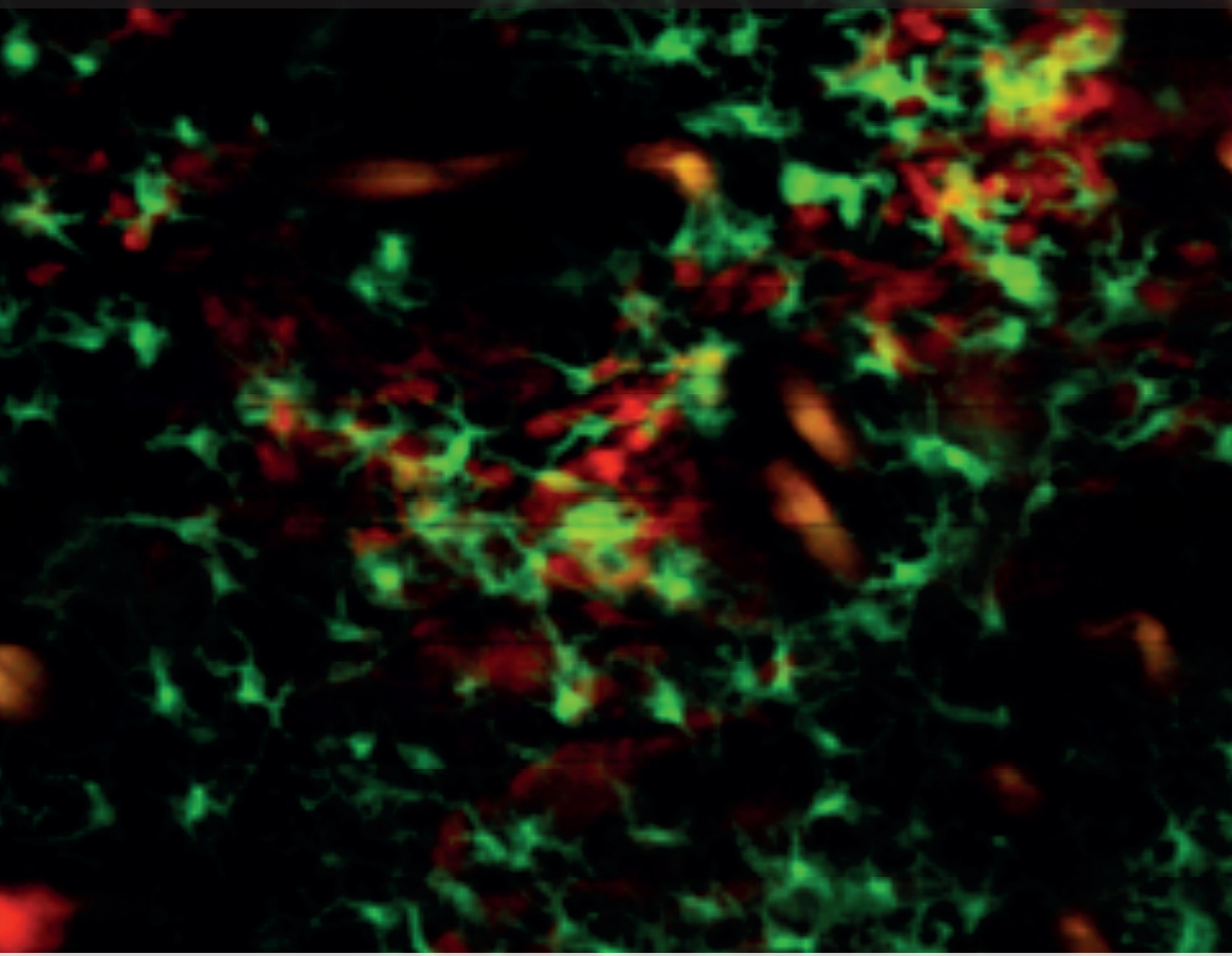
ALLORECOGNITION BY LEUKOCYTES OF THE ADAPTIVE IMMUNE SYSTEM

EDITED BY: Gilles Benichou and James Kim
PUBLISHED IN: Frontiers in Immunology



frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88945-386-3
DOI 10.3389/978-2-88945-386-3

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

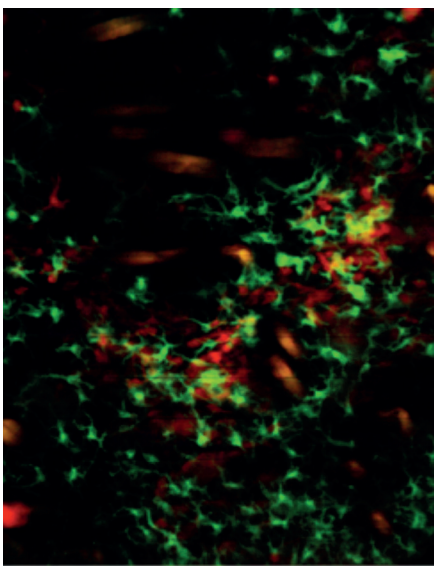
Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: **researchtopics@frontiersin.org**

ALLORECOGNITION BY LEUKOCYTES OF THE ADAPTIVE IMMUNE SYSTEM

Topic Editors:
Gilles Benichou, Massachusetts General Hospital and Harvard Medical School, United States
James Kim, Massachusetts General Hospital and Harvard Medical School, United States



This picture shows dendritic cells in a skin graft.

Image by Dr. Georges Tocco and Dr. Seok-Hyun Yun

The term allorecognition refers to the series of mechanisms used by an individual's immune system to distinguish its own cells and tissues from those of another individual belonging to the same species. During evolution, different cells and molecules of both innate and adaptive immune systems have been selected to recognize and respond to antigens expressed by allogeneic cells, but not autologous cells (alloantigens). This research topic focuses on allorecognition by lymphocytes of the adaptive immune system and its involvement in rejection or tolerance of allogeneic transplants.

T and B cells recognizing alloantigens via specific receptors become activated and undergo proliferation and differentiation into different types of effector and memory cells. Allorecognition by lymphocytes occurs regularly during pregnancy upon trafficking of both maternal and fetal cells. In this setting, allorecognition triggers an alloresponse that is protective towards the fetus thus preventing abortion. Protective alloimmunity is mediated through cooperation between different lymphocytes and antigen presenting cells (APCs), as well as regulatory mediators and receptors. Likewise, certain transplants placed in organs and tissues called immune-privileged sites such as the eye, the central nervous system and the testis elicit protective rather than destructive adaptive immune responses. Therefore, under certain circumstances, allorecognition by regulatory lymphocytes (Tregs and Bregs) can lead to tolerance of alloantigens. In contrast, allorecognition by T cells in non-immune privileged sites and under inflammatory conditions leads to a destructive immune response. Indeed, after transplantation of most allogeneic organs and tissues, activation of pro-inflammatory T cells (TH1 and TH17), which recognize donor MHC proteins (direct

pathway) or peptides derived from donor MHC and minor antigens (indirect pathway), leads to graft rejection. This inflammatory response leads to the differentiation of allospecific cytotoxic T cells as well as production of donor specific antibodies by B cells, both of which contribute to the destruction of the transplant. In this Research Topic, we describe the different pathways of allorecognition by T cells involved in allograft rejection, as well as the role of different antigen presenting cells and graft-derived microvesicles (exosomes) involved in this process.

Another aspect of this Research Topic addresses the essential role of alloreactive memory T cells in allograft rejection and resistance to transplant tolerance induction in laboratory rodents, as well as non-human primates and patients. Indeed, it has become evident that laboratory mice display very few memory alloreactive T cells pre-transplantation, essentially due to the fact that they are raised in pathogen-free facilities. In contrast, primates display high frequencies of alloreactive memory T cells, either generated through prior exposure to allogeneic MHC molecules or via cross-reactivity with microbial antigens. We and others have provided ample evidence showing that this feature accounts for differences in terms of tolerance susceptibility between laboratory rodents and patients. This implies that further investigation of tolerance protocols in laboratory mice should be performed using "dirty mice" i.e., mice raised in non-sterile conditions.

In summary, this Research Topic addresses key aspects of allorecognition by lymphocytes and alloantigen presentation by dendritic cells, and specifically how these processes shape our immune system and govern the rejection or tolerance of allogeneic tissues and organs.

Citation: Benichou, G., Kim, J., eds. (2018). Allorecognition by Leukocytes of the Adaptive Immune System. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-386-3

Table of Contents

frontiers in Immunology

EDITORIAL
published: 15 November 2017
doi: 10.3389/fimmu.2017.01555

Editorial: Allorecognition by Leukocytes of the Adaptive Immune System

Gilles Benichou and James Kim*

Center for Transplantation Sciences, Department of Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, MA, United States

Keywords: allorecognition, lymphocytes, transplantation immunology, tolerance, MHC

Editorial on the Research Topic

Allorecognition by Leukocytes of the Adaptive Immune System

Allorecognition refers to a series of mechanisms by which an individual's immune system distinguishes its own cells and tissue from those of another individual belonging to the same species. This phenomenon is responsible for self–non-self recognition in both invertebrates and vertebrates. Both cells of innate and adaptive immune systems are capable of allorecognition.

In vertebrates, allogeneic cells are recognized by lymphocytes of the adaptive immune system, through interaction of alloantigens (antigens expressed by allogeneic but not autologous cells) with specific receptors (BCR and TCR for B and T lymphocytes, respectively). Such allorecognition by lymphocytes can trigger immune responses, which can lead to either rejection or acceptance of cells, tissues, and organs displaying these alloantigens. In the case of transplantation of allogeneic organs and tissues, allorecognition generally initiates an inflammatory response, which leads to the destruction and rejection of the graft. On the other hand, a number of regulatory mechanisms have been selected through evolution to suppress deleterious alloimmunity in selected situations. For instance, during pregnancy in mammalians, alloimmunity directed to paternal antigens generally prevents immune attack and abortion of the fetus by the female's immune system. Similar to the fetus, it is now established that allorecognition within certain organs such as the testis and the brain (called immune privileged) triggers specific types of immune responses protecting allogeneic cells from rejection. This phenomenon called immunological tolerance involves active processes mediated by regulatory lymphocytes and cytokines. Therefore, the nature of the cells, the environment, and the molecular mechanisms involved in allorecognition govern the fate of the immune response to allogeneic cells (rejection or tolerance).

This Topic of Frontiers offers the reader views on key aspects of the immune mechanisms underlying allorecognition by T and B cells and its relationship to the development of pro-inflammatory and regulatory responses involved in rejection or tolerance of allografts.

T cells are considered as the main driving force behind the initiation and regulation of immunity to alloantigens. The article by Jose Marino and his colleagues reviews current knowledge regarding the different pathways of alloantigen presentation, direct, indirect, and semi-direct, and their contributions to T cell alloimmunity and allograft rejection. This paper provides evidence that the role of different allorecognition pathways in allograft rejection or tolerance varies depending upon the nature of the transplant, its site of placement, and the time after transplantation. This paper also describes recent studies describing how extracellular vesicles and donor MHC cross-dressing of recipient cells influence allorecognition and allograft rejection. The paper by Gilles Benichou et al. summarizes current knowledge regarding alloresponses by memory T cells in experimental and clinical transplantation models. The article by Sehrawat and Rouse describes the interplay of

OPEN ACCESS

Edited and Reviewed by:
Antoine Toubert, Paris Diderot University, France

****Correspondence:***
Gilles Benichou
gbenichou@partners.org

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *26 October 2017*
Accepted: *31 October 2017*
Published: *15 November 2017*

Citation:
Benichou G and Kim J (2017) Editorial: Allorecognition by Leukocytes of the Adaptive Immune System. Front. Immunol. 8:1555. doi: 10.3389/fimmu.2017.01555

regulatory T cells and $CD4^+$ TH17 cells and how it influences the fate of immune responses in humans and animals. The article by Degauque and his colleagues describes different studies designed to track and identify particular T cell clones involved in allograft rejection or tolerance. Finally, the paper by Scalea et al. summarizes current knowledge of cell therapies designed to achieve T cell tolerance in transplantation and their mechanisms of action.

The concept of immune privilege is an important aspect of transplantation immunology. Indeed, certain organ and tissue transplants are less susceptible than others to inflammation and rejection. At the same time, placement of allografts in selected body sites enjoy long-term survival with no or minimal immunosuppression. Immune privilege represents a natural form of tolerance selected through evolution to prevent potentially dangerous inflammation in selected tissues and organs, such as the central nervous system. It is also an important element of the prevention of immune attack of the fetus and its abortion by the mother during pregnancy. Therefore, a better understanding of the mechanisms underlying this phenomenon may help with the design of tolerance protocols in transplantation. The paper by A. Taylor summarizes current knowledge in the field of immune privilege and addresses specific mechanisms contributing to the development and maintenance of immune privilege in the eye.

B lymphocytes play a key role in the response to and rejection of allogeneic transplants. They contribute to this process by producing antibodies against donor MHC antigens and tissue-specific autoantibodies and by serving as antigen-presenting cells for T cell activation. In addition, there is accumulating evidence showing that donor-specific antibodies and autoantibodies are involved in chronic form of allograft rejection characterized by tissue graft fibrosis and vasculopathy, a major cause of progressive organ transplant failure in clinical settings. On the other hand, recent studies have now firmly established the existence of anti-inflammatory, tolerogenic B cells (regulatory B cells or Bregs), which contribute, along with regulatory T cells, to prevent allograft rejection. The article by Michelle Hickey and colleagues provides a comprehensive overview on the generation and functions of alloantibodies directed to HLA and their role in transplant rejection. The paper by Daniel Firl et al. discusses current knowledge of different subsets of B cells, including regulatory B cells, in rejection and tolerance of allografts in experimental and clinical transplantation models.

Antigen-presenting cells, in particular dendritic cells (DCs), initiate alloimmune responses by activating T cells through the presentation of intact donor MHC molecules (direct allorecognition) or donor MHC peptides (indirect allorecognition). It is now well established that the nature of the T cell responses depends on the nature of the DCs, their degree of maturation, and their ability to deliver selected costimulatory signals to T cells. The article by Angus Thomson and colleagues reviews current knowledge regarding the tolerogenic properties of certain DCs and their potential utilization to achieve tolerance in transplantation.

The transplantation of allogeneic bone marrow and stem cells is an important aspect of clinical transplantation as it has been used for curative treatment of hematological malignancies. Unfortunately, the desired antitumor or graft-versus-leukemia effect is often accompanied with undesired side effects against healthy tissues known as graft-versus-host disease. The article by M. Griffioen and colleagues provides insights into the composition and kinetics of *in vivo* immune responses with respect to specificity, diversity, and frequency of specific T-cells and surface expression of HLA–peptide complexes and other (accessory) molecules on the target cell. It describes how the complex interplay between these factors and their environment ultimately determines the spectrum of clinical manifestations caused by immune responses after transplantation of allogeneic stem cells.

In summary, great progress has been made with regards to the description of the cells and molecules involved in allorecognition by the adaptive immune system. Furthermore, we have acquired a better understanding of the mechanisms by which these cells and molecules influence the nature of the immune response to alloantigens in health and disease. Based on this knowledge, novel strategies are being designed in an effort to manipulate alloreactivity in clinical settings, such as organ transplantation and transplantation of allogeneic bone marrow and stem cells as well as pancreatic islet cells.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

This work was funded through an NIH grant to GB, NIH R21 AI117466.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 14 December 2016
doi: 10.3389/fimmu.2016.00582

Allorecognition by T Lymphocytes and Allograft Rejection

*Jose Marino, Joshua Paster and Gilles Benichou**

Center for Transplantation Sciences, Department of Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA

Recognition of donor antigens by recipient T cells in secondary lymphoid organs initiates the adaptive inflammatory immune response leading to the rejection of allogeneic transplants. Allospecific T cells become activated through interaction of their T cell receptors with intact allogeneic major histocompatibility complex (MHC) molecules on donor cells (direct pathway) and/or donor peptides presented by self-MHC molecules on recipient antigen-presenting cells (APCs) (indirect pathway). In addition, recent studies show that alloreactive T cells can also be stimulated through recognition of allogeneic MHC molecules displayed on recipient APCs (MHC cross-dressing) after their transfer *via* cell–cell contact or through extracellular vesicles (semi-direct pathway). The specific allorecognition pathway used by T cells is dictated by intrinsic and extrinsic factors to the allograft and can influence the nature and magnitude of the alloresponse and rejection process. Consequently, various organs and tissues such as skin, cornea, and solid organ transplants are recognized differently by pro-inflammatory T cells through these distinct pathways, which may explain why these grafts are rejected in a different fashion. On the other hand, the mechanisms by which anti-inflammatory regulatory T cells (Tregs) recognize alloantigen and promote transplantation tolerance are still unclear. It is likely that thymic Tregs are activated through indirect allorecognition, while peripheral Tregs recognize alloantigens in a direct fashion. As we gain insights into the mechanisms underlying allorecognition by pro-inflammatory and Treg cells, novel strategies are being designed to prevent allograft rejection in the absence of ongoing immunosuppressive drug treatment in patients.

Keywords: allorecognition, T cells, regulatory T cells, allograft rejection, T cell tolerance, major histocompatibility complex, exosomes

OPEN ACCESS

Edited by:
Gilles Blancho, University of Nantes, France

Reviewed by:
Thomas Wekerle, Medical University of Vienna, Austria
Marcella Franquesa, Institut d'Investigació Germans Trias i Pujol, Spain

***Correspondence:**
Gilles Benichou
gbenichou@partners.org

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *15 September 2016*
Accepted: *24 November 2016*
Published: *14 December 2016*

Citation:
Marino J, Paster J and Benichou G (2016) Allorecognition by T Lymphocytes and Allograft Rejection. Front. Immunol. 7:582. doi: 10.3389/fimmu.2016.00582

INTRODUCTION

Allorecognition relates to the detection of genetically encoded polymorphisms between individual organisms of the same species by the immune system. Allorecognition has been described in nearly all multicellular phyla, including invertebrates that are devoid of an adaptive immune system (1). Indeed, certain cells of the innate immune system such as NK cells and macrophages are capable of

Abbreviations: MHC, major histocompatibility complex; APC, antigen-presenting cell; Treg, regulatory T cell; tTreg, thymic regulatory cell; pTreg, peripheral regulatory T cell; γIFN, gamma interferon; TNFα, tumor necrosis factor alpha; DST, donor-specific transfusion; DTR, diphtheria toxin receptor; TMEM, memory T cell; CTL, cytotoxic T lymphocyte; DC, dendritic cell.

self–non-self discrimination (2, 3). In vertebrates, the adaptive immune response to allogeneic cells is initiated through recognition of polymorphic proteins by T lymphocytes through their antigen receptors. Subsequent activation of pro-inflammatory allospecific T cells initiates a cascade of reactions leading to rejection of transplanted allogeneic tissues and organs. Alternatively, under particular circumstances, deletion or inhibition of alloreactive effector T cells can result in allograft acceptance or tolerance (4, 5). In this article, we review current knowledge of the different pathways underlying alloantigen recognition by different T cells subsets and examine their contributions to rejection or tolerance of allografts.

DIFFERENT MECHANISMS INVOLVED IN T CELL RECOGNITION OF ALLOANTIGENS

The following section describes the three known pathways (direct, indirect, and semi-direct) by which recipient T cells recognize donor alloantigens [major histocompatibility complex (MHC) and minor antigens] after allotransplantation.

Direct Allorecognition

Seminal studies in skin-grafted rodents support the view that early after transplantation intra-graft dendritic cells (DCs) (passenger leukocytes) migrate through lymphatics to host regional lymph nodes (LNs) (6, 7). Naïve T cells located in these LNs become activated through recognition of allogeneic MHC molecules displayed on these donor passenger leukocytes (8). This phenomenon, known as direct T cell allorecognition, initiates an inflammatory immune response leading to rapid and acute cellular rejection of skin allografts (9). Unlike conventional T cell responses to nominal protein antigens, the direct T cell alloresponse is polyclonal in that it involves a large portion of the T cell repertoire (1–10%) (10–13). Two non-mutually exclusive mechanisms have been proposed to explain this unique feature of the T cell response against allogeneic MHC molecules: the *high determinant density* and the *multiple binary complex* models (14–16). The high determinant density model postulates that each allogeneic MHC molecule on a foreign cell can be recognized by a single T cell receptor (TCR), which is focused on exposed amino acid polymorphisms of the allogeneic MHC molecule independent of the peptide bound to it. Likewise, various T cells may be activated even if each individual receptor on a given clone displays a low affinity for its ligand. The multiple binary complex model is based on the principle that each individual alloreactive T cell clone interacts with allogeneic MHC molecules bound to a defined peptide. Allo-MHC molecules being occupied by a multitude of different peptides can create many new pMHC complexes that can serve as ligands for various T cell clones. The prevalence of either model in T cell allorecognition presumably depends upon the degree of heterogeneity (structural and/or conformational) between recipient and donor MHC molecules. Unlike conventional immune responses, T cell responses to allogeneic MHC antigens can be observed *in vitro* with T cells isolated from naïve animals cultured with allogeneic irradiated cells. This so-called mixed allogeneic reaction [mixed lymphocyte reactions (MLR)] is believed to rely on the high frequency of precursor T cells capable of recognizing allogeneic MHC molecules. It is also possible, however, that the MLR may reflect the presence of alloreactive memory T cells generated after infections through cross-reactive recognition of self-MHC molecules bound to microbial peptides mimicking an allogeneic MHC–peptide complex, a phenomenon called heterologous immunity (17, 18). For instance, T cells from individuals sensitized to EBV peptides presented by self-MHC class I HLA-B8 also recognize the HLA-B4402 allogeneic MHC molecules (19). Consequently, HLA-B8 individuals display memory T cells directed to HLA-B4402 allogeneic subjects as a result of an EBV infection. The same phenomenon has also been shown in mice after exposure to LCMV and *Leishmania* parasites (17, 20, 21).

Indirect Allorecognition

Seminal studies by Singer showed that allogeneic MHC class I antigens could be presented by self-MHC class I on antigen-presenting cells (APCs) and trigger the activation of some $CD8^+$ cytotoxic T cells *in vitro*, a phenomenon referred to as cross-presentation (22). Most importantly, Lechler and Batchelor provided evidence for an alternative pathway of T cell alloresponse *in vivo* in the early 1980s (23, 24). It was observed that allosensitization could occur in the absence of donor passenger leukocytes following retransplantation of kidney grafts in rats (23, 24). Based on the assumption that donor parenchymal cells were not capable of sensitizing naïve T cells, it was proposed that host MHC class II^+ bone marrow-derived professional APCs could present alloantigens and initiate an alloresponse. In 1992, our laboratory provided definitive evidence showing that allogeneic MHC peptides were regularly presented by self-MHC class II molecules on recipient APCs and triggered the activation of $CD4^+$ T cells in the LNs of skin-grafted mice (25). The relevance of this process, called indirect allorecognition, in solid organ transplantation was documented the same year in two subsequent studies by Fabre and Suciu-Foca's groups in rats and humans, respectively (26, 27). Subsequent studies documented indirect activation of $CD8^+$ T cells after skin transplantation; the relevance of this phenomenon in the rejection process is discussed later in this article (28–30). Determinant mapping and TCR repertoire studies showed that the initial indirect response to an allograft was oligoclonal and followed the rules of immunodominance in that it was mediated by a discrete set of T cell clones directed to a few dominant determinants usually located within polymorphic regions of allogeneic MHC proteins (31, 32). However, progressively, indirect alloresponse by T cells tend to spread to new formerly cryptic allo-MHC peptides (33). Cryptic determinants correspond to peptides that are not processed and/or presented efficiently enough to trigger a T cell response after protein immunization (34). However, T cell responses to these determinants can be elicited upon peptide immunization (34). Secondary responses to formerly cryptic determinants also called antigen spreading has been documented in autoimmune disorders (35, 36) and after allotransplantation and could be involved in chronic rejection (37).

In addition to its role in allo-MHC recognition, indirect T cell recognition is considered as the main driving force being T cell responses to minor antigens (mH), which are peptides usually derived from housekeeping proteins displaying some degree of polymorphism (38). The contributions of mH to the overall indirect alloresponse by T cells and to allograft rejection are discussed later in this article. Finally, it is important to note that it is still unclear where and through which process donor antigens are taken up and processed by recipient APCs and presented to T cells after transplantation. Acquisition of donor antigens by recipient APCs may occur in the graft itself or in the host lymphoid organs through pinocytosis of shed donor proteins, phagocytosis of dead donor cells and apoptotic bodies, or *via* transfer of donor antigens through cell–cell contact or phagocytosis of extracellular vesicles secreted by donor cells.

Semi-Direct Allorecognition

It is now well established that leukocytes exchange molecules, including RNA and proteins, either *via* cell–cell contact (trogocytosis), nanotubes, or through the release of extracellular vesicles such as exosomes (39–41). For instance, T cells were shown to acquire surface immunoglobulin molecules from B cells (42) and antigens from macrophages (43). Likewise, the transfer of MHC molecules between hematopoietic cells was originally documented by Frelinger et al. (44). Acquired peptide–MHC complexes have been shown to remain at the cell surface of APCs for more than 48 h, providing ample opportunities for T cell activation (45). There is accumulating evidence suggesting that this process plays a key role in the initiation and regulation of immunity to microbes and tumors (46). Recent studies have documented the transfer of MHC class I and II molecules (MHC cross-dressing) between recipient and donor DCs after solid organ and bone marrow transplantation (40, 47, 48). At the same time, DCs that have acquired allogeneic MHC proteins *in vitro via* cell–cell contact have been shown to stimulate allospecific T cells *in vitro*, through a mechanism often referred to as *semi-direct allorecognition* (**Figure 1**) (49–51). It is conceivable that allo-MHC cross-dressing of APCs after transplantation could occur *via* cell–cell contact and through secretion of extracellular vesicles. Lechler et al. have shown that DCs and endothelial cells can acquire MHC complexes *in vitro* and *in vivo* (after DC injections) through cell–cell contact in a temperature- and energy-dependent manner. In these studies, allo-MHC cross-dressed cells induced proliferation of Ag-specific T cells *in vitro* (49–51). On the other hand, a recent study by Marino in our laboratory shows that recipient APCs having acquired donor MHC from donor exosomes trafficking from skin and heart to host lymphoid organs are involved in T cell antigen recognition and activation after allotransplantation. Most exosomes expressed preferentially allogeneic MHC class II and were derived from donor DCs and B cells, i.e., bone marrow-derived professional APCs. However, it is important to note that a significant number of MHC class II$^+$ vesicles involved in MHC cross-dressing were not derived from these cells and could potentially be secreted by activated endothelial cells, as suggested by a previous report from Lechler's laboratory (50). Altogether, these studies involving transfer of MHC antigens provide a different view of the process by which donor passenger leukocyte cells can trigger T cell alloresponses after transplantation. It is now crucial to investigate whether exosomes and allo-MHC cross-dressing are essential elements of the overall alloresponse and allograft rejection processes.

RELATIONSHIPS BETWEEN DIFFERENT PATHWAYS

Direct and indirect allorecognition represent distinct mechanisms involving different APCs, T cells, and antigen determinants. Each of these pathways can sufficiently and exclusively lead to acute rejection of fully allogeneic skin allografts (52). In certain circumstances, T cells activated directly and indirectly could either cooperate or suppress each other, a process influencing the survival of allografts. It is plausible that in recipients of MHC class I-disparate allografts, CD4$^+$ T cells activated exclusively through indirect allorecognition provide help [*via* IL-2 and gamma interferon (γIFN) secretion] for the direct activation of other CD4$^+$ T cells (three-cell cluster model) or the differentiation of CD8$^+$ cytotoxic T cells recognizing donor MHC class I peptides in a direct fashion (four-cell cluster model) (**Figure 1**). Likewise, in the absence of bone marrow-derived donor professional APCs, T cells recognizing donor MHC class I or II directly on parenchymal cells can receive costimulatory signals *via* interaction with CD80/86 or CD40 located on recipient professional APCs (activated through indirect presentation to T cells) (trans-costimulation) (**Figure 1**). At the same time, early inflammatory direct alloresponses associated with γIFN and tumor necrosis factor alpha production and subsequent induction of donor MHC class II expression on endothelial cells presumably enhances allo-MHC antigen processing by recipient APCs and indirect activation of T cells. Therefore, the direct and indirect alloresponses can act synergistically to reject an allograft.

T CELL RECOGNITION PATHWAYS INVOLVED IN ALLOGRAFT REJECTION

Many factors either intrinsic or extrinsic to the graft influence the nature and magnitude of the T cell response induced by a defined pathway of allorecognition. Consequently, the contribution of each T cell allorecognition pathway (direct or indirect) to the rejection process varies upon the nature of the tissue or organ transplanted, the site of the body where it is placed, and the immunological status of the recipient. This section describes some of the factors governing the initiation of direct and indirect alloresponses by CD4$^+$ and CD8$^+$ pro-inflammatory T cells and the rejection of allogeneic skin, corneal, and heart grafts.

T Cell Allorecognition in Skin Transplantation

Potent direct and indirect alloresponses by CD4$^+$ T cells are induced after transplantation of fully MHC-mismatched skin allografts (13). The direct alloresponse to donor MHC class II antigens by inflammatory CD4$^+$ T cells is polyclonal and leads

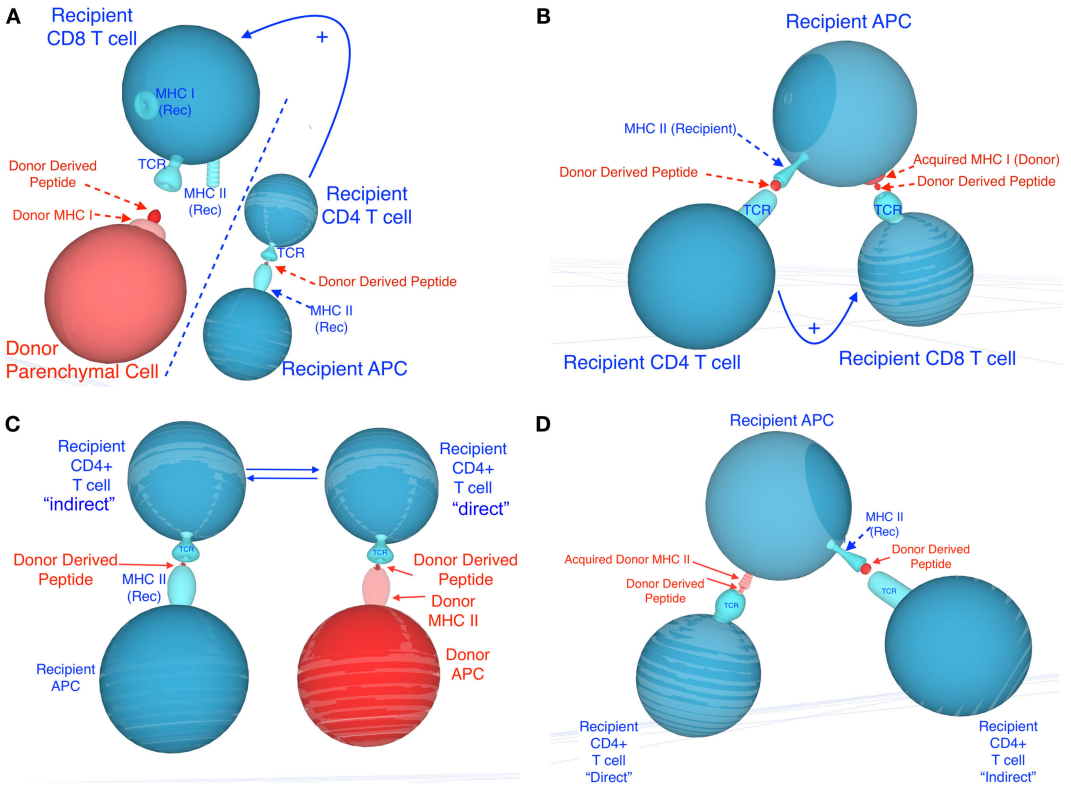


FIGURE 1 | Potential mechanisms involved in semi-direct allorecognition by recipient CD4⁺ and CD8⁺ T cells. Recipient cells are shown in blue and donor cells and peptides are shown in red. **(A)** depicts the classical four-cell cluster model in which CD4⁺ T cells activated indirectly [*via* recognition of recipient major histocompatibility complex (MHC) class II⁺ donor peptide, displayed on recipient antigen-presenting cells (APCs)] may provide help for CD8⁺ T cells activated directly (*via* recognition of donor MHC class I on donor APCs). **(C)** depicts the traditional four-cell cluster model in which CD4⁺ T cells activated indirectly (*via* recognition of recipient MHC class II⁺ donor peptide, displayed on recipient APCs) and CD4⁺ T cells activated directly (*via* recognition of donor MHC class II on donor APCs). **(B,D)** describe different mechanisms by which semi-direct allorecognition could allow for a three-cell cluster model in which different T cells recognize different antigens on the same recipient APCs. This process would optimize T cell help by CD4⁺ T cells activated indirectly for CD8⁺ T cell activated directly (recognition of acquired MHC class I) **(B)** and enhance cooperation between CD4⁺ T cells activated directly (*via* recognition of acquired donor MHC class II) and indirectly (recipient MHC class II⁺ donor peptide) **(D)**.

to the rapid rejection of skin allografts (53). Yet, studies from Auchincloss' laboratory using MHC class II-deficient skin allografts have demonstrated that the CD4⁺ T cell indirect alloresponse was sufficient on its own to cause acute skin graft rejection by providing help for the activation/differentiation of CD8⁺ cytotoxic T cells recognizing donor MHC class I directly (54, 55). This conclusion was further confirmed by experiments using recipient mice adoptively transferred with CD4⁺ T cell clones recognizing donor antigens indirectly (56). In addition, indirect responses by CD8⁺ T cells are also detectable after skin transplantation (28). Studies by Valujskikh and Heeger support the view that indirectly activated CD8⁺ T cells can reject skin allografts following recognition of self-MHC class I⁺ allopeptides present on vascular endothelial cells after replacement of donor graft vessels by recipient ones (30, 57). Therefore, both CD4⁺ and CD8⁺ T cells activated directly and indirectly are elicited after skin grafting and can lead to acute rejection of these allografts. Recent articles by Marino et al. and Smyth et al. support the view that T cells activated through direct and possibly indirect pathway after skin transplantation recognize donor MHC molecules and peptides acquired and displayed by recipient APCs (58, 59). However, the precise contribution of this phenomenon to acute rejection of these grafts remains to be evaluated. Finally, it is important to note that skin allografts that are vascularized at the time of their placement are acutely rejected at the same pace as their conventional (non-primarily vascularized) counterparts, but they do not induce an indirect alloresponse (60). This shows that graft vascularization influences the nature of the allorecognition by T cells after skin transplantation.

T Cell Allorecognition in Corneal Transplantation

In contrast to skin transplants, corneal allograft rejection is slower and is driven by minor antigens instead of MHC disparities between the host and recipient (61). This unusual feature of corneal transplantation is attributed to the facts that (1) corneal

allografts are devoid of MHC class II$^+$ APCs at the time of transplantation and (2) they are placed in the eye that is an immune-privileged site of the body (62, 63). These grafts induce indirect but no direct alloresponses by CD4$^+$ T cells, a feature presumably associated with the lack of donor MHC class II expression in the cornea (64). In addition, the indirect CD4$^+$ T cell alloresponse is directed almost exclusively to minor antigens (61). Such dominance of minor antigens is likely to rely on the low expression of MHC antigens in the cornea [absence of MHC class II and reduced MHC class I expression (65)]. Additionally, in the absence of CD4$^+$ T cell direct alloreactivity, indirect alloresponse may be biased toward mH antigens, as observed in the rejection of APC-depleted thyroid grafts (66). On the other hand, CD8$^+$ T cells activated directly against donor MHC class I are readily detected after corneal transplantation (67, 68). Although these CD8$^+$ T cells produce γIFN, they do not display cytotoxic functions (67, 68).

Only indirectly activated CD4$^+$ T cells then drive the rejection process. Interestingly, while no MHC class II$^+$ cells were originally detected in the cornea, studies by Dana's laboratory have documented the presence of DCs in the cervical LNs draining corneal allografts (69). Indeed, CD11c$^+$ DCs and CD11b$^+$ macrophages are present in the corneal epithelium (70). Interestingly, in "high-risk" recipients of corneal transplants placed in an inflamed eye bed environment (71), corneal DCs express MHC class II molecules as well as CD40, CD80, and CD86 co-receptors at the time of transplantation (71). Consequently, these allografts trigger vigorous direct alloresponses by host CD4$^+$ T cells against intact donor MHC class II molecules and are acutely rejected in a few days similar to skin grafts (71). Therefore, lack of immunogenicity of corneal DCs is not an intrinsic property of these cells, but it is due to the microenvironment of the eye. This view is supported by Niederkorn's studies showing that heterotopic corneal allografts elicit *bona fide* cytotoxic T cell (CTL) responses (72). Likewise, we have shown that corneal allografts placed subcutaneously in mice trigger CD4$^+$ T cell direct alloresponses (68). Altogether, these studies demonstrate that both intrinsic (APC contents) and extrinsic (site of placement) factors determine the fate of corneal allografts by influencing the allorecognition pathway and the nature of target alloantigens involved in the T cell response against these grafts.

T Cell Allorecognition and Rejection of Vascularized Solid Organ Transplants

Early acute rejection of cardiac and kidney allografts is essentially initiated by CD4$^+$ T cells recognizing donor MHC class II molecules in a direct fashion (73, 74). These transplants differ from skin allografts in that they are vascularized at the time of their placement (75). This is associated with a rapid trafficking of graft DCs to the host spleen presumably occurring *via* reverse transendothelial vascular migration (76, 77). In addition, some studies suggest that these allografts could be rapidly infiltrated with recipient endogenous alloreactive effector memory T cells (78, 79). These pre-existing memory T cells are present at low frequencies (5–10%) in laboratory rodents (80, 81). In contrast, primates display much higher frequencies (>50%) of alloreactive memory T cells before transplantation (82, 83). These memory T cells may be generated through mimicry with microbial antigens or prior exposure to allogeneic MHC molecules following events such as pregnancy or blood transfusion. We and others have shown that these memory T cells account for resistance to allograft tolerance induction in primates (82–85). Therefore, primarily naïve and presumably endogenous memory T cells activated in a direct fashion mediate early acute rejection of solid organ transplants. Suppression of this response by calcineurin inhibitors and other immunosuppressive agents is regularly achieved in transplanted patients, thereby allowing large-scale clinical transplantation of organs such as kidneys and livers. However, many of these transplants are ultimately lost due to chronic rejection, a process associated with progressive graft tissue fibrosis and blood vessel occlusion (86, 87). There is strong circumstantial evidence suggesting that T cells activated indirectly are responsible for chronic allograft rejection, either on their own or through the induction of alloantibody production by B cells (86–89). The relevance of this concept in clinical transplantation is supported by the detection of donor HLA DR peptide-reactive T cells in kidney-transplanted patients with chronic rejection (90). Additionally, studies by Baker et al. showed the loss of direct and maintenance of indirect alloresponses in renal allograft recipients and its implications in chronic allograft nephropathy in patients (87). Finally, recent studies by Benichou and Morelli's laboratories suggest that activation of recipient T cells through semi-direct allorecognition might represent an essential element of the immune response to and rejection of cardiac allografts in mice (58, 91). Both studies show that T cells activated *via* this pathway recognized allo-MHC molecules transferred to recipient APCs by donor exosomes released either in the heart transplant or in the recipient's lymphoid organs (58, 91). Ongoing studies are underway to assess the role of semi-direct alloreactivity in acute and chronic rejection of heart and other solid organ transplants in animal models and patients.

T CELL ALLORECOGNITION PATHWAYS IN REGULATORY TOLERANCE

Allograft tolerance, defined as long-term survival of allogeneic transplants in the absence of ongoing immunosuppressive drug treatment, can occur *via* deletion or inhibition of alloreactive T cells. This process can occur naturally, as seen in the tolerance of paternal alloantigens expressed by the fetus during pregnancy (92, 93). In addition, immune-privileged tissues such as the central nervous system and the testis are tolerogenic in that they elicit systemic tolerance to foreign antigens to which they are exposed (94–96). Various cells and mediators of the innate and adaptive immune systems have been implicated in the process of allograft tolerance (4, 96–99). Among them, regulatory T cells (Tregs) play an essential role by suppressing inflammatory responses (100–102). Tregs are CD4$^+$CD25^{high} T lymphocytes expressing FoxP3 transcription factor either constitutively (thymic Tregs or tTregs) or after

peripheral recognition of antigens (peripheral Tregs or pTregs) (100, 103, 104). In addition to their role in self-antigen tolerance, both Treg subsets can suppress inflammatory alloreactive T cells *in vitro* and *in vivo*. They inhibit alloreactivity in MLR *in vitro* (4, 96, 99, 105) and are thought to mediate transplant tolerance elicited *via* leukocyte costimulation blockade, donor-specific transfusion (4). This is supported by experiments in which inoculation of Tregs from tolerant mice to naïve mice could prolong allograft survival and even transfer tolerance (4). In addition, studies from Colvin's laboratory using FoxP3-diphtheria toxin receptor mice showed that *in vivo* deletion of Tregs abrogated ongoing tolerance to kidney allografts in mice (106). tTRegs are positively selected in the thymus medulla based on their high affinity for self-antigen pMHC complexes (107). While tTregs require TCR interaction with self-MHC class II molecules to mediate their suppress functions, they are thought to be non-antigen specific. Indeed, tTregs isolated from naïve mice can suppress T cells responding to polyclonal stimulators (anti-CD3/anti-CD28 mAbs or PMA/ionomycin) and MLR regardless of the nature of the allogeneic stimulators. The nature of the self-peptide determinants recognized for tTregs is not known. Studies from LeGuern's laboratory suggest that tTreg recognition is biased to self-MHC class II peptides bound with self-MHC class II molecules themselves (referred to as Tlo) (108). Tolerance of solid organ transplants in swine and rodents *via* allo-MHC class II transgenesis support this view (109–113). In contrast to tTregs, pTregs presumably acquire FoxP3 expression and suppressor functions through recognition of donor antigens (MHC and/or minor antigens) presented by selected APCs (immature DCs and plasmocytoid DCs) in an appropriate cytokine milieu (4, 114–117). Although activation of pTregs may be antigen specific, it is not clear whether their suppressive function follows the same rules. Therefore, both Treg subsets involved in allograft tolerance are presumably activated through recognition of peptides presented by self-MHC class II on recipient APCs, i.e., in an indirect fashion. However, the mechanisms by which they suppress alloreactive T cells and induce and/or maintain allograft tolerance are still unknown.

CONCLUDING REMARKS

It is now firmly established that the mechanisms by which T cell recognize and respond to alloantigens greatly vary upon the nature of the transplanted organ or tissue, the site of anatomical placement, and the immunological status of the host. This explains why certain transplants, such as skin allografts, which induce potent inflammatory responses by both $CD4^+$ and $CD8^+$, activated directly and indirectly, are highly immunogenic and thereby resistant to tolerance induction. In contrast, corneal allografts that elicit only indirect alloresponses by $CD4^+$ T cells are tolerogenic and often spontaneously accepted. On the other hand, early acute rejection of solid organ allografts such as hearts and kidneys is mediated essentially by T cells activated directly. While this immune response results in a potent inflammatory reaction, it is readily inhibited by calcineurin inhibitors. This explains why these drugs have been effective at achieving prolonged survival of organ allografts in patients. These treatments do not, however, efficiently suppress alloreactive memory T cells, thus precluding transplantation in patients sensitized to their potential donors (10% of patients). Most importantly, many transplanted organs are progressively lost due to chronic rejection, a process presumably initiated by indirectly activated T cells and subsequent production of cytotoxic anti-donor antibodies. For reasons that are still unclear, this response is not always efficiently suppressed by current immunosuppressive drugs. Therefore, future challenges in clinical transplantation will be to suppress or eliminate allospecific memory T cells and to prevent the development of indirect alloresponses.

AUTHOR CONTRIBUTIONS

All the authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

This study was supported by NIH grants R21AI111191 and R21AI117466 to Dr. GB.

REFERENCES

1. Rosa SF, Powell AE, Rosengarten RD, Nicotra ML, Moreno MA, Grimwood J, et al. Hydractinia allodeterminant alr1 resides in an immunoglobulin superfamily-like gene complex. *Curr Biol* (2010) 20(12):1122–7. doi:10.1016/j.cub.2010.04.050
2. Kroemer A, Edtinger K, Li XC. The innate natural killer cells in transplant rejection and tolerance induction. *Curr Opin Organ Transplant* (2008) 13(4):339–43. doi:10.1097/MOT.0b013e3283061115
3. Oberbarnscheidt MH, Zecher D, Lakkis FG. The innate immune system in transplantation. *Semin Immunol* (2011) 23(4):264–72. doi:10.1016/j.smim.2011.06.006
4. Wood KJ, Sakaguchi S. Regulatory T cells in transplantation tolerance. *Nat Rev Immunol* (2003) 3(3):199–210. doi:10.1038/nri1027
5. Wekerle T, Kurtz J, Bigenzahn S, Takeuchi Y, Sykes M. Mechanisms of transplant tolerance induction using costimulatory blockade. *Curr Opin Immunol* (2002) 14(5):592–600. doi:10.1016/S0952-7915(02)00378-3
6. Barker CF, Billingham RE. The role of afferent lymphatics in the rejection of skin homografts. *J Exp Med* (1968) 128(1):197–221. doi:10.1084/jem.128.1.197
7. Steinmuller D. Passenger leukocytes and the immunogenicity of skin allografts. *J Invest Dermatol* (1980) 75(1):107–15. doi:10.1111/1523-1747.ep12521331
8. Billingham RE. The passenger cell concept in transplantation immunology. *Cell Immunol* (1971) 2(1):1–12. doi:10.1016/0008-8749(71)90022-0
9. Rosenberg AS, Singer A. Cellular basis of skin allograft rejection: an in vivo model of immune-mediated tissue destruction. *Annu Rev Immunol* (1992) 10:333–58. doi:10.1146/annurev.iy.10.040192.002001
10. Lindahl KF, Wilson DB. Histocompatibility antigen-activated cytotoxic T lymphocytes. II. Estimates of the frequency and specificity of precursors. *J Exp Med* (1977) 145(3):508–22. doi:10.1084/jem.145.3.508
11. Ashwell JD, Chen C, Schwartz RH. High frequency and nonrandom distribution of alloreactivity in T cell clones selected for recognition of foreign antigen in association with self class II molecules. *J Immunol* (1986) 136(2):389–95.
12. Suchin EJ, Langmuir PB, Palmer E, Sayegh MH, Wells AD, Turka LA. Quantifying the frequency of alloreactive T cells in vivo: new answers to an old question. *J Immunol* (2001) 166(2):973–81. doi:10.4049/jimmunol.166.2.973
13. Benichou G, Valujskikh A, Heeger PS. Contributions of direct and indirect T cell alloreactivity during allograft rejection in mice. *J Immunol* (1999) 162(1):352–8.

14. Rogers NJ, Lechler RI. Allorecognition. *Am J Transplant* (2001) 1(2):97–102. doi:10.1034/j.1600-6143.2001.10201.x
15. Lechler RI, Lombardi G, Batchelor JR, Reinsmoen N, Bach FH. The molecular basis of alloreactivity. *Immunol Today* (1990) 11(3):83–8. doi:10.1016/0167-5699(90)90033-6
16. Hogquist KA, Jameson SC, Heath WR, Howard JL, Bevan MJ, Carbone FR. T cell receptor antagonist peptides induce positive selection. *Cell* (1994) 76(1):17–27. doi:10.1016/0092-8674(94)90169-4
17. Adams AB, Pearson TC, Larsen CP. Heterologous immunity: an overlooked barrier to tolerance. *Immunol Rev* (2003) 196:147–60. doi:10.1046/j.1600-065X.2003.00082.x
18. Taylor DK, Neujahr D, Turka LA. Heterologous immunity and homeostatic proliferation as barriers to tolerance. *Curr Opin Immunol* (2004) 16(5):558–64. doi:10.1016/j.coi.2004.07.007
19. Amir AL, D'Orsogna LJ, Roelen DL, van Loenen MM, Hagedoorn RS, de Boer R, et al. Allo-HLA reactivity of virus-specific memory T cells is common. *Blood* (2010) 115(15):3146–57. doi:10.1182/blood-2009-07-234906
20. Brehm MA, Markees TG, Daniels KA, Greiner DL, Rossini AA, Welsh RM. Direct visualization of cross-reactive effector and memory allo-specific CD8 T cells generated in response to viral infections. *J Immunol* (2003) 170(8):4077–86. doi:10.4049/jimmunol.170.8.4077
21. Pantenburg B, Heinzel F, Das L, Heeger PS, Valujskikh A. T cells primed by *Leishmania major* infection cross-react with alloantigens and alter the course of allograft rejection. *J Immunol* (2002) 169(7):3686–93. doi:10.4049/jimmunol.169.7.3686
22. Golding H, Singer A. Role of accessory cell processing and presentation of shed H-2 alloantigens in allospecific cytotoxic T lymphocyte responses. *J Immunol* (1984) 133(2):597–605.
23. Lechler RI, Batchelor JR. Immunogenicity of retransplanted rat kidney allografts. Effect of inducing chimerism in the first recipient and quantitative studies on immunosuppression of the second recipient. *J Exp Med* (1982) 156(6):1835–41. doi:10.1084/jem.156.6.1835
24. Lechler RI, Batchelor JR. Restoration of immunogenicity to passenger cell-depleted kidney allografts by the addition of donor strain dendritic cells. *J Exp Med* (1982) 155(1):31–41. doi:10.1084/jem.155.1.31
25. Benichou G, Takizawa PA, Ho PT, Killion CC, Olson CA, McMillan M, et al. Immunogenicity and tolerogenicity of self-major histocompatibility complex peptides. *J Exp Med* (1990) 172(5):1341–6. doi:10.1084/jem.172.5.1341
26. Liu Z, Braunstein NS, Suciu-Foca N. T cell recognition of allopeptides in context of syngeneic MHC. *J Immunol* (1992) 148(1):35–40.
27. Fangmann J, Dalchau R, Sawyer GJ, Priestley CA, Fabre JW. T cell recognition of donor major histocompatibility complex class I peptides during allograft rejection. *Eur J Immunol* (1992) 22(6):1525–30. doi:10.1002/eji.1830220627
28. Popov IA, Fedoseyeva EV, Orr PL, Garovoy MR, Benichou G. Direct evidence for in vivo induction of CD8+ cytotoxic T cells directed to donor MHC class I peptides following mouse allotransplantation. *Transplantation* (1995) 60(12):1621–4.
29. Valujskikh A, Hartig C, Heeger PS. Indirectly primed CD8+ T cells are a prominent component of the allogeneic T-cell repertoire after skin graft rejection in mice. *Transplantation* (2001) 71(3):418–21. doi:10.1097/00007890-200102150-00014
30. Valujskikh A, Lantz O, Celli S, Matzinger P, Heeger PS. Cross-primed CD8(+) T cells mediate graft rejection via a distinct effector pathway. *Nat Immunol* (2002) 3(9):844–51. doi:10.1038/ni831
31. Benichou G, Fedoseyeva E, Lehmann PV, Olson CA, Geysen HM, McMillan M, et al. Limited T cell response to donor MHC peptides during allograft rejection. Implications for selective immune therapy in transplantation. *J Immunol* (1994) 153(3):938–45.
32. Liu Z, Sun YK, Xi XYP, Hong B, Harris PE, Reed EF, et al. Limited usage of T cell receptor V beta genes by allopeptide-specific T cells. *J Immunol* (1993) 150(8 Pt 1):3180–6.
33. Boisgerault F, Anosova NG, Tam RC, Illigens BM, Fedoseyeva EV, Benichou G. Induction of T-cell response to cryptic MHC determinants during allograft rejection. *Hum Immunol* (2000) 61(12):1352–62. doi:10.1016/S0198-8859(00)00209-3
34. Sercarz EE, Lehmann PV, Ametani A, Benichou G, Miller A, Moudgil K. Dominance and crypticity of T cell antigenic determinants. *Annu Rev Immunol* (1993) 11:729–66. doi:10.1146/annurev.iy.11.040193.003501
35. Lehmann PV, Forsthuber T, Miller A, Sercarz EE. Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. *Nature* (1992) 358(6382):155–7. doi:10.1038/358155a0
36. Lehmann PV, Sercarz EE, Forsthuber T, Dayan CM, Gammon G. Determinant spreading and the dynamics of the autoimmune T-cell repertoire [see comments]. *Immunol Today* (1993) 14(5):203–8. doi:10.1016/0167-5699(93)90163-F
37. Suciu-Foca N, Harris PE, Cortesini R. Intramolecular and intermolecular spreading during the course of organ allograft rejection. *Immunol Rev* (1998) 164:241–6. doi:10.1111/j.1600-065X.1998.tb01224.x
38. Goulmy E. Human minor histocompatibility antigens. *Curr Opin Immunol* (1996) 8(1):75–81. doi:10.1016/S0952-7915(96)80108-7
39. Joly E, Hudrisier D. What is trogocytosis and what is its purpose? *Nat Immunol* (2003) 4(9):815. doi:10.1038/ni0903-815
40. Brown K, Sacks SH, Wong W. Extensive and bidirectional transfer of major histocompatibility complex class II molecules between donor and recipient cells in vivo following solid organ transplantation. *FASEB J* (2008) 22(11):3776–84. doi:10.1096/fj.08-107441
41. Thery C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. *Nat Rev Immunol* (2002) 2(8):569–79. doi:10.1038/nri855
42. Hudson L, Sprent J. Specific adsorption of IgM antibody onto H-2-activated mouse T lymphocytes. *J Exp Med* (1976) 143(2):444–9. doi:10.1084/jem.143.2.444
43. Bona C, Robineaux R, Anteunis A, Heuclin C, Astesano A. Transfer of antigen from macrophages to lymphocytes. II. Immunological significance of the transfer of lipopolysaccharide. *Immunology* (1973) 24(5):831–40.
44. Frelinger JA, Neiderhuber JE, David CS, Shreffler DC. Evidence for the expression of Ia (H-2-associated) antigens on thymus-derived lymphocytes. *J Exp Med* (1974) 140(5):1273–84. doi:10.1084/jem.140.5.1273
45. Dolan BP, Gibbs KD Jr, Ostrand-Rosenberg S. Dendritic cells cross-dressed with peptide MHC class I complexes prime CD8+ T cells. *J Immunol* (2006) 177(9):6018–24. doi:10.4049/jimmunol.177.9.6018
46. Brown K, Fidanboylu M, Wong W. Intercellular exchange of surface molecules and its physiological relevance. *Arch Immunol Ther Exp (Warsz)* (2010) 58(4):263–72. doi:10.1007/s00005-010-0085-y
47. Harper SJ, Ali JM, Wlodek E, Negus MC, Harper IG, Chhabra M, et al. CD8 T-cell recognition of acquired alloantigen promotes acute allograft rejection. *Proc Natl Acad Sci U S A* (2015) 112(41):12788–93. doi:10.1073/pnas.1513533112
48. Markey KA, Koyama M, Gartlan KH, Leveque L, Kuns RD, Lineburg KE, et al. Cross-dressing by donor dendritic cells after allogeneic bone marrow transplantation contributes to formation of the immunological synapse and maximizes responses to indirectly presented antigen. *J Immunol* (2014) 192(11):5426–33. doi:10.4049/jimmunol.1302490
49. Herrera OB, Golshayan D, Tibbott R, Salcido Ochoa F, James MJ, Marelli-Berg FM, et al. A novel pathway of alloantigen presentation by dendritic cells. *J Immunol* (2004) 173(8):4828–37. doi:10.4049/jimmunol.173.8.4828
50. Smyth LA, Herrera OB, Golshayan D, Lombardi G, Lechler RI. A novel pathway of antigen presentation by dendritic and endothelial cells: implications for allorecognition and infectious diseases. *Transplantation* (2006) 82(1 Suppl):S15–8. doi:10.1097/01.tp.0000231347.06149.ca
51. Russo V, Zhou D, Sartirana C, Rovere P, Villa A, Rossini S, et al. Acquisition of intact allogeneic human leukocyte antigen molecules by human dendritic cells. *Blood* (2000) 95(11):3473–7.
52. Benichou G, Yamada Y, Yun SH, Lin C, Fray M, Tocco G. Immune recognition and rejection of allogeneic skin grafts. *Immunotherapy* (2011) 3(6):757–70. doi:10.2217/imt.11.2
53. Benichou G. Direct and indirect antigen recognition: the pathways to allograft immune rejection. *Front Biosci* (1999) 4:D476–80. doi:10.2741/A442
54. Auchincloss H Jr, Lee R, Shea S, Markowitz JS, Grusby MJ, Glimcher LH. The role of "indirect" recognition in initiating rejection of skin grafts from major histocompatibility complex class II-deficient mice. *Proc Natl Acad Sci U S A* (1993) 90(8):3373–7. doi:10.1073/pnas.90.8.3373
55. Lee RS, Grusby MJ, Glimcher LH, Winn HJ, Auchincloss H Jr. Indirect recognition by helper cells can induce donor-specific cytotoxic T lymphocytes in vivo. *J Exp Med* (1994) 179(3):865–72. doi:10.1084/jem.179.3.865

56. Valujskikh A, Heeger PS. CD4+ T cells responsive through the indirect pathway can mediate skin graft rejection in the absence of interferon-gamma. *Transplantation* (2000) 69(5):1016–9. doi:10.1097/00007890-200003150-00063
57. Valujskikh A, VanBuskirk AM, Orosz CG, Heeger PS. A role for TGFbeta and B cells in immunologic tolerance after intravenous injection of soluble antigen. *Transplantation* (2001) 72(4):685–93. doi:10.1097/00007890-200108270-00022
58. Marino J, Babiker M, Crosby Bertorini P, Paster JT, LeGuern C, Germana S, et al. Donor exosomes rather than passenger leukocytes initiate alloreactive T cell responses after transplantation. *Sci Immunol* (2016) 1(1):1–12. doi:10.1126/sciimmunol.aaf8759
59. Smyth LA, Lechler RI, Lombardi G. Continuous acquisition of MHC:peptide complexes by recipient cells contributes to the generation of anti-graft CD8+ T cell immunity. *Am J Transplant* (2016) 16(11):1–9. doi:10.1111/ajt.13996
60. Kant CD, Akiyama Y, Tanaka K, Shea S, Connolly SE, Germana S, et al. Primary vascularization of allografts governs their immunogenicity and susceptibility to tolerogenesis. *J Immunol* (2013) 191(4):1948–56. doi:10.4049/jimmunol.1202092
61. Sano Y, Ksander BR, Streilein JW. Minor H, rather than MHC, alloantigens offer the greater barrier to successful orthotopic corneal transplantation in mice. *Transpl Immunol* (1996) 4(1):53–6. doi:10.1016/S0966-3274(96)80035-9
62. Streilein JW. Immunologic privilege of the eye. *Springer Semin Immunopathol* (1999) 21(2):95–111. doi:10.1007/BF00810243
63. Streilein JW, Wilbanks GA, Cousins SW. Immunoregulatory mechanisms of the eye. *J Neuroimmunol* (1992) 39(3):185–200. doi:10.1016/0165-5728(92)90253-H
64. Streilein JW, Toews GB, Bergstresser PR. Corneal allografts fail to express Ia antigens. *Nature* (1979) 282(5736):326–7. doi:10.1038/282326a0
65. Whitsett CF, Stulting RD. The distribution of HLA antigens on human corneal tissue. *Invest Ophthalmol Vis Sci* (1984) 25(5):519–24.
66. Bartlett ST, Jennings AS, Yu C, Naji A, Barker CF, Silvers WK. Influence of culturing on the survival of major histocompatibility complex-compatible and -incompatible thyroid grafts in rats. *J Exp Med* (1983) 157(1):348–52. doi:10.1084/jem.157.1.348
67. Boisgerault F, Liu Y, Anosova N, Ehrlich E, Dana MR, Benichou G. Role of CD4+ and CD8+ T cells in allorecognition: lessons from corneal transplantation. *J Immunol* (2001) 167(4):1891–9. doi:10.4049/jimmunol.167.4.1891
68. Boisgerault F, Liu Y, Anosova N, Dana R, Benichou G. Differential roles of direct and indirect allorecognition pathways in the rejection of skin and corneal transplants. *Transplantation* (2009) 87(1):16–23. doi:10.1097/TP.0b013e318191b38b
69. Liu Y, Hamrah P, Zhang Q, Taylor AW, Dana MR. Draining lymph nodes of corneal transplant hosts exhibit evidence for donor major histocompatibility complex (MHC) class II-positive dendritic cells derived from MHC class II-negative grafts. *J Exp Med* (2002) 195(2):259–68. doi:10.1084/jem.20010838
70. Hamrah P, Liu Y, Zhang Q, Dana MR. The corneal stroma is endowed with a significant number of resident dendritic cells. *Invest Ophthalmol Vis Sci* (2003) 44(2):581–9. doi:10.1167/iovs.02-0838
71. Huq S, Liu Y, Benichou G, Dana MR. Relevance of the direct pathway of sensitization in corneal transplantation is dictated by the graft bed microenvironment. *J Immunol* (2004) 173(7):4464–9. doi:10.4049/jimmunol.173.7.4464
72. Matoba AY, Peeler JS, Niederkorn JY. T cell subsets in the immune rejection of murine heterotopic corneal allografts. *Invest Ophthalmol Vis Sci* (1986) 27(8):1244–54.
73. Krieger NR, Yin DP, Fathman CG. CD4+ but not CD8+ cells are essential for allorejection. *J Exp Med* (1996) 184(5):2013–8. doi:10.1084/jem.184.5.2013
74. Trager DK, Banks BA, Rosenbaum GE, Holm BI, Shizuru JA, Strober S, et al. Cardiac allograft prolongation in mice treated with combined posttransplantation total-lymphoid irradiation and anti-L3T4 antibody therapy. *Transplantation* (1989) 47(4):587–91. doi:10.1097/00007890-198904000-00004
75. Corry RJ, Winn HJ, Russell PS. Primarily vascularized allografts of hearts in mice. The role of H-2D, H-2K, and non-H-2 antigens in rejection. *Transplantation* (1973) 16(4):343–50. doi:10.1097/00007890-197310000-00010
76. Larsen CP, Morris PJ, Austyn JM. Migration of dendritic leukocytes from cardiac allografts into host spleens. A novel pathway for initiation of rejection. *J Exp Med* (1990) 171(1):307–14. doi:10.1084/jem.171.1.307
77. Wang Z, Castellaneta A, De Creus A, Shufesky WJ, Morelli AE, Thomson AW. Heart, but not skin, allografts from donors lacking Flt3 ligand exhibit markedly prolonged survival time. *J Immunol* (2004) 172(10):5924–30. doi:10.4049/jimmunol.172.10.5924
78. Su CA, Iida S, Abe T, Fairchild RL. Endogenous memory CD8 T cells directly mediate cardiac allograft rejection. *Am J Transplant* (2014) 14(3):568–79. doi:10.1111/ajt.12605
79. Obhrai JS, Oberbarnscheidt MH, Hand TW, Diggs L, Chalasani G, Lakkis FG. Effector T cell differentiation and memory T cell maintenance outside secondary lymphoid organs. *J Immunol* (2006) 176(7):4051–8. doi:10.4049/jimmunol.176.7.4051
80. Adams AB, Williams MA, Jones TR, Shirasugi N, Durham MM, Kaech SM, et al. Heterologous immunity provides a potent barrier to transplantation tolerance. *J Clin Invest* (2003) 111(12):1887–95. doi:10.1172/JCI200317477
81. Valujskikh A, Lakkis FG. In remembrance of things past: memory T cells and transplant rejection. *Immunol Rev* (2003) 196:65–74. doi:10.1046/j.1600-065X.2003.00087.x
82. Nadazdin O, Boskovic S, Murakami T, O'Connor DH, Wiseman RW, Karl JA, et al. Phenotype, distribution and alloreactive properties of memory T cells from cynomolgus monkeys. *Am J Transplant* (2010) 10(6):1375–84. doi:10.1111/j.1600-6143.2010.03119.x
83. Nadazdin O, Boskovic S, Murakami T, Tocco G, Smith RN, Colvin RB, et al. Host alloreactive memory T cells influence tolerance to kidney allografts in nonhuman primates. *Sci Transl Med* (2011) 3(86):86ra51. doi:10.1126/scitranslmed.3002093
84. Heeger PS, Greenspan NS, Kuhlenschmidt S, Dejelo C, Hricik DE, Schulak JA, et al. Pretransplant frequency of donor-specific, IFN-gamma-producing lymphocytes is a manifestation of immunologic memory and correlates with the risk of posttransplant rejection episodes. *J Immunol* (1999) 163(4):2267–75.
85. Hricik DE, Rodriguez V, Riley J, Bryan K, Tary-Lehmann M, Greenspan N, et al. Enzyme linked immunosorbent spot (ELISPOT) assay for interferon-gamma independently predicts renal function in kidney transplant recipients. *Am J Transplant* (2003) 3(7):878–84. doi:10.1034/j.1600-6143.2003.00132.x
86. Lee RS, Yamada K, Houser SL, Womer KL, Maloney ME, Rose HS, et al. Indirect recognition of allopeptides promotes the development of cardiac allograft vasculopathy. *Proc Natl Acad Sci U S A* (2001) 98(6):3276–81. doi:10.1073/pnas.051584498
87. Baker RJ, Hernandez-Fuentes MP, Brookes PA, Chaudhry AN, Cook HT, Lechler RI. Loss of direct and maintenance of indirect alloresponses in renal allograft recipients: implications for the pathogenesis of chronic allograft nephropathy. *J Immunol* (2001) 167(12):7199–206. doi:10.4049/jimmunol.167.12.7199
88. Hornick PI, Mason PD, Baker RJ, Hernandez-Fuentes M, Frasca L, Lombardi G, et al. Significant frequencies of T cells with indirect anti-donor specificity in heart graft recipients with chronic rejection. *Circulation* (2000) 101(20):2405–10. doi:10.1161/01.CIR.101.20.2405
89. Suciu-Foca N, Liu Z, Colovai AI, Tugulea S, Reed EF, Mancini D, et al. Role of indirect allorecognition in chronic rejection of human allografts. *Transplant Proc* (1996) 28(1):404–5.
90. Najafian N, Salama AD, Fedoseyeva EV, Benichou G, Sayegh MH. Enzyme-linked immunosorbent spot assay analysis of peripheral blood lymphocyte reactivity to donor HLA-DR peptides: potential novel assay for prediction of outcomes for renal transplant recipients. *J Am Soc Nephrol* (2002) 13(1):252–9.
91. Liu Q, Rojas-Canales DM, Divito SJ, Shufesky WJ, Stolz DB, Erdos G, et al. Donor dendritic cell-derived exosomes promote allograft-targeting immune response. *J Clin Invest* (2016) 126(8):2805–20. doi:10.1172/JCI84577
92. Vacchio MS, Jiang SP. The fetus and the maternal immune system: pregnancy as a model to study peripheral T-cell tolerance. *Crit Rev Immunol* (1999) 19(5–6):461–80.
93. Ait-Azzouzene D, Gendron MC, Houdayer M, Langkopf A, Burki K, Nemazee D, et al. Maternal B lymphocytes specific for paternal histocompatibility antigens are partially deleted during pregnancy. *J Immunol* (1998) 161(6):2677–83.

94. Niederkorn JY. Immune privilege and immune regulation in the eye. *Adv Immunol* (1990) 48:191–226. doi:10.1016/S0065-2776(08)60755-5
95. Nasr IW, Wang Y, Gao G, Deng S, Diggs L, Rothstein DM, et al. Testicular immune privilege promotes transplantation tolerance by altering the balance between memory and regulatory T cells. *J Immunol* (2005) 174(10):6161–8. doi:10.4049/jimmunol.174.10.6161
96. Cobbold SP, Adams E, Graca L, Daley S, Yates S, Paterson A, et al. Immune privilege induced by regulatory T cells in transplantation tolerance. *Immunol Rev* (2006) 213:239–55. doi:10.1111/j.1600-065X.2006.00428.x
97. Wekerle T, Kurtz J, Sayegh M, Ito H, Wells A, Bensinger S, et al. Peripheral deletion after bone marrow transplantation with costimulatory blockade has features of both activation-induced cell death and passive cell death. *J Immunol* (2001) 166(4):2311–6. doi:10.4049/jimmunol.166.4.2311
98. LaRosa DF, Rahman AH, Turka LA. The innate immune system in allograft rejection and tolerance. *J Immunol* (2007) 178(12):7503–9. doi:10.4049/jimmunol.178.12.7503
99. Waldmann H, Graca L, Cobbold S, Adams E, Tone M, Tone Y. Regulatory T cells and organ transplantation. *Semin Immunol* (2004) 16(2):119–26. doi:10.1016/j.smim.2003.12.007
100. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell* (2008) 133(5):775–87. doi:10.1016/j.cell.2008.05.009
101. Sakaguchi S, Miyara M, Costantino CM, Hafler DA. FOXP3+ regulatory T cells in the human immune system. *Nat Rev Immunol* (2010) 10(7):490–500. doi:10.1038/nri2785
102. Shevach EM, McHugh RS, Piccirillo CA, Thornton AM. Control of T-cell activation by CD4+ CD25+ suppressor T cells. *Immunol Rev* (2001) 182:58–67. doi:10.1034/j.1600-065X.2001.1820104.x
103. Sakaguchi S, Sakaguchi N, Shimizu J, Yamazaki S, Sakihama T, Itoh M, et al. Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol Rev* (2001) 182:18–32. doi:10.1034/j.1600-065X.2001.1820102.x
104. Shevach EM, McHugh RS, Thornton AM, Piccirillo C, Natarajan K, Margulies DH. Control of autoimmunity by regulatory T cells. *Adv Exp Med Biol* (2001) 490:21–32. doi:10.1007/978-1-4615-1243-1_3
105. Graca L, Cobbold SP, Waldmann H. Identification of regulatory T cells in tolerated allografts. *J Exp Med* (2002) 195(12):1641–6. doi:10.1084/jem.20012097
106. Miyajima M, Chase CM, Alessandrini A, Farkash EA, Della Pelle P, Benichou G, et al. Early acceptance of renal allografts in mice is dependent on foxp3(+) cells. *Am J Pathol* (2011) 178(4):1635–45. doi:10.1016/j.ajpath.2010.12.024
107. Josefowicz SZ, Rudensky A. Control of regulatory T cell lineage commitment and maintenance. *Immunity* (2009) 30(5):616–25. doi:10.1016/j.immuni.2009.04.009
108. LeGuern C. Regulation of T-cell functions by MHC class II self-presentation. *Trends Immunol* (2003) 24(12):633–8. doi:10.1016/j.it.2003.10.010
109. LeGuern C, Shimada H, Emery DW, Germana S, Shafer GE, Sachs DH. Retrovirus-mediated transfer of MHC class II cDNA into swine bone marrow cells. *J Mol Med (Berl)* (1995) 73(6):269–78. doi:10.1007/BF00231613
110. Germana S, Shimada H, Sachs DH, LeGuern C. Retroviral vectors transfer functional MHC class II heterodimers into bone marrow: a genetic approach to tolerance induction. *Transplant Proc* (1997) 29(1–2):1129. doi:10.1016/S0041-1345(96)00463-0
111. Sonntag KC, Emery DW, Yasumoto A, Haller G, Germana S, Sablinski T, et al. Tolerance to solid organ transplants through transfer of MHC class II genes. *J Clin Invest* (2001) 107(1):65–71. doi:10.1172/JCI11015
112. LeGuern C. Potential role of major histocompatibility complex class II peptides in regulatory tolerance to vascularized grafts. *Transplantation* (2004) 77(1 Suppl):S35–7. doi:10.1097/01.TP.0000106472.91343.8D
113. LeGuern C, Akiyama Y, Germana S, Tanaka K, Fernandez L, Iwamoto Y, et al. Intracellular MHC class II controls regulatory tolerance to allogeneic transplants. *J Immunol* (2010) 184(5):2394–400. doi:10.4049/jimmunol.0803664
114. Wing K, Sakaguchi S. Regulatory T cells exert checks and balances on self tolerance and autoimmunity. *Nat Immunol* (2010) 11(1):7–13. doi:10.1038/ni.1818
115. Ochando JC, Homma C, Yang Y, Hidalgo A, Garin A, Tacke F, et al. Alloantigen-presenting plasmacytoid dendritic cells mediate tolerance to vascularized grafts. *Nat Immunol* (2006) 7(6):652–62. doi:10.1038/ni1333
116. Ezzelarab M, Thomson AW. Tolerogenic dendritic cells and their role in transplantation. *Semin Immunol* (2011) 23(4):252–63. doi:10.1016/j.smim.2011.06.007
117. McCurry KR, Colvin BL, Zahorchak AF, Thomson AW. Regulatory dendritic cell therapy in organ transplantation. *Transpl Int* (2006) 19(7):525–38. doi:10.1111/j.1432-2277.2006.00306.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 03 April 2017
doi: 10.3389/fimmu.2017.00341

Interplay of Regulatory T Cell and Th17 Cells during Infectious Diseases in Humans and Animals

Sharvan Sehrawat[1] and Barry T. Rouse[2]*

[1] Indian Institute of Science Education and Research Mohali, Mohali, Punjab, India, [2] Department of Biomedical and Diagnostic Sciences, College of Veterinary Medicine, The University of Tennessee, Knoxville, TN, USA

It is now clear that the outcome of an inflammatory process caused by infections depends on the balance of responses by several components of the immune system. Of particular relevance is the interplay between regulatory T cells (Tregs) and CD4$^+$ T cells that produce IL-17 (Th17 cells) during immunoinflammatory events. In addition to discussing studies done in mice to highlight some unresolved issues in the biology of these cells, we emphasize the need to include outbred animals and humans in analyses. Achieving a balance between Treg and Th17 cells responses represents a powerful approach to control events during immunity and immunopathology.

