still many unknowns, and more research is necessary if this is to be pharmacologically exploited. In particular, understanding ligand-specific and cell-specific interference with immune responses will be pivotal for any therapeutic approach [67].

5.2 Innate Lymphoid Cells

DC are professional antigen-presenting cells, which can sense pathogenic challenges via their Toll-like receptors. DC secrete cytokines upon antigen uptake and thereby generate either a tolerogenic or inflammatory micromilieu, adapted to the type of pathogen and the immunological situation. Their activities thus range from ensuring immune tolerance against dietary antigens to the initiation of a potent immune response upon entering bacteria into skin wounds. Of note, DC express high levels of AhR. In LC, AhR is needed for maturation and function of the cells [49]. Also, expression of the immunosuppressive enzyme indolamine-2,3-dioxygenase (IDO) by DC needs the presence of AhR, and is driven by kynurenine, a ligand of AhR which is produced by IDO;

AhR is in this case a negative regulator of immunogenicity [49, 63]. Triggering of immunosuppression via production of kynurenine as AhR ligands is even used by glioblastoma cancer cells to evade immunity [68]. Consequently, some researchers have looked at the potential of AhR ligands to manipulate immune responses. For instance, one such compound, VAF347, can suppress allergies or suppress graft rejection in a mouse model [62].

AhR signaling has recently been shown to be important for the differentiation and function of other cells of the innate immune system as well. AhR and AhR signaling is necessary for γδ T cells, innate lymphoid cells of the gut and NK cells. Its presence is required for proliferation and expansion in the respective tissues, and for secretion of IL17 and IL22. AhR-deficient mice thus lack important immune cells in their gut and skin, with potentially devastating consequences during bacterial infection and inflammation [45, 51, 53, 59, 69].

5.3 Epithelial Cells

The epithelia of the gut, skin, lung, and genitals present barriers to the environment. They are the first line of defense against unwanted chemicals, but also must allow some passage of chemicals, e.g., from the diet. Maybe not surprisingly, AhR expression is high in most cells of the epithelia, at least as far as analyzed. Immune cells and epithelial cells of the skin, gut, and lung have high AhR levels (reviewed in Esser and Rannug, 2015). For many of these cells, AhR was shown to be important for specific cell responses, such as the ultraviolet (UV) B stress response in keratinocytes of the skin [32, 70], or proliferation of ILC3 in the gut [51]. Intriguingly, ILC3 proliferation is also impaired when the AhR ligands are removed from the diet, highlighting that the AhR signaling evolved as a sensor for environmental triggers.

5.4 Therapeutic Potential

Immunotoxicology and immunopharmacology are two sides of one coin. Both analyze and describe how chemicals change immune responses. The immune system is very complex and thus the search for drugs which can be used in specific situations reflects this complexity. AhR activity is modulated by the type and affinity of ligands, as well as the target cell type. Persistent or short-term activation of the AhR by ligands can lead to changes in immunity, as known from the effects of TCDD and other polycyclic aromatic hydrocarbons, and from studies using FICZ or other endogenous ligands. As described above 4, immunosuppression is a hallmark of TCDD exposure. TCDD affects numerous immune cells, and similar effects of any AhR ligands ought to be considered carefully. A number of chemicals have been proposed as potential drugs, but so far no clinical trials have been reported. Of particular interest may be drugs, which are already marketed for certain diseases (and thus have undergone phase I and II trials), and have later been identified as AhR activators and thus maybe eligible for new fields of

application. Tranilast is one such example. Originally used as a drug for allergic diseases, it is now suggested as a breast cancer drug [71]. StemRegenin was found to promote proliferation of HSC [72], and kynurenine inhibitors as cancer drugs [68]. UVB irradiation-induced skin damage is dampened by the chemical BDDI [73], and coal tar, a mixture containing many AhR ligands, is a long-standing effective treatment for psoriasis, an inflammatory skin disease. With an ever-increasing knowledge of AhR ligands and their biochemistry and pharmacokinetics, the stage is set, however, for AhR-signaling related drugs. Conceivably, the situation in vivo integrates AhR activation in a more complex fashion than deduced from in vitro data, and in vitro data must be viewed with caution [60].

6 Major Tools

Research on AhR function and detection of novel ligands requires a range of tools. In the following text, I briefly describe such tools and how they can be used in AhR research.

6.1 Anti-AhR Antibodies

More than 200 monoclonal antibodies (mAb) against AhR are currently commercially available. In contrast, only a handful of polyclonal anti-AhR antisera are sold. The majority of anti-AhR mAbs are raised against short peptides from the N-Terminus of AhR. Fewer mAbs exist, which were developed using peptides from the C-terminus of AhR or against the phosphorylated form of AhR (e.g. against pSer-36). Phosphorylation of AhR contributes to nuclear import and DNA binding [16, 74]. Mouse and human AhR have about 80 % homology. Because of this high cross-species homology of AhR, anti-AhR antibodies are often cross-reactive and will detect AhR from human and several "laboratory" animals/rodents.

Before choosing and buying an antibody for AhR detection, it is useful to consider the way the antibody has been quality tested. Because most cells contain ARNT, another member of the PAS-bHLH family with sequence similarity to AhR, cross-reactivity is a risk. Quality tests thus should go beyond showing the size of the "anti-AhR" antibody on a Western blot. Rigorous negative controls must be done as well. The gold standard is testing the antibody or antiserum in question on AhR-negative cells or tissues. Thus Western blots should be done using cell lysates from AhR-negative/low tissues, lysates from cells from AhR knock-out mice, or siRNA knock-down cells. Similarly, for immunohistochemistry, a control with AhR-negative cells or tissues should be provided by the company and done as a routine control when using the antibody.

6.2 AhR Ligand Binding and Activation: Assays and Cell Lines

Identification of AhR ligands and their affinities/capacities to induce AhR-dependent transcription is an important tool in the search for therapeutic ligands, or evaluation of environmental exposure [16, 75]. Many endogenous and endogenous potential AhR ligands have been identified [11]. Ligand binding is the first and decisive step in AhR activation. The affinities between AhR and its ligands are relevant for the outcome of activation of AhR. Toxicological research has used this fact for developing the Toxic Equivalent Factor (TEF) concept, where 2,3,7,8-TCDD, the substance with the highest affinity to AhR, is assigned the value "1," and other substances get factors in relation to their affinity [76, 77]. This then allows describing the toxicity of a chemical mixture, albeit the metabolic stability of a substance and AhR expression levels also influences the toxicity in biological scenarios [78, 79].

Both direct affinity of an AhR ligand to AhR, and the ability to mediate transcription of AhR-dependent genes—most often *cyp1a1* is the gene of choice—can be measured. The latter has to be interpreted with greater caution, excluding indirect gene induction mechanism and the involvement of other transcription factors, such as retinoid X receptor, NF-κB, and others [80–83]. Identification of a true AhR ligand or inhibitor is not trivial [84]. Ligand binding, capacity to trigger nuclear translocation, AhRE binding, and eventually gene transcription are steps in the signaling pathway. Ideally, all of these are measured. Several methods exist, which are described briefly below. An example can be found here [85], where a new class of AhR ligands was recently identified with immune-modulating potential.

6.3 Measuring Gene Induction

For measuring activation of AhR transcriptional activity (rather than direct ligand binding), gene induction is the method of choice. The cytochrome P4501A1 (*cyp1a1*) gene has several DREs in its promoter [21], and is often used for assessing the AhR-activating ability of a ligand. Measurements are done by RT-PCR. Liver has high AhR expression levels, making liver-derived cells such as HepG2 (human), H4IIE (rat), or Hepa1c1 (mouse) suitable and common tools. For control purposes, one can also assay cell lines which have lost or deleted AhR or ARNT. A set of murine liver hepatoma cells (Hepa1c1c7 (wild-type), Hepa1c12 (AhR-deficient), Hepa1c4 (ARNT deficient)) has been published by Oliver Hankinson many years ago [86]. In addition, a transient transfection with respective siRNAs is a fast and easy alternative to prove the involvement of AhR and/or ARNT in the regulation of a certain gene of interest.

6.3.1 CYP1 Activity Assays

Rather than CYP450 induction, enzyme activity of CYP1 isoenzymes is often measured. In the so-called ethoxyresorufin-O-deethylase (EROD) assay, 7-ethoxyresorufin is preferentially converted into

resorufin by CYP1A1 won from cell lysates treated with putative AhR ligands. For a more specific measurement of CYP1A2 activity, the methoxyresorufin-*O*-deethylase (MROD) assay, with 7-methoxyresorufin serving as substrate, is often used. The EROD/MROD product—if present—can easily be measured fluorometrically, and the 50 % effective concentration (EC_{50}) values quantified.