Keywords: T regulatory cells, Th17 cells, cross-regulation, humans, animals, outbred population

Edited by:
Antoine Toubert,
Paris Diderot University, France

Reviewed by:
Karen Bieback,
Medical Faculty Mannheim, Germany
Federica Casiraghi,
Istituto Di Ricerche Farmacologiche Mario Negri, Italy

****Correspondence:***
Sharvan Sehrawat
sharvan@iisermohali.ac.in

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *03 October 2016*
Accepted: *09 March 2017*
Published: *03 April 2017*

Citation:
Sehrawat S and Rouse BT (2017) Interplay of Regulatory T Cell and Th17 Cells during Infectious Diseases in Humans and Animals. Front. Immunol. 8:341. doi: 10.3389/fimmu.2017.00341

INTRODUCTION

The realization that CD4$^+$ T cells could be differentiated in two phenotypically separate lineages, Th1 cells that predominantly produce IFN-γ and IL-2 while Th2 cells produce IL-4 and IL-10, was elucidated by Mosmann et al. (1). The idea caught on because these cell types cross-regulated each other and this phenomenon helped in explaining many observations in inflammatory and infectious diseases. Subsequently, several additional subtypes of CD4$^+$ T cells were discovered based on the transcription factor expressed, their cytokine profile and functions (2, 3). Of particular relevance was the discovery that some CD4$^+$ T cells play a regulatory role and helped to constrain the effector function of other cell types. We currently recognize at least four CD4$^+$ T cell subsets which largely play an effector function (Th1, Th2, Th9, and Th17) and another subset T follicular helper cell (T_{FH}) which plays a major role during immune induction (4). This review focuses largely on the cross play between regulatory T cells (Tregs) and Th17 cells since these two subsets often subserve opposite roles during inflammatory processes. Th17 cells are recognized as one of the predominant proinflammatory cell types and produce IL-17 to help attract other innate immune cells such as macrophages and neutrophils to further aggravate chronic inflammation. The transcription factor RAR-related orphan receptor (ROR)-γt regulates the speciation program of Th17 cells. Tregs on the other hand act to regulate the differentiation and activity of Th17 cells. In fact, several lines of evidence demonstrate that Treg and Th17 cells exhibit some key shared differentiation pathways (**Figure 1**). Thus, both cell types require TGF-β and IL-2 for their differentiation and are predominantly present in the gut to maintain homeostasis (5). Both Treg and Th17 cells exhibit specificity toward commensal-derived antigens or self-antigens and their speciation transcriptional program shows direct interaction (5). Of the two major classes of antigen-presenting cells (APCs) in the gut, dendritic cells (DCs) are known to promote Th17 cell responses while macrophages promote Treg responses (6). Treg and Th17 cells were shown to predominantly maintain gut homeostasis but their interplay in other

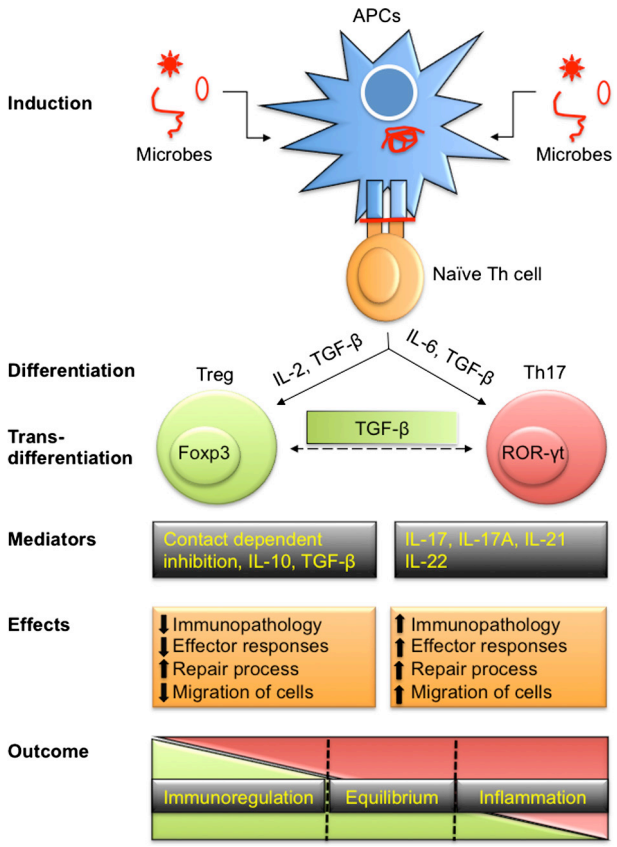


FIGURE 1 | Differentiation of T regulatory cells (Tregs) and Th17 to effect immunity and immunopathology during infections. Antigen-presenting cells (APCs) either by direct infection or by exogenously taking up antigens process polypeptides intracellularly to generate peptides. These peptides are loaded onto class II MHC molecules and presented on their surface to activate naïve Th cells. Depending upon the affinity of TCR to recognize processed peptides and the microenvironment in which such interactions take place, Th cells are polarized into pTreg and Th17 cells to maintain homeostasis. Treg and Th17 cells can also transdifferentiate depending on intrinsic as well as some extrinsic factors such as local concentration of TGF-β. Predominant products of Treg include IL-10, IL-35, and TGF-β in addition to membrane-expressed molecules while Th17 cells secrete IL-17, IL-21, IL-22, and other cytokines. Tregs cause immunoregulation while Th17 serve as proinflammatory cells during disease progression. Treg and Th17 leads to differential outcome ranging from dominant regulatory to stimulatory activity while a fine balance ensures homeostasis.

diseases that include those caused by infections is beginning to be appreciated.

The idea that T cells could suppress the function of other cells was popularized by Gershon and Kondo (7). The cells were called suppressor cells, but since there were no reliable means of identifying them, they soon fell into disrepute. Resurrection and respectability for Treg came some two decades later by the work performed by the Sakaguchi et al. and Suri-Payer et al. who had discovered a reliable way of distinguishing Treg from other cell types and also demonstrated their regulatory effects (8–10). Sakaguchi et al. and Thornton and Shevach demonstrated that 5–10% of T helper (Th) cells that expressed the high affinity IL-2 receptor alpha (α) chain (CD25) were present in naïve mice and were able to suppress the proliferation of those cells that did not similarly express this molecule (9, 11). The idea of Treg's existence helped in explaining many unsolved mysteries in immunobiology such as how tolerance is maintained and the variable outcome of autoimmune and infectious diseases is effected (12–14). The canonical transcription factor Fork head box protein 3 (Foxp3), responsible for controlling the function of Treg and acting as their identifier, was discovered in 2001 (15–17). Whereas Foxp3$^+$ Tregs are perhaps the most prominent regulatory cells, other cell types have been observed to mediate regulatory effects alongside or alternatively to Foxp3$^+$ Treg. These many alternative regulators include Tr1 cells, Th3 cells, CD8$^+$ Treg, double negative CD3$^+$ T cells, gamma delta (γδ) T cells, natural killer T cells, regulatory B cells, myeloid-derived suppressor cells, and perhaps others.

A great majority of our understanding of how the immune system works comes from studies performed in inbred mice housed in controlled environment. Our ultimate objective, however, is to understand the workings of the immune system and to apply the wisdom to manipulate the outcome of events in humans and other animals. There is still a gap in our knowledge regarding what happens in humans and outbred non-rodents and this issue is elaborated in this review. We also discuss unresolved issues in the biology as well as pathophysiology of Treg and Th17 cells during infectious diseases.

BIOLOGY OF TREG AND Th17 CELLS

The expression of the transcription factors Foxp3 and ROR-γt defines Treg and Th17 cells, respectively. Foxp3 is critically involved in the differentiation and function of Treg. Foxp3 does so by directly binding to DNA to be transcribed and in so doing regulates the transcription of more than thousand genes many of which are involved in T cell activation. Some of the Treg-specific genes directly targeted by Foxp3 are *Il2ra* (CD25), *Tnfrsf18* (GITR), *Nrp1* (neuropillin-1), and *Ccr4* among others (18–20). Foxp3 could also influence gene expression indirectly by recruiting epigenetic modifiers such as histone deacetylases (HDAC1, 2, and 3) in the complex (21). Many genes that include *Il2* are downregulated by HDACs activity. As newer mechanistic insights are emerging, clearly there is need of more studies to better define the role of Foxp3 in programming Treg and in fact different functions could be attributed to its different domains. Similarly, Foxp3 regulates the expression of some chemokine receptors suggesting that it may also control the homing of Treg. The latter effect has not received much attention and needs to be understood in greater detail. This is because immunosuppression at inflammatory sites is one of the most desirable outcomes of cell-based immunotherapies.

Tregs are broadly divided into thymically derived regulatory T cells (tTregs) and those that are induced in the periphery (pTregs). pTregs are usually more plastic than tTregs (22). Nrp1 may act as the distinguishing marker between tTreg (+) and pTreg (−) (23–25). Tregs in the thymus develop after 3 days of birth and a thymectomy at 3 days of birth abrogates Treg responses

leading to multiorgan autoimmune inflammatory diseases (26). However, some Treg that specifically home to select lymphoid organs can be detected in 3-day-old thymectomized mice (27). Therefore, it could be that the kinetics of Treg generation in the thymus is also linked to their differential homing pattern. As and when growing animals are exposed to different environmental conditions that include feed and habitation, the homing properties, functionality, and repertoire of Treg may be refined further to maintain homeostasis at different locations.

For the induction of T cell responses that include Treg, three signals comprising MHC–peptide–TCR, engagement of co-stimulatory/inhibitory molecule, and cytokines in milieu are required (28, 29). Issues such as the strength and the nature of inducing signals and the subsequent formation of either plastic or stable Treg are beginning to be investigated (30). Low to intermediate affinity interactions between the TCR expressed by developing T cells and peptides–MHC class II complexes in thymus are considered as one of the critical drivers of Treg differentiation (28). Contrary to what was considered as a paradigm that both α and β chains of the TCR are involved in peptide binding (31), a recent study demonstrated that only the β chain of TCR along with its framework regions contributed to peptide binding in Tr1 cells and thereby making it a very low affinity interaction (32, 33). However, one wonders how such a weakly interacting TCR ensures survivability of T cells during the thymic selection process. Whether or not TCRs of different types of Treg also display a similar orientation and affinity remains unexplored.

The affinity with which TCRs of Th17 cells recognize peptides has not been extensively explored. Only a few studies have demonstrated that TCRs of Th17 cells might exhibit a low affinity (34). High affinity interactions in fact might be counterproductive for gut health, a site so heavily infested by microbes. Thus, in healthy individuals a unique tripartite interaction among gut microbiota, Treg, and Th17 cells may be required to maintain gut homeostasis (35). Conceivably, Th17 cells act to control the excessive growth of microbes in the gut while Tregs regulate Th17 cell responses. Whether Th17 cells exhibit differential TCR specificity or affinity toward antigens and how it affects their pathogenicity is worth investigating and could indeed help identify Th17 cell subsets with different functions. Some studies have supported a similar idea that Th17 cells could indeed exist in different subtypes (36–38). Accordingly, a local intracellular concentration of saturated fatty acids (SFA) compared to polyunsaturated fatty acids (PUFA) favored more pathogenic Th17 cell formation (38). Differential accumulation of SFA or PUFA and their binding to intracellularly expressed CD5L led to the generation of Th17 exhibiting differential pathogenicity (38).

The stimulating antigens for Treg and perhaps for Th17 cells could also be generated during an ongoing inflammatory response caused by autoimmune diseases or infections. To support this notion, a few studies have demonstrated that Tregs isolated from draining LNs are more active and better suppressors as compared to those isolated from distal LNs (39–42). In draining LNs, APCs home from local sites and predominantly sample antigens released from these areas. This provides ample stimulation for Treg to remain better suppressors.

TGF-β is a critical cytokine required at least *in vitro* for inducing the regulatory phenotype in T cells. Depending on the concentration, context, and condition, TGF-β helps skew responses toward Treg or Th17 cells (43, 44). Thus, a greater concentration of TGF-β may be conducive for a Treg response while a lower concentration particularly in the presence of other inflammatory cytokines such as IL-6 and IL-21 could preferentially promote Th17 responses (45). In fact, some pathogens either encode for the homologs of TGF-β or help activate latent TGF-β and this may be responsible for differential proinflammatory or regulatory responses (46). Whether or not TGF-β is critical for Treg generation in the thymic environment was investigated in the absence of TGF-β signaling using complete knockout or T cell specific TGFβRII knock out mice (47–49). These studies revealed that an absence of TGF-β signaling only affected the peripheral pool of Treg and not their thymic generation (47). It could also suggest that Treg that develop in the thymus halt their proliferation and remain quiescent until they home to the periphery. The reduced proliferation of Treg in the thymus could be the consequence of limited antigen availability and the presence of abundant TGF-β, both of which could serve to induce slow proliferation of Treg (48). The thymic microenvironment could indeed provide copious amount of TGF-β for Treg differentiation or maintenance because of an ongoing process of apoptosis and disposal of such cells by phagocytic activity of DCs and macrophages (49). Another signal that has been implicated in Treg generation is retinoic acid, a metabolite of vitamin A (50). The expression of TGF-β and retinoic acid has also been demonstrated in the thymus supporting the notion that these induction pathways either alone or cooperatively could help thymic Treg generation (51–53).

IL-2 signaling is critically involved in Treg as well as Th17 cell differentiation. IL-2 is consumed preferentially by Treg since they express high affinity IL-2 receptors (54). IL-2 also acts to stabilize Foxp3 induced by TGF-β (30, 55). Treg are supposed to dampen inflammation where a mix of both pro- and anti-inflammatory cytokines constitutes the microenvironment. Therefore, the functionality of Treg needs to be evaluated in the presence of relative abundance of different cytokines. TGF-β and IL-2, if present in an environment along with other proinflammatory cytokines such as IL-1β, IL-6, IL-21, or IL-23, facilitate Th17 differentiation at the expense of Treg (56). It is worth investigating how ROR-γt in Th17 cells actually promotes their programming. Thus, whether or not the transcription factor ROR-γt in Th17 cells actually binds in the promoter region of IL-17 to modulate its expression has not been shown experimentally. However, a putative binding site of ROR- γt in IL-17 promoter has been predicted (57). Similarly, any naturally existing endogenous ligands for ROR-γt is yet to be identified. The induction kinetics of such ligands during infection could provide better insights into the differentiation of Th17 cells during an ongoing immune response and provide potential targets to block a pathogenic response. One such example is binding of an artificial ligand digoxin to ROR-γt which acts to diminish IL-17 production (58). The factors shown to favor and antagonize Treg and Th17 cells in different species are summarized in **Table 1**. In a subsequent section, we highlight technological advances that facilitated Treg or Th17 cell response investigations

TABLE 1 | A summary of positive and negative regulators of regulatory T cell (Treg) and Th17 cell response in different species.

Species	Tregs		$CD4^+$ T cells that produce IL-17	
	Promoting factors	Inhibiting factors	Promoting factors	Inhibiting factors
Mouse	IL-2 TGF-β IL-10 Lower affinity of TCR	IL-6 TNF-α IL-1β	IL-6 TGF-β (low concentration) IL-21 IL-23 Saturated fatty acids IL-1β	TGF-β (high concentration) IL-2 IL-4 IL-12 IFN-γ Polyunsaturated fatty acids Estradiol
Human	Antigen or mitogen IL-2 TGF-β	IL-6 IL-21 IL-23 IL-17 TNF-α IL-1β RANTES	IL-6 TGF-β (low concentrated) IL-21 IL-23	TGF-β IL-4 IL-12 IFN-γ
Canine (dogs)	Con-A IL-2 TGF-β IL-10	IL-6 IL-1β	IL-6 IL-1β TFG-β	TGF-β
Feline (cats)	Mitogens LPS and flagellin IL-2	IL-6 IL-1β	IL-1β IL-6 TGF-β IL-21	TGF-β IL-10
Bovine	Antigen or mitogens along with IL-10, TGF-β	IL-6	IL-23	Progesterone, IFN-γ

as well as their interplay and also make some comments about their potential therapeutic value.

HOW DO WE STUDY THE FUNCTION AND PHENOTYPE OF TREG AND Th17 CELLS?

A summary of the key technological advancements that has facilitated studies involving phenotype and function of Treg and Th17 cells is provided in **Figure 2**. One of the initial identifiers of Treg in naïve mice was surface expression of CD25 (IL-2R α chain) and this served as a marker to facilitate their isolation and characterization (10). The discovery of the *bona fide* transcription factor Foxp3 advanced the field since it is distinctive of Treg and separates them from non-Treg during ongoing infections. Foxp3 not only confers Treg with their regulatory function but also is used for monitoring Treg responses during disease progression to serve as a prognostic biomarker (15, 16, 59–62). As Foxp3 is expressed intracellulary, its detection requires cells to be permeabilized, which renders them dysfunctional, and hence limits utility. The issue was addressed in inbred mice by Bettelli et al. and Fontenot et al. who constructed a Foxp3-GFP knock-in mouse so that live cells could be recovered based on GFP positivity (63, 64). This model also allowed studying migration and localization of Treg during infections (65–67). Other transgenic mouse models such as Foxp3-diphtheria toxin receptor (DTR) also helped advance our understanding of the function and pathophysiology of Treg especially during ongoing infections and immune activation. The DTR is not naturally present in mice and, therefore, a selective depletion of Treg could be achieved by injecting minimal dose of diphtheria toxin (13, 66, 68, 69). Many studies employed this model to study the role of Treg during different stages of an ongoing infection or autoimmune disease (66, 70).

A confounding problem, however, complicated matters since it was realized that Treg might lose their expression of Foxp3 as well as their regulatory function. Moreover, such cells could even take on the function of effector T cells (4). This phenomenon is usually referred to as plasticity or the transdifferentiation. This can be investigated with the availability of so-called fate mapping mice (77, 78). Such animals are constructed in a way that desirable gene products such as Foxp3 or IL-17 are driving Cre recombinase. Crossing these animals with reporter floxed mice having a transgene for fluorescent protein generated fate-mapping mice to address plasticity issues during infections and other inflammatory situations (77, 79). Whether or not proinflammatory cells producing IL-17 could also become regulatory at a later time, triple fate mapping mice have now been created (77). Using these animals, it was demonstrated that Th17 cells could transdifferentiate into Tr1 cells in a model of parasite induced inflammatory disease (77). Thus, fate-mapping mice have become a valuable model to follow the functional changes of T cell subsets in different situations. The method of generation such as inducible vs constitutive expression of transgene/reporter, number of copies inserted, and the expression of products under non-endogenous promoters could, however, impact on the overall utility of such animal models (80). Whether or not Treg plasticity occurs in humans has been difficult to quantify and co-staining for different markers followed by multicolor flow cytometry represents one surrogate way to measure it. In order to generate phenotypically stable regulatory T cells, approaches that modify epigenetic architecture are used. For example, epigenetic modifiers such as HDACs or DNA methyltransferases (DNMTs) inhibitors are used. The use of azacytidine that inhibits DNMTs activity ameliorated herpes simplex virus 1 (HSV-1) induced ocular inflammatory lesion and enhanced Treg responses (81).

In order to gain insights into the functioning of Treg or Teffectors in lymphoid organs or in inflammatory tissues, cells need to be visualized *in vivo*. This could be achieved using two photon intravital microscopy but its accessibility is limited (82, 83). Many observations obtained using inbred strains may not translate to outbred populations for reasons such as the representation of limited MHC polymorphism in former animals. In addition, spontaneous exposures of feral animals to multiple antigens as compared to those that are housed in clean facilities may also yield confounding conclusions. That a dirty environment can make a difference is being emphasized and may include differential migration pattern of immune cells as was shown for $CD8^+$ T cells (84, 85). This led to differential outcome during a subsequent viral infection (84). There is no reason to believe that

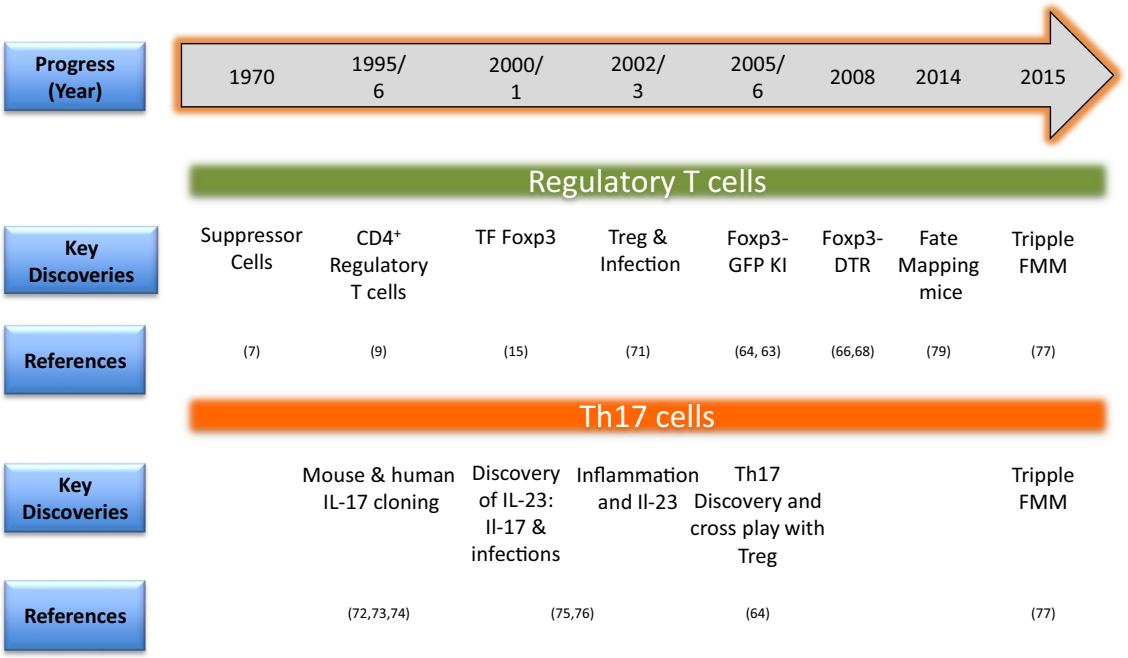


FIGURE 2 | Timeline in brief for methodological advances for investigating regulatory T cell (Treg) and Th17 responses in animals (TF, transcription factor; KI, knock-in-mice; DTR, diphtheria toxin receptor; FMM, fate mapping mice) (71–76).

such a situation would not exist for Th subsets and other types of infections.

One model that could be valuable to address such issues is the zebrafish (*Danio rerio*) (86). The model could be particularly valuable to study cellular interactions due to its anatomical visual transparency. For investigating Treg and Th17 responses in zebrafish, the genes encoding for transcription factors Foxp3 and ROR-γt have been cloned successfully (87). The immune cells and molecules known to exist in vertebrates critical for adaptive immunity are also present in zebrafish (88). Procedures such as transgenesis, nuclear reprogramming, and gene function disruption can be performed with ease in these animals as compared to mice (89–92). Therefore, rather than demonstrating immunological events with select few lines, multiple lines of zebrafish can be generated and used (93, 94). Moreover, the zebrafish is an excellent model for tracing some infectious diseases such as tuberculosis. The granuloma formed by mycobacterial infection in zebrafish exhibits similar histological and pathological features as are evident in *Mtb* infected human granuloma lesions (95, 96). The zebrafish model could surely empower immunologists to visualize the cross regulation of Treg and Th17 cells in pathophysiology of diseases.

The functionality of Treg and Th17 cells can be measured by various *in vitro* assays and *in vivo* adoptive transfer approaches. *In vitro* functional assays include isolating and co-culturing Treg with identifiable non-Treg to measure the functionality (11, 97). Whether or not suppressive activity is contact dependent can be established using trans well assays (11). The responding cells used for suppressive assays can either be stimulated in a polyclonal manner or by antigen pulsed APCs (98).

COMPARISON OF TREG AND Th17 RESPONSE IN HUMANS, RODENTS, AND NON-RODENT ANIMALS

Although there are considerable similarities in the function and phenotype of Treg as well as Th17 cells isolated from mice and humans, differences are also evident. Isoforms of Foxp3 that lack exon 2 or exon 7 exist in human, but not in mice suggesting that the differentiation pathways for Treg in humans and mice may differ (99). Stimulated $CD4^+CD25^-$ T cells in the presence of IL-2 and TGF-β, express Foxp3 and IL-2 acts to stabilize the expression (30). In the absence of TGF-β, Foxp3 could be expressed transiently in stimulated $Foxp3^-$ T cells isolated from humans and to a lesser extent in mice but human cells express latent TGF-β on their surface (100–102). Reactive oxygen species are abundantly present during the initial stages of inflammation and can activate latent TGF-β to make it available for further differentiation into either Treg or Th17 cells. The stages of human Treg generation when TGF-β and IL-2 are critically involved are not yet clearly identified and most studies have concluded that these cytokines dominantly help stabilize Foxp3 expression (49). Varying degrees of epigenetic changes in the Foxp3 locus of human and mouse Treg have been observed (103, 104). Thus, the Foxp3 locus in humans is methylated to a greater extent as compared to that in mice suggesting human Treg take longer to adopt a phenotype

similar to that of mouse Treg (30). The identification markers used for distinguishing human and mouse Treg also display discordance. Thus, even Foxp3 cannot be used for unambiguously defining Treg in humans, unlike in mice (105). Cells expressing sustained Foxp3 expression, however, are considered as suppressive cells. The recently described marker Nrp1 that distinguishes mouse tTreg from pTreg does not faithfully identify one subset or the other in humans (106).

IL-6 and TGF-β play a non-redundant role in the generation of mouse Th17, but this may not be true for human Th17 cell generation (107). IL-1β, TNF-α, and IL-23 are all effective inducers of ROR-γt in differentiating human Th17 cells (108). TGF-β may be dispensable for Th17 cell generation in humans but not in mice (101). The requirement of factors for differentiation of human Th17 cells, however, needs to be cautiously interpreted. Thus, most studies focusing on differentiation of human Th17 cells were performed using peripheral blood cells, and donors are expected to have an exposure to one or more antigens. Therefore, the starting population may not be naïve. Cells are more likely to be naïve when isolated from cord blood and for such cells to differentiate into Th17, TGF-β seems to be critically involved (107). Therefore, differentiation and transdifferentiation of human Th cells need to be fully understood for both naïve and committed cells in order to manipulate Th17 cell responses.

Regulatory T cells and to a lesser extent Th17 cells have been described to exist in most non-rodent animals as well. However, as is described for humans and mice, a mutation in Foxp3 and any subsequent phenotypic effect has not been described in other animals. This could be because of the rarity of such genetic disorders. Anti-human or mouse Foxp3 monoclonal antibodies that cross-react with xenogeneic Foxp3 molecule are used for immunophenotyping Treg in other animal species. Various domains of Foxp3 are conserved across different species and hence show appreciable cross reactivity (109). Foxp3 specific monoclonal antibodies were produced for some non-rodents such as cats and bovines to detect and measure Treg responses (110–112). In cats, an alternative splice variant of Foxp3 lacking exon 2 also exists, an observation similarly recorded for human Foxp3 (99). Surprisingly, when wild type and the variant lacking in exon 2 were expressed in a cell, the suppressive activity was enhanced, as compared a single version expressing cells suggesting a critical role of exon 2 in activity of Treg (111). Cytokines shown to promote Th17 responses in cats are IL-1β, IL-6, TGF-β, and IL-21 (113). Foxp3$^+$ Treg have been demonstrated in animals that include pigs, cows, sheep, goat, horses, baboon, macaque, chimpanzee, harbor seals, and walrus (109). The Foxp3 expression could be induced in CD4$^+$ as well as CD8$^+$ T cells isolated from lymph node of healthy dogs that were stimulated with Con-A (114). A subset of cells that express Foxp3 at intermediate level, but not Foxp3 high cells, also expressed IFN-γ suggesting a plastic nature of such stimulated Treg as well as their tendency to acquire an effector phenotype (115). This could also means that those cells that express optimal level of Foxp3 are more stable as compared to those expressing it to a lower level and the latter cells are not fully committed to Treg phenotype. CD4$^+$ T cells isolated from PBMCs could be efficiently polarized into Th17 cells using a poly-specific stimulator con-A and a combination of cytokines that include IL-6, IL-1β, and TGF-β (116). Foxp3$^+$ Tregs in other species were also described. In fact, suppressor cells in domestic animals were described even before CD25$^+$CD4$^+$ T cells description in mice. In most of these studies, PBMCs stimulated with con-A for a few days acquired suppressive activity toward autologous and allogeneic blood cells (117), but phenotypic markers of these suppressor cells were not described. More recently, Foxp3 was detected not only in bovines αβ-T cells but also in a small proportion of γδ-T cells that were stimulated with Con-A (118, 119). In fact, a recent report suggested that in ruminants that includes bovines, γδ-T cells predominantly play a regulatory role by producing copious amounts of IL-10 and the contribution of CD4$^+$CD25$^+$ Foxp3$^+$ T cells as regulatory cells is minimal (120). In small ruminants that include sheep and goats, Foxp3 expression was not only limited to CD4$^+$ T cells, but was also detectable in other cells such as CD4$^+$CD8$^+$ T cells, CD4$^-$ CD8$^+$ T cells, as well as double negative CD3$^+$ T cells (109). The proportion of non-CD4$^+$ T cells showing Foxp3 expression was variable however. The recorded variation in Treg responses could be attributed to a lack of appropriate reagents, pathophysiological condition of animals, and accessibility to tissues samples for analysis. Animals that are also used for meat purpose, the analyses could be performed using peripheral blood as well as accessing lymphoid organs from slaughtered animals.

In summary, Treg and Th17 cells are likely to be present in most vertebrate species as these cells are thought to have co-evolved (5). The contribution of Th17 cells and the cytokine IL-17 in the pathogenesis of some infectious diseases in some of the non-rodent animals has been described (116, 121). However, most of these studies are observational, and cells were isolated from peripheral blood samples only.

INTERCONVERSION OF TREG AND Th17 CELLS

Does plasticity of Th subsets confer any advantage to the host? The answer probably is in the affirmative. Thus, thymic regression with age limits T cell precursor frequency and the interconverting ability of different Th subsets could provide a facility for the generation of an appropriate helper T cell response required for an efficient adaptive immunity. The cytokines present in the milieu dictate the phenotype of cell upon differentiation, which is well appreciated (122). Functional alteration can include a loss of a useful function, gain of an undesirable activity, or a change in cell location from the site where they normally function. Naïve non-Treg (CD4$^+$Foxp3$^-$) are converted into Treg (CD4$^+$Foxp3$^+$) when stimulated in the presence of IL-2 and TGF-β (11). Similarly, the forced expression of Foxp3 converted conventional T cells into Treg that exhibited a suppressive activity (59). Treg may lose expression of Foxp3 but may not necessarily undergo functional changes (123). Alteration in a cell location is usually explained by differential expression of homing molecules and this relocation can also explain functional changes in some instances (40, 42). Relocation effects may help explain changes in Treg activity during different phases of an inflammatory response. In fact, during an acute inflammatory response, the number of Foxp3$^+$ Treg in draining lymph node is reduced dramatically while their number

increased in distal lymph nodes. This could mean that Treg prefer to stay in a non-inflammatory environment conceivably by modulating their homing receptors. This may also mean that Treg are more efficient in regulating responses that are milder in nature. Alternatively, those cells that reside in the most severe inflammatory environments and still retain the phenotype are more resilient and less likely to become non-Treg. All these issues have yet to be addressed adequately.

Among factors responsible for conferring stability and limiting, plasticity is the continuous availability of cytokines such as IL-2 (124). Treg that are deprived of IL-2 and potentially other cytokines are more inclined to change their phenotype (125). At a molecular level, this outcome can be explained in terms of epigenetic alterations in the conserved non-coding sequences (CNS) of *Foxp3* gene (126). Some have advocated that the subsets of Treg that are more plastic are those at an intermediate stage of their differentiation (122, 126). Such cells may eventually fail to establish their complete epigenetic architecture, an effect that can be influenced by the microenvironment (122). One of the most studied epigenetic modifications that is known to influence the stability of Treg is methylation of CpG islands in the CNS2 of *Foxp3* gene, also known as Treg-specific demethylated region (TSDR) (126). Thus, those Treg that have a hypomethylated Foxp3 TSDR are more stable as compared to those whose TSDR is hypermethylated (125, 126). This also relates to the expression of Foxp3 and its ability to promote expression of Treg associated genes. Accordingly, the activity of DNA methyl transferases in such cells may decide whether phenotypically stable cells will be generated or not. Nrp1 a molecules differentially expressed by tTreg is also involved in stabilization of Foxp3 expression. Signaling induced by ligation of Nrp1 with semaphorin-4a molecule in Treg-enhanced expression of transcription factors such as Foxo1 and Foxo3 to help stabilize Foxp3 expression (23). Eos is another transcription factor that impacts on the stability of Treg, but this effect could be independent of Foxp3 expression (127). Other studies indicate that Treg stability involves post translational modification of Foxp3 and the induction of its alternative splice variants (128). Treg that have enhanced phosphorylated Foxp3 (p-Foxp3) levels are more stable as compared to those that have less or no p-Foxp3 (128–130). Accordingly, phosphatases induced by a highly proinflammatory environment could dephosphorylate Foxp3 in Treg, which then are converted to become pathogenic Th17 cells (129, 130). Another study attributed the metabolic state of Th cells to their function and phenotype (131). Thus, it was shown that glycolysis in Th cells is critical for their conversion to become Treg (131). Enolase I, an enzyme, is induced when cell metabolism is switched to the glycolytic pathway (131). Enolase I plays an essential role by interacting with Foxp3 regulatory sequences to effect the expression of an alternative splice variant that utilizes exon 2 of Foxp3 (131). However, the mechanisms responsible for stability conferred by alternative splice variants of Foxp3 are not entirely clear, but could relate to their resistance to degradation or the presence of more amino acid residues that can undergo phosphorylation.

Some studies have implicated the role of certain microRNAs in regulating the stability of Treg (132–134). miRNAs are small oligonucleotides that are expressed endogenously and have critical roles in gene expression (132). In general, miRNA 29, 125a, 125b, 155, and 181 seem to affect differentiation of Th subsets (132). Some miRNAs such as miRNA 181 modulates TCR signaling and its expression alters with the maturation state of T cells (135). miRNA 155 specifically influences differentiation of Treg and Th17 cells which can affect the outcome of inflammatory diseases (136, 137).

As differentiation pathways between Treg and Th17 cells are shared, these cells exhibit greater tendency for interconversion. Some investigators have suggested that TGF-β induced Tregs as compared to natural Tregs are more likely to acquire a Th17 phenotype. Such cells are more likely to express membrane bound TGF-β and in an environment enriched in IL-6 or other inflammatory molecules, they become Th17 cells (138). Additionally, TGF-β induced cells have not established their complete epigenetic landscape and hence are more plastic in nature as compared to natural Treg. The conditions where Th17 cells can also become Foxp3 expressing Treg have not been established as yet, but the Th17 cells change to acquire other phenotypes that include Th1, Th2, Tr1, or T_{FH}. This could occur because of the relative positioning of Foxp3 and ROR-γt in a 3-diamensional space in the cell and hence a physical interaction may not occur in Th17 cells as does occur in Treg (139). For establishing plasticity issues unambiguously, fate-mapping mice as described in an earlier section are used. Not only mice but also human Treg can become Th17 cells when stimulated with IL-1β and IL-6 (140). In conclusion, the interconversion of $Foxp3^+$ Treg into Th17 cells is appreciable and well established upon the change of microenvironment but counterconversion of Th17 cells into $Foxp3^+$ Treg cells is not known currently.

CROSS REGULATION OF TREG AND Th17 CELLS DURING PATHOPHYSIOLOGY OF INFECTIOUS DISEASES

That Foxp3 is critically involved in the function of Treg has been shown in both humans and mice. A spontaneous mutation comprising a 2-bp insertion in the coding region of Foxp3 gene resulted in a truncated non-functional protein. Mutant mice, known as scurfy mice, developed spontaneous multiorgan inflammatory lesions (141–143). Male mice exhibited a pronounced phenotype as compared to females, suggesting the mutation was X-linked. Crossing scurfy mice with Foxp3 transgenics rescued the phenotype confirming the role of the mutation in disease causation (16). Similarly, patients who had immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome exhibited a mutation in the Foxp3 gene and developed autoimmune enteropathy, psoriasiform or eczematous dermatitis, nail dystrophy, and endocrinopathy. IPEX is a rare disease with a strong genetic association (144).

The balancing of response in activity of Treg and Th17 cells can influence the outcome of numerous infectious and non-infectious diseases (108). Whether or not these cells play a role in orchestrating disease due to infections in non-rodent animals is not well established and is suggested based on scanty data, which are often unconfirmed. During infections, the dominant effect of Treg perhaps is not to dampen protective immunity, but

to prevent collateral tissue damage. In some infections, such as the one caused by *Leishmania*, Tregs that were induced *de novo* and recruited to infected sites were specific to pathogen-derived antigens (14, 145). Along similar lines, it was demonstrated that parasites (*Schistosome*) and bacteria (*Helicobacter pylori*, *Mycobacterium*, *Histoplasma)* promoted the peripheral generation of Treg (146–148). A protozoan parasite, *Toxoplasma gondii*, caused enhanced immunopathological reactions by inhibiting and destabilizing Treg (14, 145). Interestingly, destabilized Treg acquired Tbet and produced IFN-γ suggesting their conversion into Th1 like cells. In this study, ROR-γt expression by these was not analyzed. Tbet controls the expression of TIM-3 and those Tregs that express TIM-3 were shown to be resistant to apoptosis when ligated with galectin-9 (149). These seemingly contradicting observations could in fact hint the existence of different subtypes of Treg some of which will eventually be eliminated while some remain in animals and serve as dual function. How acute and chronic viral infections signal Treg response has been investigated (46). The outcome of acute infections caused by viruses such as Friend retrovirus, lymphocytic choriomeningitis virus (LCMV), influenza A virus (IAV), West Nile virus (WNV), respiratory syncytial virus (RSV), hepatitis A virus (HAV), and HSV-1 is influenced to a varying degree by Treg and possible Th17 cell responses (150). Acute LCMV infection induced type I interferon that diminished Treg function and as a result anti-viral $CD4^+$ and $CD8^+$ T cell responses are enhanced (151). Treg also critically influence the outcome of infection by IAV, WNV, RSV, HAV, and HSV-1 (69). In mice infected with IAV intranasally, more $Foxp3^+$ Treg accumulate in the draining mediastinal LNs (MLNs) suggesting that virus is able to promote Treg responses (152). Mice depleted of Treg developed more severe lesion suggesting Tregs were able to control immunopathological responses. Respiratory influenza infection induced $CCR9^+CD4^+$ T helper cell generation in the MLNs. These cells, by responding to CCL25, preferentially migrated to the gut and were responsible for an inflammatory reaction mediated by Th17 cells (153). The antigen specificity and phenotype of migrating $CD4^+$ T cells in the gut are not known. Whether or not the migratory cells by themselves orchestrated gut inflammation, or induced conversion of resident $CD4^+$ T cells to become Th17 cells, remains to be elucidated. However, this study indicated that Th17 cells could in fact serve as one of the players of "common mucosal immune axis" and could influence the composition of microbiota in the gut during some infections (153). Another study demonstrated that influenza virus inhibited Th17 mediated control of a secondary bacterial infection to cause pneumonia (154). Therefore, during IAV pathogenesis, the cross play of Treg and Th17 can impact the pathogenesis. During WNV infection in humans, Treg helped control the development of clinical symptoms and fever by preventing tissue damaging inflammatory reactions because asymptomatic individuals had greater numbers of Treg in peripheral blood (155). Similarly, WNV infected mice, depleted of Treg, developed lethal encephalitis suggesting Treg response was protective in nature (155). A specific role of Th17 cells in WNV pathogenesis has not been demonstrated, but encephalitis caused by WNV was not influenced by the Th17 cell response (156). As compared to controls, Treg depleted mice upon RSV infection showed enhanced Th2 responses that led to severe pulmonary immunopathological lesions (157). Most cases of acute HAV infection resolve with efficient viral clearance and innocuous pathological consequences, which could relate to how Treg are signaled (158). HAV directly binds to its cellular receptor 1 (HAVCR1 also known as TIM-1) expressed by Treg and as a result abrogates their function to promote anti-viral $CD8^+$ T cell responses. Efficient $CD8^+$ T cells then help control virus infection (159). Whether or not Th17 cells play any role in RSV and HAV infection is not clear. The influence of Treg in HSV pathogenesis has been extensively studied by numerous approaches (160–164). Mice that were depleted of Treg prior to HSV infection mounted enhanced primary and memory anti-viral $CD8^+$ T cell responses (162) and when Treg were depleted prior to ocular infection with HSV-1 heightened $CD4^+$ T cell effector response led to an aggravated corneal inflammatory disease, as compared to those mice that had intact Treg responses (163). This observation was followed up in subsequent studies employing adoptive transfer of natural Treg as well as TGF-β induced Treg in mice before infection (161, 163). Treg recipient mice developed diminished inflammatory lesion as compared to infected controls (161). We observed that ligation of $CD4^+$ T cell expressed sphingosine 1 phosphate receptor (S1P1) by an agonist FTY720 promoted Treg responses (165, 166). These converted cells, however, were inclined to acquire a Th17 phenotype when incubated with IL-6 and exhibited an aggressive proinflammatory activity in HSV-1 infected animals (165). IL-6 neutralization diminished lesions of the disease suggesting that the converted cells might be more plastic and in fact more damaging. What stage of infection Treg responses are critical in controlling the disease severity was investigated using a DTR-Foxp3 transgenic mouse model in which Treg could be depleted using diphtheria toxin at different times post-infection (70). The results suggested that Tregs continue to regulate inflammatory responses irrespective of stage when these are depleted and that Treg might in fact be acting both in the DLN during induction phase of response and at inflammatory sites (70). Direct interaction of Treg expressed HVEM and HSV-1-gD glycoprotein provided a partial explanation as to how HSV-1 is able to signal Treg so promptly after infection (167). The role of Th17 cells in HSV-1 induced pathogenesis was also investigated using IL-17R KO mice as well as in mice lacking different subunits of cytokine IL-23 (p19 and p35), a cytokine critically involved in promoting Th17 cell responses (168, 169). These studies demonstrated that IL-17 contributed by innate immune cells, γδ T cells and Th cells, enhanced the severity of inflammatory lesions. Th17 cells were predominantly involved during the chronic phase of infection, while during the acute phase their contribution was minimal (169). This also suggests that inflammatory milieu in cornea may induce conversion of some accumulated Treg or Th1 cells into a Th17 phenotype. It would be worth investigating whether Th17 cells can further become Treg and how would that influence the lesion severity.

Most chronic viral infections were shown to influence Treg responses and eventually the outcome of chronic infections (13, 46). Notably, HIV and HCV are the most prominent chronic viral infections where Treg seems to play a critical role in pathogenesis (13, 46). Precise mechanisms how these infections trigger

Treg responses are not clear, but the microenvironments created could contribute. HIV, HCV, and IAV could all activate latent TGF-β to promote Treg and potentially Th17 responses depending on its concentration along with that of other inflammatory cytokines (43, 170, 171). During HIV infection, Tregs play multiple roles that range from an early abrogation of effector $CD4^+$ T cells to tissue repair during later stages (172). HIV promotes Treg responses by modulating the function of DCs which stimulate Treg generation (173). Tregs, in turn, control the activation of $CD4^+$ T cells to minimize their infection by the virus. Thus, activated $CD4^+$ T cells are more susceptible to HIV infection as compared to those in resting stage (174). TGF-β produced by Treg, and probably other cells, promotes collagen deposition in lymphoid organs (175). This poses a problem when the patients are given anti-retroviral therapy and immune reconstitution is required. Thus, the effective space available would be less for immune reconstitution (175). The involvement of Treg in HIV pathogenesis, therefore, is a complex issue and needs more study.

Th17 cells seem to play a crucial role in the pathogenesis of HIV infection as these cells accumulate abundantly in the gut-associated lymphoid tissues (GALT) early after infection (176). Whether the accumulated Th17 cells in GALT originate from Treg or differentiate from naïve cells is still to be established. The activated Treg in the gut could contribute to TGF-β production and HIV infection could trigger IL-6 production by innate cells. Th17 cells are known to express surface CD45RO, CCR5, and CXCR4 making them more permissive to HIV infection (177). Infected Th17 cells are cleared by the virus itself, or by cytotoxic $CD8^+$ T cells. As Th17 cells are critical for maintaining the integrity of mucosal barriers, their depletion could disrupt these barriers and initiate generalized immune activation (178, 179). The Th17 cells that influence the outcome of HIV infection may not necessarily be specific for viral antigens. The role of Th17 cells was also demonstrated in long-term non-progressers who exhibit pronounced Th17 responses as compared to those who progress rapidly to develop HIV-AIDS. Restoration of Th17 cells in patients undergoing highly active anti-retroviral therapy is an indicator of better prognosis predominantly due to efficient control of bacterial infections by these cells (180). Therefore, a balance of Treg and Th17 cell response may critically influence the pathogenesis of HIV infection.

HCV and Treg interaction is complicated to investigate, as the responses need to be evaluated in the liver, where disease occurs. This is particularly confounded by the unavailability of a rodent model and the now unavailable chimpanzee being the only reliable animal model to study HCV pathogenesis. What determines the resolution of infection in only 20% HCV infected patients is not clearly understood but is thought to be explained by an effective anti-viral $CD8^+$ and $CD4^+$ T cell response (181, 182). In those which fail to control infection, some have advocated that an induced Treg response, which blunts the activity of effector T cells, could be the explanation (183). During HCV infection, the cell types that are known to exhibit predominant regulatory activity are Tr1 cells and possibly $CD8^+$ Treg in addition to Foxp3 positive cells as suggested by some studies (170). However, it remains to be evaluated whether Tregs play a beneficial or detrimental role during chronic stages of HCV infection. Th17 cells, owing to their cytokine secretion, are thought to play a predominant role in the repair process leading to fibrosis in the liver and seem not to play a critical role early during HCV infection. Accordingly, patients treated with interferon and ribavirin therapy had decreased Treg responses but minimal effects on Th17 cells were observed (183). The Treg and Th17 cell ratio, however, was skewed toward Th17 cells with a favorable outcome of therapy. How various subsets of Th cells influence HCV pathogenesis remains a controversial issue that merits further evaluation. However, the issue is now less relevant since there is a new highly effective anti-viral that controls HCV infection.

Regulatory T cells, and to a lesser extent Th17 cells, do influence the outcome of various infections in pet animals that include dogs and cats. These animals also serve as models for various infectious and non-infectious diseases. For example, similarities in the pathogenesis of feline immunodeficiency virus (FIV) and HIV make the cat a useful animal model (184). FIV was shown to infect Treg and this made them better suppressors (113, 185). FIV infected cats exhibit an early depletion of $CD4^+$ T cells and enhanced Treg activity, which in turn compromises anti-viral adaptive immunity. This provides the virus an opportunity to establish a productive infection (186). More recent reports suggest a dysregulation of Treg and Th17 cells during FIV pathogenesis in cats during a systemic infection as well as in the placenta leading to non-viable pregnancies (113). Whether or not a similar situation exists in pregnant women infected with HIV is not known.

The canines genome revealed striking similarities in functionally related genes with humans and single nucleotide polymorphisms have been recently mapped (187). Some shared infections between dogs and humans are beginning to provide new insights in the pathophysiology of diseases (188). $Foxp3^+$ Treg responses have been studied in canine leishmania infection where a variable response pattern for Treg and Th17 cells was observed in different organs (189). Whether or not interconversion in these cell populations occurs during infection is yet to be explained.

The responsiveness of Treg during infectious diseases in bovines has been investigated (190–192). *Mycobacterium paratuberculosis,* the causative agent of debilitating Johne's disease and bovine leukemia virus (BLV) induce $CD4^+$ T cells that produced IL-10 and those that expressed Foxp3, respectively (190, 192). During BLV infections, enhanced Treg responses act to constrain anti-viral immunity and probably cause the pathogen to persist in animals (191). Johne's disease is thought to be orchestrated by Th1 cells of which some cells also produced IL-17 suggesting the plastic nature of these cells. However, as this is mainly a gut associated disease, probably the role of balance between Tregs and Th17 cells would provide better insights into its pathogenesis.

Small ruminants, such as sheep and goats, serve as major livestock for landless laborers and marginal farmers. Which cellular mediators are induced early during the response decides the efficiency of immunity to infections as well as immunization. Major pathogens that infect small ruminants are parasites such as *Teladorsagia circumcincta* and *Haemonchus contortus*, which induce an orchestrated response pattern characterized initially by Th1 and during later stages by Th2 and regulatory response (193, 194). Whether or not Th17 responses are critical for defense against parasitic infections has not been investigated. As these

parasites infest gut of these animals, it would be interesting to investigate how a balance of Th17 and Treg is affected. Rinderpest virus is the only pathogen of animals eliminated from the face of earth; however, its close relative pestes des petits ruminantium virus (PPRV) is still a major problem in many parts of the world in ruminants and cause immunosuppression in the host. Both viruses inhibit proliferation of leukocyte *in vitro* (195). Surprisingly, however, the role of Treg and Th17 during PPRV infection or during vaccination against PPRV has not been investigated and could provide better insights into their pathogenesis and eventually better management practices could be employed.

CONCLUSION

Enumerable studies performed in rodents and to some extent in humans exposed to or infected with one or more microbes revel an intricate interplay of various subsets of $CD4^+$ T cells which influences the disease outcome. Treg and Th17 response dynamics is beginning to provide new insights into the pathogenesis of various infections. However, there exist a vast gap in our understanding how these cell type are induced, maintained, and interact with each other in animals other than inbred rodents. Such insights could open new avenues of modifying their function to achieve better resolution of infection and mitigate tissue damaging reaction in humans and animals.

AUTHOR CONTRIBUTIONS

SS compiled information and discussed in context, while BR was involved in editing and logically presenting the information.

FUNDING

The work was supported by the intramural funding from the Indian Institute of Science Education and Research Mohali to SS and extramural funds from NIH to BR (Grant No AI 063365).

REFERENCES

1. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* (1986) 136:2348–57.
2. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* (1989) 7:145–73. doi:10.1146/annurev.iy.07.040189.001045
3. Zhu J, Paul WE. CD4 T cells: fates, functions, and faults. *Blood* (2008) 112:1557–69. doi:10.1182/blood-2008-05-078154
4. Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol* (2010) 28:445–89. doi:10.1146/annurev-immunol-030409-101212
5. Weaver CT, Harrington LE, Mangan PR, Gavrieli M, Murphy KM. Th17: an effector CD4 T cell lineage with regulatory T cell ties. *Immunity* (2006) 24:677–88. doi:10.1016/j.immuni.2006.06.002
6. Denning TL, Wang Y, Patel SR, Williams IR, Pulendran B. Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17 – producing T cell responses. *Nat Immunol* (2007) 8:1086–94. doi:10.1038/ni1511
7. Gershon RK, Kondo K. Cell interactions in the induction of tolerance: the role of thymic lymphocytes. *Immunology* (1970) 18:723–37.
8. Sakaguchi S, Takahashi T, Nishizuka Y. Study on cellular events in post-thymectomy autoimmune oophoritis in mice. II. Requirement of Lyt-1 cells in normal female mice for the prevention of oophoritis. *J Exp Med* (1982) 156:1577–86. doi:10.1084/jem.156.6.1565
9. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* (1995) 155:1151–64.
10. Suri-Payer E, Amar AZ, Thornton AM, Shevach EM. CD4+CD25+ T cells inhibit both the induction and effector function of autoreactive T cells and represent a unique lineage of immunoregulatory cells. *J Immunol* (1998) 160:1212–8.
11. Thornton AM, Shevach EM. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* (1998) 188:287–96. doi:10.1084/jem.188.2.287
12. Belkaid Y. Regulatory T cells and infection: a dangerous necessity. *Nat Rev Immunol* (2007) 7:875–88. doi:10.1038/nri2189
13. Belkaid Y, Rouse BT. Natural regulatory T cells in infectious disease. *Nat Immunol* (2005) 6:353–60. doi:10.1038/ni1181
14. Belkaid Y, Tarbell K. Regulatory T cells in the control of host-microorganism interactions (*). *Annu Rev Immunol* (2009) 27:551–89. doi:10.1146/annurev.immunol.021908.132723
15. Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, et al. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet* (2001) 27:68–73. doi:10.1038/83784
16. Khattri R, Kasprowicz D, Cox T, Mortrud M, Appleby MW, Brunkow ME, et al. The amount of scurfin protein determines peripheral T cell number and responsiveness. *J Immunol* (2001) 167:6312–20. doi:10.4049/jimmunol.167.11.6312
17. Ramsdell F, Ziegler SF. {FOXP3} and scurfy: how it all began. *Nat Rev Immunol* (2014) 14:343–9. doi:10.1038/nri3650
18. Marson A, Kretschmer K, Frampton GM, Jacobsen ES, Polansky JK, MacIsaac KD, et al. Foxp3 occupancy and regulation of key target genes during T-cell stimulation. *Nature* (2007) 445:931–5. doi:10.1038/nature05478
19. Rudra D, deRoos P, Chaudhry A, Niec RE, Arvey A, Samstein RM, et al. Transcription factor Foxp3 and its protein partners form a complex regulatory network. *Nat Immunol* (2012) 13:1010–9. doi:10.1038/ni.2402
20. Wu Y, Borde M, Heissmeyer V, Feuerer M, Lapan AD, Stroud JC, et al. FOXP3 controls regulatory T cell function through cooperation with NFAT. *Cell* (2006) 126:375–87. doi:10.1016/j.cell.2006.05.042
21. Xie X, Stubbington MJT, Nissen JK, Andersen KG, Hebenstreit D, Teichmann SA, et al. The regulatory T cell lineage factor Foxp3 regulates gene expression through several distinct mechanisms mostly independent of direct DNA binding. *PLoS Genet* (2015) 11:e1005251. doi:10.1371/journal.pgen.1005251
22. Ziegler SF. FOXP3: not just for regulatory T cells anymore. *Eur J Immunol* (2007) 37:21–3. doi:10.1002/eji.200636435
23. Delgoffe GM, Woo S-R, Turnis ME, Gravano DM, Guy C, Overacre AE, et al. Stability and function of regulatory T cells is maintained by a neuropilin-1-semaphorin-4a axis. *Nature* (2013) 501:252–6. doi:10.1038/nature12428
24. Weiss JM, Bilate AM, Gobert M, Ding Y, Curotto de Lafaille MA, Parkhurst CN, et al. Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not mucosa-generated induced Foxp3+ T reg cells. *J Exp Med* (2012) 209(1723–42):S1. doi:10.1084/jem.20120914
25. Yadav M, Louvet C, Davini D, Gardner JM, Martinez-Llordella M, Bailey-Bucktrout S, et al. Neuropilin-1 distinguishes natural and inducible regulatory T cells among regulatory T cell subsets in vivo. *J Exp Med* (2012) 209(1713–22):S1–19. doi:10.1084/jem.20120822
26. Asano M, Toda M, Sakaguchi N, Sakaguchi S. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J Exp Med* (1996) 184:387–96. doi:10.1084/jem.184.2.387
27. Samy ETE, Wheeler KM, Roper RJ, Teuscher C, Tung KSK. Cutting edge: autoimmune disease in day 3 thymectomized mice is controlled by endogenous disease-specific regulatory T cells. *J Immunol* (2008) 180:4366–70.

28. Josefowicz SZ, Lu L-F, Rudensky AY. Regulatory T cells: mechanisms of differentiation and function. *Annu Rev Immunol* (2012) 30:531–64. doi:10.1146/annurev.immunol.25.022106.141623
29. Rossjohn J, Gras S, Miles JJ, Turner SJ, Godfrey DI, McCluskey J. T cell antigen receptor recognition of antigen-presenting molecules. *Annu Rev Immunol* (2015) 33:169–200. doi:10.1146/annurev-immunol-032414-112334
30. Sakaguchi S, Vignali DAA, Rudensky AY, Niec RE, Waldmann H. The plasticity and stability of regulatory T cells. *Nat Rev Immunol* (2013) 13:461–7. doi:10.1038/nri3464
31. Yin L, Scott-Browne J, Kappler JW, Gapin L, Marrack P. T cells and their eons-old obsession with MHC. *Immunol Rev* (2012) 250:49–60. doi:10.1111/imr.12004
32. Beringer DX, Kleijwegt FS, Wiede F, van der Slik AR, Loh KL, Petersen J, et al. T cell receptor reversed polarity recognition of a self-antigen major histocompatibility complex. *Nat Immunol* (2015) 16:1153–61. doi:10.1038/ni.3271
33. Peakman M, Sewell AK. Reversed-polarity Treg cell TCRs provide a shock. *Nat Immunol* (2015) 16:1105–7. doi:10.1038/ni.3289
34. Purvis HA, Stoop JN, Mann J, Woods S, Kozijn AE, Hambleton S, et al. Low-strength T-cell activation promotes Th17 responses. *Blood* (2010) 116:4829–37. doi:10.1182/blood-2010-03-272153
35. Rawls JF, Mahowald MA, Ley RE, Gordon JI. Reciprocal gut microbiota transplants from zebrafish and mice to germ-free recipients reveal host habitat selection. *Cell* (2006) 127:423–33. doi:10.1016/j.cell.2006.08.043
36. Berod L, Friedrich C, Nandan A, Freitag J, Hagemann S, Harmrolfs K, et al. De novo fatty acid synthesis controls the fate between regulatory T and T helper 17 cells. *Nat Med* (2014) 20:1327–33. doi:10.1038/nm.3704
37. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-Y M, et al. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science* (2013) 341:569–73. doi:10.1126/science.1241165
38. Wang C, Yosef N, Gaublomme J, Wu C, Lee Y, Clish CB, et al. CD5L/AIM regulates lipid biosynthesis and restrains Th17 cell pathogenicity. *Cell* (2015) 163:1413–27. doi:10.1016/j.cell.2015.10.068
39. Scheinecker C, McHugh R, Shevach EM, Germain RN. Constitutive presentation of a natural tissue autoantigen exclusively by dendritic cells in the draining lymph node. *J Exp Med* (2002) 196:1079–90. doi:10.1084/jem.20020991
40. Wei S, Kryczek I, Zou W. Regulatory T-cell compartmentalization and trafficking. *Blood* (2006) 108:426–31. doi:10.1182/blood-2006-01-0177
41. Wheeler KM, Samy ET, Tung KSK. Cutting edge: normal regional lymph node enrichment of antigen-specific regulatory T cells with autoimmune disease-suppressive capacity. *J Immunol* (2009) 183:7635–8. doi:10.4049/jimmunol.0804251
42. Zhang N, Schröppel B, Lal G, Jakubzick C, Mao X, Chen D, et al. Regulatory T cells sequentially migrate from inflamed tissues to draining lymph nodes to suppress the alloimmune response. *Immunity* (2009) 30:458–69. doi:10.1016/j.immuni.2008.12.022
43. Amarnath S, Dong L, Li J, Wu Y, Chen W. Endogenous TGF-beta activation by reactive oxygen species is key to Foxp3 induction in TCR-stimulated and HIV-1-infected human CD4+CD25- T cells. *Retrovirology* (2007) 4:57. doi:10.1186/1742-4690-4-57
44. Koli K, Myllärniemi M, Keski-Oja J, Kinnula VL. Transforming growth factor-beta activation in the lung: focus on fibrosis and reactive oxygen species. *Antioxid Redox Signal* (2008) 10:333–42. doi:10.1089/ars.2007.1914
45. Li MO, Flavell RA. TGF-β: a master of all T cell trades. *Cell* (2008) 134:392–404. doi:10.1016/j.cell.2008.07.025
46. Rouse BT, Sehrawat S. Immunity and immunopathology to viruses: what decides the outcome? *Nat Rev Immunol* (2010) 10:514–26. doi:10.1038/nri2802
47. Marie JC, Letterio JJ, Gavin M, Rudensky AY. TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells. *J Exp Med* (2005) 201:1061–7. doi:10.1084/jem.20042276
48. Rubtsov YP, Rudensky AY. TGFβ signalling in control of T-cell-mediated self-reactivity. *Nat Rev Immunol* (2007) 7:443–53. doi:10.1038/nri2095
49. Wan YY, Flavell RA. "Yin-Yang" functions of transforming growth factor-beta and T regulatory cells in immune regulation. *Immunol Rev* (2007) 220:199–213. doi:10.1111/j.1600-065X.2007.00565.x
50. von Boehmer H. Oral tolerance: is it all retinoic acid? *J Exp Med* (2007) 204:1737–9. doi:10.1084/jem.20071251
51. Benson MJ, Pino-Lagos K, Rosemblatt M, Noelle RJ. All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. *J Exp Med* (2007) 204:1765–74. doi:10.1084/jem.20070719
52. Sun C-M, Hall JA, Blank RB, Bouladoux N, Oukka M, Mora JR, et al. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med* (2007) 204:1775–85. doi:10.1084/jem.20070602
53. Zhou X, Wang W, Yang Y. The expression of retinoic acid receptors in thymus of young children and the effect of all-transretinoic acid on the development of T cells in thymus. *J Clin Immunol* (2008) 28:85–91. doi:10.1007/s10875-007-9122-y
54. Vignali DAA, Collison LW, Workman CJ. How regulatory T cells work. *Nat Rev Immunol* (2008) 8:523–32. doi:10.1038/nri2343
55. Schmierer B, Hill CS. TGFβ–SMAD signal transduction: molecular specificity and functional flexibility. *Nat Rev Mol Cell Biol* (2007) 8:970–82. doi:10.1038/nrm2297
56. Ziegler SF, Buckner JH. FOXP3 and the regulation of Treg/Th17 differentiation. *Microbes Infect* (2009) 11:594–8. doi:10.1016/j.micinf.2009.04.002
57. Wang X, Zhang Y, Yang XO, Nurieva RI, Chang SH, Ojeda SS, et al. Transcription of IL17 and IL17f is controlled by conserved noncoding sequence 2. *Immunity* (2012) 36:23–31. doi:10.1016/j.immuni.2011.10.019
58. Fujita-Sato S, Ito S, Isobe T, Ohyama T, Wakabayashi K, Morishita K, et al. Structural basis of digoxin that antagonizes RORgamma t receptor activity and suppresses Th17 cell differentiation and interleukin (IL)-17 production. *J Biol Chem* (2011) 286(36):31409–17. doi:10.1074/jbc.M111.254003
59. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* (2003) 299:1057–61. doi:10.1126/science.1079490
60. Schreiber TH. The use of FoxP3 as a biomarker and prognostic factor for malignant human tumors. *Cancer Epidemiol Biomarkers Prev* (2007) 16:1931–4. doi:10.1158/1055-9965.EPI-07-0396
61. Shang B, Liu Y, Jiang S, Liu Y. Prognostic value of tumor-infiltrating FoxP3+ regulatory T cells in cancers: a systematic review and meta-analysis. *Sci Rep* (2015) 5:15179. doi:10.1038/srep15179
62. Weller P, Bankfalvi A, Gu X, Dominas N, Lehnerdt GF, Zeidler R, et al. The role of tumour FoxP3 as prognostic marker in different subtypes of head and neck cancer. *Eur J Cancer* (2014) 50:1291–300. doi:10.1016/j.ejca.2014.02.016
63. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* (2006) 441:235–8. doi:10.1038/nature04753
64. Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* (2005) 22:329–41. doi:10.1016/j.immuni.2005.01.016
65. Huter EN, Punkosdy GA, Glass DD, Cheng LI, Ward JM, Shevach EM. TGF-beta-induced Foxp3+ regulatory T cells rescue scurfy mice. *Eur J Immunol* (2008) 38:1814–21. doi:10.1002/eji.200838346
66. Kim JM, Rasmussen JP, Rudensky AY. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat Immunol* (2007) 8:191–7. doi:10.1038/ni1428
67. Punkosdy GA, Blain M, Glass DD, Lozano MM, O'Mara L, Dudley JP, et al. Regulatory T-cell expansion during chronic viral infection is dependent on endogenous retroviral superantigens. *Proc Natl Acad Sci U S A* (2011) 108(9):3677–82. doi:10.1073/pnas.1100213108
68. Lahl K, Loddenkemper C, Drouin C, Freyer J, Arnason J, Eberl G, et al. Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. *J Exp Med* (2007) 204:57–63. doi:10.1084/jem.20061852
69. Veiga-Parga T, Sehrawat S, Rouse BT. Role of regulatory T cells during virus infection. *Immunol Rev* (2013) 255:182–96. doi:10.1111/imr.12085
70. Veiga-Parga T, Suryawanshi A, Mulik S, Giménez F, Sharma S, Sparwasser T, et al. On the role of regulatory T cells during viral-induced inflammatory lesions. *J Immunol* (2012) 189:5924–33. doi:10.4049/jimmunol.1202322
71. Belkaid Y, Piccirillo CA, Mendez S, Shevach EM, Sacks DL. CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity. *Nature* (2002) 420:502–7. doi:10.1038/nature01152
72. Rouvier E, Luciani MF, Mattéi MG, Denizot F, Golstein P. CTLA-8, cloned from an activated T cell, bearing AU-rich messenger RNA instability

sequences, and homologous to a Herpesvirus saimiri gene. *J Immunol* (1993) 150:5445–56.
73. Yao Z, Painter SL, Fanslow WC, Ulrich D, Macduff BM, Spriggs MK, et al. Human IL-17: a novel cytokine derived from T cells. *J Immunol* (1995) 155:5483–6.
74. Fossiez F, Djossou O, Chomarat P, Flores-Romo L, Ait-Yahia S, Maat C, et al. T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. *J Exp Med* (1996) 183:2593–603. doi:10.1084/jem.183.6.2593
75. Oppmann B, Lesley R, Blom B, Timans JC, Xu Y, Hunte B, et al. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* (2000) 13:715–25. doi:10.1016/S1074-7613(00)00070-4
76. Ye P, Rodriguez FH, Kanaly S, Stocking KL, Schurr J, Schwarzenberger P, et al. Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. *J Exp Med* (2001) 194:519–27. doi:10.1084/jem.194.4.519
77. Gagliani N, Vesely MCA, Iseppon A, Brockmann L, Xu H, Palm NW, et al. Th17 cells transdifferentiate into regulatory T cells during resolution of inflammation. *Nature* (2015) 523:221–5. doi:10.1038/nature14452
78. Miyao T, Floess S, Setoguchi R, Luche H, Fehling HJ, Waldmann H, et al. Plasticity of Foxp3(+) T cells reflects promiscuous Foxp3 expression in conventional T cells but not reprogramming of regulatory T cells. *Immunity* (2012) 36:262–75. doi:10.1016/j.immuni.2011.12.012
79. Komatsu N, Okamoto K, Sawa S, Nakashima T, Oh-hora M, Kodama T, et al. Pathogenic conversion of Foxp3+ T cells into TH17 cells in autoimmune arthritis. *Nat Med* (2014) 20:62–8. doi:10.1038/nm.3432
80. Overacre AE, Vignali DA. T(reg) stability: to be or not to be. *Curr Opin Immunol* (2016) 39:39–43. doi:10.1016/j.coi.2015.12.009
81. Varanasi SK, Reddy PBJ, Bhela S, Jaggi U, Gimenez F, Rouse BT. Azacytidine treatment inhibits the progression of herpes stromal keratitis by enhancing regulatory T cell function. *J Virol* (2017) 91:e02367-16. doi:10.1128/JVI.02367-16
82. Cahalan MD, Parker I, Wei SH, Miller MJ. Two-photon tissue imaging: seeing the immune system in a fresh light. *Nat Rev Immunol* (2002) 2:872–80. doi:10.1038/nri935
83. Phan TG, Bullen A. Practical intravital two-photon microscopy for immunological research: faster, brighter, deeper. *Immunol Cell Biol* (2010) 88:438–44. doi:10.1038/icb.2009.116
84. Beura LK, Hamilton SE, Bi K, Schenkel JM, Odumade OA, Casey KA, et al. Normalizing the environment recapitulates adult human immune traits in laboratory mice. *Nature* (2016) 532:512–6. doi:10.1038/nature17655
85. Stappenbeck TS, Virgin HW. Accounting for reciprocal host–microbiome interactions in experimental science. *Nature* (2016) 534:191–9. doi:10.1038/nature18285
86. Sullivan C, Kim CH. Zebrafish as a model for infectious disease and immune function. *Fish Shellfish Immunol* (2008) 25:341–50. doi:10.1016/j.fsi.2008.05.005
87. Quintana FJ, Iglesias AH, Farez MF, Caccamo M, Burns EJ, Kassam N, et al. Adaptive autoimmunity and Foxp3-based immunoregulation in zebrafish. *PLoS One* (2010) 5:e9478. doi:10.1371/journal.pone.0009478
88. Renshaw SA, Trede NS. A model 450 million years in the making: zebrafish and vertebrate immunity. *Dis Model Mech* (2012) 5:38–47. doi:10.1242/dmm.007138
89. Lee K-Y, Huang H, Ju B, Yang Z, Lin S. Cloned zebrafish by nuclear transfer from long-term-cultured cells. *Nat Biotechnol* (2002) 20(8):795–9. doi:10.1038/nbt721
90. Ma C, Fan L, Ganassin R, Bols N, Collodi P. Production of zebrafish germline chimeras from embryo cell cultures. *Proc Natl Acad Sci U S A* (2001) 98:2461–6. doi:10.1073/pnas.041449398
91. Poss KD, Keating MT, Nechiporuk A. Tales of regeneration in zebrafish. *Dev Dyn* (2003) 226:202–10. doi:10.1002/dvdy.10220
92. Siripattarapravat K, Pinmee B, Venta PJ, Chang C-C, Cibelli JB. Somatic cell nuclear transfer in zebrafish. *Nat Methods* (2009) 6:733–5. doi:10.1038/nmeth.1369
93. Auer TO, Del Bene F. CRISPR/Cas9 and TALEN-mediated knock-in approaches in zebrafish. *Methods* (2014) 69:142–50. doi:10.1016/j.ymeth.2014.03.027
94. Hruscha A, Schmid B. Generation of zebrafish models by CRISPR/Cas9 genome editing. *Methods Mol Biol* (2015) 1254:341–50. doi:10.1007/978-1-4939-2152-2_24
95. Ramakrishnan L. Revisiting the role of the granuloma in tuberculosis. *Nat Rev Immunol* (2012) 12:352. doi:10.1038/nri3211
96. Swaim LE, Connolly LE, Volkman HE, Humbert O, Born DE, Ramakrishnan L. *Mycobacterium marinum* infection of adult zebrafish causes caseating granulomatous tuberculosis and is moderated by adaptive immunity. *Infect Immun* (2006) 74:6108–17. doi:10.1128/IAI.00887-06
97. Collison LW, Vignali DAA. In vitro Treg suppression assays. *Methods Mol Biol* (2011) 707:21–37. doi:10.1007/978-1-61737-979-6_2
98. Salomon B, Zaragoza B, Chen X, Oppenheim J, Baeyens A, Grégoire S, et al. Human Treg cell suppressive assays. *Protoc Exch* (2015). doi:10.1038/protex.2015.078
99. Allan SE, Passerini L, Bacchetta R, Crellin N, Dai M, Orban PC, et al. The role of 2 FOXP3 isoforms in the generation of human CD4+ Tregs. *J Clin Invest* (2005) 115:3276–84. doi:10.1172/JCI24685
100. Walker MR, Kasprowicz DJ, Gersuk VH, Benard A, Van Landeghen M, Buckner JH, et al. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25- T cells. *J Clin Invest* (2003) 112:1437–43. doi:10.1172/JCI19441
101. Das J, Ren G, Zhang L, Roberts AI, Zhao X, Bothwell ALM, et al. Transforming growth factor β is dispensable for the molecular orchestration of Th17 cell differentiation. *J Exp Med* (2009) 206:2407–16. doi:10.1084/jem.20082286
102. Sakaguchi S, Miyara M, Costantino CM, Hafler DA. Foxp3$^+$ regulatory T cells in the human immune system. *Nat Rev Immunol* (2010) 10(7):490–500. doi:10.1038/nri2785
103. Huehn J, Polansky JK, Hamann A. Epigenetic control of FOXP3 expression: the key to a stable regulatory T-cell lineage? *Nat Rev Immunol* (2009) 9:83–9. doi:10.1038/nri2474
104. Waight JD, Takai S, Marelli B, Qin G, Hance KW, Zhang D, et al. Cutting edge: epigenetic regulation of Foxp3 defines a stable population of CD4+ regulatory T cells in tumors from mice and humans. *J Immunol* (2015) 194:878–82. doi:10.4049/jimmunol.1402725
105. Ziegler SF. FOXP3: of mice and men. *Annu Rev Immunol* (2006) 24:209–26. doi:10.1146/annurev.immunol.24.021605.090547
106. Milpied ĀP, Renand ĀA, Bruneau J, Mendes-Da-Cruz DA, Jacquelin S, Asnafi V, et al. Neuropilin-1 is not a marker of human Foxp3 Treg. *Eur J Immunol* (2009) 39:1466–71. doi:10.1002/eji.200839040
107. Annunziato F, Cosmi L, Santarlasci V, Maggi L, Liotta F, Mazzinghi B, et al. Phenotypic and functional features of human Th17 cells. *J Exp Med* (2007) 204:1849–61. doi:10.1084/jem.20070663
108. Tesmer LA, Lundy SK, Sarkar S, Fox DA. Th17 cells in human disease. *Immunol Rev* (2008) 223:87–113. doi:10.1111/j.1600-065X.2008.00628.x
109. Garden OA, Pinheiro D, Cunningham F. All creatures great and small: regulatory T cells in mice, humans, dogs and other domestic animal species. *Int Immunopharmacol* (2011) 11:576–88. doi:10.1016/j.intimp.2010.11.003
110. Bolzer K, Käser T, Saalmüller A, Hammer SE. Molecular characterisation of porcine Forkhead-box p3 (Foxp3). *Vet Immunol Immunopathol* (2009) 132:275–81. doi:10.1016/j.vetimm.2009.05.014
111. Lankford S, Petty C, LaVoy A, Reckling S, Tompkins W, Dean GA. Cloning of feline FOXP3 and detection of expression in CD4+CD25+ regulatory T cells. *Vet Immunol Immunopathol* (2008) 122(1–2):159–66. doi:10.1016/j.vetimm.2007.11.007
112. Seo KS, Davis WC, Hamilton MJ, Park YH, Bohach GA. Development of monoclonal antibodies to detect bovine FOXP3 in PBMCs exposed to a staphylococcal superantigen. *Vet Immunol Immunopathol* (2009) 128:30–6. doi:10.1016/j.vetimm.2008.10.292
113. Chumbley LB, Boudreaux CE, Coats KS. Aberrant placental immune parameters in the feline immunodeficiency virus (FIV)-infected cat suggest virus-induced changes in T cell function. *Virol J* (2013) 10:238. doi:10.1186/1743-422X-10-238
114. Biller BJ, Elmslie RE, Burnett RC, Avery AC, Dow SW. Use of FoxP3 expression to identify regulatory T cells in healthy dogs and dogs with cancer. *Vet Immunol Immunopathol* (2007) 116:69–78. doi:10.1016/j.vetimm.2006.12.002
115. Figueiredo MM, Deoti B, Amorim IF, Pinto AJW, Moraes A, Carvalho CS, et al. Expression of regulatory T cells in jejunum, colon, and cervical and

mesenteric lymph nodes of dogs naturally infected with Leishmania infantum. *Infect Immun* (2014) 82:3704–12. doi:10.1128/IAI.01862-14
116. Kol A, Walker NJ, Nordstrom M, Borjesson DL. Th17 pathway as a target for multipotent stromal cell therapy in dogs: implications for translational research. *PLoS One* (2016) 11:e0148568. doi:10.1371/journal.pone.0148568
117. Smith WG, Usinger WR, Splitter GA. Bovine con A-induced suppressor cells: generation, macrophage requirements and possible mechanisms of regulatory action. *Immunology* (1981) 43:91–100.
118. Gerner W, Stadler M, Hammer SE, Klein D, Saalmüller A. Sensitive detection of Foxp3 expression in bovine lymphocytes by flow cytometry. *Vet Immunol Immunopathol* (2010) 138:154–8. doi:10.1016/j.vetimm.2010.07.009
119. Hoek A, Rutten VPMG, Kool J, Arkesteijn GJA, Bouwstra RJ, Van Rhijn I, et al. Subpopulations of bovine WC1(+) gammadelta T cells rather than CD4(+)CD25(high) Foxp3(+) T cells act as immune regulatory cells ex vivo. *Vet Res* (2009) 40:6. doi:10.1051/vetres:2008044
120. Guzman E, Hope J, Taylor G, Smith AL, Cubillos-Zapata C, Charleston B. Bovine γd T cells are a major regulatory T cell subset. *J Immunol* (2014) 193:208–22. doi:10.4049/jimmunol.1303398
121. Arzi B, Mills-Ko E, Verstraete FJM, Kol A, Walker NJ, Badgley MR, et al. Therapeutic efficacy of fresh, autologous mesenchymal stem cells for severe refractory gingivostomatitis in cats. *Stem Cells Transl Med* (2016) 5:75–86. doi:10.5966/sctm.2015-0127
122. Ohkura N, Kitagawa Y, Sakaguchi S. Development and maintenance of regulatory T cells. *Immunity* (2013) 38:414–23. doi:10.1016/j.immuni.2013.03.002
123. Delgoffe GM, Bettini ML, Vignali DAA. Identity crisis: it's not just Foxp3 anymore. *Immunity* (2012) 37:759–61. doi:10.1016/j.immuni.2012.10.012
124. Chen Q, Kim YC, Laurence A, Punkosdy GA, Shevach EM. IL-2 controls the stability of Foxp3 expression in TGF-beta-induced Foxp3+ T cells in vivo. *J Immunol* (2011) 186:6329–37. doi:10.4049/jimmunol.1100061
125. Zhou X, Bailey-Bucktrout S, Jeker LT, Bluestone JA. Plasticity of CD4(+) FoxP3(+) T cells. *Curr Opin Immunol* (2009) 21:281–5. doi:10.1016/j.coi.2009.05.007
126. Ohkura N, Hamaguchi M, Morikawa H, Sugimura K, Tanaka A, Ito Y, et al. T cell receptor stimulation-induced epigenetic changes and Foxp3 expression are independent and complementary events required for Treg cell development. *Immunity* (2012) 37:785–99. doi:10.1016/j.immuni.2012.09.010
127. Barbi J, Pardoll D, Pan F. Treg functional stability and its responsiveness to the microenvironment. *Immunol Rev* (2014) 259:115–39. doi:10.1111/imr.12172
128. Huynh A, DuPage M, Priyadharshini B, Sage PT, Quiros J, Borges CM, et al. Control of PI(3) kinase in Treg cells maintains homeostasis and lineage stability. *Nat Immunol* (2015) 16:188–96. doi:10.1038/ni.3077
129. Huang G, Wang Y, Shi LZ, Kanneganti T-D, Chi H. Signaling by the phosphatase MKP-1 in dendritic cells imprints distinct effector and regulatory T cell fates. *Immunity* (2011) 35:45–58. doi:10.1016/j.immuni.2011.05.014
130. Shrestha S, Yang K, Guy C, Vogel P, Neale G, Chi H. Treg cells require the phosphatase PTEN to restrain TH1 and TFH cell responses. *Nat Immunol* (2015) 16:178–87. doi:10.1038/ni.3076
131. De Rosa V, Galgani M, Porcellini A, Colamatteo A, Santopaolo M, Zuchegna C, et al. Glycolysis controls the induction of human regulatory T cells by modulating the expression of FOXP3 exon 2 splicing variants. *Nat Immunol* (2015) 16:1174–84. doi:10.1038/ni.3269
132. Fayyad-Kazan H, Rouas R, Fayyad-Kazan M, Badran R, El Zein N, Lewalle P, et al. MicroRNA profile of circulating CD4-positive regulatory T cells in human adults and impact of differentially expressed MicroRNAs on expression of two genes essential to their function*. *J Biol Chem* (2012) 287:9910–22. doi:10.1074/jbc.M111.337154
133. Sethi A, Kulkarni N, Sonar S, Lal G. Role of miRNAs in CD4 T cell plasticity during inflammation and tolerance. *Front Genet* (2013) 4:8. doi:10.3389/fgene.2013.00008
134. Zhou X, Jeker LT, Fife BT, Zhu S, Anderson MS, McManus MT, et al. Selective miRNA disruption in T reg cells leads to uncontrolled autoimmunity. *J Exp Med* (2008) 205:1983–91. doi:10.1084/jem.20080707
135. Fayyad-Kazan H, Hamade E, Rouas R, Najar M, Fayyad-Kazan M, El Zein N, et al. Downregulation of microRNA-24 and -181 parallels the upregulation of IFN-γ secreted by activated human CD4 lymphocytes. *Hum Immunol* (2014) 75:677–85. doi:10.1016/j.humimm.2014.01.007
136. Banerjee A, Schambach F, DeJong CS, Hammond SM, Reiner SL. MicroRNA-155 inhibits IFN-gamma signaling in CD4+ T cells. *Eur J Immunol* (2010) 40:225–31. doi:10.1002/eji.200939381
137. Yao R, Ma Y-L, Liang W, Li H-H, Ma Z-J, Yu X, et al. MicroRNA-155 modulates Treg and Th17 cells differentiation and Th17 cell function by targeting SOCS1. *PLoS One* (2012) 7:e46082. doi:10.1371/journal.pone.0046082
138. Xu L, Kitani A, Fuss I, Strober W. Cutting edge: regulatory T cells induce CD4+CD25-Foxp3- T cells or are self-induced to become Th17 cells in the absence of exogenous TGF-beta. *J Immunol* (2007) 178:6725–9. doi:10.4049/jimmunol.178.11.6725
139. Zhang F, Meng G, Strober W. Interactions among the transcription factors Runx1, RORγt and Foxp3 regulate the differentiation of interleukin 17–producing T cells. *Nat Immunol* (2008) 9:1297–306. doi:10.1038/ni.1663
140. Ayyoub M, Deknuydt F, Raimbaud I, Dousset C, Leveque L, Bioley G, et al. Human memory FOXP3+ Tregs secrete IL-17 ex vivo and constitutively express the T(H)17 lineage-specific transcription factor RORgamma t. *Proc Natl Acad Sci U S A* (2009) 106:8635–40. doi:10.1073/pnas.0900621106
141. Blair PJ, Bultman SJ, Haas JC, Rouse BT, Wilkinson JE, Godfrey VL. CD4+CD8- T cells are the effector cells in disease pathogenesis in the scurfy (sf) mouse. *J Immunol* (1994) 153:3764–74.
142. Godfrey VL, Rouse BT, Wilkinson JE. Transplantation of T cell-mediated, lymphoreticular disease from the scurfy (sf) mouse. *Am J Pathol* (1994) 145:281–6.
143. Kanangat S, Blair P, Reddy R, Daheshia M, Godfrey V, Rouse BT, et al. Disease in the scurfy (sf) mouse is associated with overexpression of cytokine genes. *Eur J Immunol* (1996) 26:161–5. doi:10.1002/eji.1830260125
144. Powell BR, Buist NR, Stenzel P. An X-linked syndrome of diarrhea, polyendocrinopathy, and fatal infection in infancy. *J Pediatr* (1982) 100:731–7. doi:10.1016/S0022-3476(82)80573-8
145. Belkaid Y. Role of Foxp3-positive regulatory T cells during infection. *Eur J Immunol* (2008) 38:918–21. doi:10.1002/eji.200738120
146. Hewitson JP, Grainger JR, Maizels RM. Helminth immunoregulation: the role of parasite secreted proteins in modulating host immunity. *Mol Biochem Parasitol* (2009) 167:1–11. doi:10.1016/j.molbiopara.2009.04.008
147. Kroetz DN, Deepe GS. CCR5 dictates the equilibrium of proinflammatory IL-17+ and regulatory Foxp3+ T cells in fungal infection. *J Immunol* (2010) 184:5224–31. doi:10.4049/jimmunol.1000032
148. Lundgren A, Suri-Payer E, Enarsson K, Svennerholm A-M, Lundin BS. Helicobacter pylori-specific CD4+ CD25high regulatory T cells suppress memory T-cell responses to H. pylori in infected individuals. *Infect Immun* (2003) 71:1755–62. doi:10.1128/IAI.71.4.1755-1762.2003
149. Sehrawat S, Suryawanshi A, Hirashima M, Rouse BT. Role of Tim-3/galectin-9 inhibitory interaction in viral-induced immunopathology: shifting the balance toward regulators. *J Immunol* (2009) 182:3191–201. doi:10.4049/jimmunol.0803673
150. Sehrawat S, Rouse BT. Tregs and infections: on the potential value of modifying their function. *J Leukoc Biol* (2011) 90:1079–87. doi:10.1189/jlb.0611271
151. Srivastava S, Koch MA, Pepper M, Campbell DJ. Type I interferons directly inhibit regulatory T cells to allow optimal antiviral T cell responses during acute LCMV infection. *J Exp Med* (2014) 211:961–74. doi:10.1084/jem.20131556
152. Betts RJ, Prabhu N, Ho AWS, Lew FC, Hutchinson PE, Rotzschke O, et al. Influenza A virus infection results in a robust, antigen-responsive, and widely disseminated Foxp3+ regulatory T cell response. *J Virol* (2012) 86:2817–25. doi:10.1128/JVI.05685-11
153. Wang J, Li F, Wei H, Lian Z-X, Sun R, Tian Z. Respiratory influenza virus infection induces intestinal immune injury via microbiota-mediated Th17 cell–dependent inflammation. *J Exp Med* (2014) 211:2397–410. doi:10.1084/jem.20140625
154. Kudva A, Scheller EV, Robinson KM, Crowe CR, Choi SM, Slight SR, et al. Influenza A inhibits Th17-mediated host defense against bacterial pneumonia in mice. *J Immunol* (2011) 186:1666–74. doi:10.4049/jimmunol.1002194
155. Lanteri MC, O'Brien KM, Purtha WE, Cameron MJ, Lund JM, Owen RE, et al. Tregs control the development of symptomatic West Nile virus infection in humans and mice. *J Clin Invest* (2009) 119:3266–77. doi:10.1172/JCI39387
156. Welte T, Aronson J, Gong B, Rachamallu A, Mendell N, Tesh R, et al. Vγ4+ T cells regulate host immune response to West Nile virus infection. *FEMS Immunol Med Microbiol* (2011) 63:183–92. doi:10.1111/j.1574-695X.2011.00840.x
157. Durant LR, Makris S, Voorburg CM, Loebbermann J, Johansson C, Openshaw PJM. Regulatory T cells prevent Th2 immune responses and pulmonary eosinophilia during respiratory syncytial virus infection in mice. *J Virol* (2013) 87:10946–54. doi:10.1128/JVI.01295-13