6.3.2 Luciferase Reporter Assay

Reporter gene assays are often used in lieu of affinity as well. AhR-expressing cells are stably or transiently transfected with a plasmid reporter vector containing *Photinus* luciferase under the control of a DRE-sequences containing promoter (e.g. derived from rat *cyp1a1*). These cells can then be treated with putative AhR ligands, and AhR activation measured as luminescence. In transient transfection experiments, parallel transfection with a plasmid coding for a different luciferase (e.g. from *Renilla* under a strong constitutive promoter) should be used. The so-called CALUX assay consists of stable transfectants of the plasmid pGudLuc1.1 into rat hepatoma cell line H4IIE [87–90]. In Fig. 2a, the scheme of such a reporter plasmid is shown. The pGudLuc plasmid contains four functional DREs from the murine *cyp1a1* gene that confer TCDD responsiveness upon a MMTV promoter and adjacent luciferase gene. A human HepG2 cell line with a luciferase reporter plasmid was described as well [89]. A dose–response curve can then be derived which gives EC_{50} values (Fig. 2b). These assays are interesting in particular because they can simultaneously assess and quantify the presence of agonizing and antagonizing AhR ligands in samples, including mixtures. They are very useful for screening and monitoring, especially in environmental studies. In a note of caution, inhibition of luciferase activity by the test substance must be excluded.

6.3.3 Nuclear Translocation

To study translocation of AhR into the nucleus, another important parameter of AhR activation upon ligand binding, an expression vector plasmid has been developed. It contains AhR fused to a fluorescent EGFP gene in the plasmid pEGFP-C1 [32]. Cells which are transfected with this plasmid can be treated with the AhR ligand in question and the translocation of the AhR-EGFP fusion protein into the nucleus can then be followed in a fluorescent microscope. Nuclear translocation can also be assessed in Western blots by comparing band intensity of nuclear versus cytosolic fractions upon ligand treatment of the cells. Finally, immunohistochemistry with a sensitive antibody can reveal nuclear translocation.

6.3.4 Competition Assay

The above methods measure indirectly, whether AhR activity leads to transcription. Varying background levels of the natural high-affinity ligand 6-formylindolo[3,2-*b*]carbazole (FICZ) in cell culture media leads to a particular problem for assays of AhR activation in cultured cells [91, 92]. FICZ binds to the AhR with very