158. Silberstein E, Konduru K, Kaplan GG. The interaction of hepatitis A virus (HAV) with soluble forms of its cellular receptor 1 (HAVCR1) share the physiological requirements of infectivity in cell culture. *Virol J* (2009) 6:175. doi:10.1186/1743-422X-6-175
159. Manangeeswaran M, Jacques J, Tami C, Konduru K, Amharref N, Perrella O, et al. Binding of hepatitis A virus to its cellular receptor 1 inhibits T-regulatory cell functions in humans. *Gastroenterology* (2012) 142:1516.e–25.e. doi:10.1053/j.gastro.2012.02.039
160. Rouse BT, Sarangi PP, Suvas S. Regulatory T cells in virus infections. *Immunol Rev* (2006) 212:272–86. doi:10.1111/j.0105-2896.2006.00412.x
161. Sehrawat S, Suvas S, Sarangi PP, Suryawanshi A, Rouse BT. In vitro-generated antigen-specific CD4+ CD25+ Foxp3+ regulatory T cells control the severity of herpes simplex virus-induced ocular immunoinflammatory lesions. *J Virol* (2008) 82:6838–51. doi:10.1128/JVI.00697-08
162. Suvas S, Kumaraguru U, Pack CD, Lee S, Rouse BT. CD4+CD25+ T cells regulate virus-specific primary and memory CD8+ T cell responses. *J Exp Med* (2003) 198:889–901. doi:10.1084/jem.20030171
163. Suvas S, Azkur AK, Kim BS, Kumaraguru U, Rouse BT. CD4+CD25+ regulatory T cells control the severity of viral immunoinflammatory lesions. *J Immunol* (2004) 172:4123–32. doi:10.4049/jimmunol.172.7.4123
164. Suvas S, Rouse BT. Treg control of antimicrobial T cell responses. *Curr Opin Immunol* (2006) 18:344–8. doi:10.1016/j.coi.2006.03.005
165. Reddy PBJ, Sehrawat S, Suryawanshi A, Rajasagi NK, Khatri M, Rouse BT. An approach to control relapse of inflammatory lesions after discontinuation of primary therapy. *PLoS One* (2014) 9:e98051. doi:10.1371/journal.pone.0098051
166. Sehrawat S, Rouse BT. Anti-inflammatory effects of FTY720 against viral-induced immunopathology: role of drug-induced conversion of T cells to become Foxp3+ regulators. *J Immunol* (2008) 180:7636–47. doi:10.4049/jimmunol.180.11.7636
167. Sharma S, Rajasagi NK, Veiga-Parga T, Rouse BT. Herpes virus entry mediator (HVEM) modulates proliferation and activation of regulatory T cells following HSV-1 infection. *Microbes Infect* (2014) 16:648–60. doi:10.1016/j.micinf.2014.06.005
168. Molesworth-Kenyon SJ, Yin R, Oakes JE, Lausch RN. IL-17 receptor signaling influences virus-induced corneal inflammation. *J Leukoc Biol* (2008) 83:401–8. doi:10.1189/jlb.0807571
169. Suryawanshi A, Veiga-Parga T, Rajasagi NK, Reddy PBJ, Sehrawat S, Sharma S, et al. Role of IL-17 and Th17 cells in herpes simplex virus-induced corneal immunopathology. *J Immunol* (2011) 187:1919–30. doi:10.4049/jimmunol.1100736
170. MacDonald AJ, Duffy M, Brady MT, McKiernan S, Hall W, Hegarty J, et al. CD4 T helper type 1 and regulatory T cells induced against the same epitopes on the core protein in hepatitis C virus–infected persons. *J Infect Dis* (2002) 185:720–7. doi:10.1086/339340
171. Schultz-Cherry S, Hinshaw VS. Influenza virus neuraminidase activates latent transforming growth factor beta. *J Virol* (1996) 70:8624–9.
172. Hsieh S-M, Chen M-Y, Pan S-C, Hung C-C, Chang S-C. Aberrant induction of regulatory activity of CD4+CD25+ T cells by dendritic cells in HIV-infected persons with amebic liver abscess. *J Acquir Immune Defic Syndr* (2007) 44:6–13. doi:10.1097/01.qai.0000242457.43392.61
173. Losikoff PT, Self AA, Gregory SH. Dendritic cells, regulatory T cells and the pathogenesis of chronic hepatitis C. *Virulence* (2012) 3:610–20. doi:10.4161/viru.21823
174. Moreno-Fernandez ME, Rueda CM, Rusie LK, Chougnet CA. Regulatory T cells control HIV replication in activated T cells through a cAMP-dependent mechanism. *Blood* (2011) 117:5372–80. doi:10.1182/blood-2010-12-323162
175. Schacker TW, Nguyen PL, Beilman GJ, Wolinsky S, Larson M, Reilly C, et al. Collagen deposition in HIV-1 infected lymphatic tissues and T cell homeostasis. *J Clin Invest* (2002) 110:1133–9. doi:10.1172/JCI16413
176. Brenchley JM, Paiardini M, Knox KS, Asher AI, Cervasi B, Asher TE, et al. Differential Th17 CD4 T-cell depletion in pathogenic and non-pathogenic lentiviral infections. *Blood* (2008) 112:2826–35. doi:10.1182/blood-2008-05-159301
177. El Hed A, Khaitan A, Kozhaya L, Manel N, Daskalakis D, Borkowsky W, et al. Susceptibility of human Th17 cells to human immunodeficiency virus and their perturbation during infection. *J Infect Dis* (2010) 201:843–54. doi:10.1086/651021
178. Blaschitz C, Raffatellu M. Th17 cytokines and the gut mucosal barrier. *J Clin Immunol* (2010) 30:196–203. doi:10.1007/s10875-010-9368-7
179. ElHed A, Unutmaz D. Th17 cells and HIV infection. *Curr Opin HIV AIDS* (2010) 5:146–50. doi:10.1097/COH.0b013e32833647a8
180. Klatt NR, Brenchley JM. Th17 cell dynamics in HIV infection. *Curr Opin HIV AIDS* (2010) 5:135–40. doi:10.1097/COH.0b013e3283364846
181. Favre D, Lederer S, Kanwar B, Ma Z-M, Proll S, Kasakow Z, et al. Critical loss of the balance between Th17 and T regulatory cell populations in pathogenic SIV infection. *PLoS Pathog* (2009) 5:e1000295. doi:10.1371/journal.ppat.1000295
182. Rouse BT. Regulatory T cells in health and disease. *J Intern Med* (2007) 262(1):78–95. doi:10.1111/j.1365-2796.2007.01836.x
183. Rowan AG, Fletcher JM, Ryan EJ, Moran B, Hegarty JE, O'Farrelly C, et al. Hepatitis C virus-specific Th17 cells are suppressed by virus-induced TGF-beta. *J Immunol* (2008) 181:4485–94. doi:10.4049/jimmunol.181.7.4485
184. Yamamoto JK, Sanou MP, Abbott JR, Coleman JK. Feline immunodeficiency virus model for designing HIV/AIDS vaccines. *Curr HIV Res* (2010) 8:14–25. doi:10.2174/157016210790416361
185. Mexas AM, Fogle JE, Tompkins WA, Tompkins MB. CD4+CD25+ regulatory T cells are infected and activated during acute FIV infection. *Vet Immunol Immunopathol* (2008) 126:263–72. doi:10.1016/j.vetimm.2008.08.003
186. de Parseval A, Chatterji U, Sun P, Elder JH. Feline immunodeficiency virus targets activated CD4+ T cells by using CD134 as a binding receptor. *Proc Natl Acad Sci U S A* (2004) 101:13044–9. doi:10.1073/PNAS.0404006101
187. Lindblad-Toh K, Wade CM, Mikkelsen TS, Karlsson EK, Jaffe DB, Kamal M, et al. Genome sequence, comparative analysis and haplotype structure of the domestic dog. *Nature* (2005) 438:803–19. doi:10.1038/nature04338
188. Day MJ. One health: the importance of companion animal vector-borne diseases. *Parasit Vectors* (2011) 4:49. doi:10.1186/1756-3305-4-49
189. Srivastava S, Shankar P, Mishra J, Singh S. Possibilities and challenges for developing a successful vaccine for leishmaniasis. *Parasit Vectors* (2016) 9:277. doi:10.1186/s13071-016-1553-y
190. de Almeida DE, Colvin CJ, Coussens PM. Antigen-specific regulatory T cells in bovine paratuberculosis. *Vet Immunol Immunopathol* (2008) 125:234–45. doi:10.1016/j.vetimm.2008.05.019
191. Coussens PM, Sipkovsky S, Murphy B, Roussey J, Colvin CJ. Regulatory T cells in cattle and their potential role in bovine paratuberculosis. *Comp Immunol Microbiol Infect Dis* (2012) 35:233–9. doi:10.1016/j.cimid.2012.01.004
192. Suzuki S, Konnai S, Okagawa T, Ikebuchi R, Shirai T, Sunden Y, et al. Expression analysis of Foxp3 in T cells from bovine leukemia virus infected cattle. *Microbiol Immunol* (2013) 57:600–4. doi:10.1111/1348-0421.12073
193. Hassan M, Hanrahan JP, Good B, Mulcahy G, Sweeney T. A differential interplay between the expression of Th1/Th2/Treg related cytokine genes in *Teladorsagia circumcincta* infected DRB1*1101 carrier lambs. *Vet Res* (2011) 42:45. doi:10.1186/1297-9716-42-45
194. McRae KM, Stear MJ, Good B, Keane OM. The host immune response to gastrointestinal nematode infection in sheep. *Parasite Immunol* (2015) 37:605–13. doi:10.1111/pim.12290
195. Heaney J, Barrett T, Cosby SL. Inhibition of in vitro leukocyte proliferation by *Morbilliviruses*. *J Virol* (2002) 76:3579–84. doi:10.1128/JVI.76.7.3579-3584.2002

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 28 February 2017
doi: 10.3389/fimmu.2017.00170

Role of Memory T Cells in Allograft Rejection and Tolerance

Gilles Benichou[1], Bruno Gonzalez[1], Jose Marino[1], Katayoun Ayasoufi[2] and Anna Valujskikh[2]**

[1] Center for Transplantation Sciences, Department of Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA, [2] Department of Immunology, Lerner Research Institute, Cleveland Clinic, Cleveland, OH, USA

Memory T cells are characterized by their low activation threshold, robust effector functions, and resistance to conventional immunosuppression and costimulation blockade. Unlike their naïve counterparts, memory T cells reside in and recirculate through peripheral non-lymphoid tissues. Alloreactive memory T cells are subdivided into different categories based on their origins, phenotypes, and functions. Recipients whose immune systems have been directly exposed to allogeneic major histocompatibility complex (MHC) molecules display high affinity alloreactive memory T cells. In the absence of any prior exposure to allogeneic MHC molecules, endogenous alloreactive memory T cells are regularly generated through microbial infections (heterologous immunity). Regardless of their origin, alloreactive memory T cells represent an essential element of the allograft rejection process and a major barrier to tolerance induction in clinical transplantation. This article describes the different subsets of alloreactive memory T cells involved in transplant rejection and examine their generation, functional properties, and mechanisms of action. In addition, we discuss strategies developed to target deleterious allospecific memory T cells in experimental animal models and clinical settings.

Keywords: memory T cells, allotransplantation, tolerance, heterologous immunity, transplant rejection, immune suppression, costimulation blockade

OPEN ACCESS

Edited by:
Alok Srivastava, Christian Medical College & Hospital, India

Reviewed by:
Régis Josien, University of Nantes, France
Ignacio Anegon, University of Nantes, France

****Correspondence:***
Gilles Benichou
gbenichou@partners.org;
Anna Valujskikh
valujsa@ccf.org

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *28 September 2016*
Accepted: *02 February 2017*
Published: *28 February 2017*

Citation:
Benichou G, Gonzalez B, Marino J, Ayasoufi K and Valujskikh A (2017) Role of Memory T Cells in Allograft Rejection and Tolerance. Front. Immunol. 8:170. doi: 10.3389/fimmu.2017.00170

INTRODUCTION

Rapid and robust protective responses against previously encountered antigens are beneficial during infections, vaccinations, and tumor surveillance. Conversely, memory immune responses against donor antigens are detrimental in the context of transplantation and are commonly associated with poor graft outcome. The danger of preexisting donor-specific alloantibody (DSA) was recognized early in transplant history, and all transplant candidates are tested for the presence of serum DSA prior to transplantation. Despite well documented harmful effects of memory T cells in transplantation (1–4), the potential impact of such cells is mostly neglected while choosing treatment regimens. In this review, we initially outline characteristics of alloreactive memory T cells and their functions. We also describe existing and emerging strategies designed to delete or suppress memory T cells in transplant recipients. To conclude, we discuss future areas of investigation that may translate experimental knowledge of alloreactive memory T cells into clinical practice and thus improve transplant outcome in sensitized recipients.

Abbreviations: MHC, major histocompatibility complex; APC, antigen-presenting cell; Treg, regulatory T cell; IFNγ, gamma interferon; TNFα, tumor necrosis factor alpha; CTL, cytotoxic T lymphocyte; DC, dendritic cell; DSAs, donor-specific antibodies.

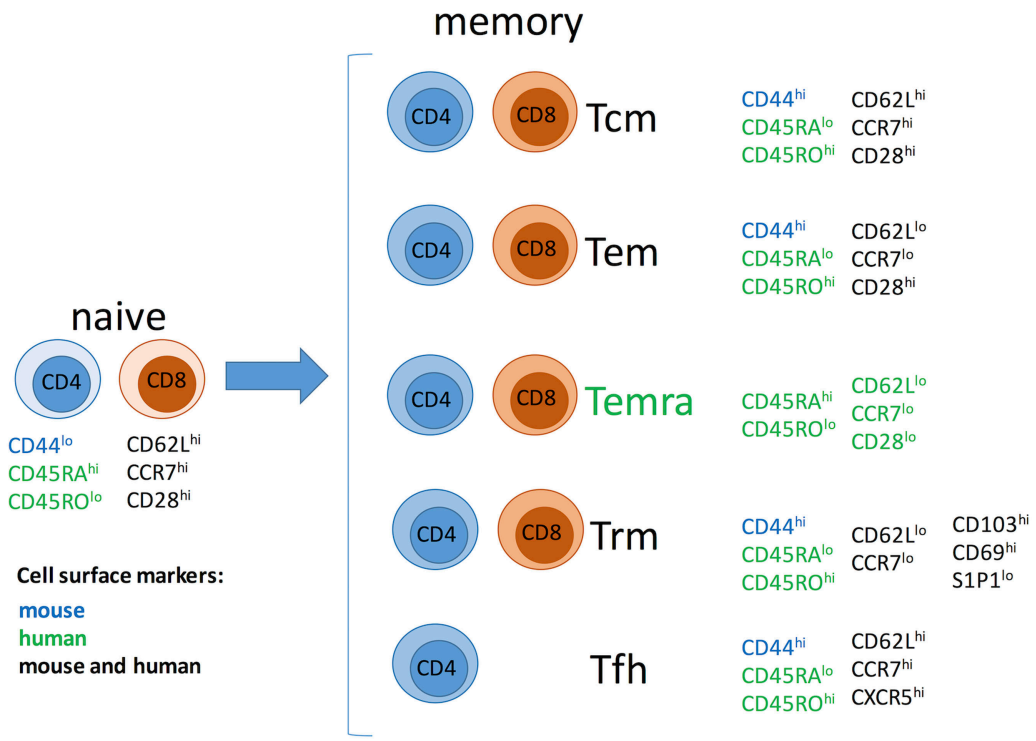


FIGURE 1 | **Memory T cell subsets**. Abbreviations: Tcm, central memory T cells; Tem, effector memory T cells; Temra, terminally differentiated effector memory T cells; Trm, resident memory T cells; Tfh, follicular helper memory T cells.

BASIC BIOLOGY OF ALLOREACTIVE MEMORY T CELLS

Origins of Alloreactive Memory T Cells

Laboratory rodents display low frequencies of memory T cells (5–10% of all T cells). In the absence of prior exposure to alloantigens, 1–10% of these memory T cells can react to allogeneic major histocompatibility complex (MHC) molecules *in vitro* (5). In mice, these cells called endogenous or natural alloreactive memory T cells recognize intact allogeneic MHC molecules through the direct allorecognition pathway (6, 7). It is likely that these memory cells are generated through the recognition of peptides from commensal bacteria or environmental antigens presented by self-MHC, which can mimic complexes formed by allogeneic MHC molecules bound to other peptides (8). Such antigen mimicry, named "heterologous immunity," is well documented in both humans and experimental animal models. Humans and non-human primates raised in a non-sterile environment are exposed to more infectious and pro-inflammatory agents during their development and thereby likely to develop potent heterologous immunity (9). For instance, following an EBV infection, HLA-B8$^+$ individuals can become sensitized to the allo-MHC molecule HLA-B4402 through antigen mimicry resulting from the presentation of some viral or parasitic peptides (10, 11).

In laboratory mice, direct sensitization with skin allografts or spleen cell immunization is a common approach for generating donor-reactive memory T cells. In humans, transplant patients can be sensitized from exposures to alloantigens such as previous transplants, pregnancies, and blood transfusions. Until now, only memory T cells recognizing intact alloantigens directly have been reported (2, 12). Yet, it is probable that sensitized patients exhibiting high titers of allospecific antibodies display memory T cells recognizing alloantigens indirectly as donor peptides–self-MHC complexes.

Memory T cells can also be generated through homeostatic proliferation in a lymphopenic environment, including potentially alloreactive and pathogenic T cells (13–15). Such homeostatically expanded memory T cells can impair tolerance induction to allografts (15–17).

The accumulation of alloreactive memory T cells may be influenced by the end stage organ disease or treatment common in transplant candidates. For example, prolonged exposure to dialysis increases the risk of developing alloreactive memory T cells (18). In addition, Sawinski et al. reported that low serum levels of 25-OH-vitamin D in dialysis patients correlates with the frequency of alloreactive memory T cells independent of age, gender, previous transplants, or time on dialysis (19).

Location of Memory T Cells

Memory T cells have been traditionally divided into two major subsets with largely overlapping functions but distinct trafficking patterns (**Figure 1**). Central memory T cells (Tcm) express lymphoid homing markers CCR7 and CD62L, whereas

effector memory T cells (Tem) are CCR7$^-$CD62L$^-$ but instead express molecules that promote migration into peripheral tissues (20–23). In humans, but not in mice, some memory T cells [terminally differentiated effector memory T cells (Temra)] re-express naive T cell surface marker CD45RA, while downregulating expression of CCR7, CD62L, and CD28, and represent a terminal stage of effector differentiation (21, 24, 25). Recent studies demonstrated that some T cells in peripheral tissues do not circulate and represent a distinct subset of tissue-resident memory T cells (Trm) (24, 26–28). Trm cells express early activation marker CD69 and αEβ7 integrin CD103 along with a number of tissue-specific chemokine receptors (26, 29–32). There is accumulating evidence that Trm cells play an important role in host protection against infections. It is conceivable that Trm cells of both donor and recipient origins may influence transplant outcome by facilitating GVHD or allograft rejection, respectively. However, the proportion of alloreactive T cells among Trm subset and the potential contribution of such cells following transplantation remain to be addressed. Another important type of memory T cells relevant to transplantation is CD4$^+$CXCR5^{hi} follicular helper (Tfh) cells that reside in B cell follicles within secondary lymphoid organs and are essential for optimal B cell responses and antibody generation (33). As memory T cells in secondary lymphoid and non-lymphoid peripheral tissues are spared by antibody-mediated lymphoablation (34) Trm cells may be harder to control compared to circulating memory T cells.

Low Activation Threshold and Resistance to Conventional Costimulatory Blockade

In the process of memory T cell differentiation, the T cell receptor and costimulatory signaling cascades are adjusted to ensure rapid activation of high magnitude upon antigen reencounter (35, 36). This results in the ability of memory T cells to respond to lower antigen doses with limited costimulation, i.e., to antigen presented by non-professional antigen-presenting cells (36–38). While this process is essential for host defense, it renders alloreactive memory T cells more dangerous in transplant settings. Numerous studies in animal models have demonstrated that donor-reactive memory T cells can induce allograft rejection despite interruption of essential costimulatory pathways, CD28/CD80/CD86 and CD40/CD154 (11, 15, 39–43).

CONTRIBUTION OF MEMORY T CELLS TO ALLOGRAFT REJECTION AND TOLERANCE

Role in Allograft Rejection

During the past decade, studies investigating CD4$^+$ versus CD8$^+$ memory T cells revealed that these subsets contribute to allograft rejection through distinct mechanisms. Indeed, memory CD4$^+$ T cells not only become effector cells upon reactivation, but also provide help for the robust activation of donor-reactive effector CD8$^+$ T cells (40). These effector CD8$^+$ T cells then are the main driving force behind allograft rejection facilitated by memory CD4$^+$ T cells in heart-transplanted mice, and CD8$^+$ T cell depletion or limiting their trafficking into the graft significantly extends allograft survival (40, 44).

While *de novo* responses by naïve T cells can be efficiently controlled by current immunosuppression, memory CD4$^+$ T cells are resistant to these therapies and can provide help for the generation of DSA leading to alloantibody-mediated graft injury (40, 44). Recent studies in a mouse model of heart transplantation identified potential therapeutic targets to control CD40-independent DSA generation by memory CD4$^+$ T cells. First, gamma interferon (IFNγ) secretion by memory helper T cells is required for *de novo* DSA generation (45). Second, CD40-independent helper functions of donor-reactive memory CD4$^+$ T cells and heart allograft rejection were markedly inhibited by neutralizing B cell activating factor and a proliferation-inducing ligand, cytokines critical for B cell survival, activation, and differentiation (46).

The fate and functions of donor-reactive memory CD8$^+$ T cells following transplantation are equally fascinating. Early direct contact of circulating memory CD8$^+$ T cells with donor endothelium upregulates the expression of adhesion molecules and chemokines thus facilitating infiltration of recipient leukocytes into the graft (47, 48). A proportion of endogenous memory CD8$^+$ T cells react to donor MHC class I molecules and can infiltrate cardiac allografts within hours after reperfusion. Once in the graft parenchyma, these memory CD8$^+$ T cells proliferate extensively, upregulate the expression of ICOS, and secrete IFNγ in ICOS-dependent manner (49, 50). Although this early expression of effector functions was found to be insufficient to mediate allograft rejection (51), the potential danger of endogenous memory CD8$^+$ T cells should not be underestimated. The approximation of clinical situation by increasing graft cold ischemia storage time enhanced effector functions of endogenous memory CD8$^+$ T cells enabling them to promptly reject a cardiac allograft despite costimulatory blockade with CTLA4-Ig (52).

Influence of Memory T Cells on Allograft Tolerance

In laboratory rodents, endogenous memory T cells generated through heterologous immunity have little ability to prevent tolerance induction given that hematopoietic chimerism and/or costimulation blockade regularly achieve tolerance of fully allogeneic transplants (53–55). In contrast, mice that have been sensitized to allogeneic MHC through transplantation or multiple viral infections become resistant to tolerance induction (11, 39, 56, 57). Moreover, naïve mice adoptively transferred with alloreactive memory T cells display similar resistance to tolerogenesis *via* hematopoietic chimerism or costimulation blockade (11, 39, 56, 57). Therefore, in laboratory rodents, antigen-induced rather than endogenous memory T cells prevent transplant tolerance. It is still unclear whether this difference relies on the low frequency of endogenous memory T cells or on the fact that these two subsets of memory T cells are different in nature.

The presence of memory T cells has been often correlated with poor outcomes in clinical transplantation. In humans, the

presence of memory T cells pretransplantation has been associated with an increased risk for acute rejection of kidney transplants (2). However, while EBV- and CMV-specific memory T cells displaying alloreactivity have been detected in human transplant recipients, so far there is no indication that the presence of "heterologous immunity" in transplant recipients correlates with worse graft outcomes (10, 58–60).

Our laboratory showed that a sizable proportion of endogenous memory T cells found in peripheral blood, and secondary lymphoid organs of naïve cynomolgus monkeys are allospecific. Most Tem were $CD8^+CD95^+CD28^-$ IFNγ-producing cells located in the spleen, peripheral blood, and bone marrow while IL-2-producing Tcm were primarily $CD4^+CD95^+CD28^+$ and limited to the lymph nodes and spleen (12). Based upon this observation, we studied the influence of pretransplant memory T cell alloreactivity on rejection versus tolerance of kidney allografts in monkeys (61). A series of cynomolgus monkeys were conditioned [whole body and thymic irradiations + horse antithymocyte globulin (ATG) treatment] and received a combined kidney and bone marrow transplantation from the same allogeneic donor (62). The animals then received a short-term immunosuppression treatment comprised of anti-CD40L antibodies and cyclosporine A (62). This procedure resulted in a transient multilineage hematopoietic chimerism and achieved long-term survival of kidney allografts (>1 year) after withdrawal of immunosuppression in 70% of the monkeys (62). On the other hand, approximately 30% of the treated monkeys rejected their allograft in an acute fashion within 100–200 days posttransplantation (61). In this model, we observed that the vast majority of tolerant animals displayed low frequencies of donor-reactive memory T cells (61). It is noteworthy that no differences between homeostatic expansion of memory T cells were observed between monkeys which rejected or accepted kidney allografts (61).

Even though memory T cells are generally viewed as pathogenic in the context of transplantation, under certain circumstances, they demonstrate regulatory capacity and suppress deleterious pro-inflammatory immune responses. Krupnick et al. have reported that early infiltration of central memory $CD8^+$ T cells is essential for lung allograft acceptance after treatment with CTLA4-Ig and anti-CD154 mAbs (63). Similarly, $CD8^+CD45RC^{lo}$ cells with regulatory properties have been described in rat models of solid organ transplantation and GVHD (64, 65). These findings raise a concern that lymphoablative approaches targeting memory T cells may interfere with allograft acceptance of certain types of transplants.

RECENT DEVELOPMENTS IN TARGETING ALLOREACTIVE T CELL MEMORY

Lymphoablation

Induction therapy is widely used in clinical transplantation to overcome the deleterious effects of preexisting donor-reactive immunity. Antibody-mediated lymphocyte depletion is most commonly used induction strategy, particularly in highly sensitized patients and in patients receiving marginal grafts (66–69). Although memory T cells are the primary targets of induction therapies, they are less susceptible to depletion than naïve T cells (70–73). T cells with an effector/memory phenotype are detectable after anti-CD52 mAb or ATG induction and are associated with acute rejection episodes in non-human primates and human transplant recipients (74, 75). In rodents, preexisting memory T cells rapidly recover following lymphocyte depletion with ATG and dominate anti-donor immune responses. The efficiency of memory $CD4^+$ T cell depletion is generally lower than that of $CD8^+$ T cells (34, 76–79). Additional depletion of residual $CD4^+$ T cells severely impairs the recovery of memory $CD8^+$ T cells after ATG treatment (80). Limiting $CD4^+$ T helper signals during lymphoablation increases the efficacy of mATG in controlling memory T cell expansion and significantly extends heart allograft survival in sensitized recipients (80). These findings are consistent with previous observations describing a synergistic effect between ATG lymphoablation and costimulatory blockade (81, 82).

Alefacept, a fusion protein combining extracellular domain of LFA-3 with constant regions of human IgG1 (83–85). LFA-3 is a ligand for CD2, a molecule that is predominantly detected on human T and NK cells. As CD2 expression is upregulated on $CD45RO^+$ effector/memory T cells, alefacept selectively depletes this subset and spares other T cell populations (86–88). Alefacept is currently being used in clinic for the treatment of severe psoriasis (89, 90) and is showing promise for targeting alloreactive effector/memory T cells in solid organ and bone marrow transplantation (91–95). Most importantly, pretransplant alefacept therapy synergizes with CTLA4-Ig presumably by targeting costimulatory blockade-resistant $CD8^+CD2^{hi}CD28^-$ effector/memory T cells (91).

In addition to direct lymphoablation, manipulating T cell survival and homeostasis by regulating cell metabolic pathways may be a promising therapeutic strategy in transplantation. Recent studies suggest that immune cells subsets use different mechanisms of energy generation, and this information can be exploited to selectively target undesirable memory T cells [reviewed in Ref. (96)].

Costimulatory Blockade

Belatacept, a second generation of CTLA4-Ig, is currently used in clinical transplantation to prevent allograft rejection and minimize the toxic side effects of calcineurin inhibitors (97). Despite reduced side effects and improved graft survival, belatacept-treated patients have higher rates of acute cellular rejection compared to CNI treatment (98, 99). As memory T cells are more resistant to the effects of CTLA4-Ig in animal transplantation models, it is possible that presensitized T cells could account for some belatacept-resistant rejection episodes. Indeed, terminally differentiated memory $CD4^+$ and $CD8^+$ T cells in humans (Temra) lose CD28 expression and become insensitive to the lack of CD28/B7 costimulation (100–104). Not surprisingly, increased numbers of both $CD4^+$ and $CD8^+$ $CD28^-$ memory T cells are associated with a poor outcome in renal and lung transplant patients (105–108). A recent report by Espinosa et al. identified yet another population of $CD57^+CD4^+$ T cells as potential mediators of belatacept-resistant renal allograft

rejection. These cells are more common in patients with kidney failure, express high levels of adhesion molecules CD2, LFA-1, and VLA-4, downregulate CD28, and produce IFNγ, tumor necrosis factor alpha (TNFα), and granzyme B consistent with effector/memory phenotype (109).

Recent reports suggest that the pedigree of alloreactive memory T cells in a given recipient may have important practical implications. Using three different pathogens to generate donor-reactive memory T cells in a mouse model of skin transplantation, Badell et al. demonstrated that the sensitivity of memory T cells to immunosuppression is dependent on their origin (110). In this study, Tcm with a less differentiated phenotype were most sensitive to the effects of costimulatory blockade. Consistent with these findings, *in vitro* comparison of CMV- and alloreactive T cells suggested that virus-specific fully differentiated T cells secreting IFNγ, TNFα, and IL-2 simultaneously are more resistant to the effects of CTLA4-Ig, whereas tacrolimus inhibits responses by both allo- and virus-specific T cells (111).

In addition to blocking CD28/B7 costimulation, CTLA4-Ig also prevents signaling through CTLA-4, which can have negative effects on generation and functions of regulatory T cells (Tregs) (112–117). To circumvent this problem, several antagonistic anti-CD28 mAbs and Ab F(ab′)2 fragments have been generated and showed promise in animal transplantation models (118–121). The selective effects of these reagents on memory T cell subsets and the potential pathogenicity of $CD28^{lo}$ Temra cells during such therapies remain to be determined. Attempts to target another major costimulatory pathway, CD40/CD154, encountered early difficulties because of thromboembolic effects of anti-CD154 (CD40L) blocking antibodies (122). To avoid cross-linking CD154 that is highly expressed on platelets, an alternative approach has been the generation of non-activating anti-CD40 antibodies. Several such reagents have been successfully tested in non-human primate recipients of renal and islet allografts (123–128).

In addition to CD28/B7 and CD40/CD154 costimulation, several other costimulatory pathways may play a role in effector/memory T cell functions. Inhibition or genetic lack of ICOS/B7RP-1, CD134/CD134L, CD70/CD27, or CD137/CD137L improved allograft survival even in donor-sensitized recipients, or after delayed administration which allowed initial priming of donor-reactive T cells [reviewed in Ref. (129)]. It was revealed that these costimulatory pathways might control distinct aspects of the alloimmune response. For example, blocking anti-CD134L mAb inhibits proliferation of effector T cells while supporting the survival of Tregs (71, 130). Conversely, signaling through CD134 inhibits immunosuppressive properties of $FoxP3^+$ Tregs and promotes allograft rejection (131, 132). ICOS/B7RP-1 blockade of resting memory $CD4^+$ T cells inhibits their helper functions and decreases alloantibody production. In contrast, circulating memory $CD8^+$ T cells are $ICOS^{lo}$, but rapidly upregulate ICOS surface expression upon graft infiltration. These examples demonstrate that the complexity of costimulatory pathways governing alloimmune responses must be considered when costimulatory blockade is used as part of immunosuppression regimen.

Limiting Trafficking of Alloreactive Memory T Cells

While preventing memory T cell entrance into graft tissue should improve transplant outcome, the attempts to neutralize chemokines or chemokine receptors such as CCR5 or CXCR3 did not live up to the initial expectations, most likely due to the redundancy of chemokine/receptor network. On other hand, reagents blocking LFA-1 (leukocyte function-associated antigen-1, an αLβ2 integrin) and VLA-4 (very late antigen-4, an α4β1 integrin) have been demonstrated to prolong allograft survival in experimental transplantation [reviewed in Ref. (133)]. Treatment with either anti-LFA-1 or anti-VLA-4 blocking mAbs prolonged skin allograft survival in a mouse model of costimulatory blockade-resistant rejection by memory $CD8^+$ T cells (134). In another study, pretransplant treatment with anti-LFA-1 mAbs inhibited early infiltration of endogenous donor-reactive memory $CD8^+$ T cells into cardiac allografts, and significantly prolonged allograft survival (135). These findings suggest that a short course of integrin blockade may be instrumental in controlling T cell memory while avoiding side effects of long-term treatments.

CONCLUDING REMARKS

While other types of immunologic memory lymphocytes such as memory B cells, preexisting alloantibodies, and "innate memory" described for NK cells and macrophages can impact transplant outcomes, in this review, we focused exclusively on T cell memory. It is now firmly established that alloreactive memory T cells accelerate allograft rejection and prevent transplant tolerance. However, the implementation of accumulated experimental knowledge in clinical transplantation is impeded by several factors. First, the diagnostics of T cell allosensitization in transplant candidates is problematic. Due to heterogeneity in phenotype and functions of memory T cells, complementary tests will be required including analyses of cytokine producing, cytotoxic, and follicular helper T cells. The resulting information is likely to be complex and hard to use in clinical decision-making. Second, memory T cells in humans are sampled only in peripheral blood. So far, there is no information on pathogenicity of tissue-resident alloreactive memory T cells. Third, memory T cell susceptibility to immunosuppression may depend on their origins. As immunological histories of individuals are difficult to trace, the situation may arise when patients with similar T cell memory profile require distinct treatment strategies. Finally, despite rapidly accumulating data on alloreactive T cell memory, the discrepancies between animal models and transplantation in human patients are profound. Ideally, animal transplantation models approximating clinical situation should take into account frequencies of total and donor-reactive memory T cells in different species, time of graft cold ischemia storage, and the presence of DSA in recipient serum. Including these considerations into experimental design will facilitate the development of novel approaches to control memory T cells and improve transplant survival in sensitized recipients.

AUTHOR CONTRIBUTIONS

GB, AV, BG, JM, and KA wrote portions of the manuscript; GB and AV edited the manuscript and prepared it for submission.

ACKNOWLEDGMENTS

This study was supported by NIH grants R21AI111191 and R21AI117466 to GB and R01AI113142 and 2PO1 AI087586-06 to AV.

REFERENCES

1. Augustine JJ, Siu DS, Clemente MJ, Schulak JA, Heeger PS, Hricik DE. Pre-transplant IFN-gamma ELISPOTs are associated with post-transplant renal function in African American renal transplant recipients. *Am J Transplant* (2005) 5(8):1971–5. doi:10.1111/j.1600-6143.2005.00958.x
2. Heeger PS, Greenspan NS, Kuhlenschmidt S, Dejelo C, Hricik DE, Schulak JA, et al. Pretransplant frequency of donor-specific, IFN-gamma-producing lymphocytes is a manifestation of immunologic memory and correlates with the risk of posttransplant rejection episodes. *J Immunol* (1999) 163(4):2267–75.
3. Hricik DE, Rodriguez V, Riley J, Bryan K, Tary-Lehmann M, Greenspan N, et al. Enzyme linked immunosorbent spot (ELISPOT) assay for interferon-gamma independently predicts renal function in kidney transplant recipients. *Am J Transplant* (2003) 3(7):878–84. doi:10.1034/j.1600-6143.2003.00132.x
4. Poggio ED, Augustine JJ, Clemente M, Danzig JM, Volokh N, Zand MS, et al. Pretransplant cellular alloimmunity as assessed by a panel of reactive T cells assay correlates with acute renal graft rejection. *Transplantation* (2007) 83(7):847–52. doi:10.1097/01.tp.0000258730.75137.39
5. Lombardi G, Sidhu S, Daly M, Batchelor JR, Makgoba W, Lechler RI. Are primary alloresponses truly primary? *Int Immunol* (1990) 2(1):9–13.
6. Benichou G, Valujskikh A, Heeger PS. Contributions of direct and indirect T cell alloreactivity during allograft rejection in mice. *J Immunol* (1999) 162(1):352–8.
7. Benichou G. Direct and indirect antigen recognition: the pathways to allograft immune rejection. *Front Biosci* (1999) 4:D476–80.
8. Lechler R, Lombardi G. Structural aspects of allorecognition. *Curr Opin Immunol* (1991) 3(5):715–21.
9. Mbitikon-Kobo FM, Vocanson M, Michallet MC, Tomkowiak M, Cottalorda A, Angelov GS, et al. Characterization of a CD44/CD122int memory CD8 T cell subset generated under sterile inflammatory conditions. *J Immunol* (2009) 182(6):3846–54. doi:10.4049/jimmunol.0802438
10. Amir AL, D'Orsogna LJ, Roelen DL, van Loenen MM, Hagedoorn RS, de Boer R, et al. Allo-HLA reactivity of virus-specific memory T cells is common. *Blood* (2010) 115(15):3146–57. doi:10.1182/blood-2009-07-234906
11. Pantenburg B, Heinzel F, Das L, Heeger PS, Valujskikh A. T cells primed by *Leishmania major* infection cross-react with alloantigens and alter the course of allograft rejection. *J Immunol* (2002) 169(7):3686–93. doi:10.4049/jimmunol.169.7.3686
12. Nadazdin O, Boskovic S, Murakami T, O'Connor DH, Wiseman RW, Karl JA, et al. Phenotype, distribution and alloreactive properties of memory T cells from cynomolgus monkeys. *Am J Transplant* (2010) 10(6):1375–84. doi:10.1111/j.1600-6143.2010.03119.x
13. Cho BK, Rao VP, Ge Q, Eisen HN, Chen J. Homeostasis-stimulated proliferation drives naive T cells to differentiate directly into memory T cells. *J Exp Med* (2000) 192(4):549–56. doi:10.1084/jem.192.4.549
14. Tchao NK, Turka LA. Lymphodepletion and homeostatic proliferation: implications for transplantation. *Am J Transplant* (2012) 12(5):1079–90. doi:10.1111/j.1600-6143.2012.04008.x
15. Wu Z, Bensinger SJ, Zhang J, Chen C, Yuan X, Huang X, et al. Homeostatic proliferation is a barrier to transplantation tolerance. *Nat Med* (2004) 10(1):87–92. doi:10.1038/nm965
16. Taylor DK, Neujahr D, Turka LA. Heterologous immunity and homeostatic proliferation as barriers to tolerance. *Curr Opin Immunol* (2004) 16(5):558–64. doi:10.1016/j.coi.2004.07.007
17. Moxham VF, Karegli J, Phillips RE, Brown KL, Tapmeier TT, Hangartner R, et al. Homeostatic proliferation of lymphocytes results in augmented memory-like function and accelerated allograft rejection. *J Immunol* (2008) 180(6):3910–8. doi:10.4049/jimmunol.180.6.3910
18. Augustine JJ, Poggio ED, Clemente M, Aeder MI, Bodziak KA, Schulak JA, et al. Hemodialysis vintage, black ethnicity, and pretransplantation antidonor cellular immunity in kidney transplant recipients. *J Am Soc Nephrol* (2007) 18(5):1602–6. doi:10.1681/ASN.2006101105
19. Sawinski D, Uribarri J, Peace D, Yao T, Wauhop P, Trzcinka P, et al. 25-OH-vitamin D deficiency and cellular alloimmunity as measured by panel of reactive T cell testing in dialysis patients. *Am J Transplant* (2010) 10(10):2287–95. doi:10.1111/j.1600-6143.2010.03264.x
20. Ahmadzadeh M, Hussain SF, Farber DL. Heterogeneity of the memory CD4 T cell response: persisting effectors and resting memory T cells. *J Immunol* (2001) 166(2):926–35. doi:10.4049/jimmunol.166.2.926
21. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol* (2004) 22:745–63. doi:10.1146/annurev.immunol.22.012703.104702
22. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* (1999) 401(6754):708–12.
23. Wherry EJ, Teichgraber V, Becker TC, Masopust D, Kaech SM, Antia R, et al. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat Immunol* (2003) 4(3):225–34. doi:10.1038/ni889
24. Sathaliyawala T, Kubota M, Yudanin N, Turner D, Camp P, Thome JJ, et al. Distribution and compartmentalization of human circulating and tissue-resident memory T cell subsets. *Immunity* (2013) 38(1):187–97. doi:10.1016/j.immuni.2012.09.020
25. Surh CD, Sprent J. Homeostasis of naive and memory T cells. *Immunity* (2008) 29(6):848–62. doi:10.1016/j.immuni.2008.11.002
26. Casey KA, Fraser KA, Schenkel JM, Moran A, Abt MC, Beura LK, et al. Antigen-independent differentiation and maintenance of effector-like resident memory T cells in tissues. *J Immunol* (2012) 188(10):4866–75. doi:10.4049/jimmunol.1200402
27. Gebhardt T, Wakim LM, Eidsmo L, Reading PC, Heath WR, Carbone FR. Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. *Nat Immunol* (2009) 10(5):524–30. doi:10.1038/ni.1718
28. Masopust D, Choo D, Vezys V, Wherry EJ, Duraiswamy J, Akondy R, et al. Dynamic T cell migration program provides resident memory within intestinal epithelium. *J Exp Med* (2010) 207(3):553–64. doi:10.1084/jem.20090858
29. Mackay LK, Rahimpour A, Ma JZ, Collins N, Stock AT, Hafon ML, et al. The developmental pathway for CD103(+)CD8+ tissue-resident memory T cells of skin. *Nat Immunol* (2013) 14(12):1294–301. doi:10.1038/ni.2744
30. Steinert EM, Schenkel JM, Fraser KA, Beura LK, Manlove LS, Igyarto BZ, et al. Quantifying memory CD8 T cells reveals regionalization of immunosurveillance. *Cell* (2015) 161(4):737–49. doi:10.1016/j.cell.2015.03.031
31. Turner DL, Gordon CL, Farber DL. Tissue-resident T cells, in situ immunity and transplantation. *Immunol Rev* (2014) 258(1):150–66. doi:10.1111/imr.12149
32. Watanabe R, Gehad A, Yang C, Scott LL, Teague JE, Schlapbach C, et al. Human skin is protected by four functionally and phenotypically discrete populations of resident and recirculating memory T cells. *Sci Transl Med* (2015) 7(279):279ra39. doi:10.1126/scitranslmed.3010302
33. Hale JS, Ahmed R. Memory T follicular helper CD4 T cells. *Front Immunol* (2015) 6:16. doi:10.3389/fimmu.2015.00016
34. Ayasoufi K, Yu H, Fan R, Wang X, Williams J, Valujskikh A. Pretransplant antithymocyte globulin has increased efficacy in controlling donor-reactive memory T cells in mice. *Am J Transplant* (2013) 13(3):589–99. doi:10.1111/ajt.12068
35. Chandok MR, Farber DL. Signaling control of memory T cell generation and function. *Semin Immunol* (2004) 16(5):285–93. doi:10.1016/j.smim.2004.08.009
36. Jameson SC, Masopust D. Diversity in T cell memory: an embarrassment of riches. *Immunity* (2009) 31(6):859–71. doi:10.1016/j.immuni.2009.11.007
37. Croft M, Bradley LM, Swain SL. Naive versus memory CD4 T cell response to antigen. Memory cells are less dependent on accessory cell costimulation

and can respond to many antigen-presenting cell types including resting B cells. *J Immunol* (1994) 152(6):2675–85.
38. Khayyamian S, Hutloff A, Buchner K, Grafe M, Henn V, Kroczek RA, et al. ICOS-ligand, expressed on human endothelial cells, costimulates Th1 and Th2 cytokine secretion by memory CD4+ T cells. *Proc Natl Acad Sci U S A* (2002) 99(9):6198–203. doi:10.1073/pnas.092576699
39. Adams AB, Williams MA, Jones TR, Shirasugi N, Durham MM, Kaech SM, et al. Heterologous immunity provides a potent barrier to transplantation tolerance. *J Clin Invest* (2003) 111(12):1887–95. doi:10.1172/JCI17477
40. Chen Y, Heeger PS, Valujskikh A. In vivo helper functions of alloreactive memory CD4+ T cells remain intact despite donor-specific transfusion and anti-CD40 ligand therapy. *J Immunol* (2004) 172(9):5456–66. doi:10.4049/jimmunol.172.9.5456
41. Valujskikh A, Pantenburg B, Heeger PS. Primed allospecific T cells prevent the effects of costimulatory blockade on prolonged cardiac allograft survival in mice. *Am J Transplant* (2002) 2(6):501–9. doi:10.1034/j.1600-6143.2002.20603.x
42. Welsh RM, Markees TG, Woda BA, Daniels KA, Brehm MA, Mordes JP, et al. Virus-induced abrogation of transplantation tolerance induced by donor-specific transfusion and anti-CD154 antibody. *J Virol* (2000) 74(5):2210–8. doi:10.1128/JVI.74.5.2210-2218.2000
43. Zhai Y, Meng L, Gao F, Busuttil RW, Kupiec-Weglinski JW. Allograft rejection by primed/memory CD8+ T cells is CD154 blockade resistant: therapeutic implications for sensitized transplant recipients. *J Immunol* (2002) 169(8):4667–73. doi:10.4049/jimmunol.169.8.4667
44. Zhang Q, Chen Y, Fairchild RL, Heeger PS, Valujskikh A. Lymphoid sequestration of alloreactive memory CD4 T cells promotes cardiac allograft survival. *J Immunol* (2006) 176(2):770–7. doi:10.4049/jimmunol.176.2.770
45. Gorbacheva V, Fan R, Wang X, Baldwin WM III, Fairchild RL, Valujskikh A. IFN-gamma production by memory helper T cells is required for CD40-independent alloantibody responses. *J Immunol* (2015) 194(3):1347–56. doi:10.4049/jimmunol.1401573
46. Gorbacheva V, Ayasoufi K, Fan R, Baldwin WM III, Valujskikh A. B cell activating factor (BAFF) and a proliferation inducing ligand (APRIL) mediate CD40-independent help by memory CD4 T cells. *Am J Transplant* (2015) 15(2):346–57. doi:10.1111/ajt.12984
47. El-Sawy T, Belperio JA, Strieter RM, Remick DG, Fairchild RL. Inhibition of polymorphonuclear leukocyte-mediated graft damage synergizes with short-term costimulatory blockade to prevent cardiac allograft rejection. *Circulation* (2005) 112(3):320–31. doi:10.1161/CIRCULATIONAHA.104.516708
48. El-Sawy T, Miura M, Fairchild R. Early T cell response to allografts occurring prior to alloantigen priming up-regulates innate-mediated inflammation and graft necrosis. *Am J Pathol* (2004) 165(1):147–57. doi:10.1016/S0002-9440(10)63283-X
49. Schenk AD, Gorbacheva V, Rabant M, Fairchild RL, Valujskikh A. Effector functions of donor-reactive CD8 memory T cells are dependent on ICOS induced during division in cardiac grafts. *Am J Transplant* (2009) 9(1):64–73. doi:10.1111/j.1600-6143.2008.02460.x
50. Schenk AD, Nozaki T, Rabant M, Valujskikh A, Fairchild RL. Donor-reactive CD8 memory T cells infiltrate cardiac allografts within 24-h posttransplant in naive recipients. *Am J Transplant* (2008) 8(8):1652–61. doi:10.1111/j.1600-6143.2008.02302.x
51. Setoguchi K, Hattori Y, Iida S, Baldwin WM III, Fairchild RL. Endogenous memory CD8 T cells are activated within cardiac allografts without mediating rejection. *Am J Transplant* (2013) 13(9):2293–307. doi:10.1111/ajt.12372
52. Su CA, Iida S, Abe T, Fairchild RL. Endogenous memory CD8 T cells directly mediate cardiac allograft rejection. *Am J Transplant* (2014) 14(3):568–79. doi:10.1111/ajt.12605
53. Wood KJ, Sakaguchi S. Regulatory T cells in transplantation tolerance. *Nat Rev Immunol* (2003) 3(3):199–210. doi:10.1038/nri1027
54. Kurtz J, Shaffer J, Lie A, Anosova N, Benichou G, Sykes M. Mechanisms of early peripheral CD4 T-cell tolerance induction by anti-CD154 monoclonal antibody and allogeneic bone marrow transplantation: evidence for anergy and deletion but not regulatory cells. *Blood* (2004) 103(11):4336–43. doi:10.1182/blood-2003-08-2642
55. Larsen CP, Elwood ET, Alexander DZ, Ritchie SC, Hendrix R, Tucker-Burden C, et al. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. *Nature* (1996) 381(6581):434–8.
56. Brehm MA, Markees TG, Daniels KA, Greiner DL, Rossini AA, Welsh RM. Direct visualization of cross-reactive effector and memory allo-specific CD8 T cells generated in response to viral infections. *J Immunol* (2003) 170(8):4077–86. doi:10.4049/jimmunol.170.8.4077
57. Macdonald WA, Chen Z, Gras S, Archbold JK, Tynan FE, Clements CS, et al. T cell allorecognition via molecular mimicry. *Immunity* (2009) 31(6):897–908. doi:10.1016/j.immuni.2009.09.025
58. Burrows SR, Silins SL, Khanna R, Burrows JM, Rischmueller M, McCluskey J, et al. Cross-reactive memory T cells for Epstein-Barr virus augment the alloresponse to common human leukocyte antigens: degenerate recognition of major histocompatibility complex-bound peptide by T cells and its role in alloreactivity. *Eur J Immunol* (1997) 27(7):1726–36.
59. Heutinck KM, Yong S, Tonneijck L, van den Heuvel H, van der Weerd NC, van der Pant KA, et al. Virus-specific CD8 T-cells cross-reactive to donor-alloantigen are transiently present in the circulation of kidney transplant recipients infected with CMV and/or EBV. *Am J Transplant* (2016) 16(5):1480–91. doi:10.1111/ajt.13618
60. Mifsud NA, Nguyen TH, Tait BD, Kotsimbos TC. Quantitative and functional diversity of cross-reactive EBV-specific CD8+ T cells in a longitudinal study cohort of lung transplant recipients. *Transplantation* (2010) 90(12):1439–49. doi:10.1097/TP.0b013e3181ff4ff3
61. Nadazdin O, Boskovic S, Murakami T, Tocco G, Smith RN, Colvin RB, et al. Host alloreactive memory T cells influence tolerance to kidney allografts in nonhuman primates. *Sci Transl Med* (2011) 3(86):86ra51. doi:10.1126/scitranslmed.3002093
62. Kawai T, Sogawa H, Boskovic S, Abrahamian G, Smith RN, Wee SL, et al. CD154 blockade for induction of mixed chimerism and prolonged renal allograft survival in nonhuman primates. *Am J Transplant* (2004) 4(9):1391–8. doi:10.1111/j.1600-6143.2004.00523.x
63. Krupnick AS, Lin X, Li W, Higashikubo R, Zinselmeyer BH, Hartzler H, et al. Central memory CD8+ T lymphocytes mediate lung allograft acceptance. *J Clin Invest* (2014) 124(3):1130–43. doi:10.1172/JCI71359
64. Guillonneau C, Picarda E, Anegon I. CD8+ regulatory T cells in solid organ transplantation. *Curr Opin Organ Transplant* (2010) 15(6):751–6. doi:10.1097/MOT.0b013e32834016d1
65. Li XL, Menoret S, Bezie S, Caron L, Chabannes D, Hill M, et al. Mechanism and localization of CD8 regulatory T cells in a heart transplant model of tolerance. *J Immunol* (2010) 185(2):823–33. doi:10.4049/jimmunol.1000120
66. Fehr T, Sykes M. Tolerance induction in clinical transplantation. *Transpl Immunol* (2004) 13(2):117–30. doi:10.1016/j.trim.2004.05.009
67. Golshayan D, Pascual M. Tolerance-inducing immunosuppressive strategies in clinical transplantation: an overview. *Drugs* (2008) 68(15):2113–30. doi:10.2165/00003495-200868150-00004
68. Haudebourg T, Poirier N, Vanhove B. Depleting T-cell subpopulations in organ transplantation. *Transpl Int* (2009) 22(5):509–18. doi:10.1111/j.1432-2277.2008.00788.x
69. Mohty M. Mechanisms of action of antithymocyte globulin: T-cell depletion and beyond. *Leukemia* (2007) 21(7):1387–94. doi:10.1038/sj.leu.2404683
70. Koyama I, Nadazdin O, Boskovic S, Ochiai T, Smith RN, Sykes M, et al. Depletion of CD8 memory T cells for induction of tolerance of a previously transplanted kidney allograft. *Am J Transplant* (2007) 7(5):1055–61. doi:10.1111/j.1600-6143.2006.01703.x
71. Kroemer A, Xiao X, Vu MD, Gao W, Minamimura K, Chen M, et al. OX40 controls functionally different T cell subsets and their resistance to depletion therapy. *J Immunol* (2007) 179(8):5584–91. doi:10.4049/jimmunol.179.8.5584
72. Neujahr DC, Chen C, Huang X, Markmann JF, Cobbold S, Waldmann H, et al. Accelerated memory cell homeostasis during T cell depletion and approaches to overcome it. *J Immunol* (2006) 176(8):4632–9. doi:10.4049/jimmunol.176.8.4632
73. Zeevi A, Husain S, Spichty KJ, Raza K, Woodcock JB, Zaldonis D, et al. Recovery of functional memory T cells in lung transplant recipients following induction therapy with alemtuzumab. *Am J Transplant* (2007) 7(2):471–5. doi:10.1111/j.1600-6143.2006.01641.x
74. Haanstra KG, Sick EA, Ringers J, Wubben JA, Kuhn EM, t Hart BA, et al. No synergy between ATG induction and costimulation blockade induced kidney allograft survival in rhesus monkeys. *Transplantation* (2006) 82(9):1194–201.

75. Pearl JP, Parris J, Hale DA, Hoffmann SC, Bernstein WB, McCoy KL, et al. Immunocompetent T-cells with a memory-like phenotype are the dominant cell type following antibody-mediated T-cell depletion. *Am J Transplant* (2005) 5(3):465–74. doi:10.1111/j.1600-6143.2005.00759.x
76. Hu Y, Turner MJ, Shields J, Gale MS, Hutto E, Roberts BL, et al. Investigation of the mechanism of action of alemtuzumab in a human CD52 transgenic mouse model. *Immunology* (2009) 128(2):260–70. doi:10.1111/j.1365-2567.2009.03115.x
77. Marco MR, Dons EM, van der Windt DJ, Bhama JK, Lu LT, Zahorchak AF, et al. Post-transplant repopulation of naive and memory T cells in blood and lymphoid tissue after alemtuzumab-mediated depletion in heart-transplanted cynomolgus monkeys. *Transpl Immunol* (2013) 29(1–4): 88–98. doi:10.1016/j.trim.2013.10.002
78. Ruzek MC, Waire JS, Hopkins D, Lacorcia G, Sullivan J, Roberts BL, et al. Characterization of in vitro antimurine thymocyte globulin-induced regulatory T cells that inhibit graft-versus-host disease in vivo. *Blood* (2008) 111(3):1726–34. doi:10.1182/blood-2007-08-106526
79. Yokota N, Daniels F, Crosson J, Rabb H. Protective effect of T cell depletion in murine renal ischemia-reperfusion injury. *Transplantation* (2002) 74(6):759–63.
80. Ayasoufi K, Fan R, Fairchild RL, Valujskikh A. CD4 T cell help via B cells is required for lymphopenia-induced CD8 T cell proliferation. *J Immunol* (2016) 196(7):3180–90. doi:10.4049/jimmunol.1501435
81. D'Addio F, Yuan X, Habicht A, Williams J, Ruzek M, Iacomini J, et al. A novel clinically relevant approach to tip the balance toward regulation in stringent transplant model. *Transplantation* (2010) 90(3):260–9. doi:10.1097/TP.0b013e3181e64217
82. Kim EJ, Kwun J, Gibby AC, Hong JJ, Farris AB III, Iwakoshi NN, et al. Costimulation blockade alters germinal center responses and prevents antibody-mediated rejection. *Am J Transplant* (2014) 14(1):59–69. doi:10.1111/ajt.12526
83. da Silva AJ, Brickelmaier M, Majeau GR, Li Z, Su L, Hsu YM, et al. Alefacept, an immunomodulatory recombinant LFA-3/IgG1 fusion protein, induces CD16 signaling and CD2/CD16-dependent apoptosis of CD2(+) cells. *J Immunol* (2002) 168(9):4462–71. doi:10.4049/jimmunol.168.9.4462
84. Majeau GR, Meier W, Jimmo B, Kioussis D, Hochman PS. Mechanism of lymphocyte function-associated molecule 3-Ig fusion proteins inhibition of T cell responses. Structure/function analysis in vitro and in human CD2 transgenic mice. *J Immunol* (1994) 152(6):2753–67.
85. Miller GT, Hochman PS, Meier W, Tizard R, Bixler SA, Rosa MD, et al. Specific interaction of lymphocyte function-associated antigen 3 with CD2 can inhibit T cell responses. *J Exp Med* (1993) 178(1):211–22.
86. Chamian F, Lowes MA, Lin SL, Lee E, Kikuchi T, Gilleaudeau P, et al. Alefacept reduces infiltrating T cells, activated dendritic cells, and inflammatory genes in psoriasis vulgaris. *Proc Natl Acad Sci U S A* (2005) 102(6): 2075–80. doi:10.1073/pnas.0409569102
87. Ortonne JP, Lebwohl M, Em Griffiths C; Alefacept Clinical Study Group. Alefacept-induced decreases in circulating blood lymphocyte counts correlate with clinical response in patients with chronic plaque psoriasis. *Eur J Dermatol* (2003) 13(2):117–23.
88. Larsen R, Ryder LP, Svejgaard A, Gniadecki R. Changes in circulating lymphocyte subpopulations following administration of the leucocyte function-associated antigen-3 (LFA-3)/IgG1 fusion protein alefacept. *Clin Exp Immunol* (2007) 149(1):23–30. doi:10.1111/j.1365-2249.2007.03380.x
89. Chamian F, Lin SL, Lee E, Kikuchi T, Gilleaudeau P, Sullivan-Whalen M, et al. Alefacept (anti-CD2) causes a selective reduction in circulating effector memory T cells (Tem) and relative preservation of central memory T cells (Tcm) in psoriasis. *J Transl Med* (2007) 5:27. doi:10.1186/1479-5876-5-27
90. Ellis CN, Krueger GG; Alefacept Clinical Study Group. Treatment of chronic plaque psoriasis by selective targeting of memory effector T lymphocytes. *N Engl J Med* (2001) 345(4):248–55. doi:10.1056/NEJM200107263450403
91. Lo DJ, Weaver TA, Stempora L, Mehta AK, Ford ML, Larsen CP, et al. Selective targeting of human alloresponsive CD8+ effector memory T cells based on CD2 expression. *Am J Transplant* (2011) 11(1):22–33. doi:10.1111/j.1600-6143.2010.03317.x
92. Shapira MY, Abdul-Hai A, Resnick IB, Bitan M, Tsirigotis P, Aker M, et al. Alefacept treatment for refractory chronic extensive GVHD. *Bone Marrow Transplant* (2009) 43(4):339–43. doi:10.1038/bmt.2008.324
93. Shapira MY, Resnick IB, Bitan M, Ackerstein A, Tsirigotis P, Gesundheit B, et al. Rapid response to alefacept given to patients with steroid resistant or steroid dependent acute graft-versus-host disease: a preliminary report. *Bone Marrow Transplant* (2005) 36(12):1097–101. doi:10.1038/sj.bmt.1705185
94. Shapira MY, Resnick IB, Dray L, Aker M, Stepensky P, Elad S, et al. A new induction protocol for the control of steroid refractory/dependent acute graft versus host disease with alefacept and tacrolimus. *Cytotherapy* (2009) 11(1):61–7. doi:10.1080/14653240802644669
95. Weaver TA, Charafeddine AH, Agarwal A, Turner AP, Russell M, Leopardi FV, et al. Alefacept promotes co-stimulation blockade based allograft survival in nonhuman primates. *Nat Med* (2009) 15(7):746–9. doi:10.1038/nm.1993
96. Priyadharshini B, Turka LA. T-cell energy metabolism as a controller of cell fate in transplantation. *Curr Opin Organ Transplant* (2015) 20(1):21–8. doi:10.1097/MOT.0000000000000149
97. Larsen CP, Pearson TC, Adams AB, Tso P, Shirasugi N, Strobert E, et al. Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties. *Am J Transplant* (2005) 5(3):443–53. doi:10.1111/j.1600-6143.2005.00749.x
98. Rostaing L, Vincenti F, Grinyo J, Rice KM, Bresnahan B, Steinberg S, et al. Long-term belatacept exposure maintains efficacy and safety at 5 years: results from the long-term extension of the BENEFIT study. *Am J Transplant* (2013) 13(11):2875–83. doi:10.1111/ajt.12460
99. Vincenti F, Larsen CP, Alberu J, Bresnahan B, Garcia VD, Kothari J, et al. Three-year outcomes from BENEFIT, a randomized, active-controlled, parallel-group study in adult kidney transplant recipients. *Am J Transplant* (2012) 12(1):210–7. doi:10.1111/j.1600-6143.2011.03785.x
100. de Graav GN, Hesselink DA, Dieterich M, Kraaijeveld R, Weimar W, Baan CC. Down-regulation of surface CD28 under belatacept treatment: an escape mechanism for antigen-reactive T-cells. *PLoS One* (2016) 11(2):e0148604. doi:10.1371/journal.pone.0148604
101. Hamann D, Kostense S, Wolthers KC, Otto SA, Baars PA, Miedema F, et al. Evidence that human CD8+CD45RA+CD27- cells are induced by antigen and evolve through extensive rounds of division. *Int Immunol* (1999) 11(7):1027–33.
102. Rufer N, Zippelius A, Batard P, Pittet MJ, Kurth I, Corthesy P, et al. Ex vivo characterization of human CD8+ T subsets with distinct replicative history and partial effector functions. *Blood* (2003) 102(5):1779–87. doi:10.1182/blood-2003-02-0420
103. Sze DM, Giesajtis G, Brown RD, Raitakari M, Gibson J, Ho J, et al. Clonal cytotoxic T cells are expanded in myeloma and reside in the CD8(+) CD57(+)CD28(-) compartment. *Blood* (2001) 98(9):2817–27. doi:10.1182/blood.V98.9.2817
104. Weekes MP, Carmichael AJ, Wills MR, Mynard K, Sissons JG. Human CD28-CD8+ T cells contain greatly expanded functional virus-specific memory CTL clones. *J Immunol* (1999) 162(12):7569–77.
105. Baeten D, Louis S, Braud C, Braudeau C, Ballet C, Moizant F, et al. Phenotypically and functionally distinct CD8+ lymphocyte populations in long-term drug-free tolerance and chronic rejection in human kidney graft recipients. *J Am Soc Nephrol* (2006) 17(1):294–304. doi:10.1681/ASN.2005020178
106. Bai Y, Liu J, Wang Y, Honig S, Qin L, Boros P, et al. L-selectin-dependent lymphoid occupancy is required to induce alloantigen-specific tolerance. *J Immunol* (2002) 168(4):1579–89. doi:10.4049/jimmunol.168.4.1579
107. Pawlik A, Florczak M, Masiuk M, Dutkiewicz G, Machalinski B, Rozanski J, et al. The expansion of CD4+CD28- T cells in patients with chronic kidney graft rejection. *Transplant Proc* (2003) 35(8):2902–4. doi:10.1016/j.transproceed.2003.10.061
108. Studer SM, George MP, Zhu X, Song Y, Valentine VG, Stoner MW, et al. CD28 down-regulation on CD4 T cells is a marker for graft dysfunction in lung transplant recipients. *Am J Respir Crit Care Med* (2008) 178(7):765–73. doi:10.1164/rccm.200701-013OC
109. Espinosa JR, Samy KP, Kirk AD. Memory T cells in organ transplantation: progress and challenges. *Nat Rev Nephrol* (2016) 12(6):339–47. doi:10.1038/nrneph.2016.9
110. Badell IR, Kitchens WH, Wagener ME, Lukacher AE, Larsen CP, Ford ML. Pathogen stimulation history impacts donor-specific CD8(+) T cell susceptibility to costimulation/integrin blockade-based therapy. *Am J Transplant* (2015) 15(12):3081–94. doi:10.1111/ajt.13399

111. Xu H, Perez SD, Cheeseman J, Mehta AK, Kirk AD. The allo- and viral-specific immunosuppressive effect of belatacept, but not tacrolimus, attenuates with progressive T cell maturation. *Am J Transplant* (2014) 14(2):319–32. doi:10.1111/ajt.12574
112. Ariyan C, Salvalaggio P, Fecteau S, Deng S, Rogozinski L, Mandelbrot D, et al. Cutting edge: transplantation tolerance through enhanced CTLA-4 expression. *J Immunol* (2003) 171(11):5673–7. doi:10.4049/jimmunol.171.11.5673
113. Fecteau S, Basadonna GP, Freitas A, Ariyan C, Sayegh MH, Rothstein DM. CTLA-4 up-regulation plays a role in tolerance mediated by CD45. *Nat Immunol* (2001) 2(1):58–63. doi:10.1038/83175
114. Iwakoshi NN, Mordes JP, Markees TG, Phillips NE, Rossini AA, Greiner DL. Treatment of allograft recipients with donor-specific transfusion and anti-CD154 antibody leads to deletion of alloreactive CD8+ T cells and prolonged graft survival in a CTLA4-dependent manner. *J Immunol* (2000) 164(1):512–21. doi:10.4049/jimmunol.164.1.512
115. Judge TA, Wu Z, Zheng XG, Sharpe AH, Sayegh MH, Turka LA. The role of CD80, CD86, and CTLA4 in alloimmune responses and the induction of long-term allograft survival. *J Immunol* (1999) 162(4):1947–51.
116. Lin H, Rathmell JC, Gray GS, Thompson CB, Leiden JM, Alegre ML. Cytotoxic T lymphocyte antigen 4 (CTLA4) blockade accelerates the acute rejection of cardiac allografts in CD28-deficient mice: CTLA4 can function independently of CD28. *J Exp Med* (1998) 188(1):199–204.
117. Markees TG, Phillips NE, Gordon EJ, Noelle RJ, Shultz LD, Mordes JP, et al. Long-term survival of skin allografts induced by donor splenocytes and anti-CD154 antibody in thymectomized mice requires CD4(+) T cells, interferon-gamma, and CTLA4. *J Clin Invest* (1998) 101(11):2446–55.
118. Poirier N, Azimzadeh AM, Zhang T, Dilek N, Mary C, Nguyen B, et al. Inducing CTLA-4-dependent immune regulation by selective CD28 blockade promotes regulatory T cells in organ transplantation. *Sci Transl Med* (2010) 2(17):17ra10. doi:10.1126/scitranslmed.3000116
119. Poirier N, Mary C, Dilek N, Hervouet J, Minault D, Blancho G, et al. Preclinical efficacy and immunological safety of FR104, an antagonist anti-CD28 monovalent Fab' antibody. *Am J Transplant* (2012) 12(10):2630–40. doi:10.1111/j.1600-6143.2012.04164.x
120. Vanhove B, Laflamme G, Coulon F, Mougin M, Vusio P, Haspot F, et al. Selective blockade of CD28 and not CTLA-4 with a single-chain Fv-alpha1-antitrypsin fusion antibody. *Blood* (2003) 102(2):564–70. doi:10.1182/blood-2002-08-2480
121. Zhang T, Fresnay S, Welty E, Sangrampurkar N, Rybak E, Zhou H, et al. Selective CD28 blockade attenuates acute and chronic rejection of murine cardiac allografts in a CTLA-4-dependent manner. *Am J Transplant* (2011) 11(8):1599–609. doi:10.1111/j.1600-6143.2011.03624.x
122. Kawai T, Andrews D, Colvin RB, Sachs DH, Cosimi AB. Thromboembolic complications after treatment with monoclonal antibody against CD40 ligand. *Nat Med* (2000) 6(2):114. doi:10.1038/72162
123. Adams AB, Shirasugi N, Jones TR, Durham MM, Strobert EA, Cowan S, et al. Development of a chimeric anti-CD40 monoclonal antibody that synergizes with LEA29Y to prolong islet allograft survival. *J Immunol* (2005) 174(1):542–50. doi:10.4049/jimmunol.174.1.542
124. Aoyagi T, Yamashita K, Suzuki T, Uno M, Goto R, Taniguchi M, et al. A human anti-CD40 monoclonal antibody, 4D11, for kidney transplantation in cynomolgus monkeys: induction and maintenance therapy. *Am J Transplant* (2009) 9(8):1732–41. doi:10.1111/j.1600-6143.2009.02693.x
125. Badell IR, Russell MC, Cardona K, Shaffer VO, Turner AP, Avila JG, et al. CTLA4Ig prevents alloantibody formation following nonhuman primate islet transplantation using the CD40-specific antibody 3A8. *Am J Transplant* (2012) 12(7):1918–23. doi:10.1111/j.1600-6143.2012.04029.x
126. Haanstra KG, Ringers J, Sick EA, Ramdien-Murli S, Kuhn EM, Boon L, et al. Prevention of kidney allograft rejection using anti-CD40 and anti-CD86 in primates. *Transplantation* (2003) 75(5):637–43. doi:10.1097/01.TP.0000054835.58014.C2
127. Imai A, Suzuki T, Sugitani A, Itoh T, Ueki S, Aoyagi T, et al. A novel fully human anti-CD40 monoclonal antibody, 4D11, for kidney transplantation in cynomolgus monkeys. *Transplantation* (2007) 84(8):1020–8. doi:10.1097/01.tp.0000286058.79448.c7
128. Pearson TC, Trambley J, Odom K, Anderson DC, Cowan S, Bray R, et al. Anti-CD40 therapy extends renal allograft survival in rhesus macaques. *Transplantation* (2002) 74(7):933–40.
129. Valujskikh A. The challenge of inhibiting alloreactive T-cell memory. *Am J Transplant* (2006) 6(4):647–51. doi:10.1111/j.1600-6143.2005.01215.x
130. Ge W, Jiang J, Liu W, Lian D, Saito A, Garcia B, et al. Regulatory T cells are critical to tolerance induction in presensitized mouse transplant recipients through targeting memory T cells. *Am J Transplant* (2010) 10(8):1760–73. doi:10.1111/j.1600-6143.2010.03186.x
131. Burrell BE, Lu G, Li XC, Bishop DK. OX40 costimulation prevents allograft acceptance induced by CD40-CD40L blockade. *J Immunol* (2009) 182(1):379–90. doi:10.4049/jimmunol.182.1.379
132. Chen M, Xiao X, Demirci G, Li XC. OX40 controls islet allograft tolerance in CD154 deficient mice by regulating FOXP3+ Tregs. *Transplantation* (2008) 85(11):1659–62. doi:10.1097/TP.0b013e3181726987
133. Kitchens WH, Larsen CP, Ford ML. Integrin antagonists for transplant immunosuppression: panacea or peril? *Immunotherapy* (2011) 3(3):305–7. doi:10.2217/imt.10.113
134. Kitchens WH, Haridas D, Wagener ME, Song M, Kirk AD, Larsen CP, et al. Integrin antagonists prevent costimulatory blockade-resistant transplant rejection by CD8(+) memory T cells. *Am J Transplant* (2012) 12(1):69–80. doi:10.1111/j.1600-6143.2011.03762.x
135. Setoguchi K, Schenk AD, Ishii D, Hattori Y, Baldwin WM III, Tanabe K, et al. LFA-1 antagonism inhibits early infiltration of endogenous memory CD8 T cells into cardiac allografts and donor-reactive T cell priming. *Am J Transplant* (2011) 11(5):923–35. doi:10.1111/j.1600-6143.2011.03492.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 24 March 2016
doi: 10.3389/fimmu.2016.00089

Cross-Reactivity of TCR Repertoire: Current Concepts, Challenges, and Implication for Allotransplantation

Nicolas Degauque[1,2,3,4]*, *Sophie Brouard*[1,2,3,4,5,6,7] *and Jean-Paul Soulillou*[1,3,7]

[1] UMR 1064, INSERM, Nantes, France, [2] ITUN, CHU de Nantes, Nantes, France, [3] Faculté de Médecine, Université de Nantes, Nantes, France, [4] LabEx IGO "Immunotherapy Graft Oncology", Nantes, France, [5] CIC Biothérapie, Nantes, France, [6] CRB, CHU Nantes, Nantes, France, [7] LabEx Transplantex, Nantes, France

Being able to track donor reactive T cells during the course of organ transplantation is a key to improve the graft survival, to prevent graft dysfunction, and to adapt the immunosuppressive regimen. The attempts of transplant immunologists have been for long hampered by the large size of the alloreactive T cell repertoire. Understanding how self-TCR can interact with allogeneic MHC is a key to critically appraise the different assays available to analyze the TCR Vβ repertoire usage. In this report, we will review conceptually and experimentally the process of cross-reactivity. We will then highlight what can be learned from allotransplantation, a situation of artificial cross-reactivity. Finally, the low- and high-resolution techniques to characterize the TCR Vβ repertoire usage in transplantation will be critically discussed.

Keywords: TCR repertoire, transplantation, cross-reactivity, alloreactivity, TCR, MHC, T cell

OPEN ACCESS

Edited by:
Gilles Benichou, Massachusetts General Hospital, USA

Reviewed by:
Ralf Dressel, University Medical Center Göttingen, Germany
Dennis O. Adeegbe, Harvard Medical School, USA

***Correspondence:**
Nicolas Degauque
nicolas.degauque@univ-nantes.fr

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *07 December 2015*
Accepted: *22 February 2016*
Published: *24 March 2016*

Citation:
Degauque N, Brouard S and Soulillou J-P (2016) Cross-Reactivity of TCR Repertoire: Current Concepts, Challenges, and Implication for Allotransplantation. Front. Immunol. 7:89. doi: 10.3389/fimmu.2016.00089

UNDERSTANDING THE CROSS-REACTIVITY

Shaping the T Lymphocyte Receptor Repertoire

Through evolution, numerous processes have been selected to generate a diverse repertoire of TCRαβ able to protect mammalian from pathogenic insults (**Figure 1**). Highly similar genes recombine to form functional genes and generate a highly diverse TCR repertoire. TCRβ chains are encoded by distinct Variable (V; TRBV), Diversity (D; TRBD), and Joining (J; TRBJ) genes, whereas TCRα chains are encoded by distinct sets of V and J genes (TRAV and TRAJ). Junctional diversification further extends the combinatorial diversity by either trimming gene ends or adding nucleotides between the recombining genes (1). In contrast to the IGHV (V genes of Immunoglobulin Heavy Chain) germline dataset compiled by the ImMunoGeneTics (IMGT) group that greatly benefit from the advanced of deep-sequencing technologies, the human TCR germline has been only minimally changed since the complete sequencing of the TCR gene loci in 1996 (2, 3). The 65 functional genes, ORFs, and pseudogenes have been reported for the TRBV, 54 for the TRAV and 2 for the TRBD dataset. The analysis of the TCR CDR3 is still a very challenging process. The identification of the TRBD genes

Abbreviations: CAMR, chronic antibody-mediated; CDR, cluster differentiation region; CMV, cytomegalovirus; CNS, central nervous system; CSF, cerebral spinal fluid; EBV, Epstein–Barr virus; GZMb, Granzym B; HLA, human leukocyte antigen; IFNg, interferon g; ITAM, immunoreceptor tyrosine activation motifs; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; NGS, next generation sequencing; PERF, perforin; TCR, T cell repertoire; TEMRA, T cell effector memory re-expressing CD45RA; TNFa, Tumor necrosis factor.

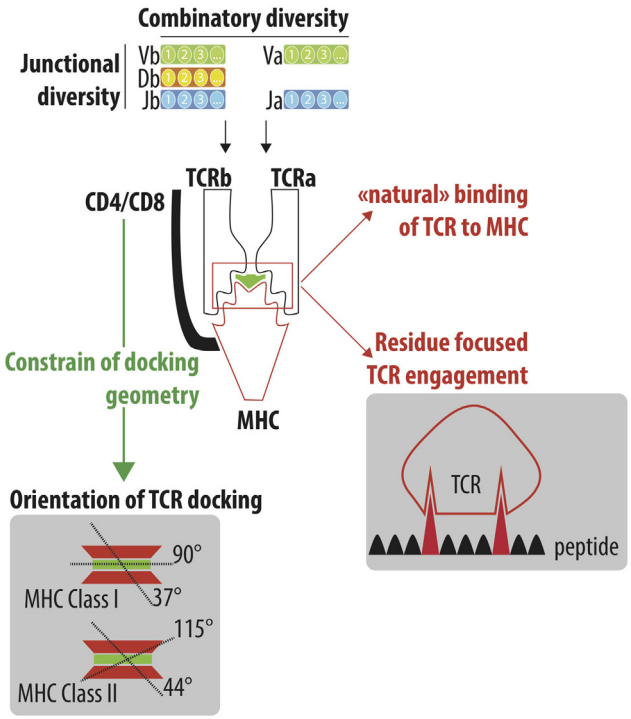


FIGURE 1 | Understand the cross-reactivity of a highly diverse TCR repertoire. A highly diverse TCRαβ repertoire is generated by iterative processes selected through evolution. Combinatory diversity results from the selection of Variable (V; TRAV and TRBV), Diversity (D; TRBD) and Joining (J; TRAJ and TRBJ) genes. Junctional diversification further extends the combinatorial diversity by either trimming gene ends or adding nucleotides between the recombining genes. Finally, the association of the TCRα and TCRβ chain constitutes the final steps of the numerous iteration processes that lead to the generation of a highly diverse TCR repertoire, which is able to efficiently protect individuals from pathogenic stimulations. TCRαβ adopts a stereotype docking geometry atop the MHC/peptide complex. This orientation leads to a spatial interaction between the germline-encoded CDR1 and CDR2 of the TCRα and β chains and the edges of the peptide-groove of MHC. The accumulation of reported crystallographic structures has challenged the stereotypic view of the angle of the TCR docking. However, the recognition of conserved motifs on the side of MHC molecules by CD4/CD8 co-receptor constrained the TCR docking geometry. Despite the high diversity of the TCR repertoire, a high degree of cross-reactivity has been reported that could be explained by the "natural" ability of TCR to interact with MHC molecules (MHC focus model) as well as the interaction of TCR to a limited number of amino acids of the peptide bound to the MHC peptide groove.

cannot be performed due to the high degree of similarities of the TRBD at their 5′ ends, the short length of the two genes, and the presence of G-rich N nucleotides at the 5′ ends that could be also added by the TdT enzyme.

It is misleading to estimate the combinatory diversity by simply multiplying together the number of V, D, and J genes (4). Rather than a random combination of the TCR genes, studies have shown that TCR genes are highly biased in their usage, and that only part of the theoretical diversity is selected (5, 6). Chromosomal recombination patterns can be explained by variations in enhancers and Recombination Signal Sequences (RSS) and organization of the TRBJ genes (a block of six and seven genes located respectively downstream from the TRDB1 and TRDB2 gene) that leads to a bias in D–J pairing. The diversity of the TCR repertoire is further broaden during the rearrangement process first by the addition of P nucleotides (Palindromic nucleotides) thanks to recombination activating gene-1 and -2 (RAG1 and RAG2) (7) that form hairpin loops at the gene end and then by the addition of N nucleotides (with a biased toward G nucleotides) by the terminal deoxynucleotidyl transferase (TdT) (8). Insertions of nucleotides have a profound impact on the diversity of the Complementary-Determining Regions 3 (CDR3) sequences and contribute to most (60%) of this diversity (9). The coding ends of the genes can be also trimmed by exonucleases. However, given the limited number of amino acids, the removal of nucleotides by exonucleases is constrained to generate a productive codon and therefore limits the contribution of exonuclease trimming to the diversity of the TCR repertoire. Finally, the association of the TCRα and TCRβ chain constitutes the final steps of the numerous iteration processes that lead to the generation of a highly diverse TCR repertoire, which is able to efficiently protect individuals from pathogenic stimulations.

Current Understanding of the Recognition of pMHC by TCR

Six CDR will engage the peptide/MHC complexes, endogenous and exogenous peptides being presented respectively by MHC class I and II molecules. MHC class I grooves constrain the length of the presented peptides (8–14 amino acids length) while the open nature of peptide-binding cleft of MHC class II molecules allow a broader range of peptides to be presented. The HLA locus is the most polymorphic region of the human genome, with more than 13,000 variant alleles (10,297 HLA Class I Alleles and 3,543 HLA Class II Alleles according to the IMGT/HLA). The high diversity of HLA conferring an almost unique signature of HLA for mankind is further extended by the combinatory diversity resulting from the association of six HLA Class I (two alleles of HLA-A, -B, and Cw) and six HLA Class II molecules (two alleles of HLA-DR, -DP, and -DQ). The high mutation level of the HLA loci is preferentially focused on the peptide-binding cleft that clustered most of the variability of the amino acid sequence. The focus of mutations underlines the function of the HLA molecules, namely being able to display a very large array of peptides.

Garcia et al. were the first to report the crystallographic structure of a murine TCR 2C bound to peptide/MHC Class I (H-2K^{B}–dEV8). The cytotoxic T cell clone 2C is one of the most well-characterized TCR and has been initially isolated from a BALB/b mouse as an allospecific T cell that recognized L^{d} on the mastocytoma P815. Beside its primary antigen (peptide p2C), the 2C TCR can bind to different antigens, including the dEV8 (10) and SIYR (11). They also showed that TCRαβ adopts a 45° diagonal orientation to the long axis of the peptide (12). This orientation leads to a spatial interaction between the germline-encoded CDR1 and CDR2 of the TCRα and β chains and the edges of the peptide-groove of MHC (**Figure 1**). The highly diverse CDR3 region is facing the central portion of the bound peptide. The multiple crystallographic structures of TCR/peptide MHC complexes [more than 120 of crystallographic structures

have been obtained (13)] have revealed that the docking angle of the TCR is conserved with a stereotype position of a 75° diagonal orientation to the long axis of the peptide (14). The conserved binding model has lead to the concept that TCR and MHC are hardwired to interact, resulting from a coevolution selection of conserved regions (codons) to lock in TCR onto MHC molecule. The stereotyped orientation of TCR atop MHC molecule is however more flexible than initially proposed, with the accumulation of crystal structures. The median docking angle of TCR is 63.2° (min–max 37–90°) with MHC class I and 76.4° (min–max 44–115°) with MHC class II (13) (**Figure 1**).

Different theories have been postulated to explain the hardwire of TCR to MHC molecules (15), including the key role of co-receptors of CD4 and CD8 that imposed steric requirements for concurrent associations of TCR, CD3, CD4/CD8, and MHC complexes allowing the appropriate signaling events to occur. Indeed, the main role of co-receptor CD4 and CD8 is to recruit the Src tyrosine kinase p65lck (lck) via its association with the cytoplasmic tail of CD4 or CD8. Lck concentration promotes phosphorylation of immunoreceptor tyrosine activation motifs (ITAMs) in the cytoplasmic tails of CD3 subunits and then initiates the cascade of signaling events leading to the full activation of the T lymphocyte. Given the key role of co-receptor CD4 and CD8 in process, it was assumed that their ability to bind, respectively, the membrane-proximal α2 and β2 domains of the MHC class II molecule and the protruding loop in the α3 domain of the MHC class I molecule will constrain the docking geometry of the TCR to the pMHC (**Figure 1**).

A recent report by Beringer et al. (16) had challenged the consensus idea of a highly stereotype docking of TCR atop MHC molecules (13). Crystallographic structures of two TCR binding to proinsulin peptide presented by HLA-DR4 (HLA-DR4^{proinsulin}) have been obtained from two clones of induced regulatory CD4 T cells. The ternary complexes revealed a 180° polarity reversal compared to all other TCR-peptide-MHC complex structures. It remains to be address whether this singular observation could be generalizable, whether the reverse docking is a unique feature of regulatory cells and whether the potential signaling differences may influence the phenotype and the function of the T cells.

Cross-Reactivity, from Intellectual Concept to a Critical Need for Immune System

Cross-reactivity can be defined by the ability of a given TCR to interact with more than one pMHC complex with different presented peptides or MHC molecules. This new concept has been presented as early as 1977 by Matzinger and Bevan (17). An alloreactive T cell clone was derived by Owens et al. in 1984 with three H2-E reactivity (allo-E^k specific, H2-E^k, DBA/B10 H2-E^d, and self H2-E^d) (18). Since then, numerous reports have provided evidence of cross-reactivity. For instance, mouse 2C TCR can interact with syngeneic MHC H-2K^b presenting dEV8 (10) and SIYR (11) and with allogeneic H-2K^{bm3} presenting dEV8/K^{bm3} (10) and allogeneic H-2L^d–p2CA (11). The study by Birnbaum et al. is an elegant attempt to quantify the cross-reactivity of a given TCR (19). Using five different CD4 TCR clones (three from mouse origin and two from human origin), high throughput screening of yeast libraries and deep sequencing, the authors demonstrate that a single TCR can interact with more than 100 different peptides.

Jerne et al. postulated in the mid-1950 that each cell exhibits a unique clonotype able to recognize only one antigen (20, 21). Don Mason has been among the first to challenge the validity of this clonal selection theory (22) showing that the immune system will be highly incompetent to protect an individual from external insult if one and only TCR was able to recognize a single peptide presented in a given HLA context. More than 10^{15} T cells, which would weigh more than 500 kg, would be needed to provide efficient coverage of the potential foreign peptides. This clearly stated that the immune system could not efficiently protect individual if one TCR interacts with a single antigen. Unlike the affinity maturation of B cell receptor, the protein sequence of TCR is fixed and naive T cells are required to recognize foreign antigens not encountered before. The number of potential antigens to be recognized is huge given the variability induced by the high diversity of peptide-binding groove of HLA class I and II molecules. From the 20 proteinogenic amino acids and given that peptides from 8- to 14-mer can be presented, an incredibly high number of peptides can be potentially generated ($>10^{15}$ peptides) (23). The diversity can be further extending by the posttranslational modifications of amino acids. In a 2012 opinion paper, Andrew Sewell elegantly presents the necessity of the cross-reactivity (23), as the number of potential foreign peptide–MHC complexes that T cells might encounter dwarfs the number of TCRs available [the number of unique TCRαβ is estimated to be in the magnitude of 10^{11} (24, 25)].

The mechanisms described previously to generate a diverse TCRαβ have to be envisioned at the population level. Given the relatively limited number of genes encoding for TCR chain α and β and the requirement of TCR to recognize the highly diverse HLA molecules, the necessity of each T cell to recognize a large array of peptides is expected (22). Before presenting the experimental approach aiming to quantify the number of peptides recognized by a single TCR, we would like to present clear evidences of the cross-reactivity involving memory T cells without previous antigen encounter. It has been described few years ago that CD8 T cells with a memory phenotype can be found in mice (26–28). CD8 T cells specific for ovalbumin and viral antigens (HSV, vaccinia) could be detected in mice despite their germ-free environment (28). Despite the absence of previous antigen encounter, these pre-existing memory CD8 T cells harbor traits of memory cells such as the ability to rapidly proliferate upon stimulation and to secrete rapidly pro-inflammatory cytokines. Homeostatic proliferation, aging, and cross-recognition of alternate ligands have been postulated to drive the accumulation of these memory-like naive CD8 T cells (27, 29). This observation has been extended to human settings in which CD4 T cells specific for HIV-1, CMV, and herpes simplex virus (HSV) epitopes were identified in healthy volunteers that had never been infected with these viruses (30). Again, these cells exhibit not only memory markers but also memory-associated features (rapid proliferation and cytokine secretion). The acquisition of memory characteristics could be a consequence of homeostatic proliferation (31) or a consequence

of the cross-reactivity to other antigens in the environment. To support the latest hypothesis, Su et al. have shown that HIV-1 specific T cells can recognize environmental peptides present in the gut and soil, bacteria and ocean algae, and plants. Of interest, T cells specific for HIV-1 can even be purified from cord-blood (30), demonstrating thereby the presence of T cells able to recognize self and non-self antigens in newborns. Of interest, the phenotype of cross-reactive T cells was different between newborns and adults, with a naive and a memory phenotype, respectively.

The concept of clonal deletion that occurred in the thymus is challenged by the aforementioned reports and compelling evidences suggest that from an evolutionary perspective, the necessity to protect an individual against pathogens is far more important than to limit the autoreactivity. A recent study from Davis team further sustained this claim (32). The frequency of CD8 T cells specific of a Y chromosome specific antigen (equivalent to HY peptide) is only threefold lower in man as compared to women (32). Of interest, whereas CD8 T cells purified for their specificity regarding a pool of six self peptides do not proliferate after stimulation with the same set of self peptides, CD8 T cells specific of a pool of six non-self peptides exhibit a potent proliferative response (32). The absence of response reported for self-specific CD8 T cells and not for foreign antigen-specific CD8 T cells has been linked to a different genetic programing as compared to the clones purified from woman, with a lower expression of IL-2R, IL-21R, and Bcl-XL (32). Thus, evolution has favored the absence of hole over autoimmune disease (about 1% of incidence). It may seem awkward that the evolution has favor the escape of anti-self specific T cells from thymic selection over a more stringent deletion of all anti-self T cells. A heavier burden of maintaining tolerance is needed to prevent the development of autoimmune diseases. However, the need to defend the immune system against pathogens, especially during childhood, is far greater than the need to prevent autoimmunity as for population's survival. By limiting the deletion of self-reactive T cells and thanks to the large cross-reactivity of T cells, the holes in the T cell repertoire that pathogens might take advantage of are constrained.

The analysis of the immune system in monozygotic twins is enlightened in many aspects as such studies allow the dissociation between the inborn and the acquired contributions. The team of Davis has recently showed that the heritability of T and B cells parameters declines very rapidly with age (33). At the age of 40 years, the heritability explained less than 10% of the variation in T and B cell parameters. CMV infection is a protypical example of the influence of non-heritable factor on the whole immune system. Indeed, 58% of all parameters measured in discordant twins were influenced by CMV infection (33). The environment carves the immune system of each single individual, with each past immune response heavily imprinting the (present) immune system.

Since the initial observation that immunity against cowpox protects individual from smallpox (34), numerous examples of cross-reactivity had been reported in mice and in human (35). For instance, infections with BCG, influenza A virus (IAV), lymphocytic choriomeningitis virus (LCMV), and murine cytomegalovirus (MCMV) all confer a level of protective immunity against Vaccinia Virus (36–38). The benefit of cross-reactivity as for pan-virus protection is more difficult to assess for obvious reasons. Nevertheless, the numerous example of a single TCR able to recognize different antigens [BCG and Poxviruses (37); Papillomavirus and Coronavirus (39); Influenza virus and Epstein–Barr virus (40)]. The large cross-reactivity of T cells confers a more efficient protection cover using a limited number of T cells that need to screen an incredibly large array of peptides that can be presented by MHC molecules. Beside the efficient use of limited T cell resource, cross-reactivity confers a spatio-temporal advantage to the immune system to scan any infected cells. Cross-reactivity could also be envisioned as an evolution strategy to limit the immune recognition escape.

ALLOTRANSPLANTATION IS NOT ONLY AN EXAMPLE OF ARTIFACTUAL CROSS-REACTIVITY BUT ALSO GIVES CLUES REGARDING THE GLOBAL ORGANIZATION OF THE IMMUNE SYSTEM

Recipient immune system can interact with foreign HLA molecules under two very different circumstances: pregnancy and transplantation. Thanks to evolution and adaptation of the maternal immune system to the presence of HLA mismatch fetuses, allorecognition during pregnancy is not harmful and could even be beneficial as for mammalian sexual reproduction. Immunological tolerance toward allogeneic fetus is obtained through a complex network of regulatory mechanisms including the lack of expression of classical MHC class I molecules by the placental trophoblast and the expression of non-classical MHC class I HLA-E and HLA-G. More surprisingly, HLA mismatches have been proposed to be beneficial for pregnancy outcome. In the 1960s, Billington reports that the placenta is larger in H-2 incompatible mouse as compared to compatible fetuses (41). HLA compatible fetuses (i.e., similar to maternal HLA) have been shown to be more prone to be aborted (42). In contrast, recipient immune system will potently eliminate an allogeneic graft in the absence of immunosuppressive therapy.

Despite the absence of thymic central selection (43) of potential graft-recipient T cells by allogeneic MHC motifs regarding their ability to recognize allogeneic potential HLA, a large pool of T cells can be activated by donor HLA molecules either through the direct pathway (i.e., donor HLA presenting donor peptides) or the expected processing of foreign MHC molecules, coined as the "indirect pathway" (i.e., recipient HLA presenting donor peptides) in transplantation immunologist jargon. The direct allorecognition pathway represents a unique example of functional and efficient cross reactivity. Two main hypothesizes have been postulated to explain the basis of alloreactivity, emphasizing the role of either MHC molecule or peptide. The polymorphism between donor and MHC molecules could act as an "innate focus" that leads to the activation of unprimed recipient T cells or the allopeptide could be recognized as foreign antigen while allogeneic and self-MHC molecules exhibit a high degree of similarity (**Figure 1**).

According to the MHC centric model, the peptide plays only a minor role in the process, and alloreactive TCRs recognize structural determinants on the MHC helices of syngeneic or allogeneic MHC. The bias of TCR to interact with MHC molecules supports this theory. Crystal structures of allo-pMHC complexes such as 2C TCR with allogeneic H-2K^{bm3} presenting dEV8/K^{bm3} (44) or BM3.3 TCR with allogeneic pBM1–H-2K^{b} (45) have shown that alloreactive TCRs interact with allogeneic MHC in a similar fashion as with syngeneic MHC. To further support the role of MHC in alloreactivity, it has been reported that some HLA mismatches between donor and recipient are associated with worse graft survival than others, leading to the notion of taboo mismatches based on shape rather than sequence differences (46). For instance, despite a single amino acid in an HLA Class I antigen, mismatches between HLA-B*4402 and HLA-B*4403 is associated with transplant rejection (47) and acute graft-versus-host disease (48). The peptide repertoire bound to HLA-B*4402 or HLA-B*4403 have been shown to be very similar (49). However, a recent report challenges this observation (50). The single amino acid mismatch induced the presentation of more unique peptides by HLA-B*4403 than HLA-B*4402, consistent with the stronger T cell alloreactivity observed toward HLA-B*4403 compared with HLA-B*4402 (50). This observation supports the notion of a peptide focus TCR allorecognition, in the same line as molecular mimicry.

Allorecognition could also involved cross-reactivity between MHC class I and MHC class II or even xeno MHC (51, 52). In 1986, Schilman et al. reported that CD8 T cell clone could be activated by both MHC class I (H-2D^{b}) and MHC class II (I-E^{k}) molecules (53). By-directional recognition of T cells between MHC class I and MHC class II have been reported later (54–56). These observations may have important implication in the attempt to minimize HLA mismatches during the process of organ allocation.

Defining the Magnitude of T Cell Response to Allostimulation

Using a mixed lymphocyte reaction (57), it has been shown that 1–10% of T cell in peripheral blood can be activated (58). As mentioned before, the number of HLA mismatches between donor and recipient is a primary driving force that mobilized a larger fraction of T cells than nominal antigens. Whether alloreactive T cells are activated by the high number of new antigens presented by donor HLA or by the large number of different allo-pHLA complexes (or both) is still under debate, and the two hypotheses are not mutually exclusives. The indirect pathway further enhances the reactivity of recipient T cells toward allogeneic graft. Indeed, peptides presented by MHC molecules derived predominantly from MHC-related molecules (59–61). The introduction of donor HLA molecules will thus lead to the introduction of great pool of new peptides that can mobilized a large fraction of recipient T cells.

It is now also well accepted that memory T cells generated prior transplantation constitute a major hurdle for long-term graft acceptance. Chronic viruses such as EBV and CMV induce the generation of a large pool of memory T cells. For instance, 10% of both the CD4 and CD8 memory compartments in blood are reactive to HCMV (62). The cross-reactivity between virus-specific T cells and allogeneic HLA has been extensively documented (63). EBV or CMV specific CD8 T cells exhibit frequently a cross-reactivity toward allogeneic MHC class I complexes (64–68). Similar observations have been reported for CD4 T cells specific for EBV or CMV (69–71). Virus-specific T cells that cross-react with alloantigens have been shown in experimental models to proliferate in response to a transplanted allograft *in vivo* (72). For instance, LCMV-specific CD8 T cells generated after infection of mice with Armstrong strain of LCMV are able to vigorously proliferate *in vivo* after skin transplantation and ultimately to mediate skin graft rejection (72).

Tracking Anti-Donor Response by the Investigation of TCR Vβ Repertoire: From Low Resolution Technique to High Throughput Sequencing

Given the size of anti-donor T cell pool, great efforts have been paid to track the immune-response using the analysis of TCR Vβ repertoire and to correlate specific usage of TCR Vβ repertoire with graft status or graft outcome. Before presenting the available reports, it is necessary to present the two major methods used to investigate TCR Vβ usage; a low resolution (spectratype alone or TcLandscape when combined with quantitative analysis) and, more recently, a high resolution (deep-sequencing of TCR Vβ region) approach (**Figure 2**). The low-resolution technique is based on the analysis of the length of the CDR3 region whereas the high-resolution technique identifies the sequence of each TCR Vβ and later quantifies the abundance of the different T cell clones.

Each TCR Vβ family is composed of T cells with various lengths of their CDR3 region. The distribution of the CDR3 length can be assessed by spectratype (73, 74). A broad spectrum of profiles can be identified ranging from a Gaussian-like profile to a highly restricted profile, highlighting the absence of selection of T cell, or the expansion of T cell clones, respectively. Different analytic tools have been used to characterize the CDR3 length distribution (75–78). The qualitative assessment of the TCR Vβ repertoire can be complemented by the quantification of the different Vβ families at the mRNA level using qRT-PCR (79–81) or at the cellular level using flow-cytometry (82). Such techniques still offer several benefits over higher resolution techniques such as their cost, the short time frame to obtain results, and the generation of a reasonable amount of data can be also displayed as "visible" pattern as an "X-ray" of the global TCR alteration in a specific pathological context (83–86). A rapid survey of the usage of the TCR Vβ repertoire can be efficiently performed, guiding further investigations focused on targeted TCR Vβ families. At the other range of the resolution spectrum, deep-sequencing of TCR Vβ obtains a full picture of the usage of T cell repertoire with deep or ultra-deep resolution. The availability of all TCR Vβ sequences allows for the precise appraisal of the distribution of the different T cell clones especially across different biological compartments (76). Furthermore, with a complete TCR Vβ sequencing, researchers can investigate the similarity of T cell

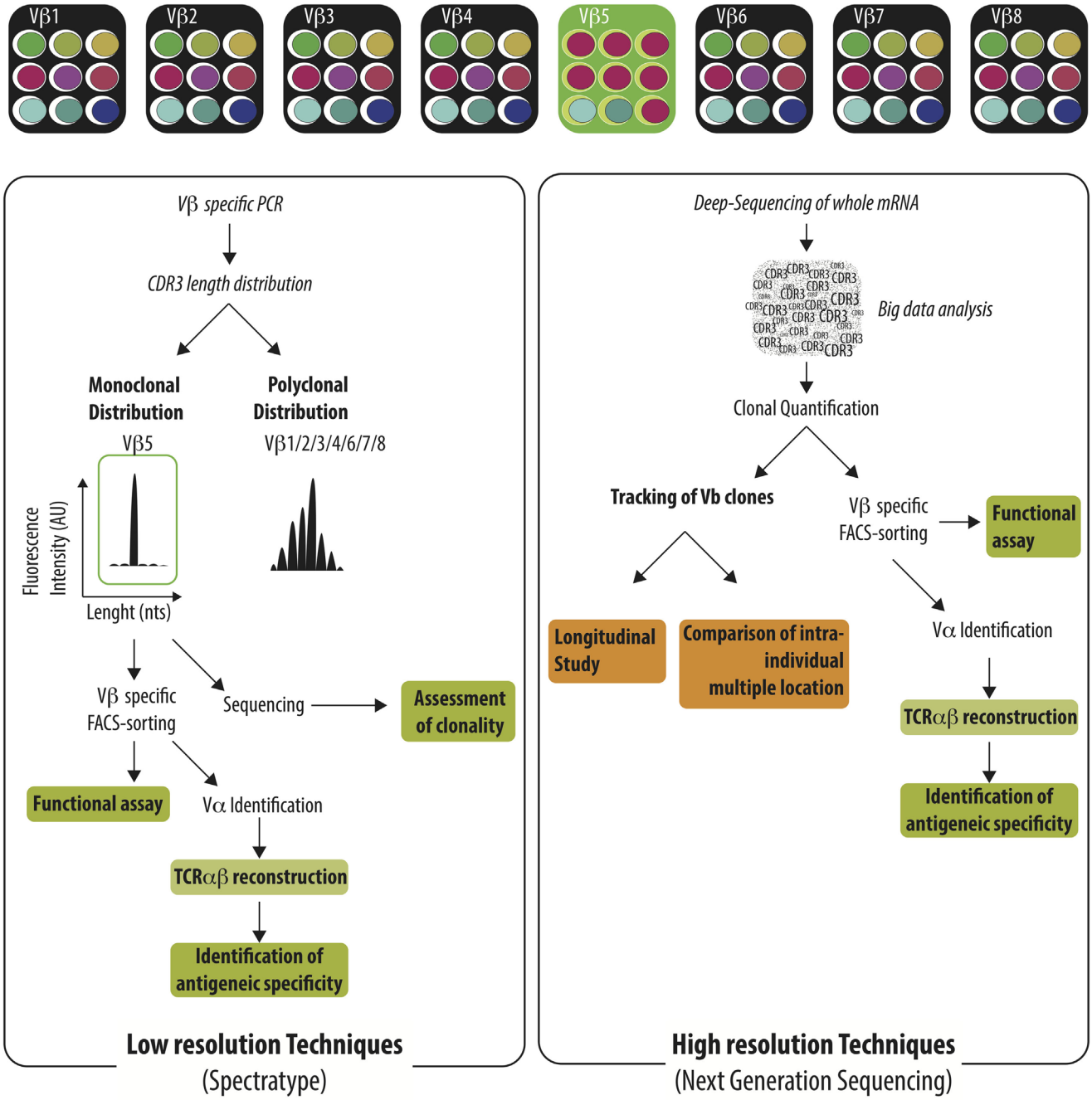


FIGURE 2 | Characterization of the TCR Vβ repertoire by low resolution and high resolution technique. Immune challenge leads to the selection of T cells harboring specific TCRαβ among the highly diverse TCRαβ repertoire. Antigen-specific T cells could be identified by low-resolution techniques (e.g., spectratype) or high-resolution techniques (e.g., NGS). Low-resolution techniques are aiming to identify Vβ families that exhibit monoclonal distribution of their CDR3 length distribution using Vβ specific PCR and spectratyping. The clonality of the identified Vβ families needs to be confirmed by the sequencing of the PCR product. Vβ-specific T cell purification enables later to perform functional assay or to reconstruct the TCRαβ in order to identify the recognized antigen. Deep-sequencing of TCR Vβ region identify the sequence of each TCR Vβ and intensive bio-informatic process is needed to quantify the abundance of the different T cell clones. Given the burden of data generated, the Next-Generation Sequencing is well-fitted to track T cell clones in time or across different anatomic sites.

sequences between biological compartments or individuals and take advantage of public repository databases to assess the specificity of a sequence and potentially to reconstruct the TCR in order to search for the recognized peptides. However, the amount of data generated using this technique is extremely high and efficient bio-informatics tools specifically devoted to the analysis are needed to identify meaningful information in the ocean of data. The accessibility of deep-sequencing is likely to be broaden in the near future thanks to the advances in bio-informatics tools and the reduction of the cost.

Low-resolution techniques have been used to investigate the usage of TCR Vβ repertoire in kidney transplant recipients with various clinical outcomes or at various time points post-transplantation (86–88). Using the combination of spectratyping and quantitative assessment of the TCR Vβ transcript, we have been able to define direct or indirect allorecognition patterns in an experiment model of allograft in congenic rats (52, 79, 80). Using the same approach, we reported that patients with biopsy-proven chronic antibody-mediated (CAMR) rejection exhibits strong alterations of their TCR Vβ repertoire correlating with the level of graft lesions classified with Banff classification (87). In contrast, operationally tolerant patients [i.e., patients off-immunosuppression for more than 12 months with a well-functioning graft (89–91)] exhibit a polyclonal TCR Vβ repertoire (87). A large cohort of patients with stable graft function for more than 5 years post-transplantation had been prospectively recruited in our center with stringent clinical and demographic inclusion criteria in order to obtain a homogeneous population. Nevertheless, we could highlight that the usage of TCR Vβ repertoire is highly heterogeneous ranging from the absence of clonal selection (similar to operational tolerance) to an accumulation of selected T cells (as for CAMR rejection) (87). The presence of altered TCR Vβ repertoire has been previously reported in a rat model of CAMR (92) in which similar CD8 clones could be identified in the blood and in the graft (93). In a large prospective study of kidney transplant recipients with a stable graft function for more than 5 years, we show that the altered TCR Vβ repertoire was due to an accumulation of TEMRA (T cell Effector Memory re-expressing CD45RA; $CD45RA^{+}CCR7^{-}$) CD8 T cells with an activated profile ($CD27^{-}CD28^{-}$), a high expression of cytotoxic molecules, perforin (PERF) and Granzym B (GZM-B), T-bet, and CD57 and the ability to secrete TNF-α and IFN-γ (88). Of interest, stable patients who have an increase in differentiated TEMRA CD8 T cells have a twofold higher risk of long-term graft dysfunction (88). Of note, using a similar strategy, Kim et al. recently reported that clonal CD8 T cell could be evidenced in human transplanted hand, with several TCR clonal selections persisting at least 100 days (among the 178 days of surveillance) (94). Collectively, these data highlight that a low-resolution technic provides key features as for the accumulation of selected T cell clones that can be used to monitor the kidney transplant recipients.

A major drawback of spectratype-based method is its intrinsic low resolution as multiple T cell clones could share the same CDR3 length. It is necessary to sequence the TCR Vβ chain with an altered CDR3 length distribution to assess the clonality of a given Vβ family. However, we recently compared spectratype or next generation sequencing (NGS) techniques to characterize the TCR Vβ repertoire in the blood, the cerebral spinal fluid (CSF), and the central nervous system (CNS) of patients with multiple sclerosis (76). Both methods were as efficient to highlight the similarity of TCR Vβ repertoire between CSF and CNS (≈80% of TCR Vβ clones identified in the CNS were also found in the CSF) and to identify ≈50% of the TCR Vβ clones using blood CD8 sample (76).

As previously discussed, the size of donor-reactive T cell repertoire is large and constitutes a limitation to the use of deep-sequencing approach. It may thus be a naive approach to perform NGS on unfractioned T cells with the aim to identify T cell clones specific to a given situation, such as kidney transplantation or viral infection; a two-step approach is needed. The first step is to purify the T cell population of interest based on the expression of phenotypic (using tetramer for instance) or functional (e.g., cytokine secretion, proliferation) markers. The in-depth characterization of TCR Vβ of T cell population of interest allows for the definition of a signature that can be later used as a tag when unpurified samples are analyzed. This approach has been used to track CMV- or BK-specific T cell clones (95) or alloreactive T cells (96, 97). The first report hypothesizing such an approach in the transplant context has been published by the group of Leventhal (96). Using healthy volunteers, this study aimed to assess breadth, clonal structure, and dynamics of the alloreactive T cell repertoire. After 7 days of MLR, the proliferating T cells were purified according to the dilution of cell division dye. By comparing the number of clones before culture and in the proliferated MLR responder, two types of alloreactive clones were identified, low- (i.e., unobserved in pre-culture sample and ≥10 T cells after MLR) and high-abundance pre-culture clones (i.e., present in pre-culture sample and ≥10× enriched after MLR). More than 11,000 low-abundant clones and more than 2,000 high-abundant clones were detected in the different experiments. These data provide new evidences of the large size of the alloreactive T cell pool.

This approach was used recently to track donor-reactive T cells in kidney transplant recipients (97). The fingerprint of donor-reactive T cell repertoire was established before transplantation by deep-sequencing of proliferating CD4 and CD8 T cells after 6 days of MLR. The fingerprint of donor-reactive T cells was monitored later after transplantation without the need to perform MLR. The team of Sykes provides evidences that tolerance induction protocol based on combined kidney and non-myeloablative bone marrow transplantation results in a reduction of donor-alloreactive T cell clones. However, this decrease was neither observed in the patient that failed to respond to the tolerant inducing protocol nor in patients with standard immunosuppressive regimens. Pre-transplant identification of donor-reactive T cell clones before transplantation could thus be a means to track the activation of the immune system by allogeneic graft. The studies of Emerson (96) and Morris (97) showed that the anti-donor fingerprint is stable over-time in healthy volunteers. Given the design of the assay, only pre-existing clones could be tracked. It would be of great value to compare the anti-donor clone repertoire before and after transplantation, starting each time from a direct MLR assay to investigate if new anti-donor T cells arise after transplantation. Indeed, infections that occurred frequently after transplantation could generate virus-specific T cells with an allogeneic cross-reactivity potential (71). Moreover, not all proliferating cells after 6 days of MLR are *per se* donor-specific as proliferation of T cells could also be linked to bystander stimulation (98).

CONCLUDING REMARKS

Will transplant immunologists be able to track the rise and the expansion of donor-specific T cells and would this approach be

widely available and useful to the clinical management are still open questions. High-through put techniques that have recently emerged are certainly an important step forward. Nevertheless, the high cross-reactivity of T cells is a major hurdle to identify the trigger of the expansion of donor-reactive T cells, as donor antigen, viral peptides, and other environmental antigens can lead to the selection of donor-specific T cells. While promising, the study of TCR alteration has not overcome the double difficulties of offering an accessible technical presentation of the data and a validated correlation with clinical outcomes. Therefore, longitudinal studies to test the reactivity of recipient T cells against donor antigens at different time points are needed.

AUTHOR CONTRIBUTIONS

ND, SB, and J-P S wrote the review.

ACKNOWLEDGMENTS

This work was realized in the context of the LabEX IGO program supported by the National Research Agency via the investment of the future program ANR-11-LABX-0016-01 and the LABEX TRANSPLANTEX [ANR-11-LABX-0070_TRANSPLANTEX], in the context of the IHU-Cesti project which received French government financial support managed by the National Research Agency via the "Investment Into The Future" program ANR-10-IBHU-005 and in the context of a CENTAURE foundation. The IHU-Cesti project is also supported by Nantes Metropole and the Pays de la Loire Region. This work was also supported by the FP7 VISICORT project that has received funding from the European Union's Seventh Framework Program for research, technological development and demonstration under grant agreement no 602470. We thank Dr. Yap and Dr. Haspot for their fruitful discussion.

REFERENCES

1. Tonegawa S. Somatic generation of antibody diversity. *Nature* (1983) **302**:575–81. doi:10.1038/302575a0
2. Rowen L, Koop BF, Hood L. The complete 685-kilobase DNA sequence of the human beta T cell receptor locus. *Science* (1996) **272**:1755–62. doi:10.1126/science.272.5269.1755
3. Boysen C, Simon MI, Hood L. Analysis of the 1.1-Mb human alpha/delta T-cell receptor locus with bacterial artificial chromosome clones. *Genome Res* (1997) **7**:330–8.
4. Zarnitsyna VI, Evavold BD, Schoettle LN, Blattman JN, Antia R. Estimating the diversity, completeness, and cross-reactivity of the T cell repertoire. *Front Immunol* (2013) **4**:485. doi:10.3389/fimmu.2013.00485
5. Quirós Roldàn E, Sottini A, Bettinardi A, Albertini A, Imberti L, Primi D. Different TCRBV genes generate biased patterns of V-D-J diversity in human T cells. *Immunogenetics* (1995) **41**:91–100.
6. Warren RL, Freeman JD, Zeng T, Choe G, Munro S, Moore R, et al. Exhaustive T-cell repertoire sequencing of human peripheral blood samples reveals signatures of antigen selection and a directly measured repertoire size of at least 1 million clonotypes. *Genome Res* (2011) **21**:790–7. doi:10.1101/gr.115428.110
7. Lafaille JJ, DeCloux A, Bonneville M, Takagaki Y, Tonegawa S. Junctional sequences of T cell receptor gamma delta genes: implications for gamma delta T cell lineages and for a novel intermediate of V-(D)-J joining. *Cell* (1989) **59**:859–70. doi:10.1016/0092-8674(89)90609-0
8. Cabaniols JP, Fazilleau N, Casrouge A, Kourilsky P, Kanellopoulos JM. Most alpha/beta T cell receptor diversity is due to terminal deoxynucleotidyl transferase. *J Exp Med* (2001) **194**:1385–90. doi:10.1084/jem.194.9.1385
9. Murugan A, Mora T, Walczak AM, Callan CG. Statistical inference of the generation probability of T-cell receptors from sequence repertoires. *Proc Natl Acad Sci U S A* (2012) **109**:16161–6. doi:10.1073/pnas.1212755109
10. Tallquist MD, Weaver AJ, Pease LR. Degenerate recognition of alloantigenic peptides on a positive-selecting class I molecule. *J Immunol* (1998) **160**:802–9.
11. Udaka K, Wiesmüller KH, Kienle S, Jung G, Walden P. Self-MHC-restricted peptides recognized by an alloreactive T lymphocyte clone. *J Immunol* (1996) **157**:670–8.
12. Garcia KC, Degano M, Stanfield RL, Brunmark A, Jackson MR, Peterson PA, et al. An alpha beta T cell receptor structure at 2.5 A and its orientation in the TCR-MHC complex. *Science* (1996) **274**:209–19. doi:10.1126/science.274.5285.209
13. Rossjohn J, Gras S, Miles JJ, Turner SJ, Godfrey DI, McCluskey J. T cell antigen receptor recognition of antigen-presenting molecules. *Annu Rev Immunol* (2015) **33**:169–200. doi:10.1146/annurev-immunol-032414-112334
14. Garcia KC. Reconciling views on T cell receptor germline bias for MHC. *Trends Immunol* (2012) **33**:429–36. doi:10.1016/j.it.2012.05.005
15. Kappler JW, Wade T, White J, Kushnir E, Blackman M, Bill J, et al. A T cell receptor V beta segment that imparts reactivity to a class II major histocompatibility complex product. *Cell* (1987) **49**:263–71. doi:10.1016/0092-8674(87)90567-8
16. Beringer DX, Kleijwegt FS, Wiede F, van der Slik AR, Loh KL, Petersen J, et al. T cell receptor reversed polarity recognition of a self-antigen major histocompatibility complex. *Nat Immunol* (2015) **16**:1153–61. doi:10.1038/ni.3271
17. Matzinger P, Bevan MJ. Hypothesis: why do so many lymphocytes respond to major histocompatibility antigens? *Cell Immunol* (1977) **29**:1–5. doi:10.1016/0008-8749(77)90269-6
18. Owens T, Liddell ME, Crispe IN. Derivation from an alloreactive T-cell line of a clone which cross-reacts with a self H2-E-restricted minor alloantigen. *Cell Immunol* (1984) **85**:436–46. doi:10.1016/0008-8749(84)90257-0
19. Birnbaum ME, Mendoza JL, Sethi DK, Dong S, Glanville J, Dobbins J, et al. Deconstructing the peptide-MHC specificity of T cell recognition. *Cell* (2014) **157**:1073–87. doi:10.1016/j.cell.2014.03.047
20. Jerne NK. The natural-selection theory of antibody formation. *Proc Natl Acad Sci U S A* (1955) **41**:849–57. doi:10.1073/pnas.41.11.849
21. Jerne NK. The somatic generation of immune recognition. *Eur J Immunol* (1971) **1**:1–9. doi:10.1002/eji.1830010102
22. Mason D. A very high level of crossreactivity is an essential feature of the T-cell receptor. *Immunol Today* (1998) **19**:395–404. doi:10.1016/S0167-5699(98)01299-7
23. Sewell AK. Why must T cells be cross-reactive? *Nat Rev Immunol* (2012) **12**:669–77. doi:10.1038/nri3279
24. Arstila TP. A direct estimate of the human T cell receptor diversity. *Science* (1999) **286**:958–61. doi:10.1126/science.286.5441.958
25. Robins HS, Campregher PV, Srivastava SK, Wacher A, Turtle CJ, Kahsai O, et al. Comprehensive assessment of T-cell receptor beta-chain diversity in alphabeta T cells. *Blood* (2009) **114**:4099–107. doi:10.1182/blood-2009-04-217604
26. Rudd BD, Venturi V, Li G, Samadder P, Ertelt JM, Way SS, et al. Nonrandom attrition of the naive CD8+ T-cell pool with aging governed by T-cell receptor:pMHC interactions. *Proc Natl Acad Sci USA* (2011) **108**:13694–9. doi:10.1073/pnas.1107594108
27. Akue AD, Lee J-Y, Jameson SC. Derivation and maintenance of virtual memory CD8 T cells. *J Immunol* (2012) **188**:2516–23. doi:10.4049/jimmunol.1102213
28. Haluszczak C, Akue AD, Hamilton SE, Johnson LDS, Pujanauski L, Teodorovic L, et al. The antigen-specific CD8+ T cell repertoire in unimmunized mice includes memory phenotype cells bearing markers of homeostatic expansion. *J Exp Med* (2009) **206**:435–48. doi:10.1084/jem.20081829
29. Rudd BD, Venturi V, Smithey MJ, Way SS, Davenport MP, Nikolich-Žugich J. Diversity of the CD8+ T cell repertoire elicited against an immunodominant epitope does not depend on the context of infection. *J Immunol* (2010) **184**:2958–65. doi:10.4049/jimmunol.0903493
30. Su LF, Kidd BA, Han A, Kotzin JJ, Davis MM. Virus-specific CD4+ memory-phenotype T cells are abundant in unexposed adults. *Immunity* (2013) **38**:373–83. doi:10.1016/j.immuni.2012.10.021
31. Sprent CDSJ. Homeostasis of naive and memory T cells. *Immunity* (2008) **29**:848–62. doi:10.1016/j.immuni.2008.11.002

32. Yu W, Jiang N, Ebert PJR, Kidd BA, Müller S, Lund PJ, et al. Clonal deletion prunes but does not eliminate self-specific αβ CD8(+) T lymphocytes. *Immunity* (2015) **42**:929–41. doi:10.1016/j.immuni.2015.05.001
33. Brodin P, Jojic V, Gao T, Bhattacharya S, Angel CJL, Furman D, et al. Variation in the human immune system is largely driven by non-heritable influences. *Cell* (2015) **160**:37–47. doi:10.1016/j.cell.2014.12.020
34. Stewart AJ, Devlin PM. The history of the smallpox vaccine. *J Infect* (2006) **52**:329–34. doi:10.1016/j.jinf.2005.07.021
35. Welsh RM, Che JW, Brehm MA, Selin LK. Heterologous immunity between viruses. *Immunol Rev* (2010) **235**:244–66. doi:10.1111/j.0105-2896.2010.00897.x
36. Selin LK, Varga SM, Wong IC, Welsh RM. Protective heterologous antiviral immunity and enhanced immunopathogenesis mediated by memory T cell populations. *J Exp Med* (1998) **188**:1705–15. doi:10.1084/jem.188.9.1705
37. Mathurin KS, Martens GW, Kornfeld H, Welsh RM. CD4 T-cell-mediated heterologous immunity between mycobacteria and poxviruses. *J Virol* (2009) **83**:3528–39. doi:10.1128/JVI.02393-08
38. Chen HD, Fraire AE, Joris I, Welsh RM, Selin LK. Specific history of heterologous virus infections determines anti-viral immunity and immunopathology in the lung. *Am J Pathol* (2003) **163**:1341–55. doi:10.1016/S0002-9440(10)63493-1
39. Nilges K, Höhn H, Pilch H, Neukirch C, Freitag K, Talbot PJ, et al. Human papillomavirus type 16 E7 peptide-directed CD8+ T cells from patients with cervical cancer are cross-reactive with the coronavirus NS2 protein. *J Virol* (2003) **77**:5464–74. doi:10.1128/JVI.77.9.5464-5474.2003
40. Clute SC, Naumov YN, Watkin LB, Aslan N, Sullivan JL, Thorley-Lawson DA, et al. Broad cross-reactive TCR repertoires recognizing dissimilar Epstein-Barr and influenza A virus epitopes. *J Immunol* (2010) **185**:6753–64. doi:10.4049/jimmunol.1000812
41. Billington WD. Influence of immunological dissimilarity of mother and foetus on size of placenta in mice. *Nature* (1964) **202**:317–8. doi:10.1038/202317a0
42. Ober C, Hyslop T, Elias S, Weitkamp LR, Hauck WW. Human leukocyte antigen matching and fetal loss: results of a 10 year prospective study. *Hum Reprod* (1998) **13**:33–8. doi:10.1093/humrep/13.1.33
43. Kappler JW, Roehm N, Marrack P. T cell tolerance by clonal elimination in the thymus. *Cell* (1987) **49**:273–80. doi:10.1016/0092-8674(87)90568-X
44. Luz JG, Huang M, Garcia KC, Rudolph MG, Apostolopoulos V, Teyton L, et al. Structural comparison of allogeneic and syngeneic T cell receptor-peptide-major histocompatibility complex complexes: a buried alloreactive mutation subtly alters peptide presentation substantially increasing V(beta) Interactions. *J Exp Med* (2002) **195**:1175–86. doi:10.1084/jem.20011644
45. Reiser JB, Darnault C, Guimezanes A, Grégoire C, Mosser T, Schmitt-Verhulst AM, et al. Crystal structure of a T cell receptor bound to an allogeneic MHC molecule. *Nat Immunol* (2000) **1**:291–7. doi:10.1038/79728
46. Doxiadis II, Smits JM, Schreuder GM, Persijn GG, van Houwelingen HC, van Rood JJ, et al. Association between specific HLA combinations and probability of kidney allograft loss: the taboo concept. *Lancet* (1996) **348**:850–3. doi:10.1016/S0140-6736(96)02296-9
47. Fleischhauer K, Kernan NA, O'Reilly RJ, Dupont B, Yang SY. Bone marrow-allograft rejection by T lymphocytes recognizing a single amino acid difference in HLA-B44. *N Engl J Med* (1990) **323**:1818–22. doi:10.1056/NEJM199012273232607
48. Keever CA, Leong N, Cunningham I, Copelan EA, Avalos BR, Klein J, et al. HLA-B44-directed cytotoxic T cells associated with acute graft-versus-host disease following unrelated bone marrow transplantation. *Bone Marrow Transplant* (1994) **14**:137–45.
49. Fleischhauer K, Avila D, Vilbois F, Traversari C, Bordignon C, Wallny HJ. Characterization of natural peptide ligands for HLA-B*4402 and -B*4403: implications for peptide involvement in allorecognition of a single amino acid change in the HLA-B44 heavy chain. *Tissue Antigens* (1994) **44**:311–7. doi:10.1111/j.1399-0039.1994.tb02401.x
50. Macdonald WA, Purcell AW, Mifsud NA, Ely LK, Williams DS, Chang L, et al. A naturally selected dimorphism within the HLA-B44 supertype alters class I structure, peptide repertoire, and T cell recognition. *J Exp Med* (2003) **198**:679–91. doi:10.1084/jem.20030066
51. Covassin L, Laning J, Abdi R, Langevin DL, Phillips NE, Shultz LD, et al. Human peripheral blood CD4 T cell-engrafted non-obese diabetic-scid IL2rγ(null) H2-Ab1 (tm1Gru) Tg (human leucocyte antigen D-related 4) mice: a mouse model of human allogeneic graft-versus-host disease. *Clin Exp Immunol* (2011) **166**:269–80. doi:10.1111/j.1365-2249.2011.04462.x
52. Guillet M, Gagne K, Lair D, Heslan J-M, Dore J-C, Soulillou J-P, et al. Different patterns of TCR beta chain regulation following allo- and xeno-transplantation. *Xenotransplantation* (2004) **11**:315–22. doi:10.1111/j.1399-3089.2004.00136.x
53. Schilham MW, Lang R, Benner R, Zinkernagel RM, Hengartner H. Characterization of an Lyt-2+ alloreactive cytotoxic T cell clone specific for H-2Db that cross-reacts with I-Ek. *J Immunol* (1986) **137**:2748–54.
54. Logunova NN, Viret C, Pobezinsky LA, Miller SA, Kazansky DB, Sundberg JP, et al. Restricted MHC-peptide repertoire predisposes to autoimmunity. *J Exp Med* (2005) **202**:73–84. doi:10.1084/jem.20050198
55. Huseby ES, White J, Crawford F, Vass T, Becker D, Pinilla C, et al. How the T cell repertoire becomes peptide and MHC specific. *Cell* (2005) **122**:247–60. doi:10.1016/j.cell.2005.05.013
56. Yin L, Huseby E, Scott-Browne J, Rubtsova K, Pinilla C, Crawford F, et al. A single T cell receptor bound to major histocompatibility complex class I and class II glycoproteins reveals switchable TCR conformers. *Immunity* (2011) **35**:23–33. doi:10.1016/j.immuni.2011.04.017
57. Albertini RJ, Bach FH. Quantitative assay of antigenic disparity at hl-a-the major histocompatibility locus in man. *J Exp Med* (1968) **128**:639–51. doi:10.1084/jem.128.4.639
58. Suchin EJ, Langmuir PB, Palmer E, Sayegh MH, Wells AD, Turka LA. Quantifying the frequency of alloreactive T cells in vivo: new answers to an old question. *J Immunol* (2001) **166**:973–81. doi:10.4049/jimmunol.166.2.973
59. Chicz RM, Urban RG, Lane WS, Gorga JC, Stern LJ, Vignali DA, et al. Predominant naturally processed peptides bound to HLA-DR1 are derived from MHC-related molecules and are heterogeneous in size. *Nature* (1992) **358**:764–8. doi:10.1038/358764a0
60. Chicz RM, Urban RG, Gorga JC, Vignali DA, Lane WS, Strominger JL. Specificity and promiscuity among naturally processed peptides bound to HLA-DR alleles. *J Exp Med* (1993) **178**:27–47. doi:10.1084/jem.178.1.27
61. Rudensky AY, Preston-Hurlburt P, Hong SC, Barlow A, Janeway CA. Sequence analysis of peptides bound to MHC class II molecules. *Nature* (1991) **353**:622–7. doi:10.1038/353622a0
62. Sylwester AW, Mitchell BL, Edgar JB, Taormina C, Pelte C, Ruchti F, et al. Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. *J Exp Med* (2005) **202**:673–85. doi:10.1084/jem.20050882
63. Smith C, Miles JJ, Khanna R. Advances in direct T-cell alloreactivity: function, avidity, biophysics and structure. *Am J Transplant* (2012) **12**:15–26. doi:10.1111/j.1600-6143.2011.03863.x
64. Burrows SR, Silins SL, Moss DJ, Khanna R, Misko IS, Argaet VP. T cell receptor repertoire for a viral epitope in humans is diversified by tolerance to a background major histocompatibility complex antigen. *J Exp Med* (1995) **182**:1703–15. doi:10.1084/jem.182.6.1703
65. Morice A, Charreau B, Neveu B, Brouard S, Soulillou J-P, Bonneville M, et al. Cross-reactivity of herpesvirus-specific CD8 T cell lines toward allogeneic class I MHC molecules. *PLoS One* (2010) **5**:e12120. doi:10.1371/journal.pone.0012120
66. Burrows SR, Khanna R, Burrows JM, Moss DJ. An alloresponse in humans is dominated by cytotoxic T lymphocytes (CTL) cross-reactive with a single Epstein-Barr virus CTL epitope: implications for graft-versus-host disease. *J Exp Med* (1994) **179**:1155–61. doi:10.1084/jem.179.4.1155
67. Burrows SR, Silins SL, Khanna R, Burrows JM, Rischmueller M, McCluskey J, et al. Cross-reactive memory T cells for Epstein-Barr virus augment the alloresponse to common human leukocyte antigens: degenerate recognition of major histocompatibility complex-bound peptide by T cells and its role in alloreactivity. *Eur J Immunol* (1997) **27**:1726–36. doi:10.1002/eji.1830270720
68. Rist M, Smith C, Bell MJ, Burrows SR, Khanna R. Cross-recognition of HLA DR4 alloantigen by virus-specific CD8+ T cells: a new paradigm for self-/nonself-recognition. *Blood* (2009) **114**:2244–53. doi:10.1182/blood-2009-05-222596
69. Landais E, Morice A, Long HM, Haigh TA, Charreau B, Bonneville M, et al. EBV-specific CD4+ T cell clones exhibit vigorous allogeneic responses. *J Immunol* (2006) **177**:1427–33. doi:10.4049/jimmunol.177.3.1427
70. Elkington R, Khanna R. Cross-recognition of human alloantigen by cytomegalovirus glycoprotein-specific CD4+ cytotoxic T lymphocytes: implications for graft-versus-host disease. *Blood* (2005) **105**:1362–4. doi:10.1182/blood-2004-07-2602

71. Amir AL, D'Orsogna LJA, Roelen DL, van Loenen MM, Hagedoorn RS, de Boer R, et al. Allo-HLA reactivity of virus-specific memory T cells is common. *Blood* (2010) **115**:3146–57. doi:10.1182/blood-2009-07-234906
72. Brehm MA, Daniels KA, Priyadharshini B, Thornley TB, Greiner DL, Rossini AA, et al. Allografts stimulate cross-reactive virus-specific memory CD8 T cells with private specificity. *Am J Transplant* (2010) **10**:1738–48. doi:10.1111/j.1600-6143.2010.03161.x
73. Gorski J, Yassai M, Zhu X, Kissela B, Kissella B corrected to Kissela B, Keever C, et al. Circulating T cell repertoire complexity in normal individuals and bone marrow recipients analyzed by CDR3 size spectratyping. Correlation with immune status. *J Immunol* (1994) **152**:5109–19.
74. Cochet M, Pannetier C, Regnault A, Darche S, Leclerc C, Kourilsky P. Molecular detection and in vivo analysis of the specific T cell response to a protein antigen. *Eur J Immunol* (1992) **22**:2639–47. doi:10.1002/eji.1830221025
75. Miqueu P, Guillet M, Degauque N, Dore J-C, Soulillou J-P, Brouard S. Statistical analysis of CDR3 length distributions for the assessment of T and B cell repertoire biases. *Mol Immunol* (2007) **44**:1057–64. doi:10.1016/j.molimm.2006.06.026
76. Salou M, Garcia A, Michel L, Gainche-Salmon A, Loussouarn D, Nicol B, et al. Expanded CD8 T-cell sharing between periphery and CNS in multiple sclerosis. *Ann Clin Transl Neurol* (2015) **2**:609–22. doi:10.1002/acn3.199
77. Degauque N, Boeffard F, Foucher Y, Ballet C, Brouard S, Soulillou J-P. The blood of healthy individuals exhibits CD8 T cells with a highly altered TCR Vb repertoire but with an unmodified phenotype. *PLoS One* (2011) **6**:e21240. doi:10.1371/journal.pone.0021240
78. Brouard S, Vanhove B, Gagne K, Neumann A, Douillard P, Moreau A, et al. T cell repertoire alterations of vascularized xenografts. *J Immunol* (1999) **162**:3367–77.
79. Sebille F, Gagne K, Guillet M, Degauque N, Pallier A, Brouard S, et al. Direct recognition of foreign MHC determinants by naive T cells mobilizes specific Vbeta families without skewing of the complementarity-determining region 3 length distribution. *J Immunol* (2001) **167**:3082–8. doi:10.4049/jimmunol.167.6.3082
80. Gagne K, Brouard S, Giral M, Sebille F, Moreau A, Guillet M, et al. Highly altered V beta repertoire of T cells infiltrating long-term rejected kidney allografts. *J Immunol* (2000) **164**:1553–63. doi:10.4049/jimmunol.164.3.1553
81. Thaunat O, Graff-Dubois S, Brouard S, Gautreau C, Varthaman A, Fabien N, et al. Immune responses elicited in tertiary lymphoid tissues display distinctive features. *PLoS One* (2010) **5**:e11398. doi:10.1371/journal.pone.0011398
82. Pilch H, Höhn H, Freitag K, Neukirch C, Necker A, Haddad P, et al. Improved assessment of T-cell receptor (TCR) VB repertoire in clinical specimens: combination of TCR-CDR3 spectratyping with flow cytometry-based TCR VB frequency analysis. *Clin Diagn Lab Immunol* (2002) **9**:257–66. doi:10.1128/CDLI.9.2.257-266.2002
83. Degauque N, Schadendorf D, Brouard S, Guillet M, Sébille F, Höhn H, et al. Blood T-cell Vbeta transcriptome in melanoma patients. *Int J Cancer* (2004) **110**:721–9. doi:10.1002/ijc.20149
84. Laplaud DA, Berthelot L, Miqueu P, Bourcier K, Moynard J, Oudinet Y, et al. Serial blood T cell repertoire alterations in multiple sclerosis patients; correlation with clinical and MRI parameters. *J Neuroimmunol* (2006) **177**:151–60. doi:10.1016/j.jneuroim.2006.05.006
85. Guillet M, Andrieu M, Braudeau C, Ruiz C, Daniel N, Pallier A, et al. Serial evolution of TCR beta chain transcript mobilization in HIV type-1-infected patients following vaccine immune stimulation and HAART interruption. *AIDS Res Hum Retroviruses* (2006) **22**:648–56. doi:10.1089/aid.2006.22.648
86. Brouard S, Dupont A, Giral M, Louis S, Lair D, Braudeau C, et al. Operationally tolerant and minimally immunosuppressed kidney recipients display strongly altered blood T-cell clonal regulation. *Am J Transplant* (2005) **5**:330–40. doi:10.1111/j.1600-6143.2004.00700.x
87. Miqueu P, Degauque N, Guillet M, Giral M, Ruiz C, Pallier A, et al. Analysis of the peripheral T-cell repertoire in kidney transplant patients. *Eur J Immunol* (2010) **40**:3280–90. doi:10.1002/eji.201040301
88. Yap M, Boeffard F, Clave E, Pallier A, Danger R, Giral M, et al. Expansion of highly differentiated cytotoxic terminally differentiated effector memory CD8+ T cells in a subset of clinically stable kidney transplant recipients: a potential marker for late graft dysfunction. *J Am Soc Nephrol* (2014) **25**:1856–68. doi:10.1681/ASN.2013080848
89. Brouard S, Mansfield E, Braud C, Li L, Giral M, Hsieh S-C, et al. Identification of a peripheral blood transcriptional biomarker panel associated with operational renal allograft tolerance. *Proc Natl Acad Sci U S A* (2007) **104**:15448–53. doi:10.1073/pnas.0705834104
90. Brouard S, Pallier A, Renaudin K, Foucher Y, Danger R, Devys A, et al. The natural history of clinical operational tolerance after kidney transplantation through twenty-seven cases. *Am J Transplant* (2012) **12**:3296–307. doi:10.1111/j.1600-6143.2012.04249.x
91. Roussey-Kesler G, Giral M, Moreau A, Subra JF, Legendre C, Noël C, et al. Clinical operational tolerance after kidney transplantation. *Am J Transplant* (2006) **6**:736–46. doi:10.1111/j.1600-6143.2006.01280.x
92. Ballet C, Renaudin K, Degauque N, Mai HL, Boëffard F, Lair D, et al. Indirect CD4+ TH1 response, antidonor antibodies and diffuse C4d graft deposits in long-term recipients conditioned by donor antigens priming. *Am J Transplant* (2009) **9**:697–708. doi:10.1111/j.1600-6143.2009.02556.x
93. Lair D, Degauque N, Miqueu P, Jovanovic V, Guillet M, Mérieau E, et al. Functional compartmentalization following induction of long-term graft survival with pregraft donor-specific transfusion. *Am J Transplant* (2007) **7**:538–49. doi:10.1111/j.1600-6143.2006.01660.x
94. Kim JY, Balamurugan A, Azari K, Hofmann C, Ng HL, Reed EF, et al. Clonal CD8+ T cell persistence and variable gene usage bias in a human transplanted hand. *PLoS One* (2015) **10**:e0136235. doi:10.1371/journal.pone.0136235
95. Dziubianau M, Hecht J, Kuchenbecker L, Sattler A, Stervbo U, Rödelsperger C, et al. TCR repertoire analysis by next generation sequencing allows complex differential diagnosis of T cell-related pathology. *Am J Transplant* (2013) **13**:2842–54. doi:10.1111/ajt.12431
96. Emerson RO, Mathew JM, Konieczna IM, Robins HS, Leventhal JR. Defining the alloreactive T cell repertoire using high-throughput sequencing of mixed lymphocyte reaction culture. *PLoS One* (2014) **9**:e111943. doi:10.1371/journal.pone.0111943
97. Morris H, DeWolf S, Robins H, Sprangers B, LoCascio SA, Shonts BA, et al. Tracking donor-reactive T cells: evidence for clonal deletion in tolerant kidney transplant patients. *Sci Transl Med* (2015) **7**:ra10–272. doi:10.1126/scitranslmed.3010760
98. Matesic D, Lehmann PV, Heeger PS. High-resolution characterization of cytokine-producing alloreactivity in naive and allograft-primed mice. *Transplantation* (1998) **65**:906–14. doi:10.1097/00007890-199804150-00008

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 15 March 2016
doi: 10.3389/fimmu.2016.00100

Autosomal Minor Histocompatibility Antigens: How Genetic Variants Create Diversity in Immune Targets

Marieke Griffioen, Cornelis A. M. van Bergen and J. H. Frederik Falkenburg*

Department of Hematology, Leiden University Medical Center, Leiden, Netherlands

Allogeneic stem cell transplantation (alloSCT) can be a curative treatment for hematological malignancies. Unfortunately, the desired anti-tumor or graft-versus-leukemia (GvL) effect is often accompanied with undesired side effects against healthy tissues known as graft-versus-host disease (GvHD). After HLA-matched alloSCT, GvL and GvHD are both mediated by donor-derived T-cells recognizing polymorphic peptides presented by HLA surface molecules on patient cells. These polymorphic peptides or minor histocompatibility antigens (MiHA) are produced by genetic differences between patient and donor. Since polymorphic peptides may be useful targets to manipulate the balance between GvL and GvHD, the dominant repertoire of MiHA needs to be discovered. In this review, the diversity of autosomal MiHA characterized thus far as well as the various molecular mechanisms by which genetic variants create immune targets and the role of cryptic transcripts and proteins as antigen sources are described. The tissue distribution of MiHA as important factor in GvL and GvHD is considered as well as possibilities how hematopoietic MiHA can be used for immunotherapy to augment GvL after alloSCT. Although more MiHA are still needed for comprehensive understanding of the biology of GvL and GvHD and manipulation by immunotherapy, this review shows insight into the composition and kinetics of *in vivo* immune responses with respect to specificity, diversity, and frequency of specific T-cells and surface expression of HLA–peptide complexes and other (accessory) molecules on the target cell. A complex interplay between these factors and their environment ultimately determines the spectrum of clinical manifestations caused by immune responses after alloSCT.

Keywords: allogeneic stem cell transplantation, hematological malignancy, graft-versus-leukemia reactivity, graft-versus-host disease, donor lymphocyte infusion, T-lymphocytes, minor histocompatibility antigens, immunotherapy

OPEN ACCESS

Edited by:
Gilles Benichou, Massachusetts General Hospital and Harvard Medical School, USA

Reviewed by:
Ralf Dressel, University Medical Center Göttingen, Germany
Emmanuel Zorn, Columbia University Medical Center, USA

****Correspondence:***
Marieke Griffioen
m.griffioen@lumc.nl

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *02 October 2015*
Accepted: *01 March 2016*
Published: *15 March 2016*

Citation:
Griffioen M, van Bergen CAM and Falkenburg JHF (2016) Autosomal Minor Histocompatibility Antigens: How Genetic Variants Create Diversity in Immune Targets. Front. Immunol. 7:100. doi: 10.3389/fimmu.2016.00100

Abbreviations: alloSCT, allogeneic stem cell transplantation; DLI, donor lymphocyte infusion; DRiP, defective ribosomal product; GvHD, graft-versus-host disease; GvL, graft-versus-leukemia reactivity; MiHA, minor histocompatibility antigen; SNP, single nucleotide polymorphism; WGAs, whole genome association scanning.

ALLOGENEIC STEM CELL TRANSPLANTATION

Allogeneic stem cell transplantation (alloSCT) can be a curative treatment for hematological malignancies. In alloSCT, the immune system from a healthy donor is transplanted into the patient to induce an effective response against the leukemic cells (1, 2). Unfortunately, desired anti-tumor or graft-versus-leukemia (GvL) reactivity is often accompanied with graft-versus-host disease (GvHD) affecting predominantly skin, gut, liver, and lungs (3–6). To reduce GvHD, donor T-cells can be (partially) depleted from the stem cell graft (7–9). T-cell depletion decreases the incidence and severity of GvHD, but increases the risk of leukemia relapse and opportunistic infections. Once toxicity of the conditioning has subsided, donor T-cells can be administered after alloSCT as donor lymphocyte infusions (DLI) to reinstall beneficial GvL (10, 11). Although DLI can induce long-lasting clinical remissions, GvHD remains a major cause of morbidity and mortality. Since DLI is applied to patients who do not receive (or only limited) immunosuppression, postponed DLI after alloSCT creates an ideal platform to study specificity, magnitude, and duration of GvL and GvHD.

MINOR HISTOCOMPATIBILITY ANTIGENS

To minimize GvHD, patients with hematological malignancies are preferably transplanted with HLA-matched donors (12, 13). After HLA-matched alloSCT, donor-derived T-cells can mediate GvL and GvHD by recognizing polymorphic peptides presented on patient cells by shared HLA molecules. In the classical dogma, intracellular proteins are degraded in the cytosol by the proteasome and peptides are presented by HLA class I to CD8 T-cells (14, 15). HLA class II molecules present peptides derived from intra- and extracellular proteins to CD4 T-cells (14, 16). In the autologous situation, peptides from normal cellular proteins cannot be recognized by the immune system due to negative selection and deletion of self-specific T-cells in the thymus (17). After HLA-matched alloSCT, however, donor T-cells recognize polymorphic peptides presented on patient cells by shared HLA as "non-self." These polymorphic peptides or so-called minor histocompatibility antigens (MiHA) can be encoded by the male-specific Y-chromosome (H-Y antigens) or other chromosomes (autosomal MiHA) and are produced by genetic differences between patient and donor (18–21). This review is focused on discovery strategies and molecular mechanisms behind autosomal MiHA.

T-CELL ISOLATION

Minor histocompatibility antigen-specific T-cells can be directly isolated from *in vivo* immune responses after alloSCT or generated *in vitro* by stimulating antigen-experienced or naive donor T-cells (22, 23). T-cells have been isolated from *in vivo* immune responses by their capacity to produce IFN-γ upon *in vitro* stimulation with patient hematopoietic cells (24, 25). A disadvantage is that not all specific T-cells produce IFN-γ during 5–24 h of incubation, resulting in low T-cell isolation efficiencies. HLA-DR is a marker that has successfully been used to isolate *in vivo* activated T-cells (26, 27). Since HLA-DR is expressed on activated T-cells for a prolonged time, its expression does not require *in vitro* stimulation and enables analysis of the *in vivo* immune response without introducing a bias. CD137 is another marker that allows direct isolation of antigen-experienced T-cells (28, 29). This marker is specific for T-cells that are recently activated and requires *in vitro* stimulation for re-expression on the cell surface.

DISCOVERY STRATEGIES

To develop strategies that allow manipulation of GvL and GvHD, the dominant repertoire of autosomal MiHA needs to be discovered. HA-1 and HA-2 are the first MiHA that have been identified as T-cell targets in a patient with GvHD (**Table 1**). The antigens have been characterized as peptides eluted from HLA surface molecules that are recognized by specific T-cells by mass spectrometry. Other MiHA characterized by this approach are HA-8, HA-3, PANE1, and LB-ADIR-1F (**Table 1**). cDNA library screening in which pools of plasmids are tested for T-cell recognition is another technique that has been used for discovery of HB-1H, UGT2B17/A29, UGT2B17/B44, ACC-4, ACC-5, ACC-6, SP110, LB-ECGF-1H, C19ORF48, TRIM22, and LB-TRIP10-1EPC (**Table 1**). In addition, five HLA class II-restricted MiHA encoded by *PI4K2B*, *PTK2B*, *LY75*, *MR1*, and *MTHFD1* have been characterized by screening a library in which recombinant bacteria are screened for T-cell recognition (**Table 1**).