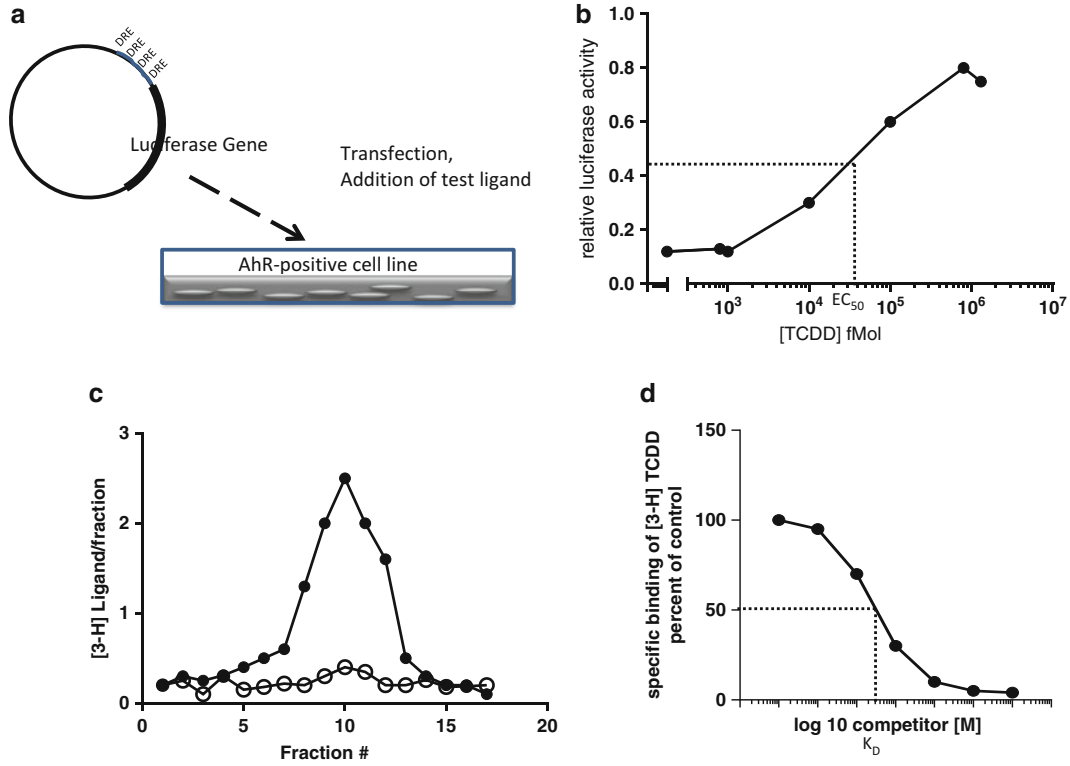

Fig. 2 Measuring AhR activity and AhR ligand affinity. A number of methods to screen for AhR activity and determine ligand binding affinity exist, as described in the text. (**a**) The luciferase reporter assay. A plasmid with the gene for luciferase under the control of a DRE-containing promoter is transfected transiently or stably into an AhR-proficient cell line. The ligand or environmental sample to be tested is added to the transfected cells. Finally, luciferase enzyme activity is measured as luminescence after addition of luciferin as the substrate. (**b**) The intensity of luminescence is correlated to ligand concentration, and can be expressed as EC_{50}. Note that the assay does not allow comparing absolute ligand affinities across labs, unless conditions were exactly the same. (**c**) Proving ligand binding—rather than induction of AhR-dependent transcription—requires biochemical competition assay. Radioactively labeled ligand, e.g. TCDD, is incubated with AhR-containing cell lysates and the amount of bound activity measured across fractions collected from a sucrose gradient. Cold TCDD is added in excess to show that binding has indeed occurred. (**d**) A graph derived from a competition assay, where binding is expressed in percentages across the molar input of ligand. The absolute affinity K_D in molar concentration can then be read off the graph, and compared between various ligands

high affinity ($<10^{-12}$ M), efficiently induces *cyp1a1* transcription and is quickly degraded by CYP1A1 enzyme activity [93]. Chemicals which inhibit CYP1A1 could thus appear as AhR activators in all the assays above [94]. Measuring direct binding of a putative ligand in competitive binding assays is therefore the only way to prove that a given chemical is an AhR ligand [95, 96]. Serial dilutions of the competitors to be tested are added to AhR-containing liver cytosol and incubated with radioactively labeled TCDD ([^{3}H]-TCDD). To control for nonspecific binding, samples are treated with radioactive TCDD together with an excess

amount of "cold" TCDD or TDCF. Unbound radioactive TCDD is removed by dextran-charcoal and the remaining solution separated on a linear sucrose gradient. Fractions are collected and the radioactivity in the fractions is determined by liquid scintillation counting. Finally, the specific binding to the AhR is calculated by subtracting the amount of radioactivity in the fractions containing radioactive TCDD together with the excess "cold" TCDD from the activity in the fractions containing radioactive TCDD [75]. Binding affinity can then be determined by plotting the specific binding relative to the concentrations of the competitor (Fig. 2c, d). Several different protocols exist and it is common that [^{125}I]2-iodo-7,8-dibromodibenzo-p-dioxin, DBDD, is used instead of [^{3}H]-TCDD. Alternatively, the hydroxyapatite (HAP) assay can be done, which was first described in 1982 [97], and has been modified since [98]. In this assay, bound radioactivity is retrieved from the mixture with hydroxyapatite. Aliquots of cytosol are incubated with [^{3}H]TCDD and different concentrations of the ligand to be tested. Thereafter, hydroxyapatite suspension is added to the different reaction mixtures and incubated. The suspension is pelleted, washed, and measured in a scintillation counter. Displacement of [^{3}H]-TCDD by the test ligand is then calculated, and corrected for nonspecific binding [99].