Due to advanced array techniques to measure single nucleotide polymorphisms (SNPs), whole genome association scanning (WGAs) became available as efficient method for MiHA discovery (39, 43). In this approach, a panel of test cells with known SNP genotypes is used to measure T-cell recognition. T-cell recognition is subsequently investigated for association with individual SNPs to identify the genomic region that encodes the MiHA. Before SNP arrays became commercially available, WGAs was performed with low-resolution genetic markers, leading to identification of large genomic regions of which all genes needed to be investigated for encoding the antigen. MiHA characterized by WGAs with low-resolution markers are ACC-1Y, ACC-2, LRH-1, and HEATR1 (**Table 1**). When high-resolution SNP data are used, WGAs enables direct identification of the MiHA-producing SNP or identification of small genomic regions with SNP(s) that are in linkage disequilibrium with the MiHA-producing SNP. MiHA identified with high-resolution SNP data are ACC-1C, SLC1A5, UGT2B17/A2, DPH1, P2RX7, LB-PRCP-1D, SSR1-1S, LB-WNK1-1I, LB-EBI3-1I, LB-BCAT2-1R, LB-ARHGDIB-1R, LB-PDCD11-1F, LB-APOBEC3B-1K, LB-GEMIN4-1V, LB-ERAP1-1R, ZAPHIR, LB-SON-1R, LB-NUP133-1R, LB-SWAP70-1Q, UTA2-1, and LB-FUCA2-1V (**Table 1**). WGAs with high-resolution SNPs also led to discovery of HLA class II-restricted MiHA encoded by *CD19*, *SLC19A1*, and *ZDHHC12* (**Table 1**). Nowadays, data for all SNPs as present in the human genome are available in the 1000 Genomes Project and the value of this dataset has recently been illustrated by discovery of UTDP4-1 (**Table 1**).

TABLE 1 | HLA class I- and II-restricted autosomal minor histocompatibility antigens.

HLA-I MiHA	MiHA/AV[a]	Gene	Variant	rs number	Location	Transcript[b]	Protein[b]	HLA	Reference
HA-3	V[**T**/M]EPGTAQY	*AKAP13*	SNP	rs2061821	Exon	Normal	Normal	A*01:01	(30)
HA-2	YIGEVLVS[**V**/M]	*MYO1G*	SNP	rs61739531	Exon	Normal	Normal	A*02:01	(31)
HA-1/A2	VL[**H**/R]DDLLEA	*HMHA1*	SNP	rs1801284	Exon	Normal	Normal	A*02:01	(32)
HA-8	**[R**/P]TLDKVLEV	*KIAA0020*	SNP	rs2173904	Exon	Normal	Normal	A*02:01	(33)
LB-ADIR-1F	SVAPALAL[**F**/S]PA	*TOR3A*	SNP	rs2296377	Exon	Normal	Alternative	A*02:01	(34)
C19ORF48	CIPPD[**S**/T]LLFPA	*C19ORF48*	SNP	rs3745526	Exon	Normal	Alternative	A*02:01	(35)
TRIM22	MAVPPC[**C**/R]IGV	*TRIM22*	SNP	rs187416296	Exon	Normal	Normal	A*02:01	(22)
LB-PRCP-1D	FMWDVAE[**D**/E]LKA	*PRCP*	SNP	rs2298668	Exon	Normal	Normal	A*02:01	(27)
LB-SSR1-1S	[**S**/L]LAVAQDLT	*SSR1*	SNP	rs10004	Exon	Normal	Normal	A*02:01	(27)
LB-WNK1-1I	RTLSPE[**I**/M]ITV	*WNK1*	SNP	rs12828016	Exon	Normal	Normal	A*02:01	(27)
T4A	GLYTYWSAG[**A**/E]	*TRIM42*	SNP	rs9876490	Exon	Normal	Normal	A*02:01	(36)
UTA2-1	QL[**L**/P]NSVLTL	*KIAA1551*	SNP	rs2166807	Exon	Normal	Normal	A*02:01	(37)
LB-HIVEP1-1S	SLPKH[**S**/N]VTI	*HIVEP1*	SNP	rs2228220	Exon	Normal	Normal	A*02:01	(38)
LB-NISCH-1A	ALAPAP[**A**/V]EV	*NISCH*	SNP	rs887515	Exon	Normal	Normal	A*02:01	(38)
UGT2B17/A2	CVATMIFMI	*UGT2B1*	Gene deletion			Polymorphic	Polymorphic	A*02:06	(39)
PANE1	RVWDLPGVLK	*CENPM*	SNP	rs5758511	Exon	Alternative	Polymorphic	A*03:01	(40)
SP110	SLP[**R**/G]GTSTPK	*SP110*	SNP	rs1365776	Exon	Normal	Normal	A*03:01	(41)
ACC-1Y	DYLQ[**Y**/C]VLQI	*BCL2A1*	SNP	rs1138357	Exon	Normal	Normal	A*24:02	(42)
ACC-1C	DYLQ[Y/**C**]VLQI	*BCL2A1*	SNP	rs1138357	Exon	Normal	Normal	A*24:02	(43)
UGT2B17/A29	AELLNIPFLY	*UGT2B17*	Gene deletion			Polymorphic	Polymorphic	A*29:02	(44)
P2RX7	WFHHC[**H**/R]PKY	*P2RX7*	SNP	rs7958311	Exon	Normal	Normal	A*29:02	(45)
ACC-4	ATLPLLCA[**R**/G]	*CTSH*	SNP	rs2289702	Exon	Normal	Normal	A*31:01	(46)
ACC-5	WATLPLLCA[**R**/G]	*CTSH*	SNP	rs2289702	Exon	Normal	Normal	A*33:03	(46)
LRH-1	TPNQRQNVC	*P2X5*	INDEL	rs3215407	Exon	Normal	Polymorphic	B*07:02	(47)
LB-ECGF-1H	RP[**H**/R]AIRRPLAL	*TYMP*	SNP	rs112723255	Exon	Normal	Alternative	B*07:02	(48)
LB-APOBEC3B-1K	[**K**/E]PQYHAEMCF	*APOBEC3B*	SNP	rs2076109	Exon	Normal	Normal	B*07:02	(27)
LB-ARHGDIB-1R	LPRACW[**R**/P]EA	*ARHGDIB*	SNP	rs4703	Exon	Normal	Alternative	B*07:02	(27)
LB-BCAT2-1R	QP[**R**/T]RALLFVIL	*BCAT2*	SNP	rs11548193	Exon	Normal	Normal	B*07:02	(27)
LB-EBI3-1I	RPRARYY[**I**/V]QV	*EBI3*	SNP	rs4740	Exon	Normal	Normal	B*07:02	(27)
LB-ERAP1-1R	HPRQEQIALLA	*ERAP1*	SNP	rs26653	Exon	Normal	Normal	B*07:02	(27)
LB-GEMIN4-1V	FPALRFVE[**V**/E]	*GEMIN4*	SNP	rs4968104	Exon	Normal	Normal	B*07:02	(27)
LB-PDCD11-1F	GPDSSKT[**F**/L]LCL	*PDCD11*	SNP	rs2986014	Exon	Normal	Normal	B*07:02	(27)
ZAPHIR	IPRDSWWVEL	*ZNF419*	SNP	rs2074071	Exon	Polymorphic	Polymorphic	B*07:02	(49)
LB-FUCA2-1V	RLRQ[**V**/M]GSWL	*FUCA2*	SNP	rs3762002	Exon	Normal	Normal	B*07:02	(50)
LB-TEP1-1S	APDGAKVA[**S**/P]L	*TEP1*	SNP	rs1760904	Exon	Normal	Normal	B*07:02	(51)
HEATR1	ISKERA[**E**/G]AL	*HEATR1*	SNP	rs2275687	Exon	Normal	Normal	B*08:01	(23)
HA-1/B60	KECVL[**H**/R]DDL	*HMHA1*	SNP	rs1801284	Exon	Normal	Normal	B*40:01	(52)
LB-NUP133-1R	SEDLILC[**R**/Q]L	*NUP133*	SNP	rs1065674	Exon	Normal	Normal	B*40:01	(53)
LB-SON-1R	SETKQ[**R**/C]TVL	*SON*	SNP	rs13047599	Exon	Normal	Normal	B*40:01	(53)
LB-SWAP70-1Q	MEQLE[**Q**/E]LEL	*SWAP70*	SNP	rs415895	Exon	Normal	Normal	B*40:01	(53)
LB-TRIP10-1EPC	G[**E**/G][**P**/S]QDL[**C**/G]TL	*TRIP10*	SNP	rs1049229 rs1049230 rs1049232	3′ UTR	Normal	Alternative	B*40:01	(53)
SLC1A5	AE[**A**/P]TANGGLAL	*SLC1A5*	SNP	rs3027956	Exon	Normal	Normal	B*40:02	(39)
HB-1H	EEKRGSL[**H**/Y]VW	*HMHB1*	SNP	rs161557	Exon	Normal	Normal	B*44:03	(54)
HB-1Y	EEKRGSL[H/**Y**]VW	*HMHB1*	SNP	rs161557	Exon	Normal	Normal	B*44:03	(55)
UGT2B17/B44	AELLNIPFLY	*UGT2B17*	Gene deletion			Polymorphic	Polymorphic	B44	(44)
ACC-2	KEFED[**D**/G]IINW	*BCL2A1*	SNP	rs3826007	Exon	Normal	Normal	B*44:03	(42)
ACC-6	MEIFIEVFSHF	*HMSD*	SNP	rs9945924	Intron	Polymorphic	Polymorphic	B*44:03	(56)
DPH1	S[**V**/L]LPEVDVW	*DPH1*	SNP	rs35394823	Exon	Normal	Normal	B*57:01	(45)
HLA-II MiHA	**MiHA/AV[a]**	**Gene**	**Variant**	**rs number**	**Location**	**Transcript[b]**	**Protein[b]**	**HLA**	**Reference**
LB-MTHFD1-1Q	SSIIAD[**Q**/R]IALKL	*MTHFD1*	SNP	rs2236225	Exon	Normal	Normal	DRB1*03:01	(26)
LB-LY75-1K	LGITYR[**N**/K]KSLMWF	*LY75*	SNP	rs12692566	Exon	Normal	Normal	DRB1*13:01	(26)
SLC19A1	[**R**/H]LVCYLCFY	*SLC19A1*	SNP	rs1051266	Exon	Normal	Normal	DRB1*15:01	(57)
LB-PTK2B-1T	VYMND[**T**/K]SPLTPEK	*PTK2B*	SNP	rs751019	Exon	Normal	Normal	DRB3*01:01	(26)
LB-MR1-1R	YFRLGVSDPI[**R**/H]G	*MR1*	SNP	rs2236410	Exon	Normal	Normal	DRB3*02:02	(26)
CD19	WEGEPPC[**L**/V]P	*CD19*	SNP	rs2904880	Exon	Normal	Normal	DQB1*02:01	(58)
LB-PI4K2B-1S	SRSS[**S**/P]AELDRSR	*PI4K2B*	SNP	rs313549	Exon	Normal	Normal	DQB1*06:03	(59)
UTDP4-1	R[**I**/N]LAHFFCGW	*ZDHHC12*	SNP	rs11539209	Exon	Normal	Normal	DPB1*04	(60)

[a]*The polymorphic amino acid in the epitope is indicated between brackets (MiHA/AV = allelic variant).*

[b]*Normal and alternative transcripts and proteins are expressed independently of the SNP in both patient and donor, whereas polymorphic transcripts and proteins are de novo created by the SNP and, thus, restricted to the patient.*

Whereas T-cells are used to identify MiHA by forward strategies; in reverse strategies, peptides are selected to search for specific T-cells. Polymorphic peptides identified in HLA-ligandomes (38, 51), hematopoiesis-restricted genes (52, 55, 61), and peptides identified based on association of SNPs with good clinical outcome after alloSCT (36) have been selected to search for specific T-cells in transplanted patients or healthy individuals.

In total, 48 HLA class I-restricted and 8 HLA class II-restricted autosomal MiHA have thus far been characterized. These numbers are expected to rapidly increase in the near future, in particular, if WGAs is performed with cell panels for which all SNPs are measured by whole genome sequencing.

MOLECULAR MECHANISMS

HLA class I-restricted autosomal MiHA are generated by different molecular mechanisms. An overview of the various mechanisms by which genetic variants create MiHA is shown in **Figure 1**. Of the 48 HLA class I-restricted MiHA, 36 antigens are encoded by SNPs in coding exons, leading to single amino acid changes in proteins that are translated from primary gene transcripts in the normal reading frame (**Figure 1A**). MiHA can, however, also be translated from normal gene transcripts in an alternative reading frame. These SNPs can be located in coding exons (C19ORF48, LB-ECGF1-1H, LB-ADIR-1F, LB-ARHGDIB-1R) (**Figure 1B**) or in 5' or 3' UTR regions (LB-TRIP10-1EPC) (**Figure 1C**). Though not yet discovered, it is expected that MiHA can also be encoded by SNP in intron regions that are retained in alternative transcripts (**Figure 1D**). Proteins translated in alternative reading frames are considered as aberrant proteins that lack any cellular function, the so-called defective ribosomal products (DRiPs). DRiPs are rapidly degraded during or shortly after translation and evidence has been found that they may be a main source of peptide precursors for T-cell immunosurveillance (62). Degradation is an important factor for HLA presentation (63), but relative abundance of normal functional proteins in the cell may counteract the actual contribution of DRiPs to the HLA-ligandome.

In addition to the proteins described above that are expressed independently of the SNP in both patient and donor, MiHA can also be derived from proteins or protein products that are *de novo* created by SNPs. Expression of these polymorphic proteins is restricted to the patient and allelic variants in the donor do not exist. As a result, the epitope that is recognized by the T-cell can be derived from another protein region than the amino acids that are directly encoded by the SNP. Examples of antigens from polymorphic proteins that are *de novo* created are LRH-1, an antigen that is produced by an insertion/deletion variant (INDEL) that induces a frameshift in protein translation (**Figure 1E**) and PANE1, which is an antigen from an elongated protein created by a SNP that disrupts the stop codon (**Figure 1F**). PANE1 and LRH-1 are both polymorphic proteins translated from transcripts that are expressed independently of the SNP in both patient and donor. However, MiHA can also be encoded by transcripts that are newly created by the SNP. These polymorphic transcripts are expressed in the patient and do not exist in the donor. Antigens encoded by polymorphic transcripts that are newly created by SNPs are ZAPHIR, which is translated from a ZNF419 transcript in which an intron is retained (**Figure 1G**), and ACC-6, which is encoded by an HMSD transcript that is generated by exon skipping (**Figure 1H**). Finally, MiHA can be encoded by polymorphic genes as illustrated by *UGT2B17*, which is present in the patient but absent in the donor genome (**Figure 1I**).

The numbers of MiHA that have been characterized for each molecular mechanism as shown in **Figure 1** probably do not reflect the actual contribution of the various mechanisms to the entire repertoire of MiHA that are recognized by specific T-cells after alloSCT. This is suggested by the finding that for various T-cell clones, associating SNPs have successfully been identified by WGAs in genomic regions outside known exons, whereas epitope discovery failed due to absence of SNP disparities in the normal gene transcript. MiHA recognized by these T-cells are probably encoded by cryptic transcripts. RNA-sequence data can be used to search for these cryptic transcripts in the genomic region that contains the associating SNPs and single RNA-sequence reads can be analyzed to determine the exact sequence composition of the transcripts, thereby facilitating discovery of these MiHA. As such, implementation of RNA-sequence analysis in a combined approach of whole genome and transcriptome analysis may increase the efficiency of MiHA discovery. The various molecular mechanisms how genetic variants create MiHA as shown in **Figure 1** are probably similar for neoantigens. Neoantigens are peptides created by tumor-specific mutations that are presented by HLA and recognized by specific T-cells (64). In cancer neoantigen discovery, research is focused on selecting peptides encoded by mutations in coding exons with single amino acid changes in the normal protein reading frame (**Figure 1A**), whereas other molecular mechanisms (**Figures 1B–H**) are often not taken into consideration. RNA-sequence analysis may, therefore, also be relevant to elucidate transcript variants for neoantigens in particular since splicing defects often occur in cancer (65).

TISSUE DISTRIBUTION

The tissue distribution of MiHA is an important factor in clinical manifestations caused by immune responses after alloSCT. Various T-cells recognize leukemic cells *in vitro* with no or minimal reactivity against non-hematopoietic cells. These T-cells are expected to mediate beneficial GvL after alloSCT without GvHD. Other T-cells are reactive with both hematopoietic and non-hematopoietic cells, suggesting a role in GvHD. Since in alloSCT, patient hematopoiesis is replaced by a blood-forming system from a healthy donor, donor T-cells for hematopoiesis-restricted MiHA eliminate the malignant cells of the patient, while sparing healthy hematopoietic cells of donor origin. Therefore, discovery of hematopoietic MiHA is an explicit research goal.

Various methods are available to investigate the tissue distribution of MiHA to estimate their efficacy and toxicity as T-cell targets. Toxicity can be analyzed by measuring T-cell reactivity against non-hematopoietic cells from organs that are targeted in GvHD. This analysis, however, requires collection of a variety of

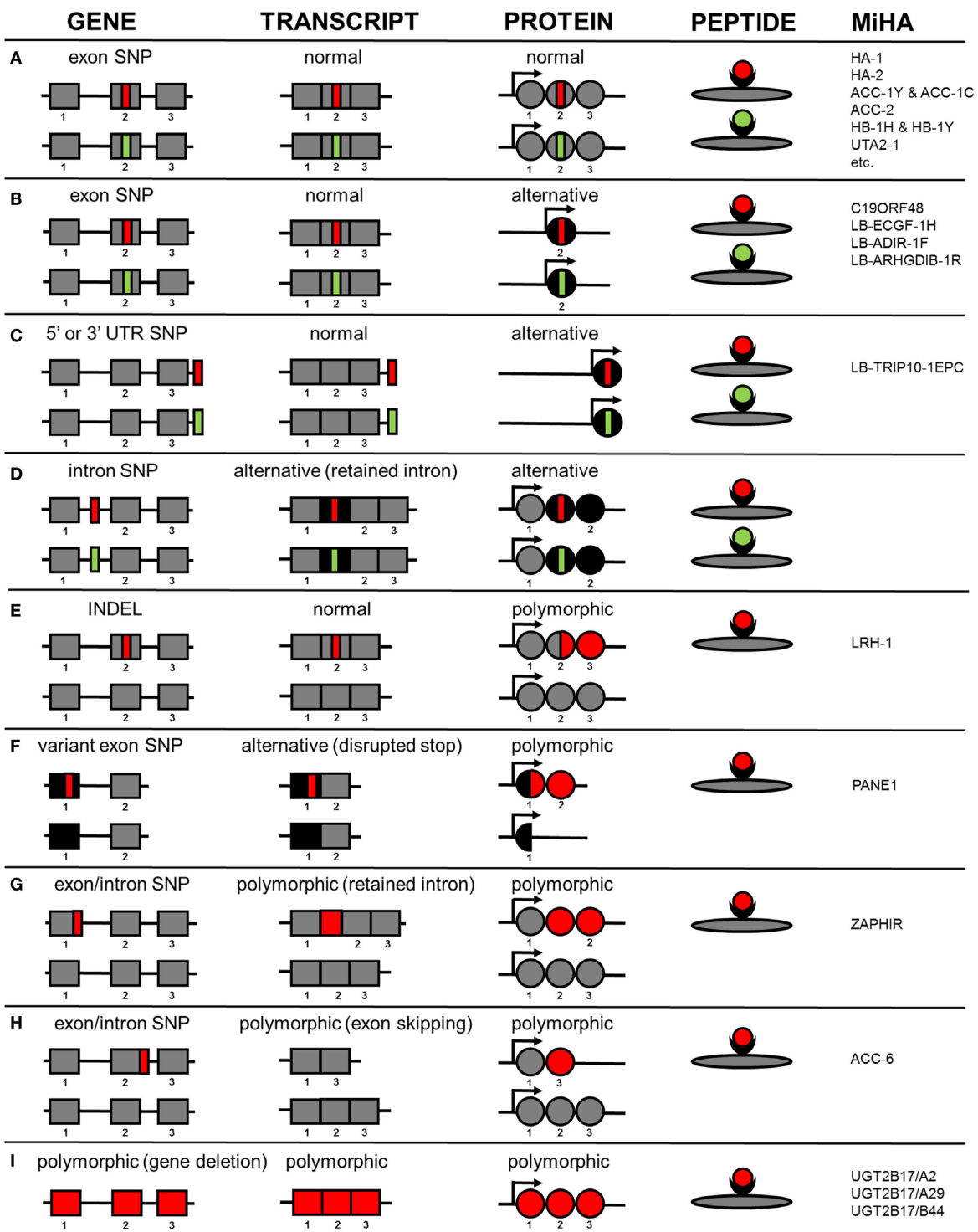


FIGURE 1 | **Molecular mechanisms by which genetic variants create autosomal MiHA**. Normal non-polymorphic sequences are indicated in gray, whereas alternative non-polymorphic sequences are shown in black. Polymorphic patient-specific sequences are shown in red and donor-specific sequences are indicated in green (if allelic variants exist). Whether the allelic variants are actually presented on the cell surface is also dependent on intracellular processing and presentation mechanisms, which are not taken into consideration in this figure. **(A)** MiHA created by SNPs in primary gene transcripts in the normal reading frame. **(B)** MiHA created by SNPs in primary gene transcripts in an alternative reading frame. **(C)** MiHA created by SNPs in 5′ or 3′ UTR of primary gene transcripts. **(D)** MiHA created by intron SNPs as retained in alternative gene transcripts. **(E)** MiHA derived from polymorphic proteins as created by frameshift insertions or deletions in primary gene transcripts. **(F)** MiHA derived from polymorphic proteins as created by SNP in alternative gene transcripts. **(G)** MiHA translated from intron sequences in polymorphic gene transcripts as created by exon or intron SNPs. **(H)** MiHA translated from polymorphic gene transcripts in which exon sequences are skipped as created by exon or intron SNPs. **(I)** MiHA encoded by polymorphic genes.

tissues expressing the relevant MiHA and HLA restriction allele. Skin fibroblasts are frequently used to estimate toxicity and have also been cultured with cytokines to mimic the inflammatory environment of the early post-transplantation period. T-cells often recognize skin fibroblasts when cultured under inflammatory conditions, what may be explained by efficient antigen processing and presentation and enhanced surface expression of HLA, costimulatory, and adhesion molecules. Other non-hematopoietic cells, however, are more difficult to culture and often not available in quantities that allow in depth T-cell analysis. Therefore, as second best option, the tissue distribution can be investigated by gene expression analysis. Thus far, only a limited number of MiHA are encoded by genes with restricted or predominant expression in (malignant) hematopoietic cells, i.e., *HMHB1* (54), *MYO1G* (66), *HMHA1* (67), *BCL2A1* (42), *P2×5* (47), *CENPM* (40), *HMSD* (56), *KIAA1551* (37), and *ARHGDIB* (68). Although gene expression analysis allows rapid selection of hematopoietic antigens, the therapeutic value of MiHA needs to be validated by demonstrating the capacity of specific T-cells to kill leukemic cells and confirming their failure to react with non-hematopoietic cells.

IN VIVO IMMUNE RESPONSES

Minor histocompatibility antigens characterization enabled *ex vivo* quantification of specific T-cells by pMHC multimers in individual patients after alloSCT. Staining with pMHC multimers demonstrated a peak in the immune response in patients who responded to DLI after HLA-matched alloSCT (27, 37, 47, 69). In these patients, high frequencies of circulating T-cells coincided with development of GvL. Detailed analysis of peak responses between 4 and 12 weeks after DLI demonstrated that a diversity of HLA class I- and II-restricted MiHA are targeted by CD8 and CD4 T-cells (26, 27, 53). These T-cells expand and retract with similar kinetics, although frequencies and timing of the peak may differ between MiHA (27, 53).

Although GvL after alloSCT is often accompanied with GvHD, strong anti-tumor responses without severe side effects are occasionally observed (69), illustrating that GvL can be separated from GvHD. In the pathophysiology of GvHD, the tissue distribution of MiHA is important as well as the frequencies of circulating T-cells, their homing behavior and capacity to destroy non-hematopoietic cells *in situ* (70). Although tissue distribution is relevant, occurrence of GvHD cannot entirely be explained by induction of T-cells targeting MiHA on non-hematopoietic tissues. This became clear when T-cells for hematopoietic and ubiquitous MiHA were simultaneously detected in patients with severe GvHD (71) and patients without GvHD (53). Since immune responses in patients with GvHD are generally strong, it can be speculated that T-cell reactivity against non-hematopoietic tissues needs to exceed a certain threshold in GvHD (72). Since T-cells for ubiquitous MiHA may stimulate development of GvL by releasing cytokines, strategies that retain reactivity against healthy tissues below the threshold may effectively separate GvL from GvHD.

THERAPEUTIC USE

As the number of characterized MiHA increases, T-cells from different patients more often recognize MiHA that are already known, suggesting that the repertoire of MiHA that are presented by HLA and recognized by specific T-cells is limited and follow rules for immunodominance that cannot be predicted by measuring only SNP disparities (73). If true, a large proportion of all MiHA with balanced population frequencies will be characterized in the coming years. Discovery of these MiHA is needed to analyze and compare *in vivo* immune responses in GvL and GvHD with respect to specificity, diversity, frequency, and dynamics of specific T-cells. Moreover, it enables to follow GvL and GvHD in large patient groups, which is essential to investigate and compare efficacy and toxicity of different alloSCT (and DLI) transplantation protocols.

With the discovery of a large proportion of common MiHA, a variety of targets become available for therapy to augment GvL after alloSCT (74, 75). One strategy is *in vitro* production and adoptive transfer of donor T-cells for hematopoietic MiHA (75, 76). Patients with leukemia who relapsed after alloSCT have been treated with *in vitro* expanded T-cells for leukemic cells (77, 78), T-cells for HA-1 (79) or MiHA-specific T-cells that lacked reactivity against fibroblasts (45). Other strategies for adoptive transfer are isolation of MiHA-specific T-cells from the DLI by pMHC multimers and T-cell receptor (TCR) gene transfer. In the latter study, patients are treated with virus-specific donor T-cells that are genetically engineered with the TCR for HA-1 (80). Besides adoptive transfer, patients with hematological malignancies can be *in vivo* vaccinated with donor (or patient) dendritic cells loaded with peptides or mRNA (81–83). In conclusion, hematopoietic MiHA (and their specific TCRs) may be easily implemented in ongoing clinical trials to increase efficacy, reduce toxicity, and broaden applicability of immunotherapy after alloSCT.

CONCLUDING REMARKS

In this review, the various molecular mechanisms how genetic variants create autosomal MiHA are described as well as the relevance of these antigens as tools to understand the biology of GvL and GvHD and as targets for immunotherapy to treat hematological cancers after alloSCT. Although more MiHA are needed for comprehensive understanding and manipulation by immunotherapy, this review shows insight into the composition and kinetics of *in vivo* immune responses with respect to specificity, diversity, and frequency of specific T-cells and surface expression of HLA-peptide complexes and other (accessory) molecules on the target cell. A complex interplay between these factors and their environment (84) ultimately determines the spectrum of clinical manifestations that are caused by immune responses after alloSCT.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct, and intellectual contribution to the work, and approved it for publication.

REFERENCES

1. Horowitz MM, Gale RP, Sondel PM, Goldman JM, Kersey J, Kolb HJ, et al. Graft-versus-leukemia reactions after bone marrow transplantation. *Blood* (1990) **75**:555–62.
2. Falkenburg JHF, Warren EH. Graft versus leukemia reactivity after allogeneic stem cell transplantation. *Biol Blood Marrow Transplant* (2011) **17**:S33–8. doi:10.1016/j.bbmt.2010.11.009
3. Gratwohl A, Brand R, Apperley J, Biezen AA, Bandini G, Devergie A, et al. Graft-versus-host disease and outcome in HLA-identical sibling transplantations for chronic myeloid leukemia. *Blood* (2002) **100**:3877–86. doi:10.1182/blood.V100.12.3877
4. Shlomchik WD. Graft-versus-host disease. *Nat Rev Immunol* (2007) **7**:340–52. doi:10.1038/nri2000
5. Ferrara JL, Levine JE, Reddy P, Holler E. Graft-versus-host disease. *Lancet* (2009) **373**:1550–61. doi:10.1016/S0140-6736(09)60237-3
6. Stern M, de Wreede LC, Brand R, van Biezen A, Dreger P, Mohty M, et al. Sensitivity of hematological malignancies to graft-versus-host effects: an EBMT megafile analysis. *Leukemia* (2014) **28**:2235–40. doi:10.1038/leu.2014.145
7. Marmont AM, Horowitz MM, Gale RP, Sobocinski K, Ash RC, van Bekkum DW, et al. T-cell depletion of HLA-identical transplants in leukemia. *Blood* (1991) **78**:2120–30.
8. Barge RM, Starrenburg CW, Falkenburg JHF, Fibbe WE, Marijt EW, Willemze R. Long-term follow-up of myeloablative allogeneic stem cell transplantation using Campath "in the bag" as T-cell depletion: the Leiden experience. *Bone Marrow Transplant* (2006) **37**:1129–34. doi:10.1038/sj.bmt.1705385
9. Hobbs GS, Perales MA. Effects of T-cell depletion on allogeneic hematopoietic stem cell transplantation outcomes in AML patients. *J Clin Med* (2015) **4**:488–503. doi:10.3390/jcm4030488
10. Kolb HJ. Graft-versus-leukemia effects of transplantation and donor lymphocytes. *Blood* (2008) **112**:4371–83. doi:10.1182/blood-2008-03-077974
11. Deol A, Lum LG. Role of donor lymphocyte infusions in relapsed hematological malignancies after stem cell transplantation revisited. *Cancer Treat Rev* (2010) **36**:528–38. doi:10.1016/j.ctrv.2010.03.004
12. Hansen JA, Petersdorf EW, Lin MT, Wang S, Chien JW, Storer B, et al. Genetics of allogeneic hematopoietic cell transplantation. Role of HLA matching, functional variation in immune response genes. *Immunol Res* (2008) **41**:56–78. doi:10.1007/s12026-007-0043-x
13. Petersdorf EW. The major histocompatibility complex: a model for understanding graft-versus-host disease. *Blood* (2013) **122**:1863–72. doi:10.1182/blood-2013-05-355982
14. Neefjes J, Jongsma ML, Paul P, Bakke O. Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat Rev Immunol* (2011) **11**:823–36. doi:10.1038/nri3084
15. Leone P, Shin EC, Perosa F, Vacca A, Dammacco F, Racanelli V. MHC class I antigen processing and presenting machinery: organization, function, and defects in tumor cells. *J Natl Cancer Inst* (2013) **105**:1172–87. doi:10.1093/jnci/djt184
16. Roche PA, Furuta K. The ins and outs of MHC class II-mediated antigen processing and presentation. *Nat Rev Immunol* (2015) **15**:203–16. doi:10.1038/nri3818
17. Klein L, Kyewski B, Allen PM, Hogquist KA. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nat Rev Immunol* (2014) **14**:377–91. doi:10.1038/nri3667
18. Falkenburg JHF, van de Corput L, Marijt EW, Willemze R. Minor histocompatibility antigens in human stem cell transplantation. *Exp Hematol* (2003) **31**:743–51. doi:10.1016/S0301-472X(03)00190-5
19. Warren EH, Zhang XC, Li S, Fan W, Storer BE, Chien JW, et al. Effect of MHC and non-MHC donor/recipient genetic disparity on the outcome of allogeneic HCT. *Blood* (2012) **120**:2796–806. doi:10.1182/blood-2012-04-347286
20. Spierings E. Minor histocompatibility antigens: past, present, and future. *Tissue Antigens* (2014) **84**:374–360. doi:10.1111/tan.12445
21. Zilberberg J, Feinman R, Korngold R. Strategies for the identification of T cell-recognized tumor antigens in hematological malignancies for improved graft-versus-tumor responses after allogeneic blood and marrow transplantation. *Biol Blood Marrow Transplant* (2015) **21**:1000–7. doi:10.1016/j.bbmt.2014.11.001
22. Wölfel C, Lennerz V, Lindemann E, Hess G, Derigs HG, Huber C, et al. Dissection and molecular analysis of alloreactive CD8+ T cell responses in allogeneic haematopoietic stem cell transplantation. *Cancer Immunol Immunother* (2008) **57**:849–57. doi:10.1007/s00262-007-0421-1
23. Bleakley M, Otterud BE, Richardt JL, Mollerup AD, Hudecek M, Nishida T, et al. Leukemia-associated minor histocompatibility antigen discovery using T-cell clones isolated by in vitro stimulation of naive CD8+ T cells. *Blood* (2010) **115**:4923–33. doi:10.1182/blood-2009-12-260539
24. Kloosterboer FM, Luxemburg-Heijs SAP, van Soest RA, van Egmond HM, Barbui AM, Strijbosch MP, et al. Minor histocompatibility antigen-specific T cells with multiple distinct specificities can be isolated by direct cloning of IFNgamma-secreting T cells from patients with relapsed leukemia responding to donor lymphocyte infusion. *Leukemia* (2005) **19**:83–90. doi:10.1038/sj.leu.2403572
25. Campbell JD, Foerster A, Lasmanowicz V, Niemoller M, Scheffold A, Fahrendorff M, et al. Rapid detection, enrichment and propagation of specific T cell subsets based on cytokine secretion. *Clin Exp Immunol* (2011) **163**:1–10. doi:10.1111/j.1365-2249.2010.04261.x
26. Stumpf AN, van der Meijden ED, van Bergen CAM, Willemze R, Falkenburg JHF, Griffioen M. Identification of 4 new HLA-DR-restricted minor histocompatibility antigens as hematopoietic targets in antitumor immunity. *Blood* (2009) **114**:3684–92. doi:10.1182/blood-2009-03-208017
27. van Bergen CAM, Rutten CE, van der Meijden ED, van Luxemburg-Heijs SAP, Lurvink EGA, Houwing-Duistermaat JJ, et al. High-throughput characterization of 10 new minor histocompatibility antigens by whole genome association scanning. *Cancer Res* (2010) **70**:9073–83. doi:10.1158/0008-5472.CAN-10-1832
28. Wehler TC, Nonn M, Brandt B, Britten CM, Grone M, Todorova M, et al. Targeting the activation-induced antigen CD137 can selectively deplete alloreactive T cells from antileukemic and antitumor donor T-cell lines. *Blood* (2007) **109**:365–73. doi:10.1182/blood-2006-04-014100
29. Wolfl M, Kuball J, Eyrich M, Schlegel PG, Greenberg PD. Use of CD137 to study the full repertoire of CD8+ T cells without the need to know epitope specificities. *Cytometry A* (2008) **73**:1043–9. doi:10.1002/cyto.a.20594
30. Spierings E, Brickner AG, Caldwell JA, Zegveld S, Tatsis N, Blokland E, et al. The minor histocompatibility antigen HA-3 arises from differential proteasome-mediated cleavage of the lymphoid blast crisis (Lbc) oncoprotein. *Blood* (2003) **102**:621–9. doi:10.1182/blood-2003-01-0260
31. den Haan JM, Sherman NE, Blokland E, Huczko E, Koning F, Drijfhout JW, et al. Identification of a graft versus host disease-associated human minor histocompatibility antigen. *Science* (1995) **268**:1476–80. doi:10.1126/science.7539551
32. den Haan JM, Meadows LM, Wang W, Pool J, Blokland E, Bishop TL, et al. The minor histocompatibility antigen HA-1: a diallelic gene with a single amino acid polymorphism. *Science* (1998) **279**:1054–7. doi:10.1126/science.279.5353.1054
33. Brickner AG, Warren EH, Caldwell JA, Akatsuka Y, Golovina TN, Zarling AL, et al. The immunogenicity of a new human minor histocompatibility antigen results from differential antigen processing. *J Exp Med* (2001) **193**:195–206. doi:10.1084/jem.193.2.195
34. van Bergen CAM, Kester MGD, Jedema I, Heemskerk MHM, van Luxemburg-Heijs SAP, Kloosterboer FM, et al. Multiple myeloma-reactive T cells recognize an activation-induced minor histocompatibility antigen encoded by the ATP-dependent interferon-responsive (ADIR) gene. *Blood* (2007) **109**:4089–96. doi:10.1182/blood-2006-08-043935
35. Tykodi SS, Fujii N, Vigneron N, Lu SM, Mito JK, Miranda MX, et al. C19orf48 encodes a minor histocompatibility antigen recognized by CD8+ cytotoxic T cells from renal cell carcinoma patients. *Clin Cancer Res* (2008) **14**:5260–9. doi:10.1158/1078-0432.CCR-08-0028
36. Armistead PM, Liang S, Li H, Lu S, van Bergen CAM, Alatrash G, et al. Common minor histocompatibility antigen discovery based upon patient clinical outcomes and genomic data. *PLoS One* (2011) **6**:e23217. doi:10.1371/journal.pone.0023217
37. Oostvogels R, Minnema MC, van Elk M, Spaapen RM, te Raa GD, Giovannone B, et al. Towards effective and safe immunotherapy after allogeneic stem cell transplantation: identification of hematopoietic-specific minor histocompatibility antigen UTA2-1. *Leukemia* (2013) **27**:642–9. doi:10.1038/leu.2012.277
38. Hombrink P, Hassan C, Kester MGD, de Ru AH, van Bergen CAM, Nijveen H, et al. Discovery of T cell epitopes implementing HLA-peptidomics into a

reverse immunology approach. *J Immunol* (2013) **190**:3869–77. doi:10.4049/jimmunol.1202351
39. Kamei M, Nannya Y, Torikai H, Kawase T, Taura K, Inamoto Y, et al. HapMap scanning of novel human minor histocompatibility antigens. *Blood* (2009) **113**:5041–8. doi:10.1182/blood-2008-07-171678
40. Brickner AG, Evans AM, Mito JK, Xuereb SM, Feng X, Nishida T, et al. The PANE1 gene encodes a novel human minor histocompatibility antigen that is selectively expressed in B-lymphoid cells and B-CLL. *Blood* (2006) **107**:3779–86. doi:10.1182/blood-2005-08-3501
41. Warren EH, Vigneron NJ, Gavin MA, Coulie PG, Stroobant V, Dalet A, et al. An antigen produced by splicing of noncontiguous peptides in the reverse order. *Science* (2006) **313**:1444–7. doi:10.1126/science.1130660
42. Akatsuka Y, Nishida T, Kondo E, Miyazaki M, Taji H, Iida H, et al. Identification of a polymorphic gene, BCL2A1, encoding two novel hematopoietic lineage-specific minor histocompatibility antigens. *J Exp Med* (2003) **197**:1489–500. doi:10.1084/jem.20021925
43. Kawase T, Nannya Y, Torikai H, Yamamoto G, Onizuka M, Morishima S, et al. Identification of human minor histocompatibility antigens based on genetic association with highly parallel genotyping of pooled DNA. *Blood* (2008) **111**:3286–94. doi:10.1182/blood-2007-10-118950
44. Murata M, Warren EH, Riddell SR. A human minor histocompatibility antigen resulting from differential expression due to a gene deletion. *J Exp Med* (2003) **197**:1279–89. doi:10.1084/jem.20030044
45. Warren EH, Fujii N, Akatsuka Y, Chaney CN, Mito JK, Loeb KR, et al. Therapy of relapsed leukemia after allogeneic hematopoietic cell transplantation with T cells specific for minor histocompatibility antigens. *Blood* (2010) **115**:3869–78. doi:10.1182/blood-2009-10-248997
46. Torikai H, Akatsuka Y, Miyazaki M, Tsujimura A, Yatabe Y, Kawase T, et al. The human cathepsin H gene encodes two novel minor histocompatibility antigen epitopes restricted by HLA-A*3101 and -A*3303. *Br J Haematol* (2006) **134**:406–16. doi:10.1111/j.1365-2141.2006.06205.x
47. de Rijke B, Horssen-Zoetbrood A, Beekman JM, Otterud B, Maas F, Woestenenk R, et al. A frameshift polymorphism in P2X5 elicits an allogeneic cytotoxic T lymphocyte response associated with remission of chronic myeloid leukemia. *J Clin Invest* (2005) **115**:3506–16. doi:10.1172/JCI24832
48. Slager EH, Honders MW, van der Meijden ED, Luxemburg-Heijs SAP, Kloosterboer FM, Kester MGD, et al. Identification of the angiogenic endothelial-cell growth factor-1/thymidine phosphorylase as a potential target for immunotherapy of cancer. *Blood* (2006) **107**:4954–60. doi:10.1182/blood-2005-09-3883
49. Broen K, Levenga H, Vos J, van Bergen CAM, Fredrix H, Greupink-Draaisma A, et al. A polymorphism in the splice donor site of ZNF419 results in the novel renal cell carcinoma-associated minor histocompatibility antigen ZAPHIR. *PLoS One* (2011) **6**:e21699. doi:10.1371/journal.pone.0021699
50. van Bergen CAM, Verdegaal EM, Honders MW, Hoogstraten C, Steijn-van Tol AQ, de Quartel L, et al. Durable remission of renal cell carcinoma in conjuncture with graft versus host disease following allogeneic stem cell transplantation and donor lymphocyte infusion: rule or exception? *PLoS One* (2014) **9**:e85198. doi:10.1371/journal.pone.0085198
51. Hombrink P, Hassan C, Kester MGD, Jahn L, Pont MJ, de Ru AH, et al. Identification of biological relevant minor histocompatibility antigens within the B-lymphocyte-derived HLA-ligandome using a reverse immunology approach. *Clin Cancer Res* (2015) **21**:2177–86. doi:10.1158/1078-0432.CCR-14-2188
52. Mommaas B, Kamp J, Drijfhout JW, Beekman N, Ossendorp F, van Veelen P, et al. Identification of a novel HLA-B60-restricted T cell epitope of the minor histocompatibility antigen HA-1 locus. *J Immunol* (2002) **169**:3131–6. doi:10.4049/jimmunol.169.6.3131
53. Griffioen M, Honders MW, van der Meijden ED, van Luxemburg-Heijs SAP, Lurvink EGA, Kester MGD, et al. Identification of 4 novel HLA-B*40:01 restricted minor histocompatibility antigens and their potential as targets for graft-versus-leukemia reactivity. *Haematologica* (2012) **97**:1196–204. doi:10.3324/haematol.2011.049478
54. Dolstra H, Fredrix H, Maas F, Coulie PG, Brasseur F, Mensink E, et al. A human minor histocompatibility antigen specific for B cell acute lymphoblastic leukemia. *J Exp Med* (1999) **189**:301–8. doi:10.1084/jem.189.2.301
55. Dolstra H, de Rijke B, Fredrix H, Balas A, Maas F, Scherpen F, et al. Bi-directional allelic recognition of the human minor histocompatibility antigen HB-1 by cytotoxic T lymphocytes. *Eur J Immunol* (2002) **32**:2748–58. doi:10.1002/1521-4141(2002010)32:10<2748::AID-IMMU2748>3.0.CO;2-T
56. Kawase T, Akatsuka Y, Torikai H, Morishima S, Oka A, Tsujimura A, et al. Alternative splicing due to an intronic SNP in HMSD generates a novel minor histocompatibility antigen. *Blood* (2007) **110**:1055–63. doi:10.1182/blood-2007-02-075911
57. Spaapen RM, de Kort RA, van den Oudenalder K, van Elk M, Bloem AC, Lokhorst HM, et al. Rapid identification of clinical relevant minor histocompatibility antigens via genome-wide zygosity-genotype correlation analysis. *Clin Cancer Res* (2009) **15**:7137–43. doi:10.1158/1078-0432.CCR-09-1914
58. Spaapen RM, Lokhorst HM, van den Oudenalder K, Otterud BE, Dolstra H, Leppert MF, et al. Toward targeting B cell cancers with CD4+ CTLs: identification of a CD19-encoded minor histocompatibility antigen using a novel genome-wide analysis. *J Exp Med* (2008) **205**:2863–72. doi:10.1084/jem.20080713
59. Griffioen M, van der Meijden ED, Slager EH, Honders MW, Rutten CE, van Luxemburg-Heijs SAP, et al. Identification of phosphatidylinositol 4-kinase type II beta as HLA class II-restricted target in graft versus leukemia reactivity. *Proc Natl Acad Sci U S A* (2008) **105**:3837–42. doi:10.1073/pnas.0712250105
60. Oostvogels R, Lokhorst HM, Minnema MC, van Elk M, van den Oudenalder K, Spierings E, et al. Identification of minor histocompatibility antigens based on the 1000 Genomes Project. *Haematologica* (2014) **99**:1854–9. doi:10.3324/haematol.2014.109801
61. Broen K, Greupink-Draaisma A, Woestenenk R, Schaap N, Brickner AG, Dolstra H. Concurrent detection of circulating minor histocompatibility antigen-specific CD8+ T cells in SCT recipients by combinatorial encoding MHC multimers. *PLoS One* (2011) **6**:e21266. doi:10.1371/journal.pone.0021266
62. Anton LC, Yewdell JW. Translating DRiPs: MHC class I immunosurveillance of pathogens and tumors. *J Leukoc Biol* (2014) **95**:551–62. doi:10.1189/jlb.1113599
63. Bassani-Sternberg M, Pletscher-Frankild S, Jensen LJ, Mann M. Mass spectrometry of human leukocyte antigen class I peptidomes reveals strong effects of protein abundance and turnover on antigen presentation. *Mol Cell Proteomics* (2015) **14**:658–73. doi:10.1074/mcp.M114.042812
64. Schumacher TN, Schreiber RD. Neoantigens in cancer immunotherapy. *Science* (2015) **348**:69–74. doi:10.1126/science.aaa4971
65. Daguenet E, Dujardin G, Valcárcel J. The pathogenicity of splicing defects: mechanistic insights into pre-mRNA processing inform novel therapeutic approaches. *EMBO Rep* (2015) **16**:1640–55. doi:10.15252/embr.201541116
66. Pierce RA, Field ED, Mutis T, Golovina TN, Kap-Herr C, Wilke M, et al. The HA-2 minor histocompatibility antigen is derived from a diallelic gene encoding a novel human class I myosin protein. *J Immunol* (2001) **167**:3223–30. doi:10.4049/jimmunol.167.6.3223
67. Wilke M, Dolstra H, Maas F, Pool J, Brouwer R, Falkenburg JHF, et al. Quantification of the HA-1 gene product at the RNA level; relevance for immunotherapy of hematological malignancies. *Hematol J* (2003) **4**:315–20. doi:10.1038/sj.thj.6200318
68. Pont MJ, Hobo W, Honders MW, van Luxemburg-Heijs SAP, Kester MGD, van Oeveren-Rietdijk AM, et al. LB-ARHGDIB-1R as novel minor histocompatibility antigen for therapeutic application. *Haematologica* (2015) **100**(10):e419–22. doi:10.3324/haematol.2015.125021
69. Marijt WAF, Heemskerk MHM, Kloosterboer FM, Goulmy E, Kester MGD, van der Hoorn MAWG, et al. Hematopoiesis-restricted minor histocompatibility antigens HA-1- or HA-2-specific T cells can induce complete remissions of relapsed leukemia. *Proc Natl Acad Sci U S A* (2003) **100**:2742–7. doi:10.1073/pnas.0530192100
70. Dickinson AM, Wang XN, Sviland L, Vyth-Dreese FA, Jackson GH, Schumacher TN, et al. In situ dissection of the graft-versus-host activities of cytotoxic T cells specific for minor histocompatibility antigens. *Nat Med* (2002) **8**:410–4. doi:10.1038/nm0402-410
71. Mutis T, Gillespie G, Schrama E, Falkenburg JHF, Moss P, Goulmy E. Tetrameric HLA class I-minor histocompatibility antigen peptide complexes demonstrate minor histocompatibility antigen-specific cytotoxic T lymphocytes in patients with graft-versus-host disease. *Nat Med* (1999) **5**:839–42. doi:10.1038/10563
72. van der Zouwen B, Kruisselbrink AB, Jordanova ES, Rutten CE, von dem Borne PA, Falkenburg JHF, et al. Alloreactive effector T cells require the local formation of a proinflammatory environment to allow crosstalk and high avidity interaction with nonhematopoietic tissues to induce GVHD

reactivity. *Biol Blood Marrow Transplant* (2012) **18**:1353–67. doi:10.1016/j.bbmt.2012.06.017

73. Jameson-Lee M, Koparde V, Griffith P, Scalora AF, Sampson JK, Khalid H, et al. In silico derivation of HLA-specific alloreactivity potential from whole exome sequencing of stem-cell transplant donors and recipients: understanding the quantitative immunobiology of allogeneic transplantation. *Front Immunol* (2014) **5**:529. doi:10.3389/fimmu.2014.00529
74. Spaapen R, Mutis T. Targeting haematopoietic-specific minor histocompatibility antigens to distinguish graft-versus-tumour effects from graft-versus-host disease. *Best Pract Res Clin Haematol* (2008) **21**:543–57. doi:10.1016/j.beha.2008.06.001
75. Bleakley M, Riddell SR. Exploiting T cells specific for human minor histocompatibility antigens for therapy of leukemia. *Immunol Cell Biol* (2011) **89**:396–407. doi:10.1038/icb.2010.124
76. Tsirigotis P, Shimoni A, Nagler A. The expanding horizon of immunotherapy in the treatment of malignant disorders: allogeneic hematopoietic stem cell transplantation and beyond. *Ann Med* (2014) **46**:384–96. doi:10.3109/07853890.2014.918463
77. Falkenburg JHF, Wafelman AR, Joosten P, Smit WM, van Bergen CAM, Bongaerts R, et al. Complete remission of accelerated phase chronic myeloid leukemia by treatment with leukemia-reactive cytotoxic T lymphocytes. *Blood* (1999) **94**:1201–8.
78. Marijt EW, Wafelman AR, van der Hoorn MAWG, van Bergen CAM, Bongaerts R, van Luxemburg-Heijs SAP, et al. Phase I/II feasibility study evaluating the generation of leukemia-reactive cytotoxic T lymphocyte lines for treatment of patients with relapsed leukemia after allogeneic stem cell transplantation. *Haematologica* (2007) **92**:72–80. doi:10.3324/haematol.10433
79. Meij P, Jedema I, van der Hoorn MAWG, Bongaerts R, Cox L, Wafelman AR, et al. Generation and administration of HA-1-specific T-cell lines for the treatment of patients with relapsed leukemia after allogeneic stem cell transplantation: a pilot study. *Haematologica* (2012) **97**:1205–8. doi:10.3324/haematol.2011.053371
80. Heemskerk MHM, Griffioen M, Falkenburg JHF. T-cell receptor gene transfer for treatment of leukemia. *Cytotherapy* (2008) **10**:108–15. doi:10.1080/14653240701883087
81. Overes IM, Fredrix H, Kester MGD, Falkenburg JHF, van der Voort R, de Witte TM, et al. Efficient activation of LRH-1-specific CD8+ T-cell responses from transplanted leukemia patients by stimulation with P2X5 mRNA-electroporated dendritic cells. *J Immunother* (2009) **32**:539–51. doi:10.1097/CJI.0b013e3181987c22
82. Rezvani K. Posttransplantation vaccination: concepts today and on the horizon. *Hematology Am Soc Hematol Educ Program* (2011) **2011**:299–304. doi:10.1182/asheducation-2011.1.299
83. Hosen N, Maeda T, Hashii Y, Tsuboi A, Nishida S, Nakata J, et al. Vaccination strategies to improve outcome of hematopoietic stem cell transplant in leukemia patients: early evidence and future prospects. *Expert Rev Hematol* (2014) **7**:671–81. doi:10.1586/17474086.2014.953925
84. Stevanovic S, van Bergen CAM, van Luxemburg-Heijs SAP, van der Zouwen B, Jordanova ES, Kruisselbrink AB, et al. HLA class II upregulation during viral infection leads to HLA-DP-directed graft-versus-host disease after CD4+ donor lymphocyte infusion. *Blood* (2013) **122**:1963–73. doi:10.1182/blood-2012-12-470872

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 02 February 2017
doi: 10.3389/fimmu.2017.00080

A Paradigm Shift on the Question of B Cells in Transplantation? Recent Insights on Regulating the Alloresponse

*Daniel J. Firl[1,2], Gilles Benichou[1], James I. Kim[1] and Heidi Yeh[1]**

[1] Transplant Center, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA, [2] Howard Hughes Medical Institute, Chevy Chase, MD, USA

B lymphocytes contribute to acute and chronic allograft rejection through their production of donor-specific antibodies (DSAs). In addition, B cells present allopeptides bound to self-MHC class II molecules and provide costimulation signals to T cells, which are essential to their activation and differentiation into memory T cells. On the other hand, both in laboratory rodents and patients, the concept of effector T cell regulation by B cells is gaining traction in the field of transplantation. Specifically, clinical trials using anti-CD20 monoclonal antibodies to deplete B cells and reverse DSA had a deleterious effect on rates of acute cellular rejection; a peculiar finding that calls into question a central paradigm in transplantation. Additional work in humans has characterized IL-10-producing B cells (IgM memory and transitional B cells), which suppress the proliferation and inflammatory cytokine productions of effector T cells *in vitro*. Understanding the mechanisms of regulating the alloresponse is critical if we are to achieve operational tolerance across transplantation. This review will focus on recent evidence in murine and human transplantation with respect to non-traditional roles for B cells in determining clinical outcomes.

Keywords: regulatory B cells, transplant tolerance, antigen presentation, allorecognition, transplant rejection, autoimmune diseases

OPEN ACCESS

Edited by:
Martin Johannes Hoogduijn, Erasmus University Rotterdam, Netherlands

Reviewed by:
Marcella Franquesa, IVECAT Group Institut d'Investigació Germans Trias i Pujol, Spain
Verônica Coelho, University of São Paulo, Brazil

****Correspondence:***
Heidi Yeh
hyeh@mgh.harvard.edu

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *03 October 2016*
Accepted: *17 January 2017*
Published: *02 February 2017*

Citation:
Firl DJ, Benichou G, Kim JI and Yeh H (2017) A Paradigm Shift on the Question of B Cells in Transplantation? Recent Insights on Regulating the Alloresponse. Front. Immunol. 8:80. doi: 10.3389/fimmu.2017.00080

INTRODUCTION

Allorecognition refers to the detection by the immune system of polymorphic determinants expressed by different individuals of the same species (alloantigens) (1–3). After transplantation of allogeneic organs or tissues, recognition of alloantigens by host leukocytes initiates an inflammatory immune response leading to graft rejection (4, 5). It is now established that certain leukocytes of the innate immune system, including NK cells and macrophages, can distinguish between self- and non-self antigens and thereby contribute to the alloresponse (6–8). However, allorecognition by T lymphocytes of the adaptive immune system is the driving force behind alloimmunity and allograft rejection in vertebrates. After transplantation, graft MHC class II$^+$ cells as well as donor-derived extracellular vesicles traffic to the recipient lymphoid organs where they

Abbreviations: AMR, antibody-mediated rejection; APC, antigen-presenting cell; Breg, B regulatory cells; cGVHD, chronic graft versus host disease; CTL, cytotoxic T lymphocytes; DSAs, donor-specific antibodies; GC, germinal center; HSCT, hematopoietic stem cell transplant; TrB, transitional B cells.

activate CD4+ allospecific T cells (9–12). This process occurs *via* two distinct pathways: *direct allorecognition* in which T cells recognize intact donor MHC molecules as well as the *semi-direct mechanism* dependent on donor-derived MHC–peptide complex, which traffics *via* extracellular vesicles to be presented upon recipient antigen-presenting cells (APCs). In this case, the recipient dendritic cell (DC) becomes chimeric for donor allopeptide–MHC complex and can present to donor responsive CD8+ T cells through the direct pathway (13). It is important to note that some complex can undergo internalization, degradation, loading, and presentation on MHC-II to CD4+ T cells in the same manner as below in the indirect pathway. Thus, a single DC can present to both CD4+ and CD8+ cells resulting in a linked activation of T cells (14). The *indirect pathway* involves T cells, which interact with donor peptides bound to recipient MHC molecules on host APCs (15–18). This process leads to the differentiation of CD8+ cytotoxic T lymphocytes (CTL) and to plasmocytes (B cells), which produce donor-specific antibodies (DSAs) (19). B cells play a key role in acute and chronic allograft rejection through their production of DSAs, a process requiring help from CD4+ T cells activated indirectly (20). In addition, B cells serve as APCs and present alloantigen peptides to T cells thereby contributing to their activation and differentiation into memory T cells (21, 22). On the other hand, certain B cell subsets can suppress inflammatory alloreactive T cells and promote allograft tolerance (23–27). In this article, we present recent data from human and animal studies that raise exciting new possibilities for B cells in antigen presentation and T cell regulation relevant to transplantation.

ALLORECOGNITION BY B CELLS

B cells have a critical role in indirect allorecognition. The traditional immunological concepts for developing an adaptive response to any given protein antigen underpin the so-called indirect pathway of allorecognition. Recipient T cells recognize processed allopeptide–self-MHC-II complexes on recipient APCs (28–30). The indirect response is primarily CD4+ T cell-driven due to the involvement of self-MHC-II molecules (31, 32). Following recognition of cognate antigen on DCs in the T cell zone, these CD4+ T cells upregulate BCL6, CXCR5, and CD40L and downregulate CCR7, which allows them to migrate to the follicle where they take on the follicular T helper cell phenotype (33). These cells can then instruct follicular B cells, which have internalized donor antigen to seed germinal centers (GCs) *via* the CD40L/CD40 axis as well as the secretion of IL-21 promoting the differentiation of CD40L stimulated B cells (34). These B cells undergo somatic hypermutation, a critical step to generating high-affinity DSA (35). They also class switch and some differentiate into plasma cells (with highest BCR signal strength) or memory B cells if density and tonicity of the B cell receptor signaling are insufficient to differentiate to a plasma or GC B cell (36). Thus, the presence of DSA can be used as a proxy measure of the activity of the indirect pathway (37, 38). In addition to alloreactive or DSA, B cells can generate antibody responses against non-HLA self-peptides, the angiotensin II receptor is an example of an activating antibody leading to a functional change following renal transplantation (39). The extent to which these antibodies contribute to rejection, especially chronic vascular type rejection is as of yet unclear; however, the mechanism of generation in the face of varying degrees of allograft tolerance (DSA levels) is intriguing (40).

B CELLS AS APCs

B cells are likely to play a role in antigen presentation associated with indirect activation of donor-specific T cells. For example, the presence of CD20+ cells in renal allografts is associated with poor outcomes and acute cellular rejection, but not necessarily antibody-mediated rejection (AMR), in renal transplantation (41). B cells present in these grafts presumably mediate their effects through alloantigen presentation and ICOS/CD28 costimulation of T cells leading to their activation and expansion (42). Graft infiltrating CD20+CD27+ memory B cells survey for cognate antigen prior to expanding and seeding GCs, a process leading to increased DSA production and subsequent acute and chronic rejection (43). These DSAs have the potential to greatly modify the interplay of donor antigen and recipient tolerance since bound antibodies have the potential to fix complement and lead to increased tissue damage and increased antigen presentation, as well as epitope spreading, leading to tissue-specific responses as in the indirect pathway described above (44).

ROLE OF B CELLS IN SUPPRESSING INFLAMMATORY ALLOIMMUNITY

B cells may not always act as pro-inflammatory players. In human renal transplantation, B cells were recently shown to have a regulatory role on T cell alloresponses *in vitro* using peripheral blood from 65 patients with biopsy-proven AMR, non-immune related graft dysfunction, or stable graft function (45). The authors found many biopsy-proven AMR samples that did not demonstrate an anti-donor IFN-gamma response unless CD25+ (regulatory T cells) and CD19+ cells (B cells) were depleted. More importantly, depletion of these cells also restored alloresponsiveness in patients with no histological signs of immune-mediated graft dysfunction. Alloresponsiveness was dependent on B–T interactions (with CD19+ cells acting as APCs *in vitro*).

A clinical trial in renal transplantation compared the efficacy of rituximab, a monoclonal anti-CD20 antibody, with daclizumab, a monoclonal anti-CD25 antibody (46) as induction therapy. This trial was halted early due to dramatically increased rates of biopsy-confirmed acute rejection (within the first 3 months post-transplant) in the rituximab-treated group compared with daclizumab (83 versus 14%; $p = 0.01$). In fact, the rate of acute rejection observed in the rituximab-treated group exceeded previously observed rates in recipients that did not receive any induction therapy (~35%), suggesting that B cell depletion actually increased alloreactivity. Another study sought to evaluate rituximab for desensitization prior to HLA-incompatible live donor renal transplantation. Rituximab-treated recipients exhibited a trend toward higher rates of acute rejection and greater

number of episodes of rejection compared with non-rituximab recipients (47). These studies' results are in line with animal models showing worsening of disease severity along several T-dependent autoimmune models including ulcerative colitis (48), psoriasis (49), and autoimmune encephalomyelitis/multiple sclerosis (EAE/MS) (50) following anti-CD20 mAb-mediated B cell depletion, despite decreases in circulating autoantibodies, underscoring the antibody-independent role of B cells in autoimmunity. However, other studies including rituximab in the induction period for ABO incompatible desensitization did not show statistically significant differences in rates of acute rejection, although they did raise the concern of possible increased risk of cardiac mortality following B cell depletion (51, 52).

The role of B cells with regulatory potential has also been explored in human hematopoietic stem cell transplantation. Chronic graft versus host disease (cGVHD) is a debilitating complication that carries a poor prognosis in patients who fail to respond to corticosteroids (53, 54). A frequent observation in GVHD is increased titers of autoantibody that demonstrates a loss of peripheral B cell tolerance (54). Khoder et al. examined the frequencies of regulatory B cells in GVHD and healthy controls and found that the ratio of IL-10$^+$ B cells to IFN-gamma CD4$^+$ T cells was greatly reduced in cGVHD patients compared to stable controls (55). They found B cells with regulatory function (Bregs) (as measured by the ability to suppress CD4$^+$ T cell proliferation and effector function *in vitro*) in both the IgM memory (CD19$^+$IgM$^+$CD27$^+$) and transitional B cell (TrB; CD19$^+$CD24^{hi}CD38^{hi}) compartments. They also demonstrated that the regulatory potential of these cells required cell–cell contact by coculturing both IgM memory and TrB cells in transwell plates with anti-CD3 and anti-CD28 antibody-activated CD4$^+$ T cells. CD80/CD86 blockade in coculture systems was also found to be deleterious to the development of full regulatory effect by Bregs, and that this effect was independent of CD80/PD-1 interactions. The necessity for cell–cell contact combined with the ability of B cells to act as APC raises the question of whether Bregs are antigen-specific *via* either the B cell receptor or MHC, although there have been no reports of direct evidence supporting either possibility.

Future work needs to be done to clarify the ontogeny of donor-specific "regulatory" B cells [current definitions rely on functional production of IL-10 (56–58)]. The regulatory B cell populations in murine models are more fully characterized and reliably defined by phenotypic markers compared with humans. Although no fewer than 10 subsets have been defined as "Bregs," most work has been done on either marginal zone precursor B2 cells or B10 cells, which are typically CD19$^+$CD1d^{hi}CD5$^+$ (a population, which overlaps with marginal zone B2 cells, marginal zone precursor B2 cells, and B1 cells) (59). However, many still perform *in vitro* assays using anti-CD40 antibodies, and PMA-ionomycin, followed by monensin or brefeldin treatment to stimulate IL-10-competent B cells to produce and retain this cytokine for intracellular staining (25). In humans, only a small percentage of cells identified as potentially regulatory by phenotypic markers produce IL-10, a finding that makes translation more difficult (60, 61).

One of the first animal models to demonstrate the regulatory role of B cells in transplantation was performed in a murine renal transplantation model where greater efficiency of tolerogenesis was observed by transplanting donor B cells at the time of renal transplantation than with donor T cells (62). Since that time, laboratory efforts have identified several subtypes of B cells with regulatory potential (63).

In a murine model of pancreatic islet allotransplantation, T cell Ig domain and mucin domain protein 1 (TIM-1), a costimulatory molecule was shown to modulate CD4$^+$ T cell reactivity and serves as a marker of Bregs (27). TIM-1 broadly marked Bregs with significant overlap with IL-10$^+$ capable cells. In fact, TIM-1 ligation actually enhanced production and secretion of IL-4 and IL-10 by B cells. Compared to other reports, this group was able to more reliably identify IL-10$^+$ cells in peripheral tissues and secondary lymphoid organs as compared to spleen using TIM-1 positivity as opposed to a non-specific CD19$^+$CD1d^{hi}CD5$^+$ gate. Finally, they were able to promote tolerogenesis *via* RMT1-10, an anti-TIM-1 mAb, which simulates CD4$^+$ binding. This work was furthered by identifying the role of Breg-derived TGF-beta in inducing Tregs and in promoting tolerance to fully MHC-mismatched pancreatic islet transplants. Tolerance induction in these mice was transferrable through injection of naïve mice with B cells from dual antibody-treated recipients (anti-CD45RB and anti-TIM-1) (24). This dual therapy promoted TGF-beta secretion by TIM-1$^+$ B cells and led to a substantial increase in Treg frequencies, which was blocked by anti-TGF-beta antibody (26).

CONCLUSION

It is clear that great strides are being made across the field of transplantation with respect to the understanding of the many roles of B cells. B cells are unique in their ability to produce antibodies, which can kill donor cells *via* antibody-dependent cell-mediated cytotoxicity and complement fixation. In addition, B cells are efficient APCs providing help to T cells thereby polarizing the T cell response and promoting the differentiation of memory T cells. However, mechanistically informed clinical trials, which sought to take advantage of the indirect pathway of allorecognition *via* CD20$^+$ antibody treatment to deplete recipient B cells, resulted in increased rates of acute cellular rejection. This peculiar result challenges the single faceted view of B cells as solely pro-inflammatory and supports the human relevance of recent laboratory work in rodents, which has demonstrated immunoregulatory roles for several B cell subsets. Future work needs to characterize the transcriptome of Bregs in an effort to identify a transcription factor necessary for function regulation such as Foxp3 in Tregs. Critical questions remain about whether the variety of reported Bregs are indeed separate cell subsets or merely different activation states of B cells across development. This would help to explain such diverse findings in B10, marginal zone precursors, and TIM-1$^+$ B cells and would open up the exploration of what cytokine environment polarizes a Breg and might be useful in clinical transplantation.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

DF is supported by a Howard Hughes Medical Institute Medical Research Fellowship. HY is supported by NIDDK grant 1K08DK094965-01A1.

REFERENCES

1. Lechler R, Lombardi G. Structural aspects of allorecognition. *Curr Opin Immunol* (1991) 3:715–21. doi:10.1016/0952-7915(91)90102-7
2. Ingulli E. Mechanism of cellular rejection in transplantation. *Pediatr Nephrol* (2010) 25:61–74. doi:10.1007/s00467-008-1020-x
3. Zakrzewski JL, van den Brink MRM, Hubbell JA. Overcoming immunological barriers in regenerative medicine. *Nat Biotechnol* (2014) 32:786–94. doi:10.1038/nbt.2960
4. Rosenberg AS, Singer A. Cellular basis of skin allograft rejection: an in vivo model of immune-mediated tissue destruction. *Annu Rev Immunol* (1992) 10:333–58. doi:10.1146/annurev.iy.10.040192.002001
5. Bharat A, Mohanakumar T. Allopeptides and the alloimmune response. *Cell Immunol* (2007) 248:31–43. doi:10.1016/j.cellimm.2007.03.010
6. Lakkis FG, Dellaporta SL, Buss LW. Allorecognition and chimerism in an invertebrate model organism. *Organogenesis* (2008) 4:236–40. doi:10.4161/org.4.4.7151
7. Oberbarnscheidt MH, Zecher D, Lakkis FG. The innate immune system in transplantation. *Semin Immunol* (2011) 23:264–72. doi:10.1016/j.smim.2011.06.006
8. Zecher D, van Rooijen N, Rothstein DM, Shlomchik WD, Lakkis FG. An innate response to allogeneic nonself mediated by monocytes. *J Immunol* (2009) 183:7810–6. doi:10.4049/jimmunol.0902194
9. Steinmuller D. Passenger leukocytes and the immunogenicity of skin allografts. *J Invest Dermatol* (1980) 75:107–15. doi:10.1111/1523-1747.ep12521331
10. Barker CF, Billingham RE. The role of afferent lymphatics in the rejection of skin homografts. *J Exp Med* (1968) 128:197–221. doi:10.1084/jem.128.1.197
11. Larsen CP, Morris PJ, Austyn JM. Migration of dendritic leukocytes from cardiac allografts into host spleens. A novel pathway for initiation of rejection. *J Exp Med* (1990) 171:307–14. doi:10.1084/jem.171.1.307
12. Brown K, Fidanboylu M, Wong W. Intercellular exchange of surface molecules and its physiological relevance. *Arch Immunol Ther Exp (Warsz)* (2010) 58:263–72. doi:10.1007/s00005-010-0085-y
13. Smyth LA, Herrera OB, Golshayan D, Lombardi G, Lechler RI. A novel pathway of antigen presentation by dendritic and endothelial cells: implications for allorecognition and infectious diseases. *Transplantation* (2006) 82:S15–8. doi:10.1097/01.tp.0000231347.06149.ca
14. Benichou G, Tocco G. The road to transplant tolerance is paved with good dendritic cells: highlights. *Eur J Immunol* (2013) 43:584–8. doi:10.1002/eji.201343361
15. Benichou G. Direct and indirect antigen recognition: the pathways to allograft immune rejection. *Front Biosci* (1999) 4:D476–80. doi:10.2741/A442
16. Leddon SA, Sant AJ. Generation of MHC class II–peptide ligands for CD4 T-cell allorecognition of MHC class II molecules. *Curr Opin Organ Transplant* (2010) 15:505–11. doi:10.1097/MOT.0b013e32833bfc5c
17. Felix NJ, Allen PM. Specificity of T-cell alloreactivity. *Nat Rev Immunol* (2007) 7:942–53. doi:10.1038/nri2200
18. Benichou G, Thomson AW. Direct versus indirect allorecognition pathways: on the right track. *Am J Transplant* (2009) 9:655–6. doi:10.1111/j.1600-6143.2009.02572.x
19. Auchincloss H, Sultan H. Antigen processing and presentation in transplantation. *Curr Opin Immunol* (1996) 8:681–7. doi:10.1016/S0952-7915(96)80086-0
20. Vongwiwatana A, Tasanarong A, Hidalgo LG, Halloran PF. The role of B cells and alloantibody in the host response to human organ allografts. *Immunol Rev* (2003) 196:197–218. doi:10.1046/j.1600-065X.2003.00093.x
21. Whitmire JK, Asano MS, Kaech SM, Sarkar S, Hannum LG, Shlomchik MJ, et al. Requirement of B cells for generating CD4+ T cell memory. *J Immunol* (2009) 182:1868–76. doi:10.4049/jimmunol.0802501
22. Ng Y-H, Oberbarnscheidt MH, Chandramoorthy HCK, Hoffman R, Chalasani G. B cells help alloreactive T cells differentiate into memory T cells. *Am J Transplant* (2010) 10:1970–80. doi:10.1111/j.1600-6143.2010.03223.x
23. Adams AB, Newell KA. B cells in clinical transplantation tolerance. *Semin Immunol* (2012) 24:92–5. doi:10.1016/j.smim.2011.08.019
24. Lee KM, Kim JI, Stott R, Soohoo J, O'Connor MR, Yeh H, et al. Anti-CD45RB/anti-TIM-1-induced tolerance requires regulatory B cells. *Am J Transplant* (2012) 12:2072–8. doi:10.1111/j.1600-6143.2012.04055.x
25. Chesneau M, Michel L, Degauque N, Brouard S. Regulatory B cells and tolerance in transplantation: from animal models to human. *Front Immunol* (2013) 4:497. doi:10.3389/fimmu.2013.00497
26. Lee KM, Stott RT, Zhao G, SooHoo J, Xiong W, Lian MM, et al. TGF-B producing regulatory B cells induce regulatory T cells and promote transplantation tolerance. *Eur J Immunol* (2014) 44:1728–36. doi:10.1002/eji.201344062
27. Ding Q, Yeung M, Camirand G, Zeng Q, Akiba H, Yagita H, et al. Regulatory B cells are identified by expression of TIM-1 and can be induced through TIM-1 ligation to promote tolerance in mice. *J Clin Invest* (2011) 121:3645–56. doi:10.1172/JCI46274
28. Fangmann J, Dalchau R, Fabre JW. Rejection of skin allografts by indirect allorecognition of donor class I major histocompatibility complex peptides. *J Exp Med* (1992) 175:1521–9. doi:10.1084/jem.175.6.1521
29. Liu Z, Sun YK, Xi YP, Maffei A, Reed E, Harris P, et al. Contribution of direct and indirect recognition pathways to T cell alloreactivity. *J Exp Med* (1993) 177:1643–50. doi:10.1084/jem.177.6.1643
30. Benichou G, Takizawa PA, Olson CA, McMillan M, Sercarz EE. Donor major histocompatibility complex (MHC) peptides are presented by recipient MHC molecules during graft rejection. *J Exp Med* (1992) 175:305–8. doi:10.1084/jem.175.1.305
31. Csencsits K, Wood S, Lu G, Magee J, Eichwald E, Chang C-H, et al. Graft rejection mediated by CD4+ T cells via indirect recognition of alloantigen is associated with a dominant Th2 response. *Eur J Immunol* (2005) 35:843–51. doi:10.1002/eji.200425685
32. Plenter RJ, Grazia TJ, Doan AN, Gill RG, Pietra BA. CD4 T cells mediate cardiac xenograft rejection via host MHC Class II. *J Heart Lung Transplant* (2012) 31:1018–24. doi:10.1016/j.healun.2012.05.018
33. Fazilleau N, McHeyzer-Williams LJ, Rosen H, McHeyzer-Williams MG. The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding. *Nat Immunol* (2009) 10:375–84. doi:10.1038/ni.1704
34. Ma CS, Deenick EK, Batten M, Tangye SG. The origins, function, and regulation of T follicular helper cells. *J Exp Med* (2012) 209:1241–53. doi:10.1084/jem.20120994
35. Victora GD, Schwickert TA, Fooksman DR, Kamphorst AO, Meyer-Hermann M, Dustin ML, et al. Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. *Cell* (2010) 143:592–605. doi:10.1016/j.cell.2010.10.032
36. Kurosaki T, Kometani K, Ise W. Memory B cells. *Nat Rev Immunol* (2015) 15:149–59. doi:10.1038/nri3802
37. Steele DJ, Laufer TM, Smiley ST, Ando Y, Grusby MJ, Glimcher LH, et al. Two levels of help for B cell alloantibody production. *J Exp Med* (1996) 183:699–703. doi:10.1084/jem.183.2.699
38. Haynes LD, Jankowska-Gan E, Sheka A, Keller MR, Hernandez-Fuentes MP, Lechler RI, et al. Donor-specific indirect pathway analysis reveals a B-cell-independent signature which reflects outcomes in kidney transplant recipients: indirect pathway in renal transplant patients. *Am J Transplant* (2012) 12:640–8. doi:10.1111/j.1600-6143.2011.03869.x
39. Dragun D, Müller DN, Bräsen JH, Fritsche L, Nieminen-Kelhä M, Dechend R, et al. Angiotensin II type 1–receptor activating antibodies in renal-allograft rejection. *N Engl J Med* (2005) 352:558–69. doi:10.1056/NEJMoa035717

40. Zhang Q, Reed EF. The importance of non-HLA antibodies in transplantation. *Nat Rev Nephrol* (2016) 12:484–95. doi:10.1038/nrneph.2016.88
41. Hippen BE, DeMattos A, Cook WJ, Kew CE, Gaston RS. Association of CD20+ infiltrates with poorer clinical outcomes in acute cellular rejection of renal allografts. *Am J Transplant* (2005) 5:2248–52. doi:10.1111/j.1600-6143.2005.01009.x
42. Du JF, Li Q-Y, Ji XQ, Chen G, Bai X, Zuo F-Y, et al. Inhibition of T-cell expansion caused by inducible costimulator/B7h costimulation blockade in direct allorecognition pathway. *Transplant Proc* (2011) 43:3960–3. doi:10.1016/j.transproceed.2011.09.044
43. Zarkhin V, Kambham N, Li L, Kwok S, Hsieh S-C, Salvatierra O, et al. Characterization of intra-graft B cells during renal allograft rejection. *Kidney Int* (2008) 74:664–73. doi:10.1038/ki.2008.249
44. Ciubotariu R, Liu Z, Colovai AI, Ho E, Itescu S, Ravalli S, et al. Persistent allopeptide reactivity and epitope spreading in chronic rejection of organ allografts. *J Clin Invest* (1998) 101:398–405. doi:10.1172/JCI1117
45. Shiu KY, McLaughlin L, Rebollo-Mesa I, Zhao J, Semik V, Cook HT, et al. B-lymphocytes support and regulate indirect T-cell alloreactivity in individual patients with chronic antibody-mediated rejection. *Kidney Int* (2015) 88:560–8. doi:10.1038/ki.2015.100
46. Clatworthy MR, Watson CJE, Plotnek G, Bardsley V, Chaudhry AN, Bradley JA, et al. B-cell–depleting induction therapy and acute cellular rejection. *N Engl J Med* (2009) 360:2683–5. doi:10.1056/NEJMc0808481
47. Jackson AM, Kraus ES, Orandi BJ, Segev DL, Montgomery RA, Zachary AA. A closer look at rituximab induction on HLA antibody rebound following HLA-incompatible kidney transplantation. *Kidney Int* (2015) 87:409–16. doi:10.1038/ki.2014.261
48. Goetz M, Atreya R, Ghalibafian M, Galle PR, Neurath MF. Exacerbation of ulcerative colitis after rituximab salvage therapy. *Inflamm Bowel Dis* (2007) 13:1365–8. doi:10.1002/ibd.20215
49. Dass S, Vital EM, Emery P. Development of psoriasis after B cell depletion with rituximab. *Arthritis Rheum* (2007) 56:2715–8. doi:10.1002/art.22811
50. Matsushita T, Yanaba K, Bouaziz J-D, Fujimoto M, Tedder T. F. Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. *J Clin Invest* (2008) 118:3420–30. doi:10.1172/JCI36030
51. Van den Hoogen MWF, Kamburova EG, Baas MC, Steenbergen EJ, Florquin S, M. Koenen HJ, et al. Rituximab as induction therapy after renal transplantation: a randomized, double-blind, placebo-controlled study of efficacy and safety: rituximab in renal transplantation. *Am J Transplant* (2015) 15:407–16. doi:10.1111/ajt.13052
52. Macklin PS, Morris PJ, Knight SR. A systematic review of the use of rituximab as induction therapy in renal transplantation. *Transplant Rev* (2015) 29:103–8. doi:10.1016/j.trre.2014.12.001
53. Flowers MED, Martin PJ. How we treat chronic graft-versus-host disease. *Blood* (2015) 125:606–15. doi:10.1182/blood-2014-08-551994
54. Socie G, Ritz J. Current issues in chronic graft-versus-host disease. *Blood* (2014) 124:374–84. doi:10.1182/blood-2014-01-514752
55. Khoder A, Sarvaria A, Alsuliman A, Chew C, Sekine T, Cooper N, et al. Regulatory B-cells are enriched within the IgM memory and transitional subsets in healthy donors but deficient in chronic graft-versus-host disease. *Blood* (2014) 124:2034–45. doi:10.1182/blood-2014-04-571125
56. Yanaba K, Bouaziz J-D, Matsushita T, Magro CM, St Clair EW, Tedder TF. B-lymphocyte contributions to human autoimmune disease. *Immunol Rev* (2008) 223:284–99. doi:10.1111/j.1600-065X.2008.00646.x
57. Yoshizaki A, Miyagaki T, DiLillo DJ, Matsushita T, Horikawa M, Kountikov EI, et al. Regulatory B cells control T-cell autoimmunity through IL-21-dependent cognate interactions. *Nature* (2012) 491:264–8. doi:10.1038/nature11501
58. Mauri C, Ehrenstein MR. The 'short' history of regulatory B cells. *Trends Immunol* (2008) 29:34–40. doi:10.1016/j.it.2007.10.004
59. Rosser EC, Mauri C. Regulatory B cells: origin, phenotype, and function. *Immunity* (2015) 42:607–12. doi:10.1016/j.immuni.2015.04.005
60. Iwata Y, Matsushita T, Horikawa M, DiLillo DJ, Yanaba K, Venturi GM, et al. Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells. *Blood* (2011) 117:530–41. doi:10.1182/blood-2010-07-294249
61. Blair PA, Noreña LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR, et al. CD19+CD24hiCD38hi B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic lupus erythematosus patients. *Immunity* (2010) 32:129–40. doi:10.1016/j.immuni.2009.11.009
62. Yan Y, van der Putten K, Bowen DG, Painter DM, Kohar J, Sharland AF, et al. Postoperative administration of donor B cells induces rat kidney allograft acceptance: lack of association with TH2 cytokine expression in long-term accepted grafts1. *Transplantation* (2002) 73:1123–30. doi:10.1097/00007890-200204150-00020
63. Nouël A, Simon Q, Jamin C, Pers J-O, Hillion S. Regulatory B cells: an exciting target for future therapeutics in transplantation. *Front Immunol* (2014) 5:11. doi:10.3389/fimmu.2014.00011

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 04 February 2016
doi: 10.3389/fimmu.2016.00030

Alloantibody Generation and Effector Function Following Sensitization to Human Leukocyte Antigen

*Michelle J. Hickey, Nicole M. Valenzuela and Elaine F. Reed**

Department of Pathology and Laboratory Medicine, UCLA Immunogenetics Center, University of California Los Angeles, Los Angeles, CA, USA

Allorecognition is the activation of the adaptive immune system to foreign human leukocyte antigen (HLA) resulting in the generation of alloantibodies. Due to a high polymorphism, foreign HLA is recognized by the immune system following transplant, transfusion, or pregnancy resulting in the formation of the germinal center and the generation of long-lived alloantibody-producing memory B cells. Alloantibodies recognize antigenic epitopes displayed by the HLA molecule on the transplanted allograft and contribute to graft damage through multiple mechanisms, including (1) activation of the complement cascade resulting in the formation of the MAC complex and inflammatory anaphylatoxins, (2) transduction of intracellular signals leading to cytoskeletal rearrangement, growth, and proliferation of graft vasculature, and (3) immune cell infiltration into the allograft via FcγR interactions with the FC portion of the antibody. This review focuses on the generation of HLA alloantibody, routes of sensitization, alloantibody specificity, and mechanisms of antibody-mediated graft damage.

Keywords: human leukocyte antigen, allorecognition, HLA antibody, non-HLA antibody, transplant, Fc receptor, complement, endothelium

OPEN ACCESS

Edited by:
Gilles Benichou, Massachusetts General Hospital, USA

Reviewed by:
Nuala Mooney, Centre National de la Recherche Scientifique, France
Emmanuel Zorn, Columbia University Medical Center, USA

***Correspondence:**
Elaine F. Reed
ereed@mednet.ucla.edu

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *18 November 2015*
Accepted: *20 January 2016*
Published: *04 February 2016*

Citation:
Hickey MJ, Valenzuela NM and Reed EF (2016) Alloantibody Generation and Effector Function Following Sensitization to Human Leukocyte Antigen. Front. Immunol. 7:30. doi: 10.3389/fimmu.2016.00030

INTRODUCTION

The immune response is designed to recognize antigens that are distinct from self – termed "non-self" or "altered self" – be they protein, lipid, or carbohydrate. Allorecognition is the activation of the transplant recipient's adaptive immune response to foreign histocompatibility antigens following transplant (1, 2). This review focuses on the recognition of allogeneic human leukocyte antigen (HLA) and non-HLA molecules by the humoral immune response in the context of transplantation. We discuss the generation of alloantibodies, and how they mediate graft injury and rejection.

Human Leukocyte Antigen: Genomic Organization, Structure, Polymorphism, and Function

The human major histocompatibility complex (MHC), located on chromosome 6, is composed of highly polymorphic HLA class I genes (HLA-A, -B, and -C), HLA class II genes (HLA-DR, -DQ, and -DP), non-classical class I genes (HLA-E, -F, and -G), and class I-like genes (MICA and MICB) (3). The HLA class I molecules function to present peptide derived from intracellular antigens to CD8+ T lymphocytes and serve as ligands for receptors on natural killer (NK) cells. The HLA class II molecules present antigens from the extracellular space to CD4+ T cells.

Human leukocyte antigen molecules are heterodimers formed by polypeptides encoded by two distinct genetic loci (**Figure 1**) (3). The HLA class I molecule consists of one heavy β-chain that is non-covalently bound to a β_2-microglobulin (β_2m) light chain at the cell surface for stability. β2m is highly conserved and does not exhibit polymorphism. The HLA class II molecule is composed of two transmembrane glycoprotein chains – an α-chain and β-chain. The α-chain shared by all HLA-DR molecules (DRA1) has limited polymorphism (seven alleles identified to date, with only two different proteins/amino acid sequences; the amino acid polymorphism is V217L in the cytoplasmic domain) and is not a known target of humoral alloresponses. By contrast, both the α- and β-chains of HLA-DP and HLA-DQ are polymorphic (3, 4).

Globular domains of the HLA Class I and II molecules form the peptide-binding cleft that accommodates peptide antigens and interacts with the T cell receptor (TCR). The remarkable polymorphism of HLA Class I and II molecules allows for the presentation of a vast array of antigenic peptides within the human population. Each HLA molecule binds distinct peptides. At the protein level, HLA molecules are defined as antigens by either low-resolution (two digit, serologic level) or high resolution (four digit, allele level) nomenclature (**Table 1**). At the serologic level, there are about 20 HLA-A, 50 HLA-B, 10 HLA-Cw, 18 HLA-DR, and 7 HLA-DQ antigens. However, at the allele level of resolution, the number of HLA antigens in each serogroup is tremendously expanded due to genetic polymorphism within each serogroup – ~2000–3000 distinct proteins for each of HLA-A, B, and C, ~500–2000 for each of DRB1, DQB1, DPB1, and ~10–50 for each of DRB3 (DR52), DRB4 (DR53), DRB5 (DR51), and DQA1(4). Amino acid differences between HLA alleles enable presentation of a diverse array of peptides, and represent the basis for alloimmune recognition of non-self HLA by both T cells and antibodies (3).

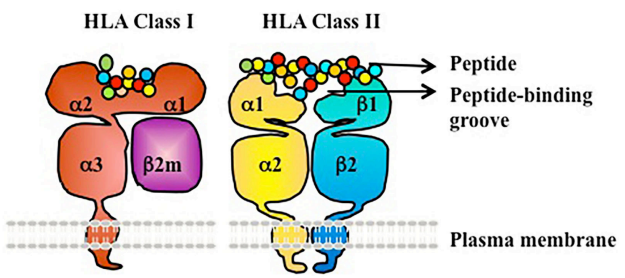


FIGURE 1 | HLA class I and II are heterodimeric transmembrane proteins. HLA Class I is made up of a heavy chain with three globular domains (a1, a2, and a3) non-covalently bound to β2m. HLA Class II is made up of two heavy chains (a-chain and b-chain) each with two globular domains (a1 and a2 or b1 and b2). The a1and a2 domains of HLA class I, and the a1 and b1 domains of HLA class II, make up the peptide-binding groove.

TABLE 1 | Typing of HLA molecules can be at low or high resolution.

Level of typing resolution	Definition	Nomenclature
Low	Serologic/antigen	A2
High	Allele	A*02:01

Mechanisms of Allorecognition and Generation of Allospecific Antibodies

Three distinct pathways of allorecognition have been defined (**Figure 2**). The direct, indirect, and semidirect pathways can occur independently or simultaneously. Activation of the recipient's CD4+ T lymphocytes is a pivotal step in the initiation of the immune response to alloantigen following transplantation leading to downstream activation of cytotoxic CD8+ T lymphocytes and antibody-producing B cells.

Indirect Allorecognition

Indirect allorecognition is the activation of the transplant recipient's CD4+ T cells by alloantigen that is processed and presented in the context of the recipients HLA as occurs with the normal immune response to foreign pathogen (2). Donor antigens, shed by the grafted organ, are processed and presented in the context of self-restricted HLA class II by the recipient's B cells. The recipient's follicular helper CD4+ T cells are then activated to provide help leading to the generation of alloreactive CD8+ effector T cells and antibody-producing B cells (1, 5, 6). The immune response engendered by this pathway is credited with driving chronic rejection and due to lower frequency of T cells with indirect allospecificity, and requirement for antigen processing, is physio-dynamically slower than the response to presentation through the direct pathway (7–10).

Direct Allorecognition

Direct allorecognition is the activation of the transplant recipient's CD4+ T cells by donor HLA:peptide complexes (2). Antigen presentation is mediated by the donor's dendritic cells that are transplanted as passengers with the organ. In the context of inflammatory signals subsequent to the transplantation surgery, the donor's DC, presenting intact donor allo-histocompatibility antigens, migrate to the secondary lymph nodes of the recipient and present antigen to the recipients CD4+ T cells (11, 12). The strength of the immune response elicited by the direct allorecognition pathway correlates to the high frequency of recipient allogeneic T cells that become activated during the first few weeks following transplant (13, 14) mediating acute rejection. The immune response weakens as the passenger DC leave the graft (15, 16). CD4+ T cells activated through the direct pathway are capable of providing help to effector CD8+ T cells, therefore, promoting rejection of the transplanted organ (5). However, activation of B cells and production of alloantibody does not occur in the context of direct allorecognition as there is no cognate interaction between the T helper cell and B cell (5).

Semi-Direct Allorecognition

The semi-direct pathway of allorecognition is presented as a hypothesis to describe events of apparent overlap between the direct and indirect pathways. Evidence from animal models of transplant rejection indicate that indirect allospecific CD4+ T cells can provide help to direct allospecific CD8+ T cells

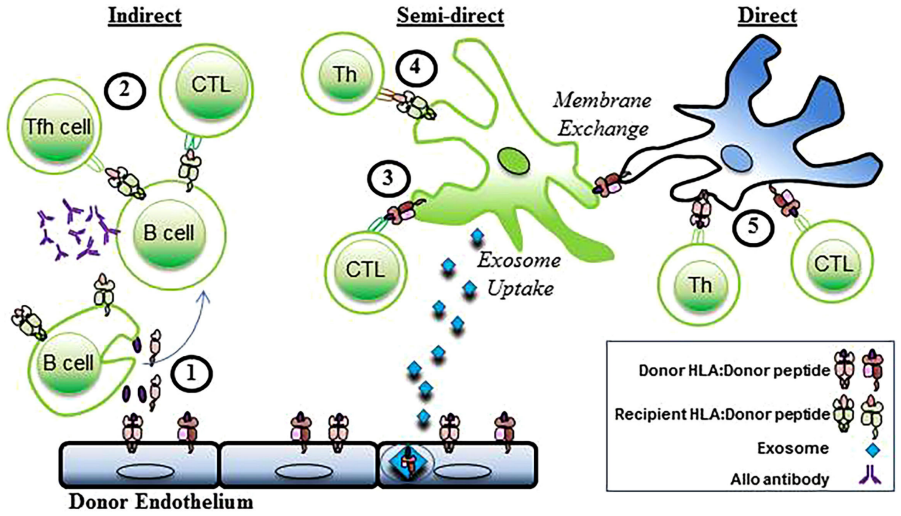


FIGURE 2 | Indirect, semidirect, and direct allorecognition. In the indirect pathway, (1) donor alloantigens are processed by recipient B cells and (2) presented to recipient T follicular helper (T_{FH}) cells and CTL. Alloantibodies are generated when alloreactive B cells interact with CD4+ T cells. The semidirect pathway involves (3) intact donor HLA class I:peptide complexes that are presented on the DC of the recipient (through either membrane exchange or exosome uptake) to recipient CD8+ T cells (CTL). Simultaneously, (4) processed donor peptide is presented in the context of the recipient's HLA class II to the recipient's helper CD4+ T cells (Th). In the direct pathway, (5) allogeneic MHC class I and II antigens are presented to recipient CD4+ and cytotoxic CD8+ T cells (CTL) by donor APCs. Recipient cells, green. Donor Cells, blue.

(17, 18). In principle, this would require a "four cell" model in which CD4+ T cells activated via the indirect pathway by processed alloantigen in the context of self-restricted HLA class II provide help to effector CD8+ T cells activated via the direct pathway by donor passenger APC bearing intact HLA:peptide. The "four cell model" challenges the dogma of the "three cell" or "linked" model whereby the primary mechanism by which activated helper T cells provide help to effector CD8+ T cells is by providing signals to the APC that result in the upregulation of presented antigens (19–21). Helper CD4+ T cells, therefore, "license" APC to more effectively present peptide in the context of HLA class I. The "three cell" model requires that both antigenic determinants recognized by CD4+ and CD8+ T cells be presented on the same APC.

However, the mechanism underling the phenomena of semi-direct allorecognition more likely lies in the exchange of membrane proteins between immune cells (22). After transplantation, the recipients DC acquire intact donor HLA class I:peptide complexes from donor passenger DC or endothelial cells through either cell–cell interactions or by uptake of exosomes containing the antigen that are shed from donor tissue (23, 24). In following, the recipients DC now bears intact donor HLA class I molecules as well as recipient HLA class II molecules, and is capable of stimulating the recipients CD4+ and CD8+ T cells via the indirect and direct pathways in a "three cell" model. Soluble MHC class I can be taken up by DC *in vitro*, and then presented leading to the production of alloantibody (25). The work by Curry et al. implies that soluble alloantigen can be taken up and presented intact to direct B cells, and can simultaneously be processed and presented to indirect CD4+ T cells.

Generation of HLA Alloantibody

Conlon et al. (6) definitively showed that production of alloantibody occurs exclusively through the indirect pathway. In a murine heart allograft model, C57B/6 mice (H-2^b) lacking intact TCRs were transplanted with a BALB/c allograft (H-$2K^d$). Subsequent reconstitution with TCR transgenic CD4+ T cells engineered to specifically recognize an immunodominant BALB/c peptide (H-$2K^d_{54–68}$) processed and presented by MHC Class II resulted in a strong anti-H-$2K^d$ IgG alloantibody response to the allograft. Furthermore, the adoptively transferred CD4+ T cells were found in germinal centers (GC), having acquired the phenotype of T follicular helper (T_{FH}) cells ($CXCR5^+CCR7^-$), and anti-H-$2K^d$ plasma cells were found in the bone marrow. By contrast, direct-pathway CD4+ T cells were unable to provide help to allospecific B cells and alloantibody was not produced.

Formation of the Germinal Center and Generation of Long-Lived Memory

B cells residing in the secondary lymphoid organs can be exposed to small antigens directly through diffusion from the lymphatic system, or to large immune complexed antigens presented by follicular dendritic cells or by macrophages. Regulation of B cell immunity and generation of antibody-secreting plasma cells is primarily dependent on interactions with T_{FH} cells in the GC of the secondary lymphoid organs (26). Antigen-specific T_{FH} cells and antigen-primed B cells migrate to follicular regions of the secondary lymph nodes and form stable contacts through the signal lymphocyte activation molecule (SLAM)-associated protein (SAP) (27). Integrins and the SLAM protein CD84 are also involved in the interaction between T_{FH} cells and pre-GC B cells

(28). These interactions ultimately lead to significant proliferation of antigen-specific B cells and the formation of the GC. CD4+ T cells recognize their cognate peptide antigen presented in HLA class II by the B cell, and provide help through costimulation and cytokines to drive activation and clonal expansion of B cells.

In the GC, B cells make contact with T_{FH} cells that are both transient and stable resulting in selection of B cells that will that enter the long-lived memory component of the immune system (29, 30). Here, through somatic hypermutation, GC B cells that have high antigen affinity differentiate into memory B cells or antibody-producing long-lived plasma cells (31). Activated B cells differentiate into low-affinity antibody-producing plasmablasts, or undergo class-switch recombination and somatic hypermutation to form affinity matured, class-switched memory B cells or plasma cells. Long-lived plasma cells residing in the bone marrow contribute to much of the circulating antigen-specific immunoglobulin and can persist for decades. There is also evidence that memory B cells can be maintained in the circulation without a requirement for continuous antigen exposure (32), ready for rapid recall upon repeated stimulation with antigen.

Recent work has aimed at detection of circulating allospecific memory B cells to predict durable sensitization and anamnestic responses in patients awaiting transplantation. One recent report (33) found that circulating HLA-specific B cells were found only in patients with a history of sensitization, and were detectable in nearly half of such patients. Interestingly, patients with circulating HLA antibodies but no known sensitization event had no detectable circulating B cells. Transfusion also resulted in little to no detectable circulating anti-HLA memory B cells, consistent with the theory that transfusion is a less vigorous sensitizing event compared with pregnancy or transplantation (see below) (34). Snanoudj et al. were able to detect circulating B cells targeting prior donor antigens many decades after transplantation and even after graft removal (33), supporting the paradigm that memory B cells do not require persistent antigen for survival. Finally, and most notably, several patients had detectable HLA antibody secreting B cells in circulation but no detectable circulating antibodies in their sera.

Kinetics of Allorecognition

Direct pathway-activated donor-specific T cells are associated with acute T cell-mediated rejection in renal transplant patients (35). CD4+ T cells isolated from the recipient's pre-transplant blood that were responsive to direct allostimulation with donor cells were also found to be predictive of early post-transplant outcomes (35, 36). However, T cells activated via the direct pathway were found to be predominantly hyporesponsive in patients with transplant coronary artery disease (TCAD), chronic allograft nephropathy (CAN), or chronic rejection following liver transplant indicating that these cells are not contributing to chronic rejection (7, 10, 37). In comparison, T cells primed by the indirect pathway are thought to mediate chronic rejection and are found in high frequency in patients with CAN, and in heart transplant patients with chronic rejection (7, 9, 10, 38).

Notably, T cells stimulated by the indirect allorecognition pathways are also capable of contributing to acute rejection during the early post-transplant period. Circulating allopeptide-reactive T cells were predictive of rejection in heart transplant patients studied during the first 10 weeks post-transplant (39). Furthermore, T cells responsive to allopeptide were found in significant quantities above that found in circulation when isolated from biopsies of graft tissue, suggesting that indirect pathway T cells can contribute directly to acute graft rejection (39).

ALLOANTIBODY ANTIGEN SPECIFICITY

Antibody Structure and Function

Antibodies are heterodimers composed of a light chain and heavy chain encoded by distinct loci on different chromosomes. Each chain contains a constant region that is invariant, and a variable region that undergoes both recombination and somatic hypermutation to yield clonally unique sequences. The variable regions of both heavy and light chain form the antigen binding region ("complementarity determining region"), or paratope, which binds its cognate epitope on the antigen. Human immunoglobulins are divided into five isotypes (IgM, IgD, IgA, IgE, and IgG). Several of these isotypes are further divided into subclasses (IgG1, IgG2, IgG3, and IgG4; IgA1 and IgA2). Antibody isotype and subclass are determined by the constant region.

The subclasses were identified and numbered according to their predominance in circulation rather than order on the genome. Early in the GC reaction, IgM+ B cells class switch first to IgG3 or IgG1, then IgG2, and rarely IgG4 [immunoglobulin sequential class switching is described in Ref. (40, 41)].

Functionally, the subclasses of IgG are distinct. IgG1 has the highest concentration in circulation, and fixes complement well. IgG2 is the next most abundant in circulation and is not an efficient complement fixer. IgG3 is unique with its long hinge region that confers the highest affinity for C1q compared with other subclasses, making it a potent effector [extensively reviewed in Ref. (42)]. However, IgG3 has the shortest half-life in circulation and, being first in order of class switching, has typically the lowest affinity for antigen but is the most potent activator of complement (43). IgG1, IgG3, and IgG4 mostly recognize protein antigens, while IgG2 is canonically efficient at recognizing carbohydrate antigens (in the absence of T cell help) and allergens. It is thought that IgG2 and IgG4 appear later after class switching and affinity maturation, as they have higher affinity for antigen but generally less effective activation of Fc-mediated effector functions, to temporally limit the immune response (41).

Antibodies Are Specific for Antigenic Epitopes

Alloantibodies can be generated against any of the polymorphic loci, i.e., HLA-A, -B, -Cw, DRB1, DRB3 (DR52), DRB4 (DR53), DRB5 (DR51), DQB1, DQA1, DPB1, and DPA1. Antibodies recognize three-dimensional arrangements of amino acids on antigens, called epitopes. Fifteen to 25 amino acid residues form epitopes that are not necessarily adjacent in linear sequence, but are generally within 4 Å (44) (**Figure 3**). Many of the amino acid polymorphisms within HLA molecules lie within and around the peptide-binding groove at exposed residues on the alpha helices of the α1 and α2 chains of HLA class I, and on the α1 and β1

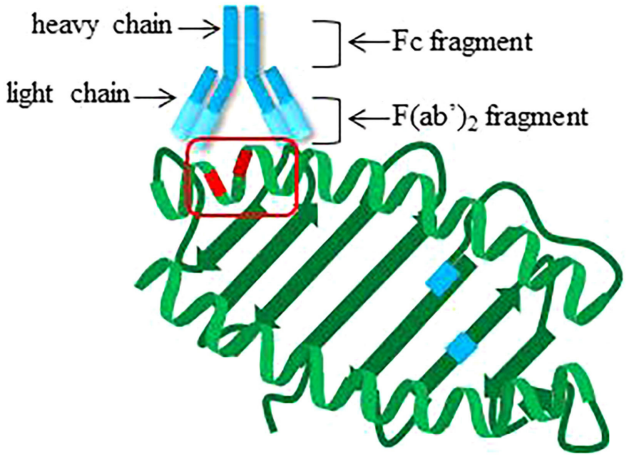


FIGURE 3 | Cartoon structure of antibody engaging epitope on HLA class I. Theoretical locations of polymorphisms impacting peptide binding are indicated in blue on the beta sheet of HLA class I. Locations of polymorphic amino acid residues available for recognition by antibodies on the alpha helices are highlighted in red. *Red box*, epitope.

chains of HLA class II, enabling presentation of diverse peptides. The host–pathogen arms race is believed to have driven this polymorphism to prevent pathogen immune escape and protect populations from epidemics (45). Interestingly, antibody reactivity may also be influenced by the bound peptide (46), which can alter the overall three-dimensional conformation of HLA.

Immunogenicity of HLA

Alloantibodies recognize three-dimensional amino acid epitopes on non-self HLA molecules. Because of the evolution of the HLA system, these epitopes can be shared by many different antigens, leading to broad antibody sensitization after exposure to a limited repertoire of non-self HLA. Antibodies are also sensitive enough to recognize single amino acid differences, resulting in intra-allele antibody production (47). Furthermore, molecular differences between HLA antigens can affect expression levels at the surface of the cell. Below, we describe in greater detail the mechanisms governing these various aspects of alloantibody recognition of epitopes.

Cross-Reactive Antibody Groups

The diversity of HLA has been driven by several genetic processes during positive selection. One major mechanism is gene conversion via homologous recombination. Gene conversion results in large segments of genetic material being shared between alleles, giving rise to multiple proteins with the same or similar amino acid epitopes that can be recognized by the alloantibody response (48). This epitope sharing also results in cross-reactive antibody groups (CREGs), and indeed phylogenetic grouping of HLA based on nucleotide sequences generally mirrors serological cross-reactivity (49). Broad sensitization against many HLA antigens can, thus, occur even when the immune system is only exposed to a single non-self HLA antigen. For example, exposure to HLA-A11 may result in the generation of an antibody that is specific to an epitope carried by multiple HLA antigens belonging to the A1 CREG including HLA-A1, A3, A23, A24, A36, and A80 as well as A11 (50, 51). In another example demonstrating inter-locus reactivity, sensitization to HLA-Cw can lead to antibody production to antigens of HLA-B (52) as HLA-B and HLA-Cw are more closely related to one another than to HLA-A. Similarly, DP antigens share epitopes with DR (53).

An extreme example of broad sensitization is in response to the mutually exclusive public epitopes Bw4 and Bw6, which are present on many different HLA-B (as well as some HLA-A, for Bw4) antigens. An individual exposed to a Bw6 positive antigen, such as B7, may produce antibodies against the Bw6 epitope that react with more than 20 different HLA-B antigens, carried by more than 50% of the population. These determinants, as well as C1 and C2 determinants on HLA-Cw molecules, are critical for NK cell receptor (KIR) binding, and so have likely been conserved through co-evolution of HLA and KIR receptors to prevent loss of self recognition (54).

In seminal work, Parham and McLean (55) described serological reactivity in relationship to known amino acid sequence data, first raising the idea of molecular matching. Differences in epitopes or "structural compatibility" between self and potential donor HLA antigens, also known as "eplets," could portend the likelihood of an antibody response. HLA typing for solid organ transplantation is generally reported at the serologic (two digit) level. However, epitope matching is best accomplished with higher resolution HLA typing such that amino acid sequences that may be different within serologically equivalent groups are defined. Several groups have advocated for the use of structural epitope or eplet matching strategies in organ allocation, over serologic level matching (56, 57). For example, Wiebe et al. reported a lower incidence of *de novo* DSA production in patients who were HLA class II epitope matched (58), and immunogenicity of HLA-DP (59) also appears to be strongly based on epitope recognition.

Allele-Specific Antibodies

Antibodies can be produced against epitopes within antigens that differ from self by as little as one amino acid. Therefore, in addition to antibodies against serologic level HLA molecules, individuals can produce antibodies to other alleles of "self" antigens, if amino acid sequences in key positions are sufficiently disparate. For example, a patient who displays HLA-DQ6 at the serologic level may also be defined through higher resolution typing methods as DQB1*06:01 at the allele level. The patient may become sensitized to other alleles of DQ6 and display allele-specific antibodies to alleles, such as DQB1*06:04, that are distinct from self (60).

Epitopes Formed by Specific DQA1/DQB1 Pairings

It is also possible for individuals to make antibodies against an epitope that is formed by the pairing of specific DQα1 and DQβ1 chains (61). The majority of HLA-DQ reactive antibodies recognize the DQβ chain, while a minority (<20%) bind DQα chain or a combination epitope formed by specific DQα/β pairings (61). Importantly, such antibodies do not produce positive crossmatches against donors who carry only one of the DQα or

DQβ alleles in a different pairing (62), emphasizing the specificity of such antibodies.

Molecular Contributions to Immunogenicity

Differences in antigen availability may necessarily influence immunogenicity. Cell surface expression levels are known to vary among different loci, and even different alleles, of HLA. Certainly, expression of HLA-Cw (63, 64) and HLA-DP (65) is less abundant than proteins of other loci on endothelial cells that make up the vascular walls of the transplanted organ. In addition, HLA-A was found to be more highly expressed than HLA-B in HEK293T cells as it is hypothesized to form a more stable interaction with β2m throughout the terminal region of the alpha 2 domain and the entire alpha 3 domain (66) of the molecules.

Furthermore, Ramsuran et al. recently reported wide variation in mRNA levels between different antigens of HLA-A; for example, individuals homozygous for HLA-A24 had higher expression of HLA-A than those homozygous for HLA-A3, which was attributed to polymorphic CpG sites and increased DNA methylation in the lower expressing alleles (67). Finally, lower expression of HLA-Cw may be the result of reduced affinity for β2 microglobulin, resulting in less stable protein at the cell surface (68, 69), increased degradation of mRNA (63), or differential regulation by miRNA (70). Accordingly, sensitization to HLA-Cw is reportedly less frequent compared with other HLA class I molecules (71).

SENSITIZING EVENTS LEADING TO HLA IMMUNIZATION: ROUTES AND RATES OF HLA SENSITIZATION

Antibody responses to allogeneic HLA molecules can occur after any exposure to non-self tissues, such as transfusion, pregnancy, or transplantation. However, the durability and nature of the sensitization may vary depending on the alloimmunizing event.

Transfusion

Interestingly, the incidence of alloimmunization in the general population with a history of prior transfusion is less than 2% (72, 73), while in comparison Hyun et al. (74) reported that one-third of transplant candidates with a history of transfusion were sensitized. The discrepancy indicates that transplant patients may have a more robust response to sensitization via transfusion, or may have more transfusions compared with non-transplant candidates.

Transfusion alone is considered poorly immunogenic. Sensitization to HLA antigens via transfusion requires very large blood volumes or multiple events to induce persistent HLA allosensitization in otherwise non-sensitized individuals (34). Paradoxically, a protective "transfusion effect" was reported in the early transplantation literature (75, 76), initially suggesting that donor-specific transfusion is immunomodulatory and improved graft outcomes. Animal models have suggested that graft passenger leukocytes are important in this process, thus, providing tolerance prior to transplant (77). However, transplant recipients sensitized by third party transfusion have poorer 1-year survival compared with non-sensitized recipients (78). A modern meta-analysis of that era concluded that higher rates of HLA sensitization are found in patients with a history of transfusion compared with those without, and that there is a neutral to negative effect on allograft outcome after sensitization by transfusion (79).

Pregnancy

Both full-term pregnancy and spontaneous miscarriage induce alloantibodies (80). Anti-paternal alloantibodies appear around or after the 28th week of gestation during pregnancy (80). Sensitized women have higher rates of parity (pregnancy) compared with non-sensitized patients (81). One-third to half of women develop HLA immunization after delivery during their first pregnancy (73, 74, 82), and immunization frequency increases with parity (82). Antibodies to HLA class I were slightly more frequent than those to class II, although both were produced.

Female patients receiving kidney allografts from their male partners or their offspring experienced higher rejection rates (83), pointing to increased immunological risk in women upon re-exposure to paternal antigens on the allograft. Generally, antibodies induced by pregnancy declined in the circulation over time. Even so, post-transplant antibody increases occurred in the pregnancy cohort even decades after the last pregnancy (84).

Transplantation

Transplantation itself is a significant alloimmunizing event (81), and previously non-donor sensitized solid organ transplant recipients develop *de novo* donor-specific HLA antibodies at a rate of about 8–10% in the first year for liver and renal transplants (85, 86), and 15–25% of renal and cardiac transplant by 10 years post-transplant (85, 87, 88). Removal (transplantectomy) of failed renal-allografts appears to stimulate a large increase in circulating DSA (89), whether from increased immune activation in response to surgical trauma, removal of the antigen "sink" provided by the allograft and/or immunosuppression, is unclear.

When evaluating a patient for re-transplantation, it is important to consider the presence of donor-specific alloantibodies that were formed via sensitization to the first allograft in relationship to the donor antigens carried by the second potential donor – the so-called "repeat mismatches." Repeat mismatched donor HLA antigens against which a recipient has preformed alloantibody, particularly to HLA-DR, were found to have a detrimental effect on renal-allograft survival (90–92). While Farney et al. did not uncover a deleterious effect on graft survival of retransplantation with donors who shared mismatches in the presence of alloantibodies with prior donors (93), a more recent study found that re-exposure to mismatched HLA class I antigens increased the risk of early graft loss in renal transplant recipients (94). Typically, repeat HLA mismatches in donors against which a recipient has made antibodies are avoided by transplant programs (95).

Allografts are also used for vascular reconstruction in many forms of congenital heart disease and have been demonstrated to cause persistent sensitization to HLA antigens (96). These findings have implications for those in whom heart transplant is considered late in the clinical course.

Ventricular Assist Devices

Ventricular Assist Devices (VADs) are associated with increased production of HLA antibodies. The current paradigm is that the VAD provides a continual antigenic or inflammatory stimulus that promotes generation of *de novo* HLA antibodies when patients are exposed to blood and/or platelet transfusions or heightens existing HLA antibody levels. In a recent study, we observed that patients implanted with the older pulsatile VAD (BiVad) showed increased HLA sensitization vs. patients implanted with the Heart MateII Axial VAD suggesting the older, pulsatile devices had greater sensitization potential (97).

Natural Antibodies

It has been suggested that HLA antibodies may be formed by means other than the typical routes of sensitization discussed above. Antibodies to HLA are found in non-transfused males at a rate of nearly 50% in one study (98) and often react with a restricted subset of HLA antigens that are uncommon in the general population. There is some evidence pointing to cross-reactivity of pathogens (especially viral) with HLA by T cells (99–102). The abundant viral-specific memory T cell repertoire may, therefore, contribute to alloantibody production. Viral cross-reactivity with HLA may also occur at the protein level. For example, antibodies to HIV-1 may recognize HLA (103), and immunization with the HepB vaccine caused HLA antibody positivity in approximately half of previously negative, healthy adults 1 month after vaccination (104).

It has been proposed that "natural antibodies" against the non-classical HLA-E molecule can cross-react with HLA class I molecules (105). Alternatively, it is possible that antibodies detected are false-positive reactions with denatured antigen, a known limitation of the single-antigen bead assay commonly used to identify HLA antibodies in the sera (106–108). Additional evidence shows these antibodies do not often react with native antigen on cells (108, 109), and the clinical significance and durability of such natural antibody responses remain unclear (110, 111).

Non-HLA Antibodies

Non-HLA antibodies can be directed toward either alloantigens, such as the major histocompatibility complex class I chain-related gene A (MICA) or B (MICB), or tissue-specific autoantigens, such as vimentin, cardiac myosin (CM), collagen V (Col V), agrin and angiotensin II receptor type I (AT1R). Additional non-HLA targets recently identified by Jackson et al. include anti-endothelial cell targets, including endoglin, EGF-like repeats, Fms-like tyrosine kinase-3 ligand, and ICAM-4. The principle antigenic targets of non-HLA antibodies are expressed on cells of the allograft, including endothelium and epithelium. Therefore, donor cells are in direct contact with the recipients circulating peripheral blood lymphocytes, and have been shown to be the major immunological targets for the pathogenesis of allograft rejection. Prevalence of anti-endothelial cell antibodies (AECA) among renal recipients was nearly one quarter in pre-transplant sera (112). AECAs correlated with post-transplant HLA DSA and AMR. Sun et al. observed that anti-endothelial cell antibodies were found in patients pre-transplant, but that they did not correlate with outcome or rejection; by contrast, *de novo* development of AECAs was significantly associated with early and severe acute rejection, but not C4d (113). AECA were implicated as the cause of acute antibody-mediated rejection (AMR) in 30% of heart transplant recipients without DSA to HLA (114).

MECHANISMS OF GRAFT DAMAGE BY HLA ANTIBODIES

High-titered pre-transplant DSA directed against HLA class I antigens can cause catastrophic hyperacute rejection and immediate graft loss (115), whereas high titer class II DSA mediate graft rejection 2–4 days after transplant, upon re-expression of HLA class II antigens on the endothelium of the allograft (116–118). By contrast, pre-transplant DSA of low titer are often associated with development of acute AMR during the first 3 months after transplantation and/or lower long-term graft survival (119). If left untreated, patients with AMR are at risk of graft loss and/or markedly shortened overall graft survival time. Patients producing *de novo* anti-HLA antibodies against their donor following transplantation are also at increased risk of graft failure unless their response can be controlled or abrogated (120).

There are three major effector functions carried out by antibodies that can impact the graft. First, bivalent IgG can dimerize or crosslink its target upon binding. Collective studies indicate that IgG binding to HLA agonistically crosslinks HLA molecules and triggers downstream activation of the target cells. Second, antibodies can activate the classical complement cascade through binding to the Fc fragment to trigger production of potent anaphylatoxins, chemoattractants, opsonins, and cell-damaging factors. Thirdly, HLA IgG bound to target cells can engage Fc receptors on myeloid and lymphoid cells, to employ a host of Fc receptor-mediated effector functions, including antibody-dependent cell cytotoxicity, antibody-dependent phagocytosis, and augment recruitment. These effector functions work in concert, and there is substantial interplay between them, as we will discuss below.

HLA Antibody-Induced Signaling in Graft Vascular Cells

Antibodies are capable of agonistically crosslinking their protein targets at the cell surface [recently reviewed in Ref. (121)]. In vascular cells, crosslinking of HLA induces intracellular signaling cascades that lead to functional changes, such as increased cell migration, cytoskeletal rearrangement, growth and proliferation, endothelial activation and exocytosis, and increased recruitment of leukocytes. These functional changes parallel the histological findings in clinical AMR, including microvascular inflammation, endothelial dysfunction, expansion of the neointima, and infiltration of mononuclear cells (**Table 2**).

HLA class I and II do not have intrinsic kinase activity and, therefore, partner with other proteins to transduce intracellular signals. Ligation of HLA class I with antibodies increases its

TABLE 2 | Function of HLA antibodies leading to histological manifestations of AMR.

Histological manifestation	Antibody function
C4d deposition	Activation of complement
Endothelial cell swelling	HLA crosslinking leading to cytoskeletal changes
Mononuclear cell infiltration	HLA crosslinking increases P-selectin and chemokines, monocyte, and neutrophil adherence Antibody Fc regions interact with FcγRs
Neointimal thickening	HLA crosslinking increases endothelial and smooth muscle cell proliferation and migration

association with integrin β4 which, in turn, activates intracellular signaling cascades (122). Integrin β4 is an important cell adhesion protein regulating cell adhesion, proliferation, migration, and survival. Blockade of integrin β4 impairs HLA antibody-stimulated signal transduction. Protein(s) that partner with HLA class II to transduce signaling are not yet reported. However, ligation of either HLA class I or class II with antibodies activates mammalian target of rapamycin (mTOR) signaling through the SRC/FAK–PI3K–AKT pathway and increases Akt-dependent cell survival signaling, through upregulation of Bcl-2 and HO-1 (123–125). Furthermore, activation of key signaling proteins in endothelium, including S6K, S6RP, and ERK, demonstrated in human cardiac allografts with AMR and in murine models of MHC antibody-mediated injury (126–128) suggests pro-survival signals that may increase endothelial persistence, stress fiber formation (129, 130), and resistance to complement-induced cell death (125), contributing to neointimal formation during chronic rejection. Additional work by Galvani et al. points to a direct effect of MHC antibodies on smooth muscle cells. Crosslinking of HLA I with antibodies provokes mitogenic signaling through matrix metalloproteinases *in vitro*, and contributes to neointimal thickening of human arterial grafts *in vivo* in murine recipients (131, 132). An additional feature of alloantibody crosslinking of HLA is increased intracellular calcium levels, leading to exocytosis of endothelial Weibel–Palade body vesicles and increased cell surface P-selectin (133–135). P-selectin captures neutrophils and monocytes (133, 134), facilitating recruitment of immune cells into the allograft.

Complement

The complement system is an ancient form of innate immunity that relies on proteolytic cleavage of active components. Complement proteins are always present in the circulation, but become rapidly activated upon exposure to target molecules. There are three main pathways of complement, which differ by the activating stimulus. The lectin pathway becomes activated upon recognition by mannose binding lectin (MBL) of pathogen-specific glycan residues on the surface of bacteria, fungi, and viruses. The alternative pathway of complement is initiated at the surface of non-host cells due to the presence of such factors as lipopolysaccharides on Gram-negative bacteria, zymosans on fungi and yeast, and other pathogen-associated molecules. Complexed human immunoglobulin has also been shown to activate the alternative pathway. The classical complement pathway is initiated exclusively by antigen-bound antibody through binding of the Fc portion of certain isotypes and subclasses to C1q. All of these pathways rely on sequential enzymatic reactions that produce active split products involved in inflammation, and all of these pathways converge on the terminal component C5.

Activation of complement by antibodies was one of the earliest methods used to detect donor-specific HLA antibodies, and positive cytotoxic crossmatch is still often considered to be a contraindication to transplant, as antibodies detected by this method can mediate hyperacute rejection of solid organ transplants (115). Although the end result of complement activation, namely deposition of MAC and cell cytotoxicity, has been a focus, it is now thought to be a rare event (136). Endothelial cells express complement regulatory proteins (CD55/DAF, CD59, Crry) that antagonize complement activation by inactivating split products. C3d and C4d are generated by such inhibitory receptors and mark early complement activation. Attention has turned to the activity and predictive value of other complement proteins. Products of complement activation, in particular C4d, have proven histological utility in detecting donor-specific antibody bound to the graft (137, 138). Other split products, including C4a, C3a, and C3b, are potent inflammatory signals that promote immune cell recruitment and opsonization.

FcγR-Bearing Immune Cells

Many cells express surface receptors that can interact with the constant region heavy chain (Fc) of antibodies. The human Fc receptor system consists of several classes that can bind to IgG (FcγR, CD64, CD32, CD16), IgA (FcαR, CD89), and IgE (FcεR, CD23). The human receptor for IgM (FcμR) had been elusive until relatively recently (139). Fc receptors serve to bridge the humoral and cellular arms of the immune system, and provide innate immune cells with a target, and are critical for a variety of functions, including antibody-dependent cell-mediated phagocytosis (ADCP), antibody-dependent cell-mediated cytotoxicity (ADCC), cell–cell tethering and degranulation.

Given that IgG is thought to be the most clinically relevant isotype of HLA antibodies, we will focus on Fc-gamma receptors (FcγR) that bind to this isotype of immunoglobulins. FcγRs are expressed broadly in both the myeloid and lymphoid compartments. There are three major classes of FcγRs, FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16). FcγRII and FcγRIII are further composed of several functionally disparate isoforms, most of which are dimorphic in the human population (140, 141). Polymorphisms in human FcγRs influence susceptibility to autoimmune disease and response to anti-tumor therapeutics (142–146), and may also influence susceptibility of transplant recipients to rejection (147, 148), although a thorough evaluation of the role of different FcγR alleles in antibody-mediated transplant rejection has not been reported.

Due to their lower affinity, the majority of FcγRs do not bind monomeric IgG very efficiently. Only the high-affinity FcγRI (CD64) is the exception, and cells with this receptor have been shown to carry monomeric IgG in circulation. FcγRs do bind to antigen-associated IgG, however, such as in immune complexes or immobilized on a (cell) surface. Once bound, FcγRs become

crosslinked as they physically colocalize at high antibody-antigen density. This promotes intracellular signaling in the FcγR-bearing cell leading to activation and maturation, and mediates effector functions such as phagocytosis or cytotoxicity.

The relevance of FcγR-bearing innate immune cells to antibody-mediated graft injury is reflected in the diagnostic criteria and histological manifestations of AMR. For example, infiltration of CD68+ macrophages is included in the AMR diagnostic criteria in cardiac transplantation (149), where macrophage staining is found intravascularly (150, 151). Indeed, increased macrophage burden is correlative with worse prognosis (152). Although not currently included in the AMR diagnostic criteria for renal transplantation, macrophage infiltration during rejection is also predictive of worse outcome in kidney allografts (153, 154). Our recent studies are consistent with these clinical findings and show that monocyte recruitment to HLA-Ab-activated endothelium is mediated by HLA-induced Weibel–Palade exocytosis and P-selectin expression (134). Blockade of P-selectin potently inhibited leukocyte recruitment to the allograft during AMR underscoring its therapeutic potential (134). Furthermore, HLA-Ab augmented monocyte recruitment by the interaction of monocyte FcγRs with the Fc portion of the HLA-Abs (135). This interaction was IgG subclass dependent and influenced by monocyte FcγRIIa allelic variants. Monocytes from donors carrying the high-affinity FcγRIIa-H131 allele had greater FcγR-dependent adhesion to ECs activated with HLA-Abs of both IgG1 and IgG2 subclasses compared with monocytes expressing only FcγRIIa-R131. These results are clinically relevant and suggest that recipients producing DSA and carrying high-affinity FcγR alleles may be pre-disposed to acute AMR accompanied by increased monocyte infiltration.

Summary

Taken together, antibodies to donor proteins, including HLA, can cause graft damage through three major mechanisms, including direct activation of endothelial, smooth muscle, and epithelial cells to promote proliferation and inflammation; activation of the complement system to generate inflammatory split products; and engagement of FcγRs on NK cells, monocytes, and neutrophils.

CONCLUSION

Allorecognition by the humoral immune system results in formation of antibodies to HLA and a variety of non-HLA proteins, and occurs after exposure to non-self tissues through pregnancy, transfusion, or transplantation. Alloantibody formation is dependent upon T cell interactions and is primarily driven by indirect allorecognition by T cells. In addition, "natural" antibodies or anti-viral antibodies may cross-react with HLA, although the clinical significance of such antibodies is not clear. Antibodies to donor HLA mediate allograft injury through Fc-dependent as well as Fc-independent mechanisms, which closely reflect the diagnostic criteria for AMR. Non-HLA antibodies can be against polymorphic proteins, such as MICA, or against autoantibodies, and also associate with worse graft outcome, although their etiology is less clear than for HLA DSA.

AUTHOR CONTRIBUTIONS

MH was responsible for outlining, research, and writing of the manuscript. NV was responsible for outlining, research, and writing of the manuscript. ER reviewed and critically examined the manuscript and shaped its final version.

FUNDING

NV is supported by a Histocompatibility Laboratory Director Trainee Fellowship from the UCLA Department of Pathology. ER is supported by NIH RO1 AI042819.

REFERENCES

1. Steele DJ, Laufer TM, Smiley ST, Ando Y, Grusby MJ, Glimcher LH, et al. Two levels of help for B cell alloantibody production. *J Exp Med* (1996) **183**(2):699–703. doi:10.1084/jem.183.2.699
2. Afzali B, Lombardi G, Lechler RI. Pathways of major histocompatibility complex allorecognition. *Curr Opin Organ Transplant* (2008) **13**(4):438–44. doi:10.1097/MOT.0b013e328309ee31
3. Zhang JQ HM, Valenzuela NM, Zhang X, Lan JH, Cecka M, Reed EF. *Histocompatibility and Immunogenetics for Solid Organ Transplantation*. London: Springer Science+Business Media (2015).
4. IMGT/HLA. Available from: http://www.ebi.ac.uk/ipd/imgt/hla/stats.html.
5. Taylor AL, Negus SL, Negus M, Bolton EM, Bradley JA, Pettigrew GJ. Pathways of helper CD4 T cell allorecognition in generating alloantibody and CD8 T cell alloimmunity. *Transplantation* (2007) **83**(7):931–7. doi:10.1097/01.tp.0000257960.07783.e3
6. Conlon TM, Saeb-Parsy K, Cole JL, Motallebzadeh R, Qureshi MS, Rehakova S, et al. Germinal center alloantibody responses are mediated exclusively by indirect-pathway CD4 T follicular helper cells. *J Immunol* (2012) **188**(6):2643–52. doi:10.4049/jimmunol.1102830
7. Hornick PI, Mason PD, Yacoub MH, Rose ML, Batchelor R, Lechler RI. Assessment of the contribution that direct allorecognition makes to the progression of chronic cardiac transplant rejection in humans. *Circulation* (1998) **97**(13):1257–63. doi:10.1161/01.CIR.97.13.1257
8. Benichou G, Valujskikh A, Heeger PS. Contributions of direct and indirect T cell alloreactivity during allograft rejection in mice. *J Immunol* (1999) **162**(1):352–8.
9. Hornick PI, Mason PD, Baker RJ, Hernandez-Fuentes M, Frasca L, Lombardi G, et al. Significant frequencies of T cells with indirect anti-donor specificity in heart graft recipients with chronic rejection. *Circulation* (2000) **101**(20):2405–10. doi:10.1161/01.CIR.101.20.2405
10. Baker RJ, Hernandez-Fuentes MP, Brookes PA, Chaudhry AN, Cook HT, Lechler RI. Loss of direct and maintenance of indirect alloresponses in renal allograft recipients: implications for the pathogenesis of chronic allograft nephropathy. *J Immunol* (2001) **167**(12):7199–206. doi:10.4049/jimmunol.167.12.7199
11. Richters CD, van Gelderop E, du Pont JS, Hoekstra MJ, Kreis RW, Kamperdijk EW. Migration of dendritic cells to the draining lymph node after allogeneic or congeneic rat skin transplantation. *Transplantation* (1999) **67**(6):828–32. doi:10.1097/00007890-199903270-00008
12. Sandner SE, Salama AD, Houser SL, Palmer E, Turka LA, Sayegh MH. New TCR transgenic model for tracking allospecific CD4 T-cell activation and tolerance in vivo. *Am J Transplant* (2003) **3**(10):1242–50. doi:10.1046/j.1600-6143.2003.00220.X
13. Kaminski E, Hows J, Man S, Brookes P, Mackinnon S, Hughes T, et al. Prediction of graft versus host disease by frequency analysis of cytotoxic T cells after unrelated donor bone marrow transplantation. *Transplantation* (1989) **48**(4):608–13.

14. Schwarer AP, Jiang YZ, Deacock S, Brookes PA, Barrett AJ, Goldman JM, et al. Comparison of helper and cytotoxic antirecipient T cell frequencies in unrelated bone marrow transplantation. *Transplantation* (1994) **58**(11):1198–203. doi:10.1097/00007890-199412270-00011
15. Lechler RI, Batchelor JR. Immunogenicity of retransplanted rat kidney allografts. Effect of inducing chimerism in the first recipient and quantitative studies on immunosuppression of the second recipient. *J Exp Med* (1982) **156**(6):1835–41. doi:10.1084/jem.156.6.1835
16. Braun MY, McCormack A, Webb G, Batchelor JR. Mediation of acute but not chronic rejection of MHC-incompatible rat kidney grafts by alloreactive CD4 T cells activated by the direct pathway of sensitization. *Transplantation* (1993) **55**(1):177–82. doi:10.1097/00007890-199301000-00033
17. Lee RS, Grusby MJ, Glimcher LH, Winn HJ, Auchincloss H Jr. Indirect recognition by helper cells can induce donor-specific cytotoxic T lymphocytes in vivo. *J Exp Med* (1994) **179**(3):865–72. doi:10.1084/jem.179.3.865
18. Wise MP, Bemelman F, Cobbold SP, Waldmann H. Linked suppression of skin graft rejection can operate through indirect recognition. *J Immunol* (1998) **161**(11):5813–6.
19. Bennett SR, Carbone FR, Karamalis F, Miller JF, Heath WR. Induction of a CD8+ cytotoxic T lymphocyte response by cross-priming requires cognate CD4+ T cell help. *J Exp Med* (1997) **186**(1):65–70. doi:10.1084/jem.186.1.65
20. Ridge JP, Di Rosa F, Matzinger P. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature* (1998) **393**(6684):474–8. doi:10.1038/30989
21. Schoenberger SP, Toes RE, van der Voort EI, Offringa R, Melief CJ. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* (1998) **393**(6684):480–3. doi:10.1038/31002
22. Smyth LA, Afzali B, Tsang J, Lombardi G, Lechler RI. Intercellular transfer of MHC and immunological molecules: molecular mechanisms and biological significance. *Am J Transplant* (2007) **7**(6):1442–9. doi:10.1111/j.1600-6143.2007.01816.x
23. Herrera OB, Golshayan D, Tibbott R, Salcido Ochoa F, James MJ, Marelli-Berg FM, et al. A novel pathway of alloantigen presentation by dendritic cells. *J Immunol* (2004) **173**(8):4828–37. doi:10.4049/jimmunol.173.8.4828
24. Smyth LA, Herrera OB, Golshayan D, Lombardi G, Lechler RI. A novel pathway of antigen presentation by dendritic and endothelial cells: implications for allorecognition and infectious diseases. *Transplantation* (2006) **82**(1 Suppl):S15–8. doi:10.1097/01.tp.0000231347.06149.ca
25. Curry AJ, Pettigrew GJ, Negus MC, Easterfield AJ, Young JL, Bolton EM, et al. Dendritic cells internalise and re-present conformationally intact soluble MHC class I alloantigen for generation of alloantibody. *Eur J Immunol* (2007) **37**(3):696–705. doi:10.1002/eji.200636543
26. McHeyzer-Williams LJ, Pelletier N, Mark L, Fazilleau N, McHeyzer-Williams MG. Follicular helper T cells as cognate regulators of B cell immunity. *Curr Opin Immunol* (2009) **21**(3):266–73. doi:10.1016/j.coi.2009.05.010
27. Qi H, Cannons JL, Klauschen F, Schwartzberg PL, Germain RN. SAP-controlled T-B cell interactions underlie germinal centre formation. *Nature* (2008) **455**(7214):764–9. doi:10.1038/nature07186
28. Cannons JL, Qi H, Lu KT, Dutta M, Gomez-Rodriguez J, Cheng J, et al. Optimal germinal center responses require a multistage T cell: B cell adhesion process involving integrins, SLAM-associated protein, and CD84. *Immunity* (2010) **32**(2):253–65. doi:10.1016/j.immuni.2010.01.010
29. Allen CD, Okada T, Cyster JG. Germinal-center organization and cellular dynamics. *Immunity* (2007) **27**(2):190–202. doi:10.1016/j.immuni.2007.07.009
30. Allen CD, Okada T, Tang HL, Cyster JG. Imaging of germinal center selection events during affinity maturation. *Science* (2007) **315**(5811):528–31. doi:10.1126/science.1136736
31. Phan TG, Paus D, Chan TD, Turner ML, Nutt SL, Basten A, et al. High affinity germinal center B cells are actively selected into the plasma cell compartment. *J Exp Med* (2006) **203**(11):2419–24. doi:10.1084/jem.20061254
32. Maruyama M, Lam KP, Rajewsky K. Memory B-cell persistence is independent of persisting immunizing antigen. *Nature* (2000) **407**(6804):636–42. doi:10.1038/35036600
33. Snanoudj R, Claas FH, Heidt S, Legendre C, Chatenoud L, Candon S. Restricted specificity of peripheral alloreactive memory B cells in HLA-sensitized patients awaiting a kidney transplant. *Kidney Int* (2015) **87**(6):1230–40. doi:10.1038/ki.2014.390
34. Scornik JC, Meier-Kriesche HU. Blood transfusions in organ transplant patients: mechanisms of sensitization and implications for prevention. *Am J Transplant* (2011) **11**(9):1785–91. doi:10.1111/j.1600-6143.2011.03705.x
35. Bestard O, Crespo E, Stein M, Lucia M, Roelen DL, de Vaal YJ, et al. Cross-validation of IFN-gamma Elispot assay for measuring alloreactive memory/effector T cell responses in renal transplant recipients. *Am J Transplant* (2013) **13**(7):1880–90. doi:10.1111/ajt.12285
36. Hricik DE, Rodriguez V, Riley J, Bryan K, Tary-Lehmann M, Greenspan N, et al. Enzyme linked immunosorbent spot (ELISPOT) assay for interferon-gamma independently predicts renal function in kidney transplant recipients. *Am J Transplant* (2003) **3**(7):878–84. doi:10.1034/j.1600-6143.2003.00132.x
37. de Haan A, van den Berg AP, Hepkema BG, van Dijk E, Haagsma EB, The TH, et al. Donor-specific hyporeactivity after liver transplantation: prominent decreases in donor-specific cytotoxic T lymphocyte precursor frequencies independent of changes in helper T lymphocyte precursor frequencies or suppressor cell activity. *Transplantation* (1998) **66**(4):516–22. doi:10.1097/00007890-199808270-00017
38. Vella JP, Spadafora-Ferreira M, Murphy B, Alexander SI, Harmon W, Carpenter CB, et al. Indirect allorecognition of major histocompatibility complex allopeptides in human renal transplant recipients with chronic graft dysfunction. *Transplantation* (1997) **64**(6):795–800. doi:10.1097/00007890-199712270-00033
39. Liu Z, Colovai AI, Tugulea S, Reed EF, Fisher PE, Mancini D, et al. Indirect recognition of donor HLA-DR peptides in organ allograft rejection. *J Clin Invest* (1996) **98**(5):1150–7. doi:10.1172/JCI118898
40. Jackson KJ, Wang Y, Collins AM. Human immunoglobulin classes and subclasses show variability in VDJ gene mutation levels. *Immunol Cell Biol* (2014) **92**(8):729–33. doi:10.1038/icb.2014.44
41. van Zelm MC. B cells take their time: sequential IgG class switching over the course of an immune response? *Immunol Cell Biol* (2014) **92**(8):645–6. doi:10.1038/icb.2014.48
42. Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: from structure to effector functions. *Front Immunol* (2014) **5**:520. doi:10.3389/fimmu.2014.00520
43. Devey ME, Bleasdale-Barr KM, Bird P, Amlot PL. Antibodies of different human IgG subclasses show distinct patterns of affinity maturation after immunization with keyhole limpet haemocyanin. *Immunology* (1990) **70**(2):168–74.
44. Duquesnoy RJ. A structurally based approach to determine HLA compatibility at the humoral immune level. *Hum Immunol* (2006) **67**(11):847–62. doi:10.1016/j.humimm.2006.08.073
45. Hertz T, Nolan D, James I, John M, Gaudieri S, Phillips E, et al. Mapping the landscape of host-pathogen coevolution: HLA class I binding and its relationship with evolutionary conservation in human and viral proteins. *J Virol* (2011) **85**(3):1310–21. doi:10.1128/JVI.01966-10
46. Mulder A, Eijsink C, Kester MG, Franke ME, Kardol MJ, Heemskerk MH, et al. Impact of peptides on the recognition of HLA class I molecules by human HLA antibodies. *J Immunol* (2005) **175**(9):5950–7. doi:10.4049/jimmunol.175.9.5950
47. Nakayama S, Kawaguchi G, Karaki S, Nagao T, Uchida H, Kashiwase K, et al. Effect of single amino acid substitution at residue 167 of HLA-B51 on binding of antibodies and recognition of T cells. *Hum Immunol* (1994) **39**(3):211–9. doi:10.1016/0198-8859(94)90262-3
48. Rodey GE, Neylan JF, Whelchel JD, Revels KW, Bray RA. Epitope specificity of HLA class I alloantibodies. I. Frequency analysis of antibodies to private versus public specificities in potential transplant recipients. *Hum Immunol* (1994) **39**(4):272–80. doi:10.1016/0198-8859(94)90270-4
49. McKenzie LM, Pecon-Slattery J, Carrington M, O'Brien SJ. Taxonomic hierarchy of HLA class I allele sequences. *Genes Immun* (1999) **1**(2):120–9. doi:10.1038/sj.gene.6363648
50. Wade JA, Hurley CK, Takemoto SK, Thompson J, Davies SM, Fuller TC, et al. HLA mismatching within or outside of cross-reactive groups (CREGs) is associated with similar outcomes after unrelated hematopoietic stem cell transplantation. *Blood* (2007) **109**(9):4064–70. doi:10.1182/blood-2006-06-032193
51. Marrari M, Mostecki J, Mulder A, Claas F, Balazs I, Duquesnoy RJ. Human monoclonal antibody reactivity with human leukocyte antigen class I epitopes

defined by pairs of mismatched eplets and self-eplets. *Transplantation* (2010) **90**(12):1468–72. doi:10.1097/TP.0b013e3182007b74
52. Lomago J, Jelenik L, Zern D, Howe J, Martell J, Zeevi A, et al. How did a patient who types for HLA-B*4403 develop antibodies that react with HLA-B*4402? *Hum Immunol* (2010) **71**(2):176–8. doi:10.1016/j.humimm.2009.11.013
53. Billen EV, Christiaans MH, Doxiadis II, Voorter CE, van den Berg-Loonen EM. HLA-DP antibodies before and after renal transplantation. *Tissue Antigens* (2010) **75**(3):278–85. doi:10.1111/j.1399-0039.2009.01428.x
54. Norman PJ, Hollenbach JA, Nemat-Gorgani N, Guethlein LA, Hilton HG, Pando MJ, et al. Co-evolution of human leukocyte antigen (HLA) class I ligands with killer-cell immunoglobulin-like receptors (KIR) in a genetically diverse population of sub-Saharan Africans. *PLoS Genet* (2013) **9**(10):e1003938. doi:10.1371/journal.pgen.1003938
55. Parham P, McLean J. Characterization, evolution, and molecular basis of a polymorphic antigenic determinant shared by HLA-A and B products. *Hum Immunol* (1980) **1**(2):131–9. doi:10.1016/0198-8859(80)90100-7
56. Tambur AR, Buckingham M, McDonald L, Luo X. Development of donor-specific and non-donor-specific HLA-DP antibodies post-transplant: the role of epitope sharing and epitope matching. *Clin Transpl* (2006):399–404.
57. Claas FH, Rahmel A, Doxiadis II. Enhanced kidney allocation to highly sensitized patients by the acceptable mismatch program. *Transplantation* (2009) **88**(4):447–52. doi:10.1097/TP.0b013e3181b04a5f
58. Wiebe C, Pochinco D, Blydt-Hansen TD, Ho J, Birk PE, Karpinski M, et al. Class II HLA epitope matching-A strategy to minimize de novo donor-specific antibody development and improve outcomes. *Am J Transplant* (2013) **13**(12):3114–22. doi:10.1111/ajt.12478
59. Laux G, Mansmann U, Deufel A, Opelz G, Mytilineos J. A new epitope-based HLA-DPB matching approach for cadaver kidney retransplants. *Transplantation* (2003) **75**(9):1527–32. doi:10.1097/01.TP.0000061759.57702.8A
60. Muro M, Gonzalez-Soriano MJ, Salgado G, Lopez R, Boix F, Lopez M, et al. Specific "intra-allele" and "intra-broad antigen" human leukocyte antigen alloantibodies in kidney graft transplantation. *Hum Immunol* (2010) **71**(9):857–60. doi:10.1016/j.humimm.2010.05.018
61. Tambur AR, Leventhal JR, Friedewald JJ, Ramon DS. The complexity of human leukocyte antigen (HLA)-DQ antibodies and its effect on virtual crossmatching. *Transplantation* (2010) **90**(10):1117–24. doi:10.1097/00007890-201007272-00536
62. Tambur AR, Leventhal JR, Zitzner JR, Walsh RC, Friedewald JJ. The DQ barrier: improving organ allocation equity using HLA-DQ information. *Transplantation* (2013) **95**(4):635–40. doi:10.1097/TP.0b013e318277b30b
63. McCutcheon JA, Gumperz J, Smith KD, Lutz CT, Parham P. Low HLA-C expression at cell surfaces correlates with increased turnover of heavy chain mRNA. *J Exp Med* (1995) **181**(6):2085–95. doi:10.1084/jem.181.6.2085
64. Apps R, Meng Z, Del Prete GQ, Lifson JD, Zhou M, Carrington M. Relative expression levels of the HLA class-I proteins in normal and HIV-infected cells. *J Immunol* (2015) **194**(8):3594–600. doi:10.4049/jimmunol.1403234
65. Muczynski KA, Ekle DM, Coder DM, Anderson SK. Normal human kidney HLA-DR-expressing renal microvascular endothelial cells: characterization, isolation, and regulation of MHC class II expression. *J Am Soc Nephrol* (2003) **14**(5):1336–48. doi:10.1097/01.ASN.0000061778.08085.9F
66. Dellgren C, Nehlin JO, Barington T. Cell surface expression level variation between two common Human Leukocyte Antigen alleles, HLA-A2 and HLA-B8, is dependent on the structure of the C terminal part of the alpha 2 and the alpha 3 domains. *PLoS One* (2015) **10**(8):e0135385. doi:10.1371/journal.pone.0135385
67. Ramsuran V, Kulkarni S, O'Huigin C, Yuki Y, Augusto DG, Gao X, et al. Epigenetic regulation of differential HLA-A allelic expression levels. *Hum Mol Genet* (2015) **24**(15):4268–75. doi:10.1093/hmg/ddv158
68. Giacomini P, Beretta A, Nicotra MR, Ciccarelli G, Martayan A, Cerboni C, et al. HLA-C heavy chains free of beta2-microglobulin: distribution in normal tissues and neoplastic lesions of non-lymphoid origin and interferon-gamma responsiveness. *Tissue Antigens* (1997) **50**(6):555–66. doi:10.1111/j.1399-0039.1997.tb02913.x
69. Sibilio L, Martayan A, Setini A, Lo Monaco E, Tremante E, Butler RH, et al. A single bottleneck in HLA-C assembly. *J Biol Chem* (2008) **283**(3):1267–74. doi:10.1074/jbc.M708068200
70. Kulkarni S, Savan R, Qi Y, Gao X, Yuki Y, Bass SE, et al. Differential microRNA regulation of HLA-C expression and its association with HIV control. *Nature* (2011) **472**(7344):495–8. doi:10.1038/nature09914
71. Bryan CF, Luger AM, Smith JL, Warady BA, Wakefield M, Schadde E, et al. Sharing kidneys across donor-service area boundaries with sensitized candidates can be influenced by HLA C. *Clin Transplant* (2010) **24**(1):56–61. doi:10.1111/j.1399-0012.2009.01167.x
72. Triulzi DJ, Kleinman S, Kakaiya RM, Busch MP, Norris PJ, Steele WR, et al. The effect of previous pregnancy and transfusion on HLA alloimmunization in blood donors: implications for a transfusion-related acute lung injury risk reduction strategy. *Transfusion* (2009) **49**(9):1825–35. doi:10.1111/j.1537-2995.2009.02206.x
73. De Clippel D, Baeten M, Torfs A, Emonds MP, Feys HB, Compernolle V, et al. Screening for HLA antibodies in plateletpheresis donors with a history of transfusion or pregnancy. *Transfusion* (2014) **54**(12):3036–42. doi:10.1111/trf.12727
74. Hyun J, Park KD, Yoo Y, Lee B, Han BY, Song EY, et al. Effects of different sensitization events on HLA alloimmunization in solid organ transplantation patients. *Transplant Proc* (2012) **44**(1):222–5. doi:10.1016/j.transproceed.2011.12.049
75. Salvatierra O Jr, Vincenti F, Amend W, Potter D, Iwaki Y, Opelz G, et al. Deliberate donor-specific blood transfusions prior to living related renal transplantation. A new approach. *Ann Surg* (1980) **192**(4):543–52. doi:10.1097/00000658-198010000-00012
76. Pfaff WW, Fennell RS, Howard RJ, Ireland JF, Scornik JC. Planned random donor blood transfusion in preparation for transplantation. Sensitization and graft survival. *Transplantation* (1984) **38**(6):701–3. doi:10.1097/00007890-198412000-00030
77. Josien R, Heslan M, Brouard S, Soulillou JP, Cuturi MC. Critical requirement for graft passenger leukocytes in allograft tolerance induced by donor blood transfusion. *Blood* (1998) **92**(12):4539–44.
78. Fuller TC, Delmonico FL, Cosimi B, Huggins CE, King M, Russell PS. Impact of blood transfusion on renal transplantation. *Ann Surg* (1978) **187**(2):211–8. doi:10.1097/00000658-197802000-00020
79. Scornik JC, Bromberg JS, Norman DJ, Bhanderi M, Gitlin M, Petersen J. An update on the impact of pre-transplant transfusions and allosensitization on time to renal transplant and on allograft survival. *BMC Nephrol* (2013) **14**:217. doi:10.1186/1471-2369-14-217
80. Regan L, Braude PR, Hill DP. A prospective study of the incidence, time of appearance and significance of anti-paternal lymphocytotoxic antibodies in human pregnancy. *Hum Reprod* (1991) **6**(2):294–8.
81. Dunn TB, Noreen H, Gillingham K, Maurer D, Ozturk OG, Pruett TL, et al. Revisiting traditional risk factors for rejection and graft loss after kidney transplantation. *Am J Transplant* (2011) **11**(10):2132–43. doi:10.1111/j.1600-6143.2011.03640.x
82. Masson E, Vidal C, Deschamps M, Bongain S, Thevenin C, Dupont I, et al. Incidence and risk factors of anti-HLA immunization after pregnancy. *Hum Immunol* (2013) **74**(8):946–51. doi:10.1016/j.humimm.2013.04.025
83. Higgins R, Lowe D, Hathaway M, Williams C, Lam FT, Kashi H, et al. Human leukocyte antigen antibody-incompatible renal transplantation: excellent medium-term outcomes with negative cytotoxic crossmatch. *Transplantation* (2011) **92**(8):900–6. doi:10.1097/TP.0b013e31822dc38d
84. Higgins R, Lowe D, Daga S, Hathaway M, Williams C, Lam FT, et al. Pregnancy-induced HLA antibodies respond more vigorously after renal transplantation than antibodies induced by prior transplantation. *Hum Immunol* (2015) **76**(8):546–52. doi:10.1016/j.humimm.2015.01.012
85. Everly MJ, Rebellato LM, Haisch CE, Ozawa M, Parker K, Briley KP, et al. Incidence and impact of de novo donor-specific alloantibody in primary renal allografts. *Transplantation* (2013) **95**(3):410–7. doi:10.1097/TP.0b013e31827d62e3
86. Kaneku H, O'Leary JG, Banuelos N, Jennings LW, Susskind BM, Klintmalm GB, et al. De novo donor-specific HLA antibodies decrease patient and graft survival in liver transplant recipients. *Am J Transplant* (2013) **13**(6):1541–8. doi:10.1111/ajt.12212
87. Smith JD, Banner NR, Hamour M, Ozawa M, Goh A, Robinson D, et al. De novo donor HLA-specific antibodies after heart transplantation are an independent predictor of poor patient survival. *Am J Transplant* (2011) **11**(2):312–9. doi:10.1111/j.1600-6143.2010.03383.x