6.3.5 Chromatin Immunoprecipitation Assay

This assay directly identifies the binding of a transcription factor to a promoter such as AhR:ARNT to the AhRE element. Briefly, cells (approximately 1×10^6 are needed per sample) are incubated with ligand, then fixed with, e.g. 1 % formaldehyde, to crosslink DNA–protein complexes. Cells are lysed and DNA is sheared to fragments of approximately 300–500 bp size. Cell debris is cleared away, and the DNA–protein complexes are precipitated by a specific antibody against AhR. The antibody can be coupled to magnetic beads, sepharose or similar to enable and enhance precipitation or isolation from the mixture. Finally, the DNA–protein complex is "decrosslinked," protein-digested with proteinase K, and the remaining DNA fragments amplified by RT-PCR [100, 101]. PCR fragments can be electrophoresed and further identified by sequencing, cloning or on a microarray (ChIP-on-chip). The quality of the assay depends of course on the quality of the antibody (see above). ChIP assay kits are commercially available by now, and ChIP technology is quickly evolving, in particular with a view to reduce the number of cells needed, and solve specificity issues.

6.3.6 EMSA Electrophoretic Mobility Shift Assay

Finally, a somewhat older method to assess the capacity of a ligand to induce AhR-DNA binding must be included. Again, cytosolic extracts are incubated with the ligand in question. The cytosolic extracts are then incubated with radioactive [γ-32P]-labeled DNA, i.e. the AhRE consensus sequence. The products are separated and visualized on a polyacrylamide gel. Specificity of the binding of

Table 2
TCDD-resistant and susceptible rodent strains

	High susceptibility	Low susceptibility
Rats (*Rattus norvegicus*)	Long-Evans (*Turku*/AB) (inbred) LnC Sprague–Dawley (outbred)	Han/Wistar (*Kuopio*, closed colony) LnA
Mice (*Mus musculus*, *Mus spretus*)	AhRb-1 C57BL/6 AhRb-2 CH3/HeJ A/J BALB/c AhRb-3 *Mus spretus*	AhRd 129 DBA/2 NZB AKR SJL/J

AhR:ligand can be confirmed by using unlabeled AhREs as competitors. Addition of anti-AhR or anti-ARNT antibodies to induce a supershift on the gel (i.e. a slower electrophoretic movement due to the larger complex) is a further proof of the specificity of the AhR:ligand:DNA complex [98, 99].

6.4 Rats and Mice

Mice and rats with AhR alleles leading to different dioxin sensitivity are known [102, 103] (Table 2). High-susceptibility rat strains are Long-Evans (Turku/AB), Sprague–Dawley, and LnC (bred from L-ExH/W). Low-susceptibility strains are Han/Wistar and LnA [103, 104]. In rats, the difference in susceptibility is caused by a deletion in the transactivation domain, resulting in the loss of transcriptional induction of genes important for TCDD toxicity [104]. High-affinity alleles ($AhR^{b\text{-}1}$, $AhR^{b\text{-}2}$, and $AhR^{b\text{-}3}$) and a low-affinity allele (AhR^{d}) exist in mice. The d allele (e.g. found in DBA/2) differs from the b-1 allele found in C57BL by 10 nucleotides, five of which represent amino acid changes. Affinity of these AhR proteins to TCDD differs approximately by a factor of 100. Congenic C57BL/6 strains have been bred, i.e. strains which differ only at the AhR locus. Several strains of AhR gene-deleted mice have been developed independently in the 1990s. In addition, ARNT-deficient, AhR repressor-deficient, AhR repressor-EGFP reporter strains, humanized transgenics, constitutively active AhR, nuclear translocation signal (NLS) hypomorphs, floxed AhR or ARNT mice and others were developed, offering a comprehensive "zoo" of mice related to AhR signaling. Many of these lines are commercially available. For a discussion of the phenotypes see [102]. In addition, conditional mouse strains, where the AhR is deleted only in certain cell types are increasingly used in research. These mice are generated by breeding $AhR^{flox/flox}$ mice (which were generated by Christopher

Bradfield, University of Wisconsin) with an appropriate Cre-expressing mouse strain. For instance, this has been done for keratinocytes, liver cells, gut epithelial cells, recombination activation gene (RAG)-positive cells, and more [45, 51, 105, 106].

Acknowledgement

I am indebted to Agneta and Ulf Rannug, Nancy Kerkvliet, and Thomas Haarmann-Stemmann for comments and suggestions, and to the Deutsche Forschungsgemeinschaft for financial support.

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Maria Cristina Cuturi and Ignacio Anegon (eds.), *Suppression and Regulation of Immune Responses: Methods and Protocols, Volume II*, Methods in Molecular Biology, vol. 1371, DOI 10.1007/978-1-4939-3139-2, © Springer Science+Business Media New York 2016

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