88. Wiebe C, Gibson W, Blydt-Hansen TD, Karpinski M, Ho J, Storsley LJ, et al. Evolution and clinical pathologic correlations of de novo donor-specific HLA antibody post kidney transplant. *Am J Transplant* (2012) **12**(5):1157–67. doi:10.1111/j.1600-6143.2012.04013.x
89. Billen EV, Christiaans MH, Lee J, van den Berg-Loonen EM. Donor-directed HLA antibodies before and after transplantectomy detected by the luminex single antigen assay. *Transplantation* (2009) **87**(4):563–9. doi:10.1097/TP.0b013e3181949e37
90. Cecka JM, Terasaki PI. Repeating HLA antigen mismatches in renal retransplants – a second class mistake? *Transplantation* (1994) **57**(4):515–9. doi:10.1097/00007890-199402000-00007
91. Doxiadis II, de Lange P, D'Amaro J, de Meester J, Schreuder GM, Claas FH. Repeated HLA mismatches in cadaveric renal transplantation: is it safe to transplant? *Transplant Proc* (1997) **29**(1–2):1408–9. doi:10.1016/S0041-1345(96)00612-4
92. Fuller A, Profaizer T, Roberts L, Fuller TC. Repeat donor HLA-DR mismatches in renal transplantation: is the increased failure rate caused by noncytotoxic HLA-DR alloantibodies? *Transplantation* (1999) **68**(4):589–91. doi:10.1097/00007890-199907270-00002
93. Farney AC, Matas AJ, Noreen HJ, Reinsmoen N, Segall M, Schmidt WJ, et al. Does re-exposure to mismatched HLA antigens decrease renal re-transplant allograft survival? *Clin Transplant* (1996) **10**(2):147–56.
94. House AA, Chang PC, Luke PP, Leckie SH, Howson WT, Ball EJ, et al. Re-exposure to mismatched HLA class I is a significant risk factor for graft loss: multivariable analysis of 259 kidney retransplants. *Transplantation* (2007) **84**(6):722–8. doi:10.1097/01.tp.0000281398.41670.1f
95. Coupel S, Giral-Classe M, Karam G, Morcet JF, Dantal J, Cantarovich D, et al. Ten-year survival of second kidney transplants: impact of immunologic factors and renal function at 12 months. *Kidney Int* (2003) **64**(2):674–80. doi:10.1046/j.1523-1755.2003.00104.x
96. O'Connor MJ, Lind C, Tang X, Gossett J, Weber J, Monos D, et al. Persistence of anti-human leukocyte antibodies in congenital heart disease late after surgery using allografts and whole blood. *J Heart Lung Transplant* (2013) **32**(4):390–7. doi:10.1016/j.healun.2012.12.009
97. Kwon MH, Zhang JQ, Schaenman JM, Cadeiras M, Gjertson DW, Krystal CA, et al. Characterization of ventricular assist device-mediated sensitization in the bridge-to-heart-transplantation patient. *J Thorac Cardiovasc Surg* (2015) **149**(4):1161–6. doi:10.1016/j.jtcvs.2015.01.003
98. Morales-Buenrostro LE, Terasaki PI, Marino-Vazquez LA, Lee JH, El-Awar N, Alberu J. "Natural" human leukocyte antigen antibodies found in nonalloimmunized healthy males. *Transplantation* (2008) **86**(8):1111–5. doi:10.1097/01.tp.0000331682.91699.7b
99. Burrows SR, Silins SL, Khanna R, Burrows JM, Rischmueller M, McCluskey J, et al. Cross-reactive memory T cells for Epstein-Barr virus augment the alloresponse to common human leukocyte antigens: degenerate recognition of major histocompatibility complex-bound peptide by T cells and its role in alloreactivity. *Eur J Immunol* (1997) **27**(7):1726–36. doi:10.1002/eji.1830270126
100. Amir AL, D'Orsogna LJ, Roelen DL, van Loenen MM, Hagedoorn RS, de Boer R, et al. Allo-HLA reactivity of virus-specific memory T cells is common. *Blood* (2010) **115**(15):3146–57. doi:10.1182/blood-2009-07-234906
101. Morice A, Charreau B, Neveu B, Brouard S, Soulillou JP, Bonneville M, et al. Cross-reactivity of herpesvirus-specific CD8 T cell lines toward allogeneic class I MHC molecules. *PLoS One* (2010) **5**(8):e12120. doi:10.1371/journal.pone.0012120
102. D'Orsogna LJ, van der Meer-Prins EM, Zoet YM, Roelen DL, Doxiadis II, Claas FH. Detection of allo-HLA cross-reactivity by virus-specific memory T-cell clones using single HLA-transfected K562 cells. *Methods Mol Biol* (2012) **882**:339–49. doi:10.1007/978-1-61779-842-9_19
103. de Santis C, Lopalco L, Robbioni P, Longhi R, Rappocciolo G, Siccardi AG, et al. Human antibodies to immunodominant C5 region of HIV-1 gp120 cross-react with HLA class I on activated cells. *AIDS Res Hum Retroviruses* (1994) **10**(2):157–62. doi:10.1089/aid.1994.10.157
104. Alberu J, Morales-Buenrostro LE, de Leo C, Vargas-Rojas MI, Marino-Vazquez LA, Crispin JC. A non-allogeneic stimulus triggers the production of de novo HLA antibodies in healthy adults. *Transpl Immunol* (2007) **18**(2):166–71. doi:10.1016/j.trim.2007.06.001
105. Ravindranath MH, Kaneku H, El-Awar N, Morales-Buenrostro LE, Terasaki PI. Antibodies to HLA-E in nonalloimmunized males: pattern of HLA-Ia reactivity of anti-HLA-E-positive sera. *J Immunol* (2010) **185**(3):1935–48. doi:10.4049/jimmunol.1000424
106. El-Awar N, Terasaki PI, Nguyen A, Sasaki N, Morales-Buenrostro LE, Saji H, et al. Epitopes of HLA antibodies found in sera of normal healthy males and cord blood. *Clin Transpl* (2008):199–214. doi:10.1016/j.humimm.2009.06.020
107. Roberts T, Tumer G, Gebel HM, Bray RA. Solid-phase assays for the detection of alloantibody against human leukocyte antigens: panacea or Pandora? *Int J Immunogenet* (2014) **41**(5):362–9. doi:10.1111/iji.12138
108. Visentin J, Guidicelli G, Bachelet T, Jacquelinet C, Audry B, Nong T, et al. Denatured class I human leukocyte antigen antibodies in sensitized kidney recipients: prevalence, relevance, and impact on organ allocation. *Transplantation* (2014) **98**(7):738–44. doi:10.1097/TP.0000000000000315
109. In JW, Rho EY, Shin S, Park KU, Song EY. False-positive reactions against HLA class II molecules detected in Luminex single-antigen bead assays. *Ann Lab Med* (2014) **34**(5):408–10. doi:10.3343/alm.2014.34.5.408
110. Cai J, Terasaki PI, Anderson N, Lachmann N, Schonemann C. Intact HLA not beta2m-free heavy chain-specific HLA class I antibodies are predictive of graft failure. *Transplantation* (2009) **88**(2):226–30. doi:10.1097/TP.0b013e3181ac6198
111. Otten HG, Verhaar MC, Borst HP, van Eck M, van Ginkel WG, Hene RJ, et al. The significance of pretransplant donor-specific antibodies reactive with intact or denatured human leucocyte antigen in kidney transplantation. *Clin Exp Immunol* (2013) **173**(3):536–43. doi:10.1111/cei.12127
112. Jackson AM, Sigdel TK, Delville M, Hsieh SC, Dai H, Bagnasco S, et al. Endothelial cell antibodies associated with novel targets and increased rejection. *J Am Soc Nephrol* (2015) **26**(5):1161–71. doi:10.1681/ASN.2013121277
113. Sun Q, Cheng Z, Cheng D, Chen J, Ji S, Wen J, et al. De novo development of circulating anti-endothelial cell antibodies rather than pre-existing antibodies is associated with post-transplant allograft rejection. *Kidney Int* (2011) **79**(6):655–62. doi:10.1038/ki.2010.437
114. Zhang Q, Cecka JM, Gjertson DW, Ge P, Rose ML, Patel JK, et al. HLA and MICA: targets of antibody-mediated rejection in heart transplantation. *Transplantation* (2011) **91**(10):1153–8. doi:10.1097/TP.0b013e3182157d60
115. Patel R, Terasaki PI. Significance of the positive crossmatch test in kidney transplantation. *N Engl J Med* (1969) **280**(14):735–9. doi:10.1056/NEJM196904032801401
116. Suitters A, Rose M, Higgins A, Yacoub MH. MHC antigen expression in sequential biopsies from cardiac transplant patients – correlation with rejection. *Clin Exp Immunol* (1987) **69**(3):575–83.
117. Hubscher SG, Adams DH, Elias E. Changes in the expression of major histocompatibility complex class II antigens in liver allograft rejection. *J Pathol* (1990) **162**(2):165–71. doi:10.1002/path.1711620115
118. Facoetti A, Nano R, Zelini P, Morbini P, Benericetti E, Ceroni M, et al. Human leukocyte antigen and antigen processing machinery component defects in astrocytic tumors. *Clin Cancer Res* (2005) **11**(23):8304–11. doi:10.1158/1078-0432.CCR-04-2588
119. Lefaucheur C, Loupy A, Hill GS, Andrade J, Nochy D, Antoine C, et al. Preexisting donor-specific HLA antibodies predict outcome in kidney transplantation. *J Am Soc Nephrol* (2010) **21**(8):1398–406. doi:10.1681/ASN.2009101065
120. Loupy A, Jordan SC. Transplantation: donor-specific HLA antibodies and renal allograft failure. *Nat Rev Nephrol* (2013) **9**(3):130–1. doi:10.1038/nrneph.2013.18
121. Valenzuela NM, Reed EF. Antibodies to HLA molecules mimic agonistic stimulation to trigger vascular cell changes and induce allograft injury. *Curr Transplant Rep* (2015) **2**(3):222–32. doi:10.1007/s40472-015-0065-6
122. Zhang X, Rozengurt E, Reed EF. HLA class I molecules partner with integrin beta4 to stimulate endothelial cell proliferation and migration. *Sci Signal* (2010) **3**(149):ra85. doi:10.1126/scisignal.2001158
123. Jin YP, Fishbein MC, Said JW, Jindra PT, Rajalingam R, Rozengurt E, et al. Anti-HLA class I antibody-mediated activation of the PI3K/Akt signaling pathway and induction of Bcl-2 and Bcl-xL expression in endothelial cells. *Hum Immunol* (2004) **65**(4):291–302. doi:10.1016/j.humimm.2004.07.014
124. Le Bas-Bernardet S, Coupel S, Chauveau A, Soulillou JP, Charreau B. Vascular endothelial cells evade apoptosis triggered by human leukocyte

antigen-DR ligation mediated by allospecific antibodies. *Transplantation* (2004) **78**(12):1729–39. doi:10.1097/01.TP.0000147339.31581.99

125. Narayanan K, Jaramillo A, Phelan DL, Mohanakumar T. Pre-exposure to sub-saturating concentrations of HLA class I antibodies confers resistance to endothelial cells against antibody complement-mediated lysis by regulating Bad through the phosphatidylinositol 3-kinase/Akt pathway. *Eur J Immunol* (2004) **34**(8):2303–12. doi:10.1002/eji.200324843
126. Lepin EJ, Zhang Q, Zhang X, Jindra PT, Hong LS, Ayele P, et al. Phosphorylated S6 ribosomal protein: a novel biomarker of antibody-mediated rejection in heart allografts. *Am J Transplant* (2006) **6**(7):1560–71. doi:10.1111/j.1600-6143.2006.01355.x
127. Jindra PT, Hsueh A, Hong L, Gjertson D, Shen XD, Gao F, et al. Anti-MHC class I antibody activation of proliferation and survival signaling in murine cardiac allografts. *J Immunol* (2008) **180**(4):2214–24. doi:10.4049/jimmunol.180.4.2357
128. Li F, Wei J, Valenzuela NM, Lai C, Zhang Q, Gjertson D, et al. Phosphorylated S6 kinase and S6 ribosomal protein are diagnostic markers of antibody-mediated rejection in heart allografts. *J Heart Lung Transplant* (2015) **34**(4):580–7. doi:10.1016/j.healun.2014.09.047
129. Ziegler ME, Jin YP, Young SH, Rozengurt E, Reed EF. HLA class I-mediated stress fiber formation requires ERK1/2 activation in the absence of an increase in intracellular Ca2+ in human aortic endothelial cells. *Am J Physiol Cell Physiol* (2012) **303**(8):C872–82. doi:10.1152/ajpcell.00199.2012
130. Ziegler ME, Souda P, Jin YP, Whitelegge JP, Reed EF. Characterization of the endothelial cell cytoskeleton following HLA class I ligation. *PLoS One* (2012) **7**(1):e29472. doi:10.1371/journal.pone.0029472
131. Galvani S, Auge N, Calise D, Thiers JC, Canivet C, Kamar N, et al. HLA class I antibodies provoke graft arteriosclerosis in human arteries transplanted into SCID/beige mice. *Am J Transplant* (2009) **9**(11):2607–14. doi:10.1111/j.1600-6143.2009.02804.x
132. Galvani S, Trayssac M, Auge N, Thiers JC, Calise D, Krell HW, et al. A key role for matrix metalloproteinases and neutral sphingomyelinase-2 in transplant vasculopathy triggered by anti-HLA antibody. *Circulation* (2011) **124**(24):2725–34. doi:10.1161/CIRCULATIONAHA.111.021790
133. Yamakuchi M, Kirkiles-Smith NC, Ferlito M, Cameron SJ, Bao C, Fox-Talbot K, et al. Antibody to human leukocyte antigen triggers endothelial exocytosis. *Proc Natl Acad Sci U S A* (2007) **104**(4):1301–6. doi:10.1073/pnas.0602035104
134. Valenzuela NM, Hong L, Shen XD, Gao F, Young SH, Rozengurt E, et al. Blockade of p-selectin is sufficient to reduce MHC I antibody-elicited monocyte recruitment in vitro and in vivo. *Am J Transplant* (2013) **13**(2):299–311. doi:10.1111/ajt.12016
135. Valenzuela NM, Mulder A, Reed EF. HLA class I antibodies trigger increased adherence of monocytes to endothelial cells by eliciting an increase in endothelial P-selectin and, depending on subclass, by engaging FcgammaRs. *J Immunol* (2013) **190**(12):6635–50. doi:10.4049/jimmunol.1201434
136. Wehner J, Morrell CN, Reynolds T, Rodriguez ER, Baldwin WM III. Antibody and complement in transplant vasculopathy. *Circ Res* (2007) **100**(2):191–203. doi:10.1161/01.RES.0000255032.33661.88
137. Nickeleit V, Mihatsch MJ. Kidney transplants, antibodies and rejection: is C4d a magic marker? *Nephrol Dial Transplant* (2003) **18**(11):2232–9. doi:10.1093/ndt/gfg304
138. Rodriguez ER, Skojec DV, Tan CD, Zachary AA, Kasper EK, Conte JV, et al. Antibody-mediated rejection in human cardiac allografts: evaluation of immunoglobulins and complement activation products C4d and C3d as markers. *Am J Transplant* (2005) **5**(11):2778–85. doi:10.1111/j.1600-6143.2005.01074.x
139. Kubagawa H, Oka S, Kubagawa Y, Torii I, Takayama E, Kang DW, et al. Identity of the elusive IgM Fc receptor (FcmuR) in humans. *J Exp Med* (2009) **206**(12):2779–93. doi:10.1084/jem.20091107
140. Bruhns P, Iannascoli B, England P, Mancardi DA, Fernandez N, Jorieux S, et al. Specificity and affinity of human Fcgamma receptors and their polymorphic variants for human IgG subclasses. *Blood* (2009) **113**(16):3716–25. doi:10.1182/blood-2008-09-179754
141. Nimmerjahn F, Ravetch JV. FcgammaRs in health and disease. *Curr Top Microbiol Immunol* (2011) **350**:105–25. doi:10.1007/82_2010_86
142. Manger K, Repp R, Spriewald BM, Rascu A, Geiger A, Wassmuth R, et al. Fcgamma receptor IIa polymorphism in Caucasian patients with systemic lupus erythematosus: association with clinical symptoms. *Arthritis Rheum* (1998) **41**(7):1181–9. doi:10.1002/1529-0131(199807)41:7<1181::AID-ART6>3.0.CO;2-C
143. Karassa FB, Trikalinos TA, Ioannidis JP. The role of FcgammaRIIA and IIIA polymorphisms in autoimmune diseases. *Biomed Pharmacother* (2004) **58**(5):286–91. doi:10.1016/j.biopha.2004.04.004
144. Diamantopoulos PT, Kalotychou V, Polonyfi K, Sofotasiou M, Anastasopoulou A, Galanopoulos A, et al. Correlation of Fc-gamma RIIA polymorphisms with latent Epstein-Barr virus infection and latent membrane protein 1 expression in patients with low grade B-cell lymphomas. *Leuk Lymphoma* (2013) **54**(9):2030–4. doi:10.3109/10428194.2012.762512
145. Mellor JD, Brown MP, Irving HR, Zalcberg JR, Dobrovic A. A critical review of the role of Fc gamma receptor polymorphisms in the response to monoclonal antibodies in cancer. *J Hematol Oncol* (2013) **6**:1. doi:10.1186/1756-8722-6-1
146. Maiga B, Dolo A, Toure O, Dara V, Tapily A, Campino S, et al. Fc gamma receptor IIa-H131R polymorphism and malaria susceptibility in sympatric ethnic groups, Fulani and Dogon of Mali. *Scand J Immunol* (2014) **79**(1):43–50. doi:10.1111/sji.12122
147. Pawlik A, Florczak M, Bak L, Domanski L, Rozanski J, Dabrowska-Zamojcin E, et al. The Fc gamma RIIa polymorphism in patients with acute kidney graft rejection. *Ann Transplant* (2003) **8**(4):24–6.
148. Yuan FF, Watson N, Sullivan JS, Biffin S, Moses J, Geczy AF, et al. Association of Fc gamma receptor IIA polymorphisms with acute renal-allograft rejection. *Transplantation* (2004) **78**(5):766–9. doi:10.1097/01.TP.0000132560.77496.CB
149. Berry GJ, Burke MM, Andersen C, Bruneval P, Fedrigo M, Fishbein MC, et al. The 2013 International Society for Heart and Lung Transplantation Working Formulation for the standardization of nomenclature in the pathologic diagnosis of antibody-mediated rejection in heart transplantation. *J Heart Lung Transplant* (2013) **32**(12):1147–62. doi:10.1016/j.healun.2012.11.005
150. Fishbein GA, Fishbein MC. Morphologic and immunohistochemical findings in antibody-mediated rejection of the cardiac allograft. *Hum Immunol* (2012) **73**(12):1213–7. doi:10.1016/j.humimm.2012.07.011
151. Fedrigo M, Feltrin G, Poli F, Frigo AC, Benazzi E, Gambino A, et al. Intravascular macrophages in cardiac allograft biopsies for diagnosis of early and late antibody-mediated rejection. *J Heart Lung Transplant* (2013) **32**(4):404–9. doi:10.1016/j.healun.2012.12.017
152. Xu L, Collins J, Drachenberg C, Kukuruga D, Burke A. Increased macrophage density of cardiac allograft biopsies is associated with antibody-mediated rejection and alloantibodies to HLA antigens. *Clin Transplant* (2014) **28**(5):554–60. doi:10.1111/ctr.12348
153. Magil AB, Tinckam K. Monocytes and peritubular capillary C4d deposition in acute renal allograft rejection. *Kidney Int* (2003) **63**(5):1888–93. doi:10.1046/j.1523-1755.2003.00921.x
154. Tinckam KJ, Djurdjev O, Magil AB. Glomerular monocytes predict worse outcomes after acute renal allograft rejection independent of C4d status. *Kidney Int* (2005) **68**(4):1866–74. doi:10.1111/j.1523-1755.2005.00606.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 08 February 2016
doi: 10.3389/fimmu.2016.00037

Ocular Immune Privilege and Transplantation

*Andrew W. Taylor**

Department of Ophthalmology, Boston University School of Medicine, Boston, MA, USA

Allografts are afforded a level of protection from rejection within immune-privileged tissues. Immune-privileged tissues involve mechanisms that suppress inflammation and promote immune tolerance. There are anatomical features, soluble factors, membrane-associated proteins, and alternative antigen-presenting cells (APC) that contribute to allograft survival in the immune-privileged tissue. This review presents the current understanding of how the mechanism of ocular immune privilege promotes tolerogenic activity by APC, and T cells in response to the placement of foreign antigen within the ocular microenvironment. Discussed will be the unique anatomical, cellular, and molecular mechanisms that lessen the chance for graft destroying immune responses within the eye. As more is understood about the molecular mechanisms of ocular immune privilege greater is the potential for using these molecular mechanisms in therapies to prevent allograft rejection.

Keywords: immune privilege, anterior chamber-associated immune deviation, immune tolerance, regulatory T cells

OPEN ACCESS

Edited by:
James I. Kim,
Harvard Medical School, USA

Reviewed by:
Philippe Saas,
Etablissement Français du Sang BFC, France
Karen Bieback,
Institute of Transfusion Medicine and Immunology, Germany

***Correspondence:**
Andrew W. Taylor
awtaylor@bu.edu

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *14 October 2015*
Accepted: *25 January 2016*
Published: *08 February 2016*

Citation:
Taylor AW (2016) Ocular Immune Privilege and Transplantation. Front. Immunol. 7:37. doi: 10.3389/fimmu.2016.00037

WHAT IS IMMUNE PRIVILEGE

The phrase immune privilege is a transplantation term defined by Peter Medawar and colleagues in the 1940s (1). They demonstrated that skin allografts placed within the anterior chamber of the eye survive indefinitely in contrast to their rapid rejection in other more conventional tissues such as the skin. This happened even when the recipient is already immunized against the alloantigens, but only if the blood–ocular barrier was maintained. It had been observed that once there is vascular leakage into the anterior chamber the graft is rejected. Another characteristic of allograft placement into the anterior chamber is that it does not immunize the recipient (2). Since the eye has no observable direct lymphatic drainage, it had suggested that alloantigen could not reach the regional lymph nodes and initiate an immune response. Such mechanisms of sequestration of antigen and antigen-expressing tissues has erroneously led some to think that the ocular microenvironment should be devoid of all immune cells and immune responses. This is clearly not the case (3).

There are resident immune cells with the potential of being antigen-presenting cells (APC) within the cornea, iris, ciliary body, and the retina. In the retina, they are the resident macrophage-like microglial cells (4–8), and there is very little evidence of cellular migration from blood circulation into the healthy ocular microenvironment (9). There is some speculation that resident macrophages and microglial cells are turned over, but this has only been seen in irradiated mice (10–13). It is also possible that the microglia of the retina are like microglial in the rest of the CNS are long-lived and are not initially bone marrow derived (9, 14–16). When there is inflammation, such as with uveitis, it is clear that the blood–ocular barrier is leaking, and that most of the infiltrating immune cells are coming through breaches in the barrier (13, 17, 18).

The blood–ocular barrier is made from the tight junctions of the pigmented epithelial cell layer of the uveal track, of the endothelial cells of the inner-retina capillaries and the avascular cornea (19). This enclosed space allows for the eye to form its own microenvironment to regionally suppress the activation of inflammation and to control the functionality of immune cells. Locally produced soluble factors found in aqueous humor, and the soluble and membrane-bound factors of the pigmented epithelial cells described in detail later suppress the activation of inflammation (20–39). These factors are a defined group of proteins, neuropeptides, and biochemicals that modify the behavior, differentiation, and survival of immune cells within the ocular microenvironment. Their combined actions make the ocular microenvironment highly anti-inflammatory; moreover, the mechanism of immune privilege makes immune cells (monocytes, macrophages, dendritic cells, microglial cells, and T cells) to contribute to the anti-inflammatory microenvironment (3, 40–42). This promotes a self-perpetuating anti-inflammatory immune response, and induction of immune tolerance, which protects the eye from the irreversible collateral damage of inflammation that can lead to blindness.

It has very much been demonstrated that immune cell activity is present, but it is driven within the ocular microenvironment toward anti-inflammatory and tolerogenic immune responses. In addition, the placement of alloantigen-expressing grafts into the anterior chamber or within the retina induces alloantigen-specific systemic tolerance (1, 31, 41, 43–47). This has shown that the presence of foreign antigen with the eye is not hidden.

WHAT ARE THE IMMUNE RESPONSES TO THE PLACEMENT OF FOREIGN ANTIGEN WITHIN THE EYE

The prolonged survival of incompatible grafts in the eye is the definition of immune privilege (1). The mechanism of how this is achieved is through induction of systemic tolerance to the alloantigens and the regional suppression of inflammation (40). These mechanisms establish a strong blockade in activating a graft destructive immune response. Although this is not an absolute suppression of immunity, understanding the mechanisms of immune privilege has led to understand that a large part is to regulate APC activity in a manner that activates regulatory T (Treg) cells (6, 47–49).

The placement of foreign antigen into the anterior chamber, vitreous, or in the sub-retinal space induces systemic tolerance to the antigen. The initial experiments demonstrating this phenomenon were done by placing MHC-mismatched tumor cells into the anterior chamber of the eye, resulted in graft survival of skin from the same MHC-mismatched mouse strain (50, 51). By contrast, mice that had the tumor cells placed into the skin rejected both the tumor cells and the subsequent skin graft. The induction of systemic tolerance was considered a deviation from the expected hypersensitivity immune response and was called anterior chamber-associated immune deviation (ACAID). Also, the same ACAID-like response is seen when any foreign antigen is placed in the vitreous, or sub-retinal space (44, 52, 53). Like the immune response to allografts, it is unclear what is the evolutionary advantage of ACAID unless it is either a byproduct of the anti-inflammatory environment of the eye or part of controlling the immune response to presented autoantigens within the eye.

The tolerance induced in ACAID is efferent suppression mediated by a tolerogenic $CD8^+$ T cell. It is antigen specific, and it suppresses the activation of effector T cells responding to the same source of antigen. Within hours after injecting antigen into the eye, the antigen disseminates almost throughout the body. This suggested for a long time that the tolerogenic mechanism of the eye was similar to inject antigen directly into the blood circulation; however, the tolerance induced by antigen placed into the eye is dependent on the spleen, and the presentation of antigen by a $F4/80^+$ macrophage (54, 55). The induction of the ACAIDogenic APC can be done by treating cultured macrophages with aqueous humor or with the aqueous humor factor TGF-β2 while providing antigen or a source of antigen, like cells expressing alloantigens (56–59). These ACAIDogenic APC leave the eye *via* the blood circulation and home to the marginal zones of the spleen. They form cellular clusters with NKT cells as well as $CD4^+$ and $CD8^+$ T cells (60). Also, in these clusters are B cells that take up antigen directly from the ACAIDogenic APC and present the antigen (61). These clusters are mediated by the production of RANTES made by NKT cells stimulated by CD1d on the ACAIDogenic APC and this brings in $CD8^+$ T cells (60, 62). The result is the induction and expansion of antigen-specific efferent suppressor $CD8^+$ T cells. These cells are responsible for the antigen-specific systemic prevention of graft rejection and hypersensitivity (63–65). Since the mechanism of inducing ACAIDogenic APC is a local effect of the immune-privileged ocular microenvironment, it is possible that presentation of CD1d in the eye would also locally activate tolerogenic NKT cells. It has been shown that cornea allograft survival is associated with CD1d stimulation tolerogenic NKT cells like in the ACAID response (66, 67). By contrast, failure to stimulate the NKT cells to promote Treg cell activation may be associated with corneal allograft rejection. Therefore, from understanding the mechanisms of the ACAID, it is possible to speculate that APC in the ocular microenvironment are also influenced by ocular TGF-β2 to CD1d-stimulated NKT cells that are anti-inflammatory, and mediators of Treg cell activation.

Similar tolerogenic APC induced by TGF-β2 is seen when antigen is placed into the sub-retinal space (68). The study of sub-retinal induction of ACAIDogenic APC has shown that part of the induction of immune deviation is a cascade of TGF-β2 activation from latent to active mediated by thrombospondin-1, and it receptor CD38 on the $F4/80^+$ macrophages (68). Therefore, antigen, either soluble or shed from transplanted cells, is processed by APC under the influence of the ocular microenvironment, and that these antigen-loaded APC migrate to the spleen to initiate tolerance, or remain within the eye to mediate anti-inflammatory activity and stimulate Treg cells.

MOLECULAR MECHANISMS OF OCULAR IMMUNE PRIVILEGE

One of the original observations about ocular immunobiology was that placement of foreign antigen into the eye of a recipient

with an already established effector immune response does not elicit an inflammatory response (1). An additional element of immune regulation is the anti-inflammatory mechanisms of the ocular microenvironment itself that works to prevent induction of inflammation and suppress the activity of effector immune cells (69, 70). This is seen as the mechanisms of immune suppression mediated by soluble molecules of aqueous humor, and membrane expressed molecules of cells within the ocular microenvironment.

The most understood immunosuppressive mechanisms of ocular immunobiology are the effects of aqueous humor on immune cells. Since the blood barrier does not inhibit effector T cell migration into the eye (71, 72), there are several mechanisms regulating T cell activity within the immune-privileged eye. When effector T cells with APC-presenting antigen are injected into the anterior chamber, the inflammation mediated by the antigen-activated effector T cells is suppressed (69). Also, the T cell-mediated inflammation is suppressed when the APC and the T cells are first treated with aqueous humor, and adoptively transferred into tissues other than the eye. Molecular analysis of aqueous humor shows that TGF-β2 has the possibly of being the major regulatory molecule; however, it is in a latent form and rarely found active in fresh aqueous humor of healthy eyes (73–76). The first reports of aqueous humor suppression of T cell activation used pooled, frozen aqueous humor samples (77). The freezing and thawing of aqueous humor activate the TGF-β2, and because of the overwhelming potency of TGF-β2 on T cell activity, the first descriptions of aqueous humor suppressive activity were more of a study on the effects of TGF-β2 on immune reactions (78). One of these is the induction of the ACAIDogenic APC (59). Careful collection of aqueous humor, and its immediate use in assays, keeping TGF-β2 in its latent form, has revealed a wealth of other soluble immunomodulating molecules dominated by neuropeptides such as alpha-melanocyte-stimulating hormone (α-MSH) (20).

Each of the molecules of aqueous humor target different cells of the immune response and different activities (41). The result is the induction of $CD4^+$ Treg cells from an already established population of effector T cells (79). This induction of $CD4^+$ Treg cells is mediated mostly by the activity of the neuropeptide α-MSH. This is enhanced by the suppression of effector T cell activity by the other neuropeptides vasoactive intestinal peptide, somatostatin, and also by TGF-β2 when activated (21, 24, 79). The APC are also converted from presenting antigen that promote effector T cell activity to present antigen that activates Treg cells (80, 81). This is mediated by α-MSH, neuropeptide Y, and TGF-β2 that activate suppressive APC, and antigen-activated Treg cells. This means that molecules within the eye prevent immune-mediated inflammation while promoting the immune response to regulate itself. Since the immune response is an already established effector response, the activated Treg cells are inducible Treg (iTregs) cells meaning that the healthy ocular microenvironment is a site of immune reeducation. Therefore, immune privilege maybe more than suppressing inflammation, and that its immunosuppressive mechanisms can be used as a molecular approach to therapeutically promote long-term allograft survival through the induction of tolerance.

The cells of the cornea and the retina express on their membrane surfaces molecules that interact with immune cells to promote regulatory activity or apoptosis in the T cells. Many of the cells of cornea constitutively express FasL and PD-1 family of molecules (38, 82–84). The encounter between activated T cells and corneal endothelial cells leads to apoptosis of the T cells. The expression of B7-2 on pigmented epithelial cells lining the uveal track is associated with the conversation of naive T cells into Treg cells (35). This action is compounded by the fact that the pigmented epithelial cells are a source of many soluble immunomodulating molecules, such as TGF-β2, α-MSH, and neuropeptide Y (25, 28). Since naive T cells rarely migrate into peripheral tissues, the induction of apoptosis in the effector T cells is an important mechanism in preventing targeted immune attacks within the ocular tissues. Also, this could be a selective mechanism to allow for Treg cells to function within the eye, since they are more resistant to FasL-induced apoptosis (85), and that PD-1 is an activation signal for Treg cells (81, 86). This indicates that even transplanted ocular tissues, such as the cornea, carry molecules with the potential to mediate immunosuppression and tolerance.

The retina expresses not only FasL like the cornea but also molecules unique to the regulation of microglial cells or migrating macrophages. Neurons of the retina express CD200 that binds to CD200L and suppresses microglial cell-mediated inflammation (87). Mice with CD200:CD200L interaction knocked out are more susceptible to uveitis (87). Along with this regulation, soluble molecules from the retinal pigment epithelial cells (RPE) alternatively activate the microglia cells and macrophages (28, 80). This alternative activation makes these potential APC act and appear like myeloid-derived suppressor cells (MDSC) (88). The most we can understand of MSC is that they prevent effector T cell activation and suppress inflammation. Their presence in tumors has blocked many attempts at anti-cancer immunotherapy (89). Having such cells as part of the healthy retina is a potential advantage for preventing autoimmune attack and inflammation (81, 90, 91). The major molecular mediators of this are the neuropeptides α-MSH and NPY (28).

The placement of allogeneic neuroretinal cells or stem cells into the retina shows protection but is eventually rejected (92). This rejection is devoid of inflammation, and how the cells are eliminated is unknown. There is no rejection if the cells differentiate into neuronal cells and make connections with other retinal cells (93). This suggest that while immune privilege can prevent an inflammatory response non-integrated neurons must some how be targeted for removal, and an alloimmune response accelerates this clearance.

Experimental conditions that alter the ocular microenvironment to make it no different from conventional tissues, such as creating a high-risk cornea graft bed, or wounding RPE monolayers, demonstrate the importance of maintaining immune privilege to the success of ocular allografts. High-risk cornea graft beds have elevated levels of dendritic cells and vascularization with in the cornea stoma, and allograft rejection is almost assured (6, 94, 95). Experimentally designed high-risk corneas in rodents do not support ACAID, suggesting that changes in the cornea are most likely opening a barrier, probably through corneal neovascularization. Also, ACAID is lost in eyes with laser and sodium iodate wounded RPE monolayers (95, 96). The microglial cells change under these conditions from acting as suppressor cells

into proinflammatory cells (28). This further demonstrates that changes in the barrier that defines the ocular microenvironment have a profound influence on APC activity. The activity changes from supporting a blockade of inflammation and effector T cell activation to one where the APC themselves may contribute to the destructive immune response. How they change and what mediates the change is unknown. It is not clear which of the molecules and mechanisms of the ocular immune privilege is no longer active in high-risk ocular tissues.

USING THE MECHANISM OF IMMUNE PRIVILEGE TO PROMOTE ALLOGRAFT SURVIVAL

It still remains to be seen if it is possible to use the molecular mechanisms of immune privilege to promote allograft survival. Some serendipitous discoveries suggest that it maybe possible involving ACAID, anti-inflammatory activity of aqueous humor, and ocular induction of Treg cells. Although there are several proposals, it will be awhile before any can be practical and administered in the clinic, but there are a few that can be done as a process of preparing and treating the allograft.

One issue of ocular immune privilege is whether it rests with the cells of eye or solely with the molecules within the healthy ocular microenvironment. Arguing that immune privilege is with the cells is the finding that allogeneic retinal progenitor cells (RPC) exhibited limited immunogenicity and may produce immunosuppressive factors that promote their survival when implanted. One idea of delivering RPC to remodel retinas is to place them in a degradable scaffolding (97). When the RPC are seeded on poly(lactic-*co*-glycolic acid) polymer, and grafted under allogeneic kidney capsules they survive, and cells begin to differentiate into neurons and astrocytes. This happens even after the grafts are treated with IFN-γ to stimulate immunogenicity. When allogeneic RPC-containing polymers are seeded with syngeneic APC, the APC acted like ACAIDogenic APC and promote alloantigen-specific tolerance. This suggests that it is possible to create a localized immune-privileged site using cells of immune-privileged tissues within a defined structural microenvironment.

It is clear that soluble immunomodulating molecules of ocular immune privilege drive the induction of regulatory immunity. It is possible to use these molecules to suppress allograft rejection. The aqueous humor neuropeptide α-MSH is one of these soluble molecules of ocular immune privilege that has been used to generate retinal autoantigen-specific Treg cells *in vitro* (98). When these α-MSH-induced Treg cells are adoptively transferred into recipients with sub-retinal neonatal retinal allografts the grafts survive and the retinal cells begin to differentiate (99). This has demonstrated that the autoantigen-activated Treg cells within the retina provided the necessary immune protection needed for neonatal retinal cell development. Corneal allografts treated with eye drops containing α-MSH promote graft survival (100). These two studies have suggested that the use of the soluble molecules of immune privilege could be a new therapeutic approach in promoting allograft survival. Whether the survival is because of α-MSH suppression of inflammation by inhibiting proinflammatory cytokine production, or in the activation of Treg cells is not known. Use of α-MSH to treat models of autoimmune uveitis suggests that both may be its action (101).

CONCLUSION

There is a need to continue to understand the molecular nature of ocular immune privilege. There is a unique molecular relationship between the ocular microenvironment and immune cells to suppress inflammation and promote regulatory immunity. Although the benefits of ocular immune privilege have been seen with corneal allografts, understanding the mechanisms of this benefit means extending it to other allografts in other tissues. The potential exists that as more is understood about the molecular building blocks of ocular immune privilege that these molecules can be applied to extend the survival of all allografts. Such a possibility would result in less need for tissue typing, and systemic anti-rejection drugs, while increasing the pool of potential allogeneic donors.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

FUNDING

This work is supported in part by the Massachusetts Lions Eye Research Foundation.

REFERENCES

1. Medawar P. Immunity to homologous grafted skin. III. The fate of skin homografts transplanted to the brain to subcutaneous tissue, and to the anterior chamber of the eye. *Br J Exp Pathol* (1948) **29**:58–69.
2. Barker CF, Billingham RE. The role of afferent lymphatics in the rejection of skin homografts. *J Exp Med* (1968) **128**(1):197–221. doi:10.1084/jem.128.1.197
3. Stein-Streilein J. Immune regulation and the eye. *Trends Immunol* (2008) **29**(11):548–54. doi:10.1016/j.it.2008.08.002
4. McMenamin PG, Holthouse I, Holt PG. Class II major histocompatibility complex (Ia) antigen-bearing dendritic cells within the iris and ciliary body of the rat eye: distribution, phenotype, and relation to retinal microglia. *Immunology* (1992) **77**:385–93.
5. Paques M, Simonutti M, Augustin S, Goupille O, El Mathari B, Sahel JA. In vivo observation of the locomotion of microglial cells in the retina. *Glia* (2010) **58**(14):1663–8. doi:10.1002/glia.21037
6. Hamrah P, Dana MR. Corneal antigen-presenting cells. *Chem Immunol Allergy* (2007) **92**:58–70. doi:10.1159/000099254
7. Knickelbein JE, Watkins SC, McMenamin PG, Hendricks RL. Stratification of antigen-presenting cells within the normal cornea. *Ophthalmol Eye Dis* (2009) **1**:45–54.
8. Hamrah P, Zhang Q, Liu Y, Dana MR. Novel characterization of MHC class II-negative population of resident corneal Langerhans cell-type dendritic cells. *Invest Ophthalmol Vis Sci* (2002) **43**(3):639–46.
9. Albini TA, Wang RC, Reiser B, Zamir E, Wu GS, Rao NA. Microglial stability and repopulation in the retina. *Br J Ophthalmol* (2005) **89**(7):901–3. doi:10.1136/bjo.2004.060293

10. Dullforce PA, Garman KL, Seitz GW, Fleischmann RJ, Crespo SM, Planck SR, et al. APCs in the anterior uveal tract do not migrate to draining lymph nodes. *J Immunol* (2004) **172**(11):6701–8. doi:10.4049/jimmunol.172.11.6701
11. Chen L, Yang P, Kijlstra A. Distribution, markers, and functions of retinal microglia. *Ocul Immunol Inflamm* (2002) **10**(1):27–39. doi:10.1076/ocii.10.1.27.10328
12. Xu H, Chen M, Mayer EJ, Forrester JV, Dick AD. Turnover of resident retinal microglia in the normal adult mouse. *Glia* (2007) **55**(11):1189–98. doi:10.1002/glia.20535
13. Kaneko H, Nishiguchi KM, Nakamura M, Kachi S, Terasaki H. Characteristics of bone marrow-derived microglia in the normal and injured retina. *Invest Ophthalmol Vis Sci* (2008) **49**(9):4162–8. doi:10.1167/iovs.08-1738
14. Ajami B, Bennett JL, Krieger C, Tetzlaff W, Rossi FM. Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. *Nat Neurosci* (2007) **10**(12):1538–43. doi:10.1038/nn2014
15. Ransohoff RM. Microgliosis: the questions shape the answers. *Nat Neurosci* (2007) **10**(12):1507–9. doi:10.1038/nn1207-1507
16. Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, et al. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science* (2010) **330**(6005):841–5. doi:10.1126/science.1194637
17. Eter N, Engel DR, Meyer L, Helb HM, Roth F, Maurer J, et al. In vivo visualization of dendritic cells, macrophages, and microglial cells responding to laser-induced damage in the fundus of the eye. *Invest Ophthalmol Vis Sci* (2008) **49**(8):3649–58. doi:10.1167/iovs.07-1322
18. McMenamin PG, Crewe J. Endotoxin-induced uveitis. Kinetics and phenotype of the inflammatory cell infiltrate and the response of the resident tissue macrophages and dendritic cells in the iris and ciliary body. *Invest Ophthalmol Vis Sci* (1995) **36**(10):1949–59.
19. Campbell M, Humphries P. The blood-retina barrier: tight junctions and barrier modulation. *Adv Exp Med Biol* (2012) **763**:70–84.
20. Taylor AW, Streilein JW, Cousins SW. Identification of alpha-melanocyte stimulating hormone as a potential immunosuppressive factor in aqueous humor. *Curr Eye Res* (1992) **11**(12):1199–206. doi:10.3109/02713689208999545
21. Taylor AW, Streilein JW, Cousins SW. Immunoreactive vasoactive intestinal peptide contributes to the immunosuppressive activity of normal aqueous humor. *J Immunol* (1994) **153**(3):1080–6.
22. Goslings W, Prodeus A, Streilein J, Carroll M, Jager M, Taylor A. Small molecular weight factor in aqueous humor acts on C1q to prevent antibody-dependent complement activation. *Invest Ophthalmol Vis Sci* (1998) **39**(6):989–95.
23. Taylor A, Yee D, Streilein J. Suppression of nitric oxide generated by inflammatory microphages by calcitonin gene-related peptide in aqueous humor. *Invest Ophthalmol Vis Sci* (1998) **39**(8):1372–8.
24. Taylor A, Yee D. Somatostatin is an imunosuppressive factor in aqueous humor. *Invest Ophthalmol Vis Sci* (2003) **44**(6):2644–9. doi:10.1167/iovs.02-1216
25. Zamiri P, Masli S, Kitaichi N, Taylor A, Streilein J. Thrombospondin plays a vital role in the immune privilege of the eye. *Invest Ophthalmol Vis Sci* (2005) **46**(3):908–19. doi:10.1167/iovs.04-0362
26. Zamiri P, Masli S, Streilein JW, Taylor AW. Pigment epithelial growth factor suppresses inflammation by modulating macrophage activation. *Invest Ophthalmol Vis Sci* (2006) **47**(9):3912–8. doi:10.1167/iovs.05-1267
27. Zamiri P, Masli S, Kitaichi N, Taylor AW, Streilein JW. Thrombospondin plays a vital role in the immune privilege of the eye. *Ocul Immunol Inflamm* (2007) **15**(3):279–94. doi:10.1080/09273940701382432
28. Kawanaka N, Taylor AW. Localized retinal neuropeptide regulation of macrophage and microglial cell functionality. *J Neuroimmunol* (2011) **232**(1–2):17–25. doi:10.1016/j.jneuroim.2010.09.025
29. Apte RS, Sinha D, Mayhew E, Wistow GJ, Niederkorn JY. Cutting edge: role of macrophage migration inhibitory factor in inhibiting NK cell activity and preserving immune privilege. *J Immunol* (1998) **160**(12):5693–6.
30. D'Orazio TJ, DeMarco BM, Mayhew ES, Niederkorn JY. Effect of aqueous humor on apoptosis of inflammatory cell types. *Invest Ophthalmol Vis Sci* (1999) **40**(7):1418–26.
31. Wenkel H, Streilein JW. Evidence that retinal pigment epithelium functions as an immune-privileged tissue. *Invest Ophthalmol Vis Sci* (2000) **41**(11):3467–73.
32. Ishida K, Panjwani N, Cao Z, Streilein JW. Participation of pigment epithelium in ocular immune privilege. 3. Epithelia cultured from iris, ciliary body, and retina suppress T-cell activation by partially non-overlapping mechanisms. *Ocul Immunol Inflamm* (2003) **11**(2):91–105. doi:10.1076/ocii.11.2.91.15914
33. Sugita S, Keino H, Futagami Y, Takase H, Mochizuki M, Stein-Streilein J, et al. B7+ iris pigment epithelial cells convert T cells into CTLA-4+, B7-expressing CD8+ regulatory T cells. *Invest Ophthalmol Vis Sci* (2006) **47**(12):5376–84. doi:10.1167/iovs.05-1354
34. Kawazoe Y, Sugita S, Keino H, Yamada Y, Imai A, Horie S, et al. Retinoic acid from retinal pigment epithelium induces T regulatory cells. *Exp Eye Res* (2012) **94**(1):32–40. doi:10.1016/j.exer.2011.11.002
35. Sugita S, Futagami Y, Smith SB, Naggar H, Mochizuki M. Retinal and ciliary body pigment epithelium suppress activation of T lymphocytes via transforming growth factor beta. *Exp Eye Res* (2006) **83**(6):1459–71. doi:10.1016/j.exer.2006.08.005
36. Sugita S, Horie S, Nakamura O, Maruyama K, Takase H, Usui Y, et al. Acquisition of T regulatory function in cathepsin L-inhibited T cells by eye-derived CTLA-2alpha during inflammatory conditions. *J Immunol* (2009) **183**(8):5013–22. doi:10.4049/jimmunol.0901623
37. Sugita S, Usui Y, Horie S, Futagami Y, Aburatani H, Okazaki T, et al. T-cell suppression by programmed cell death 1 ligand 1 on retinal pigment epithelium during inflammatory conditions. *Invest Ophthalmol Vis Sci* (2009) **50**(6):2862–70. doi:10.1167/iovs.08-2846
38. Sugita S, Usui Y, Horie S, Futagami Y, Yamada Y, Ma J, et al. Human corneal endothelial cells expressing programmed death-ligand 1 (PD-L1) suppress PD-1+ T helper 1 cells by a contact-dependent mechanism. *Invest Ophthalmol Vis Sci* (2009) **50**(1):263–72. doi:10.1167/iovs.08-2536
39. Bora NS, Gobleman CL, Atkinson JP, Pepose JS, Kaplan HJ. Differential expression of the complement regulatory proteins in the human eye. *Invest Ophthalmol Vis Sci* (1993) **34**(13):3579–84.
40. Streilein JW. Ocular immune privilege: therapeutic opportunities from an experiment of nature. *Nat Rev Immunol* (2003) **3**(11):879–89. doi:10.1038/nri1224
41. Taylor AW. Ocular immune privilege. *Eye (Lond)* (2009) **23**(10):1885–9. doi:10.1038/eye.2008.382
42. Niederkorn JY, Stein-Streilein J. History and physiology of immune privilege. *Ocul Immunol Inflamm* (2010) **18**(1):19–23. doi:10.3109/09273940903564766
43. Streilein JW, Niederkorn JY, Shadduck JA. Systemic immune unresponsiveness induced in adult mice by anterior chamber presentation of minor histocompatibility antigens. *J Exp Med* (1980) **152**(4):1121–5. doi:10.1084/jem.152.4.1121
44. Jiang LQ, Streilein JW. Immunologic privilege evoked by histoincompatible intracameral retinal transplants. *Reg Immunol* (1990) **3**(3):121–30.
45. Niederkorn JY. The immune privilege of corneal grafts. *J Leukoc Biol* (2003) **74**(2):167–71. doi:10.1189/jlb.1102543
46. Liu Y, Hamrah P, Zhang Q, Taylor AW, Dana MR. Draining lymph nodes of corneal transplant hosts exhibit evidence for donor major histocompatibility complex (MHC) class II-positive dendritic cells derived from MHC class II-negative grafts. *J Exp Med* (2002) **195**(2):259–68. doi:10.1084/jem.20010838
47. Sano Y, Okamoto S, Streilein JW. Induction of donor-specific ACAID can prolong orthotopic corneal allograft survival in "high-risk" eyes. *Curr Eye Res* (1997) **16**(11):1171–4. doi:10.1076/ceyr.16.11.1171.5109
48. Ma N, Streilein JW. Contribution of microglia as passenger leukocytes to the fate of intraocular neuronal retinal grafts. *Invest Ophthalmol Vis Sci* (1998) **39**(12):2384–93.
49. Streilein JW, Ma N, Wenkel H, Ng TF, Zamiri P. Immunobiology and privilege of neuronal retina and pigment epithelium transplants. *Vision Res* (2002) **42**(4):487–95. doi:10.1016/S0042-6989(01)00185-7
50. Kaplan HJ, Streilein JW, Stevens TR. Transplantation immunology of the anterior chamber of the eye. II. Immune response to allogeneic cells. *J Immunol* (1975) **115**(3):805–10.
51. Kaplan HJ, Streilein JW. Immune response to immunization via the anterior chamber of the eye. I. F. Lymphocyte-induced immune deviation. *J Immunol* (1977) **118**(3):809–14.
52. Wenkel H, Chen PW, Ksander BR, Streilein JW. Immune privilege is extended, then withdrawn, from allogeneic tumor cell grafts placed in the subretinal space. *Invest Ophthalmol Vis Sci* (1999) **40**(13):3202–8.
53. Sonoda KH, Sakamoto T, Qiao H, Hisatomi T, Oshima T, Tsutsumi-Miyahara C, et al. The analysis of systemic tolerance elicited by antigen inoculation

into the vitreous cavity: vitreous cavity-associated immune deviation. *Immunology* (2005) **116**(3):390–9. doi:10.1111/j.1365-2567.2005.02239.x
54. Lin HH, Faunce DE, Stacey M, Terajewicz A, Nakamura T, Zhang-Hoover J, et al. The macrophage F4/80 receptor is required for the induction of antigen-specific efferent regulatory T cells in peripheral tolerance. *J Exp Med* (2005) **201**(10):1615–25. doi:10.1084/jem.20042307
55. Streilein JW, Niederkorn JY. Induction of anterior chamber-associated immune deviation requires an intact, functional spleen. *J Exp Med* (1981) **153**(5):1058–67. doi:10.1084/jem.153.5.1058
56. Hsu SM, Mathew R, Taylor AW, Stein-Streilein J. Ex-vivo tolerogenic F4/80(+) antigen-presenting cells (APC) induce efferent CD8(+) regulatory T cell-dependent suppression of experimental autoimmune uveitis. *Clin Exp Immunol* (2014) **176**(1):37–48. doi:10.1111/cei.12243
57. Wilbanks GA, Streilein JW. Fluids from immune privileged sites endow macrophages with the capacity to induce antigen-specific immune deviation via a mechanism involving transforming growth factor-beta. *Eur J Immunol* (1992) **22**(4):1031–6. doi:10.1002/eji.1830220423
58. Hara Y, Okamoto S, Rouse B, Streilein JW. Evidence that peritoneal exudate cells cultured with eye-derived fluids are the proximate antigen-presenting cells in immune deviation of the ocular type. *J Immunol* (1993) **151**(10):5162–71.
59. Wilbanks GA, Mammolenti M, Streilein JW. Studies on the induction of anterior chamber-associated immune deviation (ACAID). III. Induction of ACAID depends upon intraocular transforming growth factor-beta. *Eur J Immunol* (1992) **22**(1):165–73. doi:10.1002/eji.1830220125
60. Faunce DE, Stein-Streilein J. NKT cell-derived RANTES recruits APCs and CD8+ T cells to the spleen during the generation of regulatory T cells in tolerance. *J Immunol* (2002) **169**(1):31–8. doi:10.4049/jimmunol.169.1.31
61. D'Orazio TJ, Niederkorn JY. Splenic B cells are required for tolerogenic antigen presentation in the induction of anterior chamber-associated immune deviation (ACAID). *Immunology* (1998) **95**(1):47–55. doi:10.1046/j.1365-2567.1998.00581.x
62. Sonoda KH, Stein-Streilein J. Ocular immune privilege and CD1d-reactive natural killer T cells. *Cornea* (2002) **21**(2 Suppl 1):S33–8. doi:10.1097/00003226-200203001-00008
63. Wilbanks GA, Streilein JW. Characterization of suppressor cells in anterior chamber-associated immune deviation (ACAID) induced by soluble antigen. Evidence of two functionally and phenotypically distinct T-suppressor cell populations. *Immunology* (1990) **71**(3):383–9.
64. Jiang L, He H, Yang P, Lin X, Zhou H, Huang X, et al. Splenic CD8+ T cells secrete TGF-beta1 to exert suppression in mice with anterior chamber-associated immune deviation. *Graefes Arch Clin Exp Ophthalmol* (2009) **247**(1):87–92. doi:10.1007/s00417-008-0947-8
65. Paunicka K, Chen PW, Niederkorn JY. Role of IFN-gamma in the establishment of anterior chamber-associated immune deviation (ACAID)-induced CD8+ T regulatory cells. *J Leukoc Biol* (2012) **91**(3):475–83. doi:10.1189/jlb.0311173
66. Sonoda KH, Stein-Streilein J. CD1d on antigen-transporting APC and splenic marginal zone B cells promotes NKT cell-dependent tolerance. *Eur J Immunol* (2002) **32**(3):848–57. doi:10.1002/1521-4141(200203)32:3<848::AID-IMMU848>3.0.CO;2-I
67. Sonoda KH, Taniguchi M, Stein-Streilein J. Long-term survival of corneal allografts is dependent on intact CD1d-reactive NKT cells. *J Immunol* (2002) **168**(4):2028–34. doi:10.4049/jimmunol.168.4.2028
68. Masli S, Turpie B, Hecker KH, Streilein JW. Expression of thrombospondin in TGFbeta-treated APCs and its relevance to their immune deviation-promoting properties. *J Immunol* (2002) **168**(5):2264–73. doi:10.4049/jimmunol.168.5.2264
69. Cousins SW, Trattler WB, Streilein JW. Immune privilege and suppression of immunogenic inflammation in the anterior chamber of the eye. *Curr Eye Res* (1991) **10**(4):287–97. doi:10.3109/02713689108996334
70. Streilein JW, Cousins SW. Aqueous humor factors and their effect on the immune response in the anterior chamber. *Curr Eye Res* (1990) **9**:175–82. doi:10.3109/02713689008999439
71. Kim MK, Caspi RR, Nussenblatt RB, Kuwabara T, Palestine AG. Intraocular trafficking of lymphocytes in locally induced experimental autoimmune uveoretinitis (EAU). *Cell Immunol* (1988) **112**(2):430–6. doi:10.1016/0008-8749(88)90312-7
72. Chan CC, Caspi RR, Roberge FG, Nussenblatt RB. Dynamics of experimental autoimmune uveoretinitis induced by adoptive transfer of S-antigen-specific T cell line. *Invest Ophthalmol Vis Sci* (1988) **29**(3):411–8.
73. Cousins SW, McCabe MM, Danielpour D, Streilein JW. Identification of transforming growth factor-beta as an immunosuppressive factor in aqueous humor. *Invest Ophthalmol Vis Sci* (1991) **32**(8):2201–11.
74. Connor TB Jr, Roberts AB, Sporn MB, Danielpour D, Dart LL, Michels RG, et al. Correlation of fibrosis and transforming growth factor-beta type 2 levels in the eye. *J Clin Invest* (1989) **83**(5):1661–6. doi:10.1172/JCI114065
75. Jampel HD, Roche N, Stark WJ, Roberts AB. Transforming growth factor-beta in human aqueous humor. *Curr Eye Res* (1990) **9**(10):963–9. doi:10.3109/02713689009069932
76. Knisely TL, Bleicher PA, Vibbard CA, Granstein RD. Production of latent transforming growth factor-beta and other inhibitory factors by cultured murine iris and ciliary body cells. *Curr Eye Res* (1991) **10**(8):761–71. doi:10.3109/02713689109013870
77. Kaiser CJ, Ksander BR, Streilein JW. Inhibition of lymphocyte proliferation by aqueous humor. *Reg Immunol* (1989) **2**(1):42–9.
78. Taylor AW. Review of the activation of TGF-beta in immunity. *J Leukoc Biol* (2009) **85**(1):29–33. doi:10.1189/jlb.0708415
79. Taylor AW, Alard P, Yee DG, Streilein JW. Aqueous humor induces transforming growth factor-beta (TGF-beta)-producing regulatory T-cells. 1997. *Ocul Immunol Inflamm* (2007) **15**(3):215–24. doi:10.1080/09273940701382234
80. Lau CH, Taylor AW. The immune privileged retina mediates an alternative activation of J774A.1 cells. *Ocul Immunol Inflamm* (2009) **17**(6):380–9. doi:10.3109/09273940903118642
81. Lee DJ, Taylor AW. Both MC5r and A2Ar are required for protective regulatory immunity in the spleen of post-experimental autoimmune uveitis in mice. *J Immunol* (2013) **191**(8):4103–11. doi:10.4049/jimmunol.1300182
82. Stuart PM, Griffith TS, Usui N, Pepose J, Yu X, Ferguson TA. CD95 ligand (FasL)-induced apoptosis is necessary for corneal allograft survival. *J Clin Invest* (1997) **99**(3):396–402. doi:10.1172/JCI119173
83. Griffith TS, Brunner T, Fletcher SM, Green DR, Ferguson TA. Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science* (1995) **270**(5239):1189–92. doi:10.1126/science.270.5239.1189
84. Hori J, Wang M, Miyashita M, Tanemoto K, Takahashi H, Takemori T, et al. B7-H1-induced apoptosis as a mechanism of immune privilege of corneal allografts. *J Immunol* (2006) **177**(9):5928–35. doi:10.4049/jimmunol.177.9.5928
85. Weiss EM, Schmidt A, Vobis D, Garbi N, Lahl K, Mayer CT, et al. Foxp3-mediated suppression of CD95L expression confers resistance to activation-induced cell death in regulatory T cells. *J Immunol* (2011) **187**(4):1684–91. doi:10.4049/jimmunol.1002321
86. Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. *Immunol Rev* (2010) **236**:219–42. doi:10.1111/j.1600-065X.2010.00923.x
87. Copland DA, Calder CJ, Raveney BJ, Nicholson LB, Phillips J, Cherwinski H, et al. Monoclonal antibody-mediated CD200 receptor signaling suppresses macrophage activation and tissue damage in experimental autoimmune uveoretinitis. *Am J Pathol* (2007) **171**(2):580–8. doi:10.2353/ajpath.2007.070272
88. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* (2009) **9**(3):162–74. doi:10.1038/nri2506
89. Marvel D, Gabrilovich DI. Myeloid-derived suppressor cells in the tumor microenvironment: expect the unexpected. *J Clin Invest* (2015) **125**(9):3356–64. doi:10.1172/JCI80005
90. Crook KR, Jin M, Weeks MF, Rampersad RR, Baldi RM, Glekas AS, et al. Myeloid-derived suppressor cells regulate T cell and B cell responses during autoimmune disease. *J Leukoc Biol* (2015) **97**(3):573–82. doi:10.1189/jlb.4A0314-139R
91. Li H, Dai F, Peng Q, Gan H, Zheng J, Xia Y, et al. Myeloid-derived suppressor cells suppress CD4(+) and CD8(+) T cell responses in autoimmune hepatitis. *Mol Med Rep* (2015) **12**(3):3667–73. doi:10.3892/mmr.2015.3791
92. Jiang LQ, Streilein JW. Immune responses elicited by transplantation and tissue-restricted antigens expressed on retinal tissues implanted subconjunctivally. *Transplantation* (1991) **52**(3):513–9. doi:10.1097/00007890-199109000-00025

93. Jiang C, Klassen H, Zhang X, Young M. Laser injury promotes migration and integration of retinal progenitor cells into host retina. *Mol Vis* (2010) **16**:983–90.
94. Ross J, He YG, Pidherney M, Mellon J, Niederkorn JY. The differential effects of donor versus host Langerhans cells in the rejection of MHC-matched corneal allografts. *Transplantation* (1991) **52**(5):857–61. doi:10.1097/00007890-199111000-00020
95. Wenkel H, Streilein JW. Analysis of immune deviation elicited by antigens injected into the subretinal space. *Invest Ophthalmol Vis Sci* (1998) **39**(10):1823–34.
96. Qiao H, Lucas K, Stein-Streilein J. Retinal laser burn disrupts immune privilege in the eye. *Am J Pathol* (2009) **174**(2):414–22. doi:10.2353/ajpath.2009.080766
97. Ng TF, Lavik E, Keino H, Taylor AW, Langer RS, Young MJ. Creating an immune-privileged site using retinal progenitor cells and biodegradable polymers. *Stem Cells* (2007) **25**(6):1552–9. doi:10.1634/stemcells.2006-0780
98. Taylor A, Namba K. In vitro induction of CD25+ CD4+ regulatory T cells by the neuropeptide alpha-melanocyte stimulating hormone (alpha-MSH). *Immunol Cell Biol* (2001) **79**(4):358–67. doi:10.1046/j.1440-1711.2001.01022.x
99. Ng TF, Kitaichi N, Taylor AW. In vitro generated autoimmune regulatory T cells enhance intravitreous allogeneic retinal graft survival. *Invest Ophthalmol Vis Sci* (2007) **48**(11):5112–7. doi:10.1167/iovs.07-0175
100. Hamrah P, Haskova Z, Taylor AW, Zhang Q, Ksander BR, Dana MR. Local treatment with alpha-melanocyte stimulating hormone reduces corneal allorejection. *Transplantation* (2009) **88**(2):180–7. doi:10.1097/TP.0b013e3181ac11ea
101. Taylor AW, Lee D. Applications of the role of alpha-MSH in ocular immune privilege. *Adv Exp Med Biol* (2010) **681**:143–9. doi:10.1007/978-1-4419-6354-3_12

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 28 January 2016
doi: 10.3389/fimmu.2016.00015

Prospective Clinical Testing of Regulatory Dendritic Cells in Organ Transplantation

Angus W. Thomson[1,2], Alan F. Zahorchak[1], Mohamed B. Ezzelarab[1], Lisa H. Butterfield[2,3], Fadi G. Lakkis[1,2] and Diana M. Metes[1,2]*

[1] Department of Surgery, Starzl Transplantation Institute, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA, [2] Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA, [3] Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Dendritic cells (DC) are rare, professional antigen-presenting cells with ability to induce or regulate alloimmune responses. Regulatory DC (DCreg) with potential to down-modulate acute and chronic inflammatory conditions that occur in organ transplantation can be generated *in vitro* under a variety of conditions. Here, we provide a rationale for evaluation of DCreg therapy in clinical organ transplantation with the goal of promoting sustained, donor-specific hyporesponsiveness, while lowering the incidence and severity of rejection and reducing patients' dependence on anti-rejection drugs. Generation of donor- or recipient-derived DCreg that suppress T cell responses and prolong transplant survival in rodents or non-human primates has been well-described. Recently, good manufacturing practice (GMP)-grade DCreg have been produced at our Institution for prospective use in human organ transplantation. We briefly review experience of regulatory immune therapy in organ transplantation and describe our experience generating and characterizing human monocyte-derived DCreg. We propose a phase I/II safety study in which the influence of donor-derived DCreg combined with conventional immunosuppression on subclinical and clinical rejection and host alloimmune responses will be examined in detail.

Keywords: dendritic cells, immune regulation, renal transplantation

OPEN ACCESS

Edited by:
Gilles Benichou, Massachusetts General Hospital, USA

Reviewed by:
Nuala Mooney, Centre National pour la Recherche Scientifique, France
Régis Josien, Nantes University Medical School, France

****Correspondence:***
Angus W. Thomson
thomsonaw@upmc.edu

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *28 October 2015*
Accepted: *12 January 2016*
Published: *28 January 2016*

Citation:
Thomson AW, Zahorchak AF, Ezzelarab MB, Butterfield LH, Lakkis FG and Metes DM (2016) Prospective Clinical Testing of Regulatory Dendritic Cells in Organ Transplantation. Front. Immunol. 7:15. doi: 10.3389/fimmu.2016.00015

INTRODUCTION

While rates of acute renal transplant rejection have improved dramatically since the advent of calcineurin inhibition (CNI) >30 years ago, similar improvement in long-term graft survival has not been achieved. This reflects the inability of conventional immunosuppressive agents to prevent late graft dysfunction leading to transplant failure (1, 2). Moreover, conventional immunosuppression is associated with significant morbidity and mortality due to cardiovascular, infectious, and pro-neoplastic side effects. Attempts to improve long-term survival, while reducing the burden of immunosuppression, have not been particularly fruitful to date. While the recent introduction of co-stimulation blockade, although renal-sparing, has resulted in an increased incidence of acute rejection (3), use of depleting antibody (Ab) as induction therapy at the time of transplantation has also failed to guarantee safe withdrawal of CNI, even in patients with stable graft function (4, 5). Furthermore, efforts to induce donor-specific tolerance using hematopoietic stem cell transplantation, an approach first shown to be successful many years ago in mice (6), have yielded promising

results, but many hurdles remain in terms of safety and widespread applicability (7).

Our long-term goal is to develop a novel, safe, donor-specific induction (pre-conditioning) approach that will promote sustained, donor-specific immune hyporesponsiveness, while lowering the incidence and severity of acute and chronic rejection and reducing patients' dependence on anti-rejection drugs. There is recent evidence that, by exploiting inherent mechanisms of immune regulation, it may be possible to achieve this goal. Rare, naturally occurring regulatory immune cells, either innate [regulatory dendritic cells (DCreg)] or adaptive [regulatory T cells (Treg)], critically regulate immunity, can promote antigen (Ag)-specific T cell hyporesponsiveness, and prevent adverse immune reactions (self-tolerance) in the healthy steady-state (8, 9). Moreover, in small animals, the adoptive transfer of DCreg (10–13) or Treg (14) can prolong allograft survival and induce donor-specific tolerance to organ transplants (15). Other regulatory immune cells with potential therapeutic applications include regulatory macrophages [Mreg; (16–18)], myeloid-derived suppressor cells [MDSC; (19)], T regulatory type-1 cells [Tr1 cells; (20)], and regulatory B cells (21). In addition to *ex vivo*-expanded Treg, now entering phase I/II trials in organ transplantation[1], a compelling rationale has emerged for clinical testing of DCreg, i.e., donor or recipient blood monocyte-derived DC generated and modified *ex vivo* to promote their inherent regulatory properties (13, 22–24). Thus, we and others have shown that, in rodents, infusion of DCreg of donor or recipient origin before or after transplantation, including their use in combination with conventional immunosuppressive agents, can promote indefinite organ allograft survival. More importantly and uniquely, using a robust, clinically relevant, non-human primate (NHP) model with minimal immunosuppression, we have shown that infusion of donor-derived DCreg, 1 week before transplant, safely prolongs major histocompatibility complex (MHC)-mismatched, life-sustaining renal allograft survival, with no evidence of host sensitization (25). Equally significant is our demonstration that this therapeutic effect is associated with selective attenuation of donor-reactive memory T cell (Tmem) responses (25, 26), an important barrier to improvement of long-term graft survival (27, 28).

We have now generated good manufacturing practice (GMP) grade human DCreg from elutriated peripheral blood monocytes and demonstrated both their stable resistance to maturation under inflammatory conditions and their ability to negatively regulate alloreactive T cell responses. We have also established release criteria for clinical testing and plan to conduct a safety trial of donor-derived DCreg in adult, *de novo*, live-donor renal transplantation. To our knowledge, this promising donor-specific induction approach to regulatory immune cell therapy in clinical organ transplantation is unique. It is distinct from the testing of recipient blood monocyte-derived DCreg in live-donor renal transplantation currently being conducted at the University of Nantes, France, as part of The ONE Study (29, 30).

[1]https://clinicaltrials.gov

THE CASE FOR DCreg THERAPY IN ORGAN TRANSPLANTATION

Extensive pre-clinical studies that we and others have conducted in rodents and human surrogate models provide compelling evidence of the potential of regulatory immune cell therapy to improve allograft outcomes and, in many instances, promote donor-specific tolerance (15). The case for testing DCreg generated *ex vivo* in human transplantation is particularly compelling (13, 23, 24) for the following reasons. First, DC are uniquely well-equipped, professional Ag-presenting cells (APC) that potently regulate innate and adaptive immunity (31, 32). Second, in many animal studies, DCreg adoptively transferred to graft recipients *before* transplant induce Ag-specific T cell unresponsiveness (13) and promote indefinite organ allograft survival. Moreover, this beneficial effect on graft survival does not appear to depend on the *in vivo* persistence of intact DCreg (33–35). Indeed, the apparent independence of efficacy and regulatory mechanisms on the persistence of intact donor DCreg may be a distinct advantage over other cell therapy approaches. Thus, e.g., Treg therapy may require costly repeated infusion of very large numbers of expanded cells (36, 37) and their sustained viability/replication may be required to achieve a therapeutic effect. *Third*, an important attribute of DCreg is their ability to regulate, in addition to *de novo*-primed effectors, preformed Tmem responses (38–40) that, either due to preformed memory to alloAgs or due to molecular mimicry and cross-reactivity with human leukocyte antigens (HLA) (41), represent a major barrier to long-term graft survival in humans (27, 28, 42, 43). *Fourth,* in normal humans, local adoptive transfer of monocyte-derived DCreg has been shown to induce Ag-specific unresponsiveness to nominal Ags (44, 45). *Fifth,* using minimal immunosuppression in a robust NHP model, we have reported that a single infusion ($3.5-10 \times 10^6$/kg) of donor-derived DCreg, 1 week before transplant, safely prolongs renal allograft survival, with no evidence of host sensitization (25). Importantly, this effect is associated with attenuation of donor-specific, alloreactive Tmem responses (25, 26).

The unique phase I/II trial of donor-derived DCreg that we now propose in live-donor renal transplantation is essentially a dose-escalation safety trial in which the cell product will be administered, once only, concomitant with mycophenolic acid (MPA), 1 week before transplantation to patients receiving standard immunosuppression (CNI, MPA, and steroids). Successful safety evaluation of our strategy and any evidence of inhibition of early, acute subclinical or clinical rejection, and/or attenuation of long-term anti-donor immunity would justify broader evaluation of DCreg efficacy in renal transplantation. This would potentially address unmet needs of CNI-free immunosuppression and/or realize the unmet goal of improving long-term allograft survival, without increasing the burden of immunosuppression.

Thus, in future studies, it would be of interest to evaluate the influence of DCreg combined with co-stimulation blockade (Co-B) to ascertain whether the incidence of rejection episodes encountered with Co-B (3) can be reduced. Furthermore, evidence of a beneficial effect of DCreg pre-conditioning in early clinical trials might justify evaluation of immunosuppressive drug curtailment. It is likely that the DCreg approach can be

applied readily in the clinic since, based on pre-clinical testing, a single infusion of a relatively small number of DCreg is sufficient to achieve the salutary effect. Therefore, neither expensive expansion of the cell product, nor repeated infusion may be necessary. It is also probable that donor-derived DCreg will have broader clinical applications to encompass recipients of renal and other organ transplants from *deceased* donors. Indeed, rodent studies have shown that delaying DCreg infusion until 7 or 14 days post transplant is (still) effective in prolonging graft survival (46, 47), thus providing ample time to prepare DCreg from deceased donors.

NOVELTY OF THE APPROACH

Several closely interrelated aspects of our proposed clinical trial of DCreg in live-donor renal transplantation are highly innovative. *First*, we have generated a highly-purified GMP cell product (allograft donor blood monocyte-derived DCreg) distinct from those [autologous tolerogenic DC (not pulsed with donor antigen), Treg, Type-1 regulatory T cells (Tr1) cells, Mreg, and mesenchymal stem cells] being investigated by other groups, which satisfies phenotypic and functional release criteria. The manufacturing process is relatively simple, comparatively short and highly reproducible. Second, while early pilot studies have begun to examine the safety of *autologous* DCreg in human autoimmune diseases (48–50) and organ transplantation (29), this will be the first study to test *allogeneic* (donor-derived) DCreg in human organ transplantation. *Third*, our proposed mechanistic studies will address our *hypothesis* that, in addition to inhibition of *de novo* T cell priming and memory reactivation against donor HLA Ags, DCreg infusion will selectively undermine early inflammation that fuels anti-donor effector/Tmem responses and promote specific T cell unresponsiveness to donor that we will monitor sequentially in blood and protocol biopsies. We will also generate novel insight into the persistence/longevity of donor-derived DCreg in graft recipients. Of particular relevance, based on our NHP transplant data, will be analyses of *de novo*-primed T cell and Tmem phenotype and function and the potential of establishing new biomarkers of donor-specific hyporesponsiveness based on the profile of donor-reactive T cells. *Fourth*, since protocol biopsies will be performed, we will gain preliminary insight into the influence of DCreg on the incidence of subclinical rejection, an important predictor of long-term graft outcomes by analyzing graft-infiltrating T lymphocytes. By contrast, traditional immunosuppression trials have focused on the incidence of clinically evident rejection as a principal endpoint.

RATIONALE FOR TESTING DCreg IN HUMAN KIDNEY TRANSPLANTATION

Dendritic cells are highly specialized, bone marrow-derived APC [first described >40 year ago (51)] that induce or regulate innate and adaptive immunity (13, 32, 52–54). While DCreg play a crucial role in maintaining self-tolerance in the healthy steady-state (8, 55, 56) over the past 20 year, our research and others have revealed that these cells can subvert naïve T cell and Tmem responses by various mechanisms (13, 22, 57–59) and that DCreg can induce or restore T cell tolerance in animal models of autoimmune disease (60–63) or organ transplant rejection (12, 13, 22, 64). In experimental transplantation, both donor-derived allogeneic DCreg and donor Ag-pulsed host autologous DCreg are effective. Importantly, our work has also confirmed that adoptive transfer of donor-derived DCreg can safely regulate T cell responses in clinically relevant NHP models, including MHC mis-matched organ allograft recipients (25, 65), an important bridge to clinical testing. There is also well-documented evidence that adoptive transfer of DCreg (*in vitro*-generated autologous DC) via local administration can control T cell responses to model Ags (flu matrix peptide and keyhole limpet hemacyamin) in human healthy volunteers (44, 45). Important insights gained from *in vitro* studies and animal models have driven the recent development of clinical grade human DCreg (66–70), with the potential to treat autoimmune disease or enhance transplant survival, while reducing patients' dependence on immunosuppressive drugs. Phase I safety trials, in which autologous DCreg have been administered locally, have been conducted in type-1 diabetes (48) and rheumatoid arthritis (RA) (49, 50), with results that emphasize the feasibility, safety, and potential efficacy of DCreg therapy.

Based on these findings, we hypothesize that DCreg infusion, as an adjunct to conventional immunosuppression, can improve long-term renal allograft and patient outcomes, with minimal early adverse events, by targeting both innate immunity and preformed memory responses. It also carries the prospect of enabling immunosuppression reduction in stable patients or converting to CNI-free immunosuppression, without increasing the incidence of rejection.

Our laboratory has had a major focus on the characterization and therapeutic efficacy of DCreg, especially in experimental pancreatic islet, skin, and organ transplantation (46, 64, 71–79). These studies include the first observations that these regulatory innate immune cells, deficient in MHC and co-stimulatory molecule expression and in the production of pro-inflammatory cytokines, could subvert alloAg-specific T cell responses, *in vitro* and *in vivo* (72, 80). In addition, we have extensive experience in the characterization and immune profiling of human T lymphocytes, including the contribution of naïve T cell and Tmem subsets to the alloimmune response, and the effects of induction therapy on regulatory T cell and Tmem subsets in relation to clinical outcome in kidney transplantation (41, 81).

EVIDENCE IN SUPPORT OF DCreg THERAPY IN TRANSPLANTATION

We summarize below evidence from rodent, NHP, and human studies that support the safety and, in the case of pre-clinical models, the efficacy of DCreg in solid-organ transplantation.

Rodent Observations

We and others have shown that combination of pre-transplant (day −7) infusion of donor-derived DCreg, either alone or with low doses of immunosuppressive agents, can induce donor-specific organ transplant tolerance in rodents (12, 74, 82–85). The route of administration, dosage, dosage regimen, and duration of dosing

TABLE 1 | Promotion of indefinite heart or renal allograft survival in rodents by infusion of donor-derived DCreg.

DC source	Species	DC culture conditions	Route of injection	When administered[a]	Additional host treatment	MST	Reference
MoDC	rat	GM-CSF	i.v.	Day + 14/15	None	>160 days	Hayamizu et al. (86)
BMDC	mouse	GM-CSF + TGFβ	i.v.	Day-7	Anti-CD40L mAb	>100 days (40%)	Lu et al. (73)
BMDC	mouse	Low GM-CSF	i.v.	Day-7	None	>100 days	Lutz et al. (12)
BMDC	mouse	GM-CSF + IL-4 + NF-κB ODN + Ad CTLA4Ig	i.v.	Day-7	None	>100 days (40%)	Bonham et al. (74)
BMDC	rat	GM-CSF + IL-4	i.v.	Day-7	ALS	>200 days (50%)	DePaz et al. (85)
BMDC	mouse	Low GM-CSF	i.v.	Day-7	Anti-CD54 mAb + CTLA4Ig	>100 days	Wang et al. (83)
BMDC	Rat[b]	GM-CSF + IL-4 + dexamethasone	i.v.	Day-10	CTLA4Ig + cyclosporine	>100 days	Mirenda et al. (84)

[a]*In relation to transplantation on d0.*

[b]*Renal transplant.*

Ad, adenoviral vector; ALS, anti-lymphocyte serum; BMDC, bone marrow-derived dendritic cells; i.v., intravenous; MoDC, monocyte-derived DC; MST, mean graft survival time; ODN, oligodeoxynucleotides decoys.

TABLE 2 | Evidence that use of standard-of-care immunosuppressive agents (corticosteroid, MMF, and CNI) together with DCreg promote long-term allograft survival in rodents.

Agent	Type of allograft (species)	Reference
MMF	Pancreatic islet (mouse)	Adorini et al. (87)
Dexamathasone	Renal (rat)	Mirenda et al. (84)
Tacrolimus	Composite tissue (rat)	Eun et al. (88)
Cyclosporine	Composite tissue (rat)	Ikeguchi et al. (46)
	Renal (rat)	Mirenda et al. 2004 (84)

MMF, mycophenolate mofetil; CNI, calcineurin inhibitor.

(single i.v. infusion of up to 5×10^6 per kg of donor DCreg, 7 days prior to transplantation) that we propose in a phase I clinical trial are, therefore, supported by experiments in rodents [**Table 1** and (12, 73, 74, 82–86)] and NHP (25). It is also important to note that use of conventional "standard of care" (SOC) immunosuppressive agents (MPA, CNI, or steroids), together with DCreg, promotes long-term allograft survival in rodents [**Table 2** and (46, 84, 87, 88)]. This is of direct relevance to the use of SOC immunosuppressive therapy in our proposed clinical trial.

NHP Observations

Non-human primate transplant models are considered important predictors of the safety and efficacy of experimental immunosuppressive/tolerogenic regimens since the NHP immune system more closely resembles that of humans than mice, and since, as in humans (but not in mice), Tmem present an important and difficult to overcome barrier to induction of donor-specific tolerance (41, 89–91). We have used a robust, MHC-mismatched, life-sustaining rhesus macaque renal transplant model to evaluate the safety and efficacy of donor-derived DCreg therapy (25). In these studies, DCreg were generated from CD14 immunobead-isolated blood monocytes in a single leukapheresis product of the prospective kidney donor in granulocyte macrophage-colony-stimulating factor (GM-CSF) and IL-4. During the 7-day culture period, vitamin D3 (VitD3), a nuclear factor κβ inhibitor that impairs DC differentiation and maturation (92, 93) and IL-10, that converts immature DC into tolerogenic APC (94), were added to promote the maturation-resistant DCreg phenotype (95). We tested whether DCreg of donor origin, infused prospectively (once only) 7 days before transplant, could safely prolong graft survival using a minimal immunosuppressive regimen of co-stimulation blockade [CTLA4Ig = cytotoxic T lymphocyte Ag 4:Ig (abatacept)] and mechanistic target of rapamycin (mTOR) inhibition [rapamycin (sirolimus)]. Our findings (25) clearly show that (1) no adverse effects were encountered, (2) no evidence of host sensitization was detected, as determined by circulating anti-donor alloAb levels, (3) graft survival time was prolonged significantly (threefold increase) in the group given DCreg compared to those recipients that did not receive the cell infusion, (4) weight loss and proteinuria were less marked in DCreg-infused monkeys, and (5) evidence was obtained of significant, donor-specific attenuation (exhaustion) of Tmem responses as evidenced by upregulation of concomitant programed death (PD)-1 and CTLA4 expression (**Figure 1**), reduced memory:regulatory T cell ratios in peripheral blood, and reduced $CD8^+$ effector T cell responses in the transplant (25, 26).

Human Observations

Several pharmacologic agents and cytokines have been used to generate GMP grade autologous human DCreg for prospective clinical use in chronic inflammatory diseases, including type-1 diabetes, RA, and multiple sclerosis (48–50, 66–68). The safety of locally administered, autologous, monocyte-derived DCreg in type-1 diabetes or RA patients has been reported (48–50). To our knowledge, there has been no human experience with donor-derived DCreg in human organ transplantation. However, clinical experience with a closely related, donor-derived myeloid lineage cell product in either deceased- or live-donor renal transplantation is relevant to the proposed investigation of DCreg in organ transplantation. Thus, "immunoregulatory macrophages" (Mreg) or "transplant acceptance-inducing cells" have been investigated by Hutchinson and colleagues in Germany as immune-conditioning therapy in human renal transplantation (96). The phenotype of these cells identifies them as a subtype of partially mature macrophages (96). Initially, they were generated from deceased-donor splenic mononuclear cells cultured in macrophage (M)-CSF and IFNγ for 5 days and administered i.v. on post-transplant day 5. All patients ($n = 12$; with 3–5 total

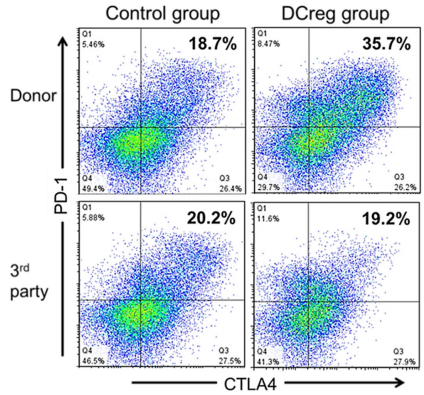


FIGURE 1 | DCreg infusion enhances programed death (PD)-1 and cytotoxic T lymphocyte antigen-4 (CTLA4) expression by donor-reactive CD4⁺Tmem in renal allograft recipient monkeys. Incidences of PD1⁺ CTLA4⁺ populations in *ex vivo*-stimulated CD95⁺CD4⁺Tmem from representative control and DCreg-treated monkeys ($n = 4$ monkeys analyzed/group). Recipient PBMC obtained 28 days after transplantation, were co-cultured with either donor or third party stimulators (T cell-depleted PBMC) for 5 days before flow cytometric analysis. The enhanced incidence of PD1⁺CTLA4⁺Tmem in response to donor, but not third party stimulation suggests selective attenuation (exhaustion) of donor-reactive Tmem. According to Ezzelarab et al. (25), Figure 5.

MHC-mis-matches) received ≥0.55×10^6 viable Mreg/kg (range $0.55–7.52 \times 10^6$/kg) and were immunosuppressed at the outset with tacrolimus, sirolimus, and glucocorticoids. They were then weaned from steroid therapy, if clinically appropriate, on day 28 post-transplant. Administration of comparatively large numbers of these cells (up to 5×10^8 viable cells) via a central line was safe, with no evidence of graft-versus-host reactions induced by the Mreg or contaminating lymphocyte populations. Furthermore, as in our NHP DCreg studies, there was no evidence that human Mreg sensitized the recipients to donor Ags, or that the cells themselves could otherwise accelerate rejection. Importantly, none of the study participants experienced any delayed complications from Mreg infusion (mean follow-up time 36 months). Thus, it was concluded that the infusion of (donor-derived) Mreg was practicable and safe in the acute and medium term.

The same group of investigators have also infused donor-derived Mreg to live-donor kidney transplant recipients ($n = 5$), 5 days before renal transplantation (97). A larger number of Mreg and a different immunosuppressive regimen [anti-thymocyte globulin (ATG), tacrolimus, and steroids] were employed. PBMC were isolated from donor leukapheresis products 14 days before transplant. On day 9 pre-transplant, non-adherent PBMC from leukapheresis products of the prospective graft recipients were added (2.10^7/ml) to the donor-derived Mreg and the co-cultures of donor origin Mreg and recipient PBMC maintained for a further 4 days until infused ($1.74–10.39 \times 10^7$ Mreg/kg) 5 days before transplant. No complications were observed. Moreover, there was no evidence that infusion of donor-derived Mreg prior to transplantation could sensitize recipients to donor Ags or otherwise accelerate graft rejection. As in the earlier study, it was concluded that preoperative treatment of live-donor kidney transplant recipients with Mreg was clinically practicable and safe in the acute and medium term.

In a further (2011) publication (98), the same group (plus additional authors) reported on two live-donor renal transplant patients who were given donor-derived Mreg (99) cultured for 6 days with M-CSF before stimulation with IFNγ for a further 24 h, and then administered 6 or 7 days before transplant. In this case, the Mreg were $CD14^{-/lo}$, $HLA\text{-}DR^+$, $CD30^{-/lo}$, $CD86^+$, $CD16^-$, toll-like receptor (TLR)2^-, and $CD163^{-/lo}$. One patient (single HLA-B and HLA-DR mismatches) received 8.0×10^6 cells/kg and the other (fully HLA-mismatched) received 7.1×10^6 cells/kg. Labeling of a proportion of the infused Mreg with [^{111}In]-oxine in one patient and whole-body single photon emission computed tomography imaging (SPECT) revealed that the Mreg located initially in the lungs, but after 2.5 h were evident in the circulation and had begun to accumulate in the liver and spleen. Twenty-four hours after Mreg infusion, signal from the lung had diminished substantially and the cells had accumulated in the liver, spleen, and bone marrow. Absence of signal from the patient's urinary tract throughout the 30 h follow-up suggested that the majority of labeled infused cells remained alive. No unexpected adverse events were observed in either patient. At 3 and 2 year, respectively, post-transplant, the patients were taking once-daily or twice-weekly tacrolimus. Despite early minimization of immunosuppressive therapy, neither patient underwent an acute rejection episode during the 3-year follow-up period.

POTENTIAL MECHANISMS OF THE LONG-TERM MAINTENANCE OF SUPPRESSION AFTER DCreg ADMINISTRATION

The *in vivo* mechanisms whereby infusion of donor (or recipient)-derived DCreg restrains alloimmunity and promotes long-term survival of experimental organ allografts are not well understood. In mice, there is evidence that donor-derived DCreg infused before transplantation are targeted by host NK cells and, thus, short-lived (35). They are reprocessed by quiescent host splenic DCs for presentation of alloAg to indirect pathway $CD4^+$ T cells. This results in abortive activation and deletion of T effector cells without impairing the incidence of indirect $CD4^+$ $Foxp3^+$ Treg, thus enhancing the regulatory to effector T cell ratio (33, 100). It appears, therefore, that mechanisms that sustain long-term graft survival are not dependent on persistence of intact donor DCreg.

PROPOSED CLINICAL TESTING OF DCreg IN RENAL TRANSPLANTATION

Here, we propose a protocol for the generation and testing of donor-derived DCreg in a phase I clinical trial in renal transplant recipients receiving conventional immunosuppressive therapy.

Generation, Purity, and Yield of hu DCreg

To ensure sufficient DCreg yields, blood monocytes will be obtained and banked in high purity by elutriation from

cryopreserved leukapheresis products of the prospective transplant donors approximately 28–15 days before scheduled transplantation (**Figure 2**). Fourteen days before transplantation, monocytes will be thawed and DCreg generated for infusion into the prospective graft recipient on day-7 (**Figure 1**). In our experience, whole individual leukapheresis products from non-mobilized, healthy adult volunteers yield $4.3 \pm 1.05 \times 10^9$ PBMC. Recovery of monocytes post-elutriation [consistently ≥90% pure with <1% $CD3^+$ T cell contamination ($n = 4$)] represents, on average, 25% of the total PBMC. The phenotype of the purified monocytes, determined by flow cytometry, is HLA-DR^+ $CD40^{lo}$ $CD80^{lo}$ $CD86^+$, programed death ligand (PD-L) 1^{lo}, $CD14^+$.

The DCreg are generated from thawed monocytes in serum-free Cell Genix (Cellgro) medium, supplemented with 5% certified human AB serum and recombinant human (rhu) GM-CSF (1000 units/ml) and rhu IL-4. These cytokines are added at the start of culture (day 0) and on day 4. VitD3 and rhu IL-10, which suppress DC maturation (94, 101, 102), are also added on day 4. The culture period is 7 days. We consistently generate sufficient, highly purified DCreg from elutriated peripheral blood monocytes (yield = 17 ± 7% of starting monocyte number) from a single whole leukapheresis product to administer up to $2.0-2.5 \times 10^6$ per kg to a 70 kg recipient. To obtain larger numbers of DCreg for a higher dose, a second donor leukapheresis may be required.

The DCreg harvested at day 7 of culture are consistently >94% pure, with ≤0.1% contaminating $CD3^+$T lymphocytes determined by flow cytometry. It is especially significant that the incidence of T cells is so low since these are the cells that are of concern regarding risk of graft-versus-host disease. The DCreg consistently exhibit an immature phenotype compared to control DC (i.e., immature DC generated in DC media without VitD3 and IL-10) and are HLA-DR^+, $CD11c^+$, $CD14^-$, $CD40^{lo}$, $CD80^{lo}$, $CD86^{lo}$, PD-$L1^{hi}$, $CCR7^+$, $CD83^{lo}$. High expression of PD-L1(= B7-H1), a negative regulator of T cell responses and a consistently high PD-L1:CD86 ratio [determined as: PD-L1 mean fluorescence intensity (MFI) ÷ isotype control/CD86 (MFI) ÷ isotype control] conforms to that of DCreg with potential to subvert alloreactive T cell responses.

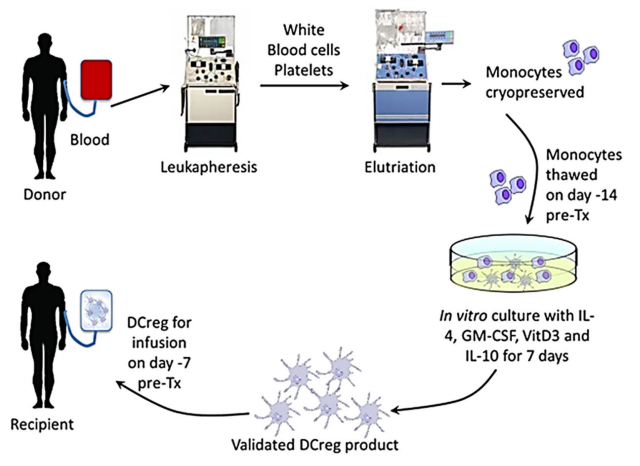


FIGURE 2 | Generation of DCreg from elutriated blood monocytes of the prospective renal allograft donor in GM-CSF, VitD3, and IL-10, and infusion of the validated cell product into the graft recipient 7 days before transplant.

Function of DCreg

Investigation of the function of hu DCreg harvested at 7 days of culture, including their responses to the TLR4 ligand bacterial lipopolysaccharide (LPS), CD40 ligation, or a pro-inflammatory cytokine cocktail, will ensure their regulatory properties. The DCreg we have generated display robust resistance to phenotypic and functional maturation in response to factors that promote the maturation of control DC, confirming that they are refractory to stimulation under inflammatory conditions. In particular, the inability of DCreg exposed to LPS to release pro-inflammatory and immunostimulatory cytokines (tumor necrosis factor [TNF]α and IL-12p70) is profoundly inhibited, while production of anti-inflammatory IL-10 is preserved, resulting in marked reversal of the IL-12:IL-10 and TNFα:IL-10 ratios.

The ability of the DCreg exposed to LPS to induce hyporesponsiveness of normal allogeneic T cells is of paramount importance. Therefore, DCreg (ratio 1 DCreg:40 T cells) should induce minimal CD4 and CD8 T cell proliferation, IFNγ and IL-17 release, or granzyme B production in the responder $CD4^+$ T cells over 4 days in culture. This analysis provides further assurance of the inability of the DCreg, despite exposure to a potent pro-inflammatory stimulus (LPS), to stimulate allogeneic effector T cell responses. Similarly, marked attenuation of alloreactive $CD8^+$ T cell responses is observed.

Thus, we contend that infusion of DCreg that are (1) phenotypically immature, (2) resistant to maturation under inflammatory conditions, and (3) able to induce allogeneic T cell hyporesponsiveness *in vitro* will not induce sensitization of prospective recipients following their adoptive *in vivo* cell transfer and rather, will induce donor-specific T cell hyporesponsiveness. Our plan to closely monitor study patients for evidence of development of donor-specific alloAb production and anti-donor T cell reactivity will allow detection of any increase in anti-donor immune effector activity in the unlikely event it should occur.

Release Criteria for DCreg

The DCreg generated for infusion will undergo rigorous testing at specified time points during their manufacture from blood monocytes. The following release criteria will be considered as crucially important: DCreg yield (sufficient cells to allow infusion of the target number per kilogram), percent purity (>95% DC, <1% T cells), and viability (>70%); sterility; DCreg phenotype: phenotypic characterization will be performed by flow cytometry to monitor CD86 and PD-L1 expression before and after LPS stimulation, compared to conventional DC cultured in GM-CSF + IL-4 and not VitD3 and IL-10. High PD-L1 and low CD86 expression, before and after LPS stimulation, with a PD-L1:CD86 ratio >3.5 (based on pre-clinical results) will be used as a release criterion. The 3.5 ratio is based on many analyses in which a ratio of 3.5 or above was associated with a cytokine profile and T cell stimulatory profile consistent with the induction of alloreactive T cell hyporesponsiveness. DCreg function:

supernatants from cultures (DCreg exposed or not to LPS) will be tested by ELISA to assess the lack of IL-12p70/TNF-α and the presence of IL-10 production, consistent with their regulatory properties and their resistance to maturation. We consider this, in addition to the tests above, a simple, reproducible, release criterion that can be applied before release of the DCreg product for infusion.

DCreg Infusion

We plan to test three dose levels of DCreg in three separate groups of recipients ($n = 5$/group, with 4 receiving DCreg and 1 "control" subject receiving concomitant pre- and post-transplant immunosuppression without DCreg): dose 1: 0.5×10^6 cells/kg body weight; dose 2: 2.5×10^6 cells/kg body weight; dose 3: 5×10^6 cells/kg body weight.

Concurrent Immunosuppressive Drug Regimen

The renal transplant recipients will receive combination immunosuppressive medications according to SOC at our Institute, with two exceptions. First, MPA (that blocks DNA synthesis in T and B cells) will be initiated 7 days before transplant, at the time of donor DCreg infusion, instead of on the day of transplantation. This is in order to minimize any risk of sensitizing the patient. Historically, pre-treatment of kidney transplant recipients with unmodified donor-specific transfusions and low-dose azathioprine (that acts similarly to MPA) significantly reduced the risk of sensitization (103–107). Furthermore, MPA augments and maintains the regulatory function of DC (108, 109), additionally minimizing any safety concern that DCreg could convert to a stimulatory phenotype after infusion. Second, Ab induction therapy will not be administered at the time of transplant. Patients will be maintained on triple immunosuppressive therapy with MPA, tacrolimus, and prednisone after transplantation, a combination regimen widely applied as SOC at many transplant centers, both in North America and elsewhere worldwide and in The ONE Study of regulatory immune cell therapy in renal transplantation[2], a trans-Atlantic (European and North American) trial utilizing a unified approach to evaluating immune cell therapy in renal transplantation for the reasons outlined above. The immunosuppressive drug regimen that we propose differs from the regimen (belatacept and rapamycin) that we used together with DCreg in NHP (25). This is because belatacept plus rapamycin is not SOC in human renal transplantation and it is important to assess the safety and efficacy of DCreg in humans in comparison with current SOC, as being evaluated in The ONE study, including the testing of autologous DCreg.

The rationale for not using ATG, alemtuzumab (anti-CD52 mAb) or basiliximab (anti-IL-2Rα mAb) as induction therapy at transplant, is to avoid potential targeting of DCreg infused 7 days before transplant or dampening of immunoregulatory pathways triggered in host T cells by DCreg. In our NHP study, we established that such an approach (pre-transplant immunosuppression at the time of DCreg infusion and avoidance of lymphocyte-depleting induction agents) is both safe and effective. We have opted, however, not to use a Co-B and/or mTOR inhibition-based immunosuppressive regimen, such as that employed in our NHP study, because of the high incidence of acute rejection episodes, including higher grade rejection, in patients receiving Co-B (belatacept), MPA, and steroid therapy and increased side effects in clinical trials of rapamycin-based regimens, either with CNI or MPA (3, 5). Since our initial proposed clinical trial is a *safety* trial, we have chosen to adhere to a safe and proven immunosuppression regimen that does not interfere with DCreg action.

Persistence of Donor DCreg after Infusion

Monitoring DCreg persistence in the circulation and their tissue homing is essential for understanding their survival and distribution. Flow cytometry techniques to detect donor T cells in peripheral blood of transplant recipients with a threshold sensitivity of one donor cell in 1000 recipient cells (0.1%) are readily available (110). We plan to identify donor DCreg in whole blood at various time points post-transplant**,** by flow staining for Lin^-, $HLA\text{-}DR^+$, $BDCA1(CD1c)^+$, $CD209\ (DC\ SIGN)^+$, $CD11c^+$ DC, in conjunction with staining for a miss-matched donor MHC allele. This approach will allow us distinguish between recipient and donor-derived DCreg. Others (98) have used [^{111}In]-oxine to label allogeneic donor-derived myeloid cells (Mreg) for short-term tracking by SPECT imaging following their infusion in renal transplant patients and we will consider using this as a complementary approach.

Mechanistic and Immunological Monitoring Analyses of Transplant Recipients

Cellular pathways engaged after organ transplantation are complex and involve coordinated interactions between DC as APC and distinct effector and regulatory T cell subsets, which can lead to a state of Ag-cognate effector cell hyporesponsiveness (graft acceptance or quiescence). While it is believed that DCreg are effective in blunting Tmem responses (25, 38–40) and *de novo*-primed naive T cells (13, 34), it is unclear how long after infusion donor-derived DCreg persist in the peripheral blood or in lymphoid tissue of transplant patients, and which mechanism(s) (clonal deletion, anergy, regulation, or exhaustion) may contribute to inducing donor-specific T cell hyporesponsiveness. Our hypothesis is that infusion of donor-derived DCreg (even if their survival is short-lived) (35) will induce donor-specific T cell hyporesponsiveness in the recipient, while nominal T cell recall responses [such as those to anti-Epstein–Barr virus (EBV) or tetanus toxoid (TT)] will be preserved. This could be mediated by decreased donor allo-specific Tmem frequencies and result in residual low allo-specific Tmem proliferation, IFN-γ and Granzyme B/Perforin production in response to donor Ag stimulation, but with preserved responses to EBV and TT stimulation.

To address these questions, we will collect blood samples pre-transplant on day −7 (pre DCreg infusion), on day 0, and at 3 months, 1 year, and 2 year post-transplant. We will (1) characterize the phenotype, memory differentiation, and function of

[2]http://www.onestudy.org

different T cell subsets, (2) assess donor-reactive T cell clonality and function, (3) identify effector and regulatory cells and molecules in for-cause and protocol biopsy samples. While no single immunologic test can identify peripheral hyporesponsiveness after organ transplantation, we will attempt to assess multiple essential T cell immune parameters methodically at the same time, an approach expected to provide a possible signature and mechanism of peripheral anti-donor hyporesponsiveness after DCreg infusion.

We will assess T cell expression of co-stimulatory receptors [e.g., CD28, inducible costimulator (ICOS) and CD40L], which are critical for cross-talk with DC, as well as co-inhibitory receptors [PD-1, TIM3 (T cell immunoglobulin mucin domain 3) and cytotoxic T lymphocyte Ag (CTLA)-4] that are up-regulated on recently activated/exhausted T cells in conjunction with expression of Annexin V/7-AAD to track apoptosis. We will also track EBV-specific and anti-TT T cells as controls for recall responses. We will correlate the levels of memory $CD8^+$, $CD4^+$ T_{FH}, and $CD4^+$ Tconv effectors with Treg, DSA titer, plasma cytokines and effector and regulatory cell, IgG, and complement (C4d) deposition in the allograft. Age-matched healthy controls and renal transplant patients who did not receive DCreg infusion will serve as controls.

CONCLUSION

There is extensive evidence that DCreg of donor origin can regulate alloimmune responses and promote long-term organ transplant survival in rodents. The recent observation that DCreg can safely prolong renal transplant survival in a robust, pre-clinical NHP model, in which the graft recipients received a minimal immunosuppressive regimen, provides further justification for a clinical trial. Appropriate culture conditions, leading to the manufacture of GMP grade DCreg, which are resistant to maturation and have potential to regulate host alloimmunity, have been developed for clinical testing.

AUTHOR CONTRIBUTIONS

AT, AZ, ME, LB, FL, and DM each contributed to the conception and writing of the work, revised it critically, finally approved the submitted version of the manuscript, and agreed to be accountable for all aspects of the accuracy and integrity of the work.

ACKNOWLEDGMENTS

The work was supported by National Institutes of Health (NIH) grants 5 UL1 TR0000005-09 (AT) and R21 AI116746 (DM) and by the UPCI Immunologic Monitoring and Cellular Products Laboratory that is supported, in part, by NIH award P30CA047904. We thank our colleagues, Drs. Sundaram Hariharan, Paul Szabolcs, Amit Tevar, and Mindi Styn for their contributions to the conception and design of the work. We thank Dr. Dalia Raich-Regue for her input in formatting of **Figure 2**.

FUNDING

The work was supported by National Institutes of Health (NIH) grants 5 UL1 TR0000005-09 (AWT) and R21 AI116746 (DM) and by the UPCI Immunologic Monitoring and Cellular Products Laboratory that is supported, in part, by NIH award P30CA047904.

REFERENCES

1. Meier-Kriesche HU, Schold JD, Kaplan B. Long-term renal allograft survival: have we made significant progress or is it time to rethink our analytic and therapeutic strategies? *Am J Transplant* (2004) **4**:1289–95. doi:10.1111/j.1600-6143.2004.00515.x
2. Lamb KE, Lodhi S, Meier-Kriesche HU. Long-term renal allograft survival in the United States: a critical reappraisal. *Am J Transplant* (2011) **11**:450–62. doi:10.1111/j.1600-6143.2010.03283.x
3. Vincenti F, Charpentier B, Vanrenterghem Y, Rostaing L, Bresnahan B, Darji P, et al. A phase III study of belatacept-based immunosuppression regimens versus cyclosporine in renal transplant recipients (BENEFIT study). *Am J Transplant* (2010) **10**:535–46. doi:10.1111/j.1600-6143.2009.03005.x
4. Hricik DE, Formica RN, Nickerson P, Rush D, Fairchild RL, Poggio ED, et al. Adverse outcomes of tacrolimus withdrawal in immune-quiescent kidney transplant recipients. *J Am Soc Nephrol* (2015) **26**:3114–22. doi:10.1681/ASN.2014121234
5. Ekberg H, Tedesco-Silva H, Demirbas A, Vitko S, Nashan B, Gurkan A, et al. Reduced exposure to calcineurin inhibitors in renal transplantation. *N Engl J Med* (2007) **357**:2562–75. doi:10.1056/NEJMoa067411
6. Billingham RE, Brent L, Medawar PB. Actively acquired tolerance of foreign cells. *Nature* (1953) **172**:603–6. doi:10.1038/172603a0
7. Kawai T, Leventhal J, Madsen JC, Strober S, Turka LA, Wood KJ. Tolerance: one transplant for life. *Transplantation* (2014) **98**:117–21. doi:10.1097/TP.0000000000000260
8. Ohnmacht C, Pullner A, King SB, Drexler I, Meier S, Brocker T, et al. Constitutive ablation of dendritic cells breaks self-tolerance of CD4 T cells and results in spontaneous fatal autoimmunity. *J Exp Med* (2009) **206**:549–59. doi:10.1084/jem.20082394
9. Sakaguchi S. Naturally arising CD4+ regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* (2004) **22**:531–62. doi:10.1146/annurev.immunol.21.120601.141122
10. Beriou G, Peche H, Guillonneau C, Merieau E, Cuturi MC. Donor-specific allograft tolerance by administration of recipient-derived immature dendritic cells and suboptimal immunosuppression. *Transplantation* (2005) **79**:969–72. doi:10.1097/01.TP.0000158277.50073.35
11. Hill M, Thebault P, Segovia M, Louvet C, Beriou G, Tilly G, et al. Cell therapy with autologous tolerogenic dendritic cells induces allograft tolerance through interferon-gamma and epstein-barr virus-induced gene 3. *Am J Transplant* (2011) **11**:2036–45. doi:10.1111/j.1600-6143.2011.03651.x
12. Lutz MB, Suri RM, Niimi M, Ogilvie AL, Kukutsch NA, Rossner S, et al. Immature dendritic cells generated with low doses of GM-CSF in the absence of IL-4 are maturation resistant and prolong allograft survival in vivo. *Eur J Immunol* (2000) **30**:1813–22. doi:10.1002/1521-4141(200007)30:7<1813::AID-IMMU1813>3.0.CO;2-8
13. Morelli AE, Thomson AW. Tolerogenic dendritic cells and the quest for transplant tolerance. *Nat Rev Immunol* (2007) **7**:610–21. doi:10.1038/nri2132
14. Kingsley C, Karim M, Bushell A, Wood K. CD25+CD4+ regulatory T cells prevent graft rejection:CTLA-4- and IL-10-dependent immunoregulation of alloresponses. *J Immunol* (2002) **168**:1080–6. doi:10.4049/jimmunol.168.3.1080
15. Wood KJ, Bushell A, Hester J. Regulatory immune cells in transplantation. *Nat Rev Immunol* (2012) **12**:417–30. doi:10.1038/nri3227
16. Brem-Exner BG, Sattler C, Hutchinson JA, Koehl GE, Kronenberg K, Farkas S, et al. Macrophages driven to a novel state of activation have

anti-inflammatory properties in mice. *J Immunol* (2008) **180**:335–49. doi:10.4049/jimmunol.180.1.335
17. Fleming BD, Mosser DM. Regulatory macrophages: setting the threshold for therapy. *Eur J Immunol* (2011) **41**:2498–502. doi:10.1002/eji.201141717
18. Broichhausen C, Riquelme P, Geissler EK, Hutchinson JA. Regulatory macrophages as therapeutic targets and therapeutic agents in solid organ transplantation. *Curr Opin Organ Transplant* (2012) **17**:332–42. doi:10.1097/MOT.0b013e328355a979
19. Ochando J, Conde P, Bronte V. Monocyte-derived suppressor cells in transplantation. *Curr Transplant Rep* (2015) **2**:176–83. doi:10.1007/s40472-015-0054-9
20. Roncarolo MG, Gregori S, Bacchetta R, Battaglia M. Tr1 cells and the counter-regulation of immunity: natural mechanisms and therapeutic applications. *Curr Top Microbiol Immunol* (2014) **380**:39–68. doi:10.1007/978-3-662-43492-5_3
21. Sicard A, Koenig A, Morelon E, Defrance T, Thaunat O. Cell therapy to induce allograft tolerance: time to switch to plan B? *Front Immunol* (2015) **6**:149. doi:10.3389/fimmu.2015.00149
22. Ezzelarab M, Thomson AW. Tolerogenic dendritic cells and their role in transplantation. *Semin Immunol* (2011) **23**:252–63. doi:10.1016/j.smim.2011.06.007
23. Beriou G, Moreau A, Cuturi MC. Tolerogenic dendritic cells: applications for solid organ transplantation. *Curr Opin Organ Transplant* (2012) **17**:42–7. doi:10.1097/MOT.0b013e32834ee662
24. Vassalli G. Dendritic cell-based approaches for therapeutic immune regulation in solid-organ transplantation. *J Transplant* (2013) **2013**:761429. doi:10.1155/2013/761429
25. Ezzelarab MB, Zahorchak AF, Lu L, Morelli AE, Chalasani G, Demetris AJ, et al. Regulatory dendritic cell infusion prolongs kidney allograft survival in nonhuman primates. *Am J Transplant* (2013) **13**:1989–2005. doi:10.1111/ajt.12310
26. Ezzelarab MB, Lu L, Guo H, Zahorchak AF, Shufesky WJ, Cooper DK, et al. Eomesodermin^{lo} CTLA4^{hi} alloreactive CD8^{+} memory T cells are associated with prolonged renal transplant survival induced by regulatory dendritic cell infusion in CTLA4Ig-treated non-human primates. *Transplantation* (2016) **100**:91–102.
27. Lakkis FG, Sayegh MH. Memory T cells: a hurdle to immunologic tolerance. *J Am Soc Nephrol* (2003) **14**:2402–10. doi:10.1097/01.ASN.0000085020.78117.70
28. Valujskikh A. The challenge of inhibiting alloreactive T-cell memory. *Am J Transplant* (2006) **6**:647–51. doi:10.1111/j.1600-6143.2005.01215.x
29. Moreau A, Varey E, Bouchet-Delbos L, Cuturi MC. Cell therapy using tolerogenic dendritic cells in transplantation. *Transplant Res* (2012) **1**:13. doi:10.1186/2047-1440-1-13
30. Geissler EK, The ONE. Study compares cell therapy products in organ transplantation: introduction to a review series on suppressive monocyte-derived cells. *Transplant Res* (2012) **1**:11. doi:10.1186/2047-1440-1-10
31. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* (1998) **392**:245–52. doi:10.1038/32588
32. Steinman RM, Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. *Annu Rev Immunol* (2003) **21**:685–711. doi:10.1146/annurev.immunol.21.120601.141040
33. Wang Z, Divito SJ, Shufesky WJ, Sumpter T, Wang H, Tkacheva OA, et al. Dendritic cell therapies in transplantation revisited: deletion of recipient DCs deters the effect of therapeutic DCs. *Am J Transplant* (2012) **12**:1398–408. doi:10.1111/j.1600-6143.2012.04060.x
34. Morelli AE, Thomson AW. Orchestration of transplantation tolerance by regulatory dendritic cell therapy or in-situ targeting of dendritic cells. *Curr Opin Organ Transplant* (2014) **19**:348–56. doi:10.1097/MOT.0000000000000097
35. Divito SJ, Wang Z, Shufesky WJ, Liu Q, Tkacheva OA, Montecalvo A, et al. Endogenous dendritic cells mediate the effects of intravenously injected therapeutic immunosuppressive dendritic cells in transplantation. *Blood* (2010) **116**:2694–705. doi:10.1182/blood-2009-10-251058
36. Riley JL, June CH, Blazar BR. Human T regulatory cell therapy: take a billion or so and call me in the morning. *Immunity* (2009) **30**:656–65. doi:10.1016/j.immuni.2009.04.006
37. Tang Q, Lee K. Regulatory T-cell therapy for transplantation: how many cells do we need? *Curr Opin Organ Transplant* (2012) **17**:349–54. doi:10.1097/MOT.0b013e328355a992
38. Nasreen M, Waldie TM, Dixon CM, Steptoe RJ. Steady-state antigen-expressing dendritic cells terminate CD4+ memory T-cell responses. *Eur J Immunol* (2010) **40**:2016–25. doi:10.1002/eji.200940085
39. Anderson AE, Sayers BL, Haniffa MA, Swan DJ, Diboll J, Wang XN, et al. Differential regulation of naive and memory CD4+ T cells by alternatively activated dendritic cells. *J Leukoc Biol* (2008) **84**:124–33. doi:10.1189/jlb.1107744
40. Kenna TJ, Waldie T, McNally A, Thomson M, Yagita H, Thomas R, et al. Targeting antigen to diverse APCs inactivates memory CD8+ T cells without eliciting tissue-destructive effector function. *J Immunol* (2010) **184**:598–606. doi:10.4049/jimmunol.0900032
41. Macedo C, Orkis EA, Popescu I, Elinoff BD, Zeevi A, Shapiro R, et al. Contribution of naive and memory T-cell populations to the human alloimmune response. *Am J Transplant* (2009) **9**:2057–66. doi:10.1111/j.1600-6143.2009.02742.x
42. Ford ML, Kirk AD, Larsen CP. Donor-reactive T-cell stimulation history and precursor frequency: barriers to tolerance induction. *Transplantation* (2009) **87**:S69–74. doi:10.1097/TP.0b013e3181a2a701
43. Ford ML, Larsen CP. Overcoming the memory barrier in tolerance induction: molecular mimicry and functional heterogeneity among pathogen-specific T-cell populations. *Curr Opin Organ Transplant* (2010) **15**:405–10. doi:10.1097/MOT.0b013e32833b7916
44. Dhodapkar MV, Steinman RM, Krasovsky J, Munz C, Bhardwaj N. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. *J Exp Med* (2001) **193**:233–8. doi:10.1084/jem.193.2.233
45. Dhodapkar MV, Steinman RM. Antigen-bearing immature dendritic cells induce peptide-specific CD8(+) regulatory T cells in vivo in humans. *Blood* (2002) **100**:174–7. doi:10.1182/blood.V100.1.174
46. Ikeguchi R, Sacks JM, Unadkat JV, Solari M, Horibe EK, Thomson AW, et al. Long-term survival of limb allografts induced by pharmacologically conditioned, donor alloantigen-pulsed dendritic cells without maintenance immunosuppression. *Transplantation* (2008) **85**:237–46. doi:10.1097/TP.0b013e31815e870e
47. Horibe EK, Sacks J, Unadkat J, Raimondi G, Wang Z, Ikeguchi R, et al. Rapamycin-conditioned, alloantigen-pulsed dendritic cells promote indefinite survival of vascularized skin allografts in association with T regulatory cell expansion. *Transpl Immunol* (2008) **18**:307–18. doi:10.1016/j.trim.2007.10.007
48. Giannoukakis N, Phillips B, Finegold D, Harnaha J, Trucco M. Phase I (safety) study of autologous tolerogenic dendritic cells in type 1 diabetic patients. *Diabetes Care* (2011) **34**:2026–32. doi:10.2337/dc11-0472
49. Thomas R, Street S, Ramnoruth N. Safety and preliminary evidence of efficacy in a phase I clinical trial of autologous tolerizing dendritic cells exposed to citrullinated peptides (Rheumavax) in patients with rheumatoid arthritis. *Ann Rheum Dis* (2011) **70**:169.
50. Benham H, Nel HJ, Law SC, Mehdi AM, Street S, Ramnoruth N, et al. Citrullinated peptide dendritic cell immunotherapy in HLA risk genotype-positive rheumatoid arthritis patients. *Sci Transl Med* (2015) **7**:290ra87. doi:10.1126/scitranslmed.aaa9301
51. Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med* (1973) **137**:1142–62. doi:10.1084/jem.137.5.1142
52. Steinman RM. The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol* (1991) **9**:271–96. doi:10.1146/annurev.iy.09.040191.001415
53. Steptoe RJ, Thomson AW. Dendritic cells and tolerance induction. *Clin Exp Immunol* (1996) **105**:397–402. doi:10.1046/j.1365-2249.1996.d01-779.x
54. Maldonado RA, von Andrian UH. How tolerogenic dendritic cells induce regulatory T cells. *Adv Immunol* (2010) **108**:111–65. doi:10.1016/B978-0-12-380995-7.00004-5
55. Steinman RM, Hawiger D, Liu K, Bonifaz L, Bonnyay D, Mahnke K, et al. Dendritic cell function in vivo during the steady state: a role in peripheral tolerance. *Ann N Y Acad Sci* (2003) **987**:15–25. doi:10.1111/j.1749-6632.2003.tb06029.x

56. Probst HC, Muth S, Schild H. Regulation of the tolerogenic function of steady-state DCs. *Eur J Immunol* (2014) **44**:927–33. doi:10.1002/eji.201343862
57. Coates PT, Thomson AW. Dendritic cells, tolerance induction and transplant outcome. *Am J Transplant* (2002) **2**:299–307. doi:10.1034/j.1600-6143.2002.20403.x
58. Thomson AW, Knolle PA. Antigen-presenting cell function in the tolerogenic liver environment. *Nat Rev Immunol* (2010) **10**:753–66. doi:10.1038/nri2858
59. Rogers NM, Isenberg JS, Thomson AW. Plasmacytoid dendritic cells: no longer an enigma and now key to transplant tolerance? *Am J Transplant* (2013) **13**:1125–33. doi:10.1111/ajt.12229
60. Thomson AW, Robbins PD. Tolerogenic dendritic cells for autoimmune disease and transplantation. *Ann Rheum Dis* (2008) **67**(Suppl 3):iii90–6. doi:10.1136/ard.2008.099176
61. Hilkens CM, Isaacs JD, Thomson AW. Development of dendritic cell-based immunotherapy for autoimmunity. *Int Rev Immunol* (2010) **29**:156–83. doi:10.3109/08830180903281193
62. Stoop JN, Robinson JH, Hilkens CM. Developing tolerogenic dendritic cell therapy for rheumatoid arthritis: what can we learn from mouse models? *Ann Rheum Dis* (2011) **70**:1526–33. doi:10.1136/ard.2011.151654
63. Van Brussel I, Lee WP, Rombouts M, Nuyts AH, Heylen M, De Winter BY, et al. Tolerogenic dendritic cell vaccines to treat autoimmune diseases: can the unattainable dream turn into reality? *Autoimmun Rev* (2014) **13**:138–50. doi:10.1016/j.autrev.2013.09.008
64. Eto M, Hackstein H, Kaneko K, Nomoto K, Thomson AW. Promotion of skin graft tolerance across MHC barriers by mobilization of dendritic cells in donor hemopoietic cell infusions. *J Immunol* (2002) **169**:2390–6. doi:10.4049/jimmunol.169.5.2390
65. Zahorchak AF, Kean LS, Tokita D, Turnquist HR, Abe M, Finke J, et al. Infusion of stably immature monocyte-derived dendritic cells plus CTLA4Ig modulates alloimmune reactivity in rhesus macaques. *Transplantation* (2007) **84**:196–206. doi:10.1097/01.tp.0000268582.21168.f6
66. Harry RA, Anderson AE, Isaacs JD, Hilkens CM. Generation and characterisation of therapeutic tolerogenic dendritic cells for rheumatoid arthritis. *Ann Rheum Dis* (2010) **69**:2042–50. doi:10.1136/ard.2009.126383
67. Naranjo-Gomez M, Raich-Regue D, Onate C, Grau-Lopez L, Ramo-Tello C, Pujol-Borrell R, et al. Comparative study of clinical grade human tolerogenic dendritic cells. *J Transl Med* (2011) **9**:89. doi:10.1186/1479-5876-9-89
68. Boks MA, Kager-Groenland JR, Haasjes MS, Zwaginga JJ, van Ham SM, ten Brinke A. IL-10-generated tolerogenic dendritic cells are optimal for functional regulatory T cell induction – a comparative study of human clinical-applicable DC. *Clin Immunol* (2012) **142**:332–42. doi:10.1016/j.clim.2011.11.011
69. Raich-Regue D, Naranjo-Gomez M, Grau-Lopez L, Ramo C, Pujol-Borrell R, Martinez-Caceres E, et al. Differential effects of monophosphoryl lipid A and cytokine cocktail as maturation stimuli of immunogenic and tolerogenic dendritic cells for immunotherapy. *Vaccine* (2012) **30**:378–87. doi:10.1016/j.vaccine.2011.10.081
70. Raich-Regue D, Grau-Lopez L, Naranjo-Gomez M, Ramo-Tello C, Pujol-Borrell R, Martinez-Caceres E, et al. Stable antigen-specific T-cell hyporesponsiveness induced by tolerogenic dendritic cells from multiple sclerosis patients. *Eur J Immunol* (2012) **42**:771–82. doi:10.1002/eji.201141835
71. Rastellini C, Lu L, Ricordi C, Starzl TE, Rao AS, Thomson AW. Granulocyte/macrophage colony-stimulating factor-stimulated hepatic dendritic cell progenitors prolong pancreatic islet allograft survival. *Transplantation* (1995) **60**:1366–70.
72. Fu F, Li Y, Qian S, Lu L, Chambers F, Starzl TE, et al. Costimulatory molecule-deficient dendritic cell progenitors (MHC class II^+, $CD80^{dim}$, $CD86^-$) prolong cardiac allograft survival in nonimmunosuppressed recipients. *Transplantation* (1996) **62**:659–65. doi:10.1097/00007890-199609150-00021
73. Lu L, Li W, Fu F, Chambers FG, Qian S, Fung JJ, et al. Blockade of the CD40-CD40 ligand pathway potentiates the capacity of donor-derived dendritic cell progenitors to induce long-term cardiac allograft survival. *Transplantation* (1997) **64**:1808–15. doi:10.1097/00007890-199712270-00031
74. Bonham CA, Peng L, Liang X, Chen Z, Wang L, Ma L, et al. Marked prolongation of cardiac allograft survival by dendritic cells genetically engineered with NF-kappa B oligodeoxyribonucleotide decoys and adenoviral vectors encoding CTLA4-Ig. *J Immunol* (2002) **169**:3382–91. doi:10.4049/jimmunol.169.6.3382
75. Wang Z, Morelli AE, Hackstein H, Kaneko K, Thomson AW. Marked inhibition of transplant vascular sclerosis by in vivo-mobilized donor dendritic cells and anti-CD154 mAb. *Transplantation* (2003) **76**:562–71. doi:10.1097/01.TP.0000068901.11693.C3
76. Taner T, Hackstein H, Wang Z, Morelli AE, Thomson AW. Rapamycin-treated, alloantigen-pulsed host dendritic cells induce Ag-specific T cell regulation and prolong graft survival. *Am J Transplant* (2005) **5**:228–36. doi:10.1046/j.1600-6143.2004.00673.x
77. Abe M, Wang Z, De Creus A, Thomson AW. Plasmacytoid dendritic cell precursors induce allogeneic T cell hyporesponsiveness and prolong heart graft survival. *Am J Transplant* (2005) **5**:1808–19. doi:10.1111/j.1600-6143.2005.00954.x
78. Lan YY, Wang Z, Raimondi G, Wu W, Colvin BL, DeCreus A, et al. "Alternatively-activated" dendritic cells preferentially secrete IL-10, expand $Foxp3^+$ $CD4^+$ T cells and induce long-term organ allograft survival in combination with CTLA4-Ig. *J Immunol* (2006) **177**:5868–77. doi:10.4049/jimmunol.177.9.5868
79. Turnquist H, Raimondi G, Zahorchak AF, Fischer RT, Wang Z, Thomson AW. Rapamycin-conditioned dendritic cells are poor stimulators of allogeneic $CD4^+$ T cells, but enrich for antigen-specific $Foxp3^+$ T regulatory cells and promote organ transplant tolerance. *J Immunol* (2007) **178**:7018–31. doi:10.4049/jimmunol.178.11.7018
80. Lu L, McCaslin D, Starzl TE, Thomson AW. Bone marrow-derived dendritic cell progenitors (NLDC 145^+, MHC class II^+, $B7\text{-}1^{dim}$, $B7\text{-}2^-$) induce alloantigen-specific hyporesponsiveness in murine T lymphocytes. *Transplantation* (1995) **60**:1539–45. doi:10.1097/00007890-199560120-00028
81. Macedo C, Walters JT, Orkis EA, Isse K, Elinoff BD, Fedorek SP, et al. Long-term effects of alemtuzumab on regulatory and memory T-cell subsets in kidney transplantation. *Transplantation* (2012) **93**:813–21. doi:10.1097/TP.0b013e318247a717
82. Lu L, Li W, Zhong C, Qian S, Fung JJ, Thomson AW, et al. Increased apoptosis of immunoreactive host cells and augmented donor leukocyte chimerism, not sustained inhibition of B7 molecule expression are associated with prolonged cardiac allograft survival in mice preconditioned with immature donor dendritic cells plus anti-CD40L mAb. *Transplantation* (1999) **68**:747–57.
83. Wang Q, Zhang M, Ding G, Liu Y, Sun Y, Wang J, et al. Anti-ICAM-1 antibody and CTLA-4Ig synergistically enhance immature dendritic cells to induce donor-specific immune tolerance in vivo. *Immunol Lett* (2003) **90**:33–42. doi:10.1016/S0165-2478(03)00160-3
84. Mirenda V, Berton I, Read J, Cook T, Smith J, Dorling A, et al. Modified dendritic cells coexpressing self and allogeneic major histocompatibility complex molecules: an efficient way to induce indirect pathway regulation. *J Am Soc Nephrol* (2004) **15**:987–97. doi:10.1097/01.ASN.0000119575.98696.1D
85. DePaz HA, Oluwole OO, Adeyeri AO, Witkowski P, Jin MX, Hardy MA, et al. Immature rat myeloid dendritic cells generated in low-dose granulocyte macrophage-colony stimulating factor prolong donor-specific rat cardiac allograft survival. *Transplantation* (2003) **75**:521–8. doi:10.1097/01.TP.0000048380.84355.4A
86. Hayamizu K, Huie P, Sibley RK, Strober S. Monocyte-derived dendritic cell precursors facilitate tolerance to heart allografts after total lymphoid irradiation. *Transplantation* (1998) **66**:1285–91. doi:10.1097/00007890-199809150-00006
87. Adorini L, Penna G, Giarratana N, Uskokovic M. Tolerogenic dendritic cells induced by vitamin D receptor ligands enhance regulatory T cells inhibiting allograft rejection and autoimmune diseases. *J Cell Biochem* (2003) **88**:227–33. doi:10.1002/jcb.10340
88. Eun SC, Baek RM, Park CG. Prolongation of the rat composite tissue allograft survival by the combination of tolerogenic immature dendritic cells and short-term treatment with FK506. *Transplant Proc* (2013) **45**:1792–6. doi:10.1016/j.transproceed.2013.01.021
89. Valujskikh A, Lakkis FG. In remembrance of things past: memory T cells and transplant rejection. *Immunol Rev* (2003) **196**:65–74. doi:10.1046/j.1600-065X.2003.00087.x
90. Bingaman AW, Farber DL. Memory T cells in transplantation: generation, function, and potential role in rejection. *Am J Transplant* (2004) **4**:846–52. doi:10.1111/j.1600-6143.2004.00453.x
91. Nadazdin O, Boskovic S, Murakami T, Tocco G, Smith RN, Colvin RB, et al. Host alloreactive memory T cells influence tolerance to kidney allografts in nonhuman primates. *Sci Transl Med* (2011) **3**:86ra51. doi:10.1126/scitranslmed.3002093

92. Gauzzi MC, Purificato C, Donato K, Jin Y, Wang L, Daniel KC, et al. Suppressive effect of 1alpha,25-dihydroxyvitamin D3 on type I IFN-mediated monocyte differentiation into dendritic cells: impairment of functional activities and chemotaxis. *J Immunol* (2005) **174**:270–6. doi:10.4049/jimmunol.174.1.270
93. D'Ambrosio D, Cippitelli M, Cocciolo MG, Mazzeo D, Di Lucia P, Lang R, et al. Inhibition of IL-12 production by 1,25-dihydroxyvitamin D3. Involvement of NF-kappaB downregulation in transcriptional repression of the p40 gene. *J Clin Invest* (1998) **101**:252–62.
94. Steinbrink K, Wolfl M, Jonuleit H, Knop J, Enk AH. Induction of tolerance by IL-10-treated dendritic cells. *J Immunol* (1997) **159**:4772–80.
95. Gordon JR, Ma Y, Churchman L, Gordon SA, Dawicki W. Regulatory dendritic cells for immunotherapy in immunologic diseases. *Front Immunol* (2014) **5**:7. doi:10.3389/fimmu.2014.00007
96. Hutchinson JA, Riquelme P, Brem-Exner BG, Schulze M, Matthai M, Renders L, et al. Transplant acceptance-inducing cells as an immune-conditioning therapy in renal transplantation. *Transpl Int* (2008) **21**:728–41. doi:10.1111/j.1432-2277.2008.00680.x
97. Hutchinson JA, Brem-Exner BG, Riquelme P, Roelen D, Schulze M, Ivens K, et al. A cell-based approach to the minimization of immunosuppression in renal transplantation. *Transpl Int* (2008) **21**:742–54. doi:10.1111/j.1432-2277.2008.00692.x
98. Hutchinson JA, Riquelme P, Sawitzki B, Tomiuk S, Miqueu P, Zuhayra M, et al. Cutting Edge: immunological consequences and trafficking of human regulatory macrophages administered to renal transplant recipients. *J Immunol* (2011) **187**:2072–8. doi:10.4049/jimmunol.1100762
99. Hutchinson JA, Riquelme P, Geissler EK, Fandrich F. Human regulatory macrophages. *Methods Mol Biol* (2011) **677**:181–92. doi:10.1007/978-1-60761-869-0_13
100. Divito SJ, Ferris LK. Advances and short comings in the early diagnosis of melanoma. *Melanoma Res* (2010) **20**:450–8. doi:10.1097/CMR.0b013e32833faf7e
101. Penna G, Adorini L. 1 Alpha,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation. *J Immunol* (2000) **164**:2405–11. doi:10.4049/jimmunol.164.5.2405
102. Hackstein H, Thomson AW. Dendritic cells: emerging pharmacological targets of immunosuppressive drugs. *Nat Rev Immunol* (2004) **4**:24–35. doi:10.1038/nri1256
103. Scornik JC, Meier-Kriesche HU. Blood transfusions in organ transplant patients: mechanisms of sensitization and implications for prevention. *Am J Transplant* (2011) **11**:1785–91. doi:10.1111/j.1600-6143.2011.03705.x
104. Anderson CB, Tyler JD, Sicard GA, Anderman CK, Rodey GE, Etheredge EE. Pretreatment of renal allograft recipients with immunosuppression and donor-specific blood. *Transplantation* (1984) **38**:664–8. doi:10.1097/00007890-198412000-00023
105. Salvatierra O Jr, Melzer J, Potter D, Garovoy M, Vincenti F, Amend WJ, et al. A seven-year experience with donor-specific blood transfusions. Results and considerations for maximum efficacy. *Transplantation* (1985) **40**:654–9. doi:10.1097/00007890-198512000-00016
106. Cheigh JS, Suthanthiran M, Fotino M, Riggio RR, Schechter N, Stubenbord WT, et al. Minimal sensitization and excellent renal allograft outcome following donor-specific blood transfusion with a short course of cyclosporine. *Transplantation* (1991) **51**:378–81. doi:10.1097/00007890-199102000-00021
107. Lederer SR, Friedrich N, Banas B, Welser G, Albert ED, Sitter T. Effects of mycophenolate mofetil on donor-specific antibody formation in renal transplantation. *Clin Transplant* (2005) **19**:168–74. doi:10.1111/j.1399-0012.2005.00261.x
108. Mehling A, Grabbe S, Voskort M, Schwarz T, Luger TA, Beissert S. Mycophenolate mofetil impairs the maturation and function of murine dendritic cells. *J Immunol* (2000) **165**:2374–81. doi:10.4049/jimmunol.165.5.2374
109. Lagaraine C, Hoarau C, Chabot V, Velge-Roussel F, Lebranchu Y. Mycophenolic acid-treated human dendritic cells have a mature migratory phenotype and inhibit allogeneic responses via direct and indirect pathways. *Int Immunol* (2005) **17**:351–63. doi:10.1093/intimm/dxh215
110. Metes D, Logar A, Rudert WA, Zeevi A, Woodward J, Demetris AJ, et al. Four-color flow cytometric analysis of peripheral blood donor cell chimerism. *Hum Immunol* (2003) **64**:787–95. doi:10.1016/S0198-8859(03)00113-7

Conflict of Interest Statement: Angus W. Thomson is co-inventor of a US patent for generation of dendritic cells to enhance transplant tolerance. The remaining co authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 07 March 2016
doi: 10.3389/fimmu.2016.00087

Transplantation Tolerance Induction: Cell Therapies and Their Mechanisms

Joseph R. Scalea[1], Yusuke Tomita[1], Christopher R. Lindholm[2] and William Burlingham[1]*

[1] Department of Surgery, Division of Transplantation, University of Wiconsin, Madison, WI, USA, [2] School of Medicine, University of Wisconsin, Madison, WI, USA

Cell-based therapies have been studied extensively in the context of transplantation tolerance induction. The most successful protocols have relied on transfusion of bone marrow prior to the transplantation of a renal allograft. However, it is not clear that stem cells found in bone marrow are required in order to render a transplant candidate immunologically tolerant. Accordingly, mesenchymal stem cells, regulatory myeloid cells, T regulatory cells, and other cell types are being tested as possible routes to tolerance induction, in the absence of donor-derived stem cells. Early data with each of these cell types have been encouraging. However, the induction regimen capable of achieving consistent tolerance, while avoiding unwanted sided effects, and which is scalable to the human patient, has yet to be identified. Here, we present the status of investigations of various tolerogenic cell types and the mechanistic rationale for their use in tolerance induction protocols.

Keywords: Treg, MDSC, regulatory mechanisms, regulatory myeloid cells, transplantation tolerance, HSCT, CD34$^+$ cells

OPEN ACCESS

Edited by:
Gilles Benichou, Massachusetts General Hospital, USA

Reviewed by:
Reem Al-Daccak, Institut National de Sante et de Recherche Medical (INSERM), France
Philippe Saas, Etablissement Français du Sang Bourgogne-Franche-Comté, France

****Correspondence:***
Joseph R. Scalea
scalea@surgery.wisc.edu

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *03 November 2015*
Accepted: *22 February 2016*
Published: *07 March 2016*

Citation:
Scalea JR, Tomita Y, Lindholm CR and Burlingham W (2016) Transplantation Tolerance Induction: Cell Therapies and Their Mechanisms. Front. Immunol. 7:87. doi: 10.3389/fimmu.2016.00087

INTRODUCTION

Cell-based therapies lie at the root of transplantation tolerance induction protocols. Ray Owen at the University of Wisconsin made the early observation that a shared, naturally occurring neonatal blood supply was associated with the presence of chimeric red blood cell populations in adult cows (1). This, and other, observation prompted Peter Medawar to explore the possibility that donor chimerism would allow for acceptance of skin grafts from the same donor through which chimerism was established (2, 3). These findings, which led to the Noble Prize in 1960, were exploited by Dr. David Sachs (4) and Dr. Sam Strober (5) such that preclinical models (6) for tolerance to solid organ transplants could be developed (7–9). These preclinical models led to human clinical trials, which have since yielded encouraging results (10, 11).

Indeed, the mechanisms underlying tolerance development are still not clear. Since the completion of Medawar's experiments, investigators have sought to identify the cell populations responsible for tolerance induction. Even today, however these cell types and their mechanisms remain elusive. Here, we will review some of the cell types, which have demonstrated tolerogenicity in both experimental and in preclinical models, focusing on the potential for tolerance induction in man.

DONOR BONE MARROW FOR MIXED CHIMERISM ESTABLISHMENT

Based on the notion that outcomes in human transplantation were unacceptable due to the requirement for long-term pharmacologic immunosuppression, and building on significant preclinical data, investigators at Massachusetts General Hospital attempted to achieve tolerance in humans. Their approach was to first establish lymphohematopoietic chimerism using the hematopoietic stem cells of the intended kidney donor, in order to establish a milieu where the donor and the recipient existed as a "mixed chimera" (10). In their seminal work published in the New England Journal of Medicine in 2008, investigators described the clinical course of five patients who received conditioning, bone marrow transplantation, and subsequent renal transplantation. Transplant recipients were conditioned using two preoperative doses of cyclophosphamide, as well as peritransplantation anti-CD2, cyclosporine, and thymic irradiation. The five patients also underwent bone marrow transplantation and renal transplantation. In the group's original description of the bone marrow procurement (11), investigators removed bone marrow from the donor's iliac crest on the day of the transplant such that 2.7×10^8 cells/kg were infused into an intended recipient (11).

As per their initial description, four of the five patients included in this study were tolerant, and off all immunosuppression at last recorded follow-up (between 2 and 5 years) (10). Interestingly, while chimerism was pan-detectable in the first week, four of five patients had no detectable chimerism as of day 14, and in the remaining one patient only 3.5% chimerism in the granulocyte lineage remained until day 21. In this respect, the attempt to achieve sustained mixed chimerism failed. Despite this, the authors observed excellent clinical results. Given the non-specific nature of the bone marrow transplantation, it is difficult to know what elements of the cell transplant (bone marrow in this case), conditioning regimen, and the organ itself in this early study were responsible for long-lasting tolerance. Irrespective of the mechanistic aspects of this initial study, these observations laid down the foundation for multiple pursuant studies, which have helped to address the tolerogenicity of cell-based transplants aimed at tolerance induction (10, 12).

Using donors and recipients who were HLA-matched siblings investigators at Stanford University employed a similar cell-based tolerance induction protocol for renal transplant recipients. Also published in the New England Journal of Medicine, Scandling et al. presented a series of 10 patients who underwent treatment with anti-thymocyte globulin, cyclosporine, and total lymphoid irradiation. Differing somewhat from the Massachusetts General Hospital (MGH) experience, an immunomagnetic bead column was used to enrich the bone marrow transplant for $CD34^+$ hematopoietic stem cells. The bone marrow donor was first mobilized with a 5-day course of subcutaneous G-CSF 6 weeks prior to procurement. Their patient then received 8×10^6 $CD34^+$ hematopoietic stem cells in addition to 1×10^6 $CD3^+$ lymphocytes. The cell transplant was cryopreserved and administered on day 14, following completion of total lymphoid irradiation (13, 14). In more recent publications, the Stanford University group has shown that 8 of 15 patients completing the tolerance induction protocol were chimeric for 6 months or greater and successfully weaned from immunosuppression (14). Only four patients were not withdrawn from immunosuppression secondary to underlying disease or episodes or rejection (14). Thus, in a well-matched cohort, both sustained mixed chimerism and renal transplantation tolerance could be achieved using this approach.

A third group at Northwestern University has successfully implemented human tolerance induction protocols using a distinct, yet similar cell-based protocol. Again, T cell depletion was utilized, however with two doses of alemtuzumab (anti-CD52) (15, 16). Tacrolimus in addition to mycophenolate mofetil was initiated at the time of transplantation. The first of four bone marrow transfusions obtained via iliac crest aspiration were given on posttransplantation day 5, followed by repeat transfusions at months 3, 6, and 9 (16). Bone marrow donors were mobilized with Neuopogen prior to donation, and bone marrow infusions were enriched for $CD34^+$ hematopoietic stem cells. Encouragingly, five of the institution's first eight patients were stably tolerant of their renal allografts at 1-year posttransplantation (16). The Northwestern group has also employed the use of "facilitator cells" to augment the chimeric state and tolerogenic milieu, although the details of these $CD8^+$ non-T cell types are largely unknown as they are considered proprietary (15, 17).

Taken together, it is clear that bone marrow infusions, likely through the action of $CD34^+$ hematopoietic stem cells can lead to tolerance induction in humans. Importantly, and consistent with the initial observations of Starzl and Demetris (18), it may not be absolutely necessary for a high-level of chimerism to last indefinitely, in order for the transplanted graft to remain tolerated (8, 10, 12).

In fact, the loss of chimerism (>1% donor cells) may coincide with a totally chimerism-free state, wherein tolerance is sustained solely by anergy and immunoregulation induced by the kidney graft parenchyma, as suggested by Sachs et al. (7, 8) Alternatively, the loss of macro-chimerism may coincide with the onset of micro-chimerism (<0.1% donor cells), a setting in which the "two-way" model of transplant tolerance, as proposed by Starzl and Demetris, is sustained (18, 19). Although Starzl's theory was based on mutual HvG/GvH reactions, and not on Regulatory T cells, a recent report indicates that Treg cells induced in the offspring during the transient chimerism stage of pregnancy are maintained by constant contact with rare maternal hematopoetic cells, indicating a key role for maternal microchimerism in tolerance (20).

In addition to the above descriptions of chimerism establishment, exciting new reports have promulgated an alternative hypothesis underlying the mechanisms of tolerance induction through bone marrow infusion. Authors have shown that $CD34^+$ monocytes are capable of inducting apoptosis of donor reactive T cells, and that through Treg expansion, this leads to tolerance. Regardless of the underlying mechanisms, immune tolerance through bone marrow infusion has proven efficacy in humans. However, additional potentially less morbid cell-based therapies are in development as well (21).

MESENCHYMAL STEM CELLS

Adapted from bone-marrow transplantation efforts to reduce the rate of bone-marrow graft failure following haplo-identical transplantation, mesenchymal stem cells may be capable of tolerance induction (22, 23). Pluripotent mesenchymal stem cells are naturally occurring and exist within the bone marrow (24–28). Mesenchymal stem cells are precursors to bone, fat, and other connective tissues. Additionally, however, mesenchymal stem cells have been shown to support normal hematopoiesis and to demonstrate immunosuppressive qualities (22, 25, 27, 28). Mesenchymal stem cells can rapidly expand *ex vivo*, yet they do not lose potential to differentiate into multiple cell types (23, 24, 28). Partially explaining augmentation of haplo-identical bone-marrow transplantation, mesenchymal stem cells also assist with engraftment of hematopoietic stem cells (23).

It has been hypothesized that mesenchymal stem cells partly explain the tolerogenic nature of bone marrow transplantation for tolerance induction. Accordingly, small and large animal models of attempted tolerance induction using these cells have been studied (23). In a rodent model of heterotopic heart transplantation, investigators observed that rapamycin alone led to rejection of haplo-mismatched cardiac grafts by 3 weeks. In contrast, mesenchymal stem cell infusion as monotherapy inhibited acute rejection, and when infusion of mesenchymal stem cells was coadministered with rapamycin, recipients enjoyed long-term, and rejection-free graft survival (23). Recipients of mesenchymal stem cell infusion also displayed minimal antibody production. Investigators observed deposition of mesenchymal stem cells into the cardiac grafts, as well as increased number of FoxP3$^+$ T regulatory cells (23). Mechanistically, authors offered that the intra-graft mesenchymal stem cells might (1) protect the donor heart from exposure of alloantigens, and (2) provide local immunomodulation for alloreactive T cell clones (23). While mesenchymal stem cells are certainly immunosuppressive, infusion of mesenchymal stem cells alone was insufficient to overcome the alloreactive host responses, suggesting that other factors intrinsic to the bone marrow (beyond mesenchymal stem cells) are potentially required for tolerance induction. Corroborating these findings, other authors have shown that mesenchymal stem cell infusions prolonged baboon skin graft survival (29) as well as survival of liver, kidney, and heart allografts in small animal models (23, 30–32).

The immunomodulatory effects of mesenchymal stem cells have been studied and their interplay with other immunological cell types has begun to be characterized (25–27). Indeed, authors have recently shown that the differential efficacy of mesenchymal stem cells is based on the cell source, suggesting that not all mesenchymal stem cells are created equally (33). While a complete understanding of the responsible mechanisms is incomplete, there is a clear upregulation of FoxP3$^+$ Regulatory T cells resulting from mesenchymal stem infusion (34). In addition, the suppressive functions of mesenchymal stem cells are thought to be mediated by both cell-to-cell contact as well as through the action of soluble factors (35). Additionally, mesenchymal stem cells have been shown to down regulate MHC class II and costimulatory molecules, resulting expansion of regulatory dendritic cells and impaired alloreactive T cell homing, respectively (30, 35–37). Perhaps important to clinical applications, recent reports suggest that the timing of mesenchymal stem cell administration is important to graft survival. In addition, the immunosuppressive effects of mesenchymal stem cells have been shown to overcome the effects of graft versus host disease (GVHD) in man (38, 39). Indeed, in a rodent renal tolerance model, when mesenchymal stem cells were infused after kidney transplantation (versus prior), graft dysfunction and neutrophilic infiltration were observed within the graft. Unfortunately, however, at present it appears that the lifespan of mesenchymal stems cells is limited (28, 40). In contrast, significant graft survival prolongation was observed with the mesenchymal stem cell administration preceded organ transplantation (36). More recently, human studies of mesenchymal stem cell administration in living donor kidney transplantation demonstrated reduced doses of tacrolimus were required for those receiving cell therapy in addition to calcineurin inhibition (41), and improved graft function at 1 year. In 2015, investigators published of a human pilot study of renal transplantation, in which pre- and posttransplantation administration of autologous mesenchymal stem cells was found to be not only safe, but the infusion lead to upregulation of Tregulatory cells in recipients (42). Taken together, mesenchymal stem cells seem capable of significant immunosuppression; however, the immunosuppressive effects appear incomplete, suggesting that additional elements need to be addressed for tolerance induction via mesenchymal stem cell adminsitration (41, 43).

EX VIVO EXPANDED REGULATORY T CELLS

Regulatory T Cells

Regulatory T cells are perhaps the most widely discussed cell type with regard to tolerance induction and their biology has driven much of the recent research in transplantation tolerance (12, 13, 44–50). Regulatory T cells, of which there are many subsets, are naturally occurring, and are required for self-tolerance. Additionally, Regulatory T cells have been implicated in the immunosuppressive mechanisms described for each of the cell types presented in this manuscript (51–60). While some investigators have reasoned that Regulatory T cells may be a marker of tolerance rather than the unifying mechanism by which tolerance to organ transplants is mediated, few will argue with the idea that Regulatory T cells are critical to the success of tolerance protocols. Accordingly, recent data show that microchimerism may itself sustain antigen-specific Regulatory T cells in a mouse model (20). Indeed, the hypothesis that Regulatory T cells represent a marker of tolerance is gaining traction among the tolerance community (20).

From the standpoint of cell-based tolerance induction protocols, Regulatory T cells can be expanded *ex vivo* and administered exogenously, or transplanted as part of a tolerated graft (intra-graft Regulatory T cells; for caveats, see Section "Intragraft Regulatory T cells"). Endogenous Regulatory T cells have been studied extensively and are conventionally defined as thymic derived (tRegulatory T cells) or peripherally derived (pRegulatory T cells). tRegulatory T cells and pRegulatory

T cells can be distinguished by different cell surface identifiers (CD39, CTLA-4, etc.) and by the soluble factors produced (IL-35, etc.). Notably, both tRegulatory T cells and pRegulatory T cells populations express intranuclear FoxP3, a transcription factor thought to be the most specific marker for Regulatory T cells (61–63). Additionally, helios, a member of the Ikaros family of transcription factors, has been shown to distinguish thymic from peripheral Regulatory T cells (64). While helios is expressed in 100% of thymocytes, naive rodent and human FoxP3 cells T cells generated peripherally via TCR stimulation failed to express helios (64). While the exact function of FoxP3 itself it not fully known, it is thought to downregulate the nuclear factor of activated T cells (NFAT) (62).

The mechanisms of Regulatory T cells have been extensively studied and recently reviewed (65). There are four primary actions, which are thought to mediate the inhibitory function of Regulatory T cells: (1) release of soluble, inhibitory factors, (2) cytolysis, (3) metabolic dysregulation, and (4) manipulation of the function of dendritic cells (65). The soluble factors IL-10 and TGF-beta have garnered significant interest in the Treg literature as the primary cytokines by which negative inhibition is mediated (66, 67). However, it is unclear if the cytokine profile for tRegulatory T cells and pRegulatory T cells is similar (65, 68). Building data from our laboratory and others have also suggested that IL-35 (Tomita et al., unpublished data) (69). It is also becoming clear that like natural killer cells and like cytotoxic T cells ($CD8^+$), and regulatory T cells inhibit anti-donor responses via cytolysis through the activity of perforin and granzyme A (65, 70). While not widely discussed as a primary Treg function, regulatory T cells are also known to deplete IL-2 from the microenvironment, resulting in metabolic dysregulation of target T cells (71, 72). The interaction of Regulatory T cells and dendritic cells is bidirectional. Below in the review, we will discuss tolerogenic monocytes, which are upstream to Regulatory T cells, however Regulatory T cells themselves may also affect the maturation of suppressive monocytes through the action of CTLA-4 and other inhibitory signals (65, 73).

Given their known suppressive role *in vivo* following protocols of tolerance induction, much interest has focused on *ex vivo* expansion of Regulatory T cells such that subsequent administration might lead to tolerance induction. Regulatory T cells may be generated (induced Regulatory T cells or iRegulatory T cells) *ex vivo*, in the presence of IL-2 and TGF-beta (61, 67). Indeed preclinical and recent human trials have demonstrated that massive expansion of Regulatory T cells is possible, *ex vivo*. For such expansions, costimulation of purified Regulatory T cells ($CD4^+CD25^+CD127^{lo}$) with CD28 in the presence of rapamycin has been associated with a 1000-fold increase in Regulatory T cells over approximately 3 weeks (74, 75). These protocols were extended to humans for the treatment of GVHD, with encouraging results. Notably, rapid expansion of Regulatory T cells *ex vivo* is associated with reduction is Regulatory T cells' suppressive qualities, despite the production of FoxP3 (74, 75). Similar expansion rates (also using CD28 costimulation) and findings were observed in human studies of autoimmune hepatitis (76, 77) and other autoimmune diseases (51).

Ex vivo expansion of Regulatory T cells has been attempted in both preclinical and clinical settings (78, 79). In a mouse model, investigators were able to expand antigen-specific $CD4^+CD25^+$ Regulatory T cells using antigen-primed, immature dendritic cells (79). Authors then adoptively transferred these antigen-specific Regulatory T cells into skin-graft recipients (78). Investigators found that CFSE-labeled Regulatory T cells migrated into the transplanted grafts, that survival was prolonged (stable appearance and hear growth at >150 days), and that animals displayed evidence of transplantation tolerance (78). In a preclinical humanized mouse model of skin transplantation, investigators recently demonstrated that exogenous antigen-specific Treg administration significantly prolonged skin-graft survival. Importantly, the Treg expansion protocol utilizing CD69 and CD71 enrichment was thought to be scalable to the clinic (80). In a phase 1 2011 study, Regulatory T cells were expanded *ex vivo* from umbilical cord blood and administered to partially HLA-matched patients with hematologic malignancy. Not only did this prove to be safe but also it provided preliminary evidence that recipients of these Regulatory T cells had decreased risk of acute GVHD (59). Another 2011 study was able to show that Regulatory T cells coinfused with conventional T cells prevented GVHD without the use of posttransplant immunosuppressive therapy (60).

According to the National Institutes of Health, there are four open-active trials and one closed-active trial utilizing the infusion of *ex vivo* generated Regulatory T cells. A European group focused on cellular immunotherapy in organ transplantation has a phase 2 study in process in which autologous Regulatory T cells are removed from living donor renal transplant recipients, and after 5 days of expansion, they are reinfused into the recipient. In a second approved human trial, through the University of Minnesota, investigators are using autologous, donor alloantigen-specific Regulatory T cells produced from expanded Regulatory T cells obtained from pre-liver transplant patients. The Regulatory T cells are then infused back into the recipient at regular intervals with the goal of achieving tolerance. A group from the University of California San Francisco is using *ex vivo* generated and expanded Regulatory T cells to assess the effect on beta cell function and the autoimmune response in type 1 diabetes. Another phase 1 trial is investigating the safety, tolerability, and effect of three different doses of *ex vivo* expanded polyclonal Regulatory T cells in the cutaneous manifestation of patients affected with lupus erythematosus. Another phase 1 trial is using *ex vivo* Regulatory T cells for the prevention of acute GVHD in patients with hematological malignancies following hematopoietic stem cell transplantation. Another group from the University of California San Francisco is investigating the role of *ex vivo* expanded Regulatory T cells as a therapy for subclinical inflammation in kidney transplant patients.

INTRAGRAFT REGULATORY T CELLS

It is widely accepted that immunomodulatory cell types home to areas of acute inflammation, and that these cell types establish a local, tolerogenic milieu (at least partly) through direct cell-to-cell interaction (44, 46, 48, 81–86). In a miniature swine animal model of MHC class-I disparate tolerance induction, authors

have shown that a short course of calcineurin inhibition via cyclosporine leads to robust, long-lasting tolerance, which is not abrogated by infusion of pro-inflammatory cytokines, removal of the tolerated graft, or leukapheresis of peripheral T regulatory cells (44–46, 82, 87–89). Indeed, only when the tolerated kidney was removed for more than 3 months in this model, during which time the animal is kept alive by renal-transplantation with a recipient-matched kidney, did tolerance begin to wane (45, 90). These data are supported by mechanistic data in small animal models of heart transplantation (91). This abrogation of tolerance was hastened by sensitization with donor-derived peptide (45).

Given that Regulatory T cells are known to mediate both tolerance induction and tolerance maintenance in the model, investigators hypothesized that adoptive transfer of recipient-derived Regulatory T cells (both peripherally and from within the graft) could lead to stable tolerance in a naive recipient (44, 46). While adoptive transfer of leukapheresed Regulatory T cells alone did not lead to tolerance induction, transplantation of the tolerated kidney (with or without peripheral Treg infusion) did lead to stable tolerance in the naive recipient (44). These data suggested that the intra-graft regulatory components, widely thought to be $CD4^+CD25^+FoxP3^+$ Regulatory T cells, were capable of overcoming the intrinsic alloreactive responses from the naive recipient (86, 88, 89, 92). In this way, adoptive transfer of intra-graft Regulatory T cells is thought to be capable of tolerance induction (46). While important mechanistically, this model itself has little direct applicability to the clinic. However, these data strongly support the notion that tolerance is mediated by immunoregulatory cells and that, were these cells clinically available, transplantation tolerance might be readily achieved. There are questions surrounding this cell population. For example, it is unclear what percentage of intagraft cells are antigen specific, in contrast to tRegulatory T cells and pRegulatory T cells. If, for example, intragraft Regulatory T cells are enriched with donor-specific Regulatory T cells, these mechanisms by which this occurs might be exploited and extrapolated to the clinic.

CD40L(CD154)/CD40 is one of the key costimulatory mechanisms required for T-cell activation. CD40L(CD154) monoclonal antibody has used as a blocker of this costimulation pathway. After the clinical failure of CD40L(CD154) blockade in humans and non-human primates (NHP), the interest in the CD40L(CD154)/CD40 axis has reemerged due to promising results with CD40 blockade. In mice, donor-specific transfusion (DST) plus CD40L(CD154) blockade is a standard and successful protocol to induce donor-specific transplant tolerance, involving apoptosis, acquisition of regulatory cells, and suppression of proliferation of effector cells (93, 94).

Abbas and colleagues (95) have shown that there can be many resident T cells in transplanted organs and tissues, including both pro-inflammatory memory T cells and memory Regulatory T cells. On day 30–40 after resolution of an inflammatory response in the skin, activated T cells, which had migrated from central lymphoid tissue, were maintained in the target tissue, thus developing "Treg memory" to that tissue. This period roughly corresponds to the kinetics of development of allo-specific, linked suppression responses observed in DST and CD40 blockade tolerization model (Tomita et al., submitted). Mechanistically, it is thought that anti-CD40L(CD154) leads to rapid changes in lymph node architecture and to the migration of Regulatory T cells and T effector cells through high-endothelial venules (96).

While capable of tolerance induction, the kinetics of peripheral allo-specific regulatory T memory cells into tissues (other than the lymphoid tissue) are unknown. In mice, approximately 5 weeks after DST and CD40 blockade, treatment was sufficient for allo-specific regulation to manifest itself in both the lymphoid tissue and the non-lymphoid organ (liver) (Tomita et al., submitted). The regulatory phenomenon was mediated by TGF-beta and IL-35, and the proportion of regulatory cytokine-producing CD4 T cells increased in lymphoid tissues and liver over time. However, TGF-beta producing and IL-35 producing cells had different migratory kinetics.

Whether Regulatory T cells (intra-graft or otherwise) induce tolerance directly or by virtue of facilitating other cell populations is unclear. Indeed, recently groups have reported that plasmacytoid dendritic cells are capable of facilitating hematopoietic cell engraftment. Below, we will address several addition cell populations, which may induce tolerance; however, it remains unclear if their function is by virtue of facilitation or by direct tolerogenic effects (17).

REGULATORY MYELOID CELLS

Myeloid cells derive from hematopoietic stem cells. Rather than a rigidly defined group of progressively matured cell types, myeloid cells are better conceptualized as a network of cells, which can differentiate into various subsets (52). Regulatory myeloid cells (RMCs) include three broad classes of cells: regulatory macrophages (Mregs), dendritic regulatory cells (DCregs), and myeloid derived regulatory cells. *In vitro* models using human cells demonstrate each class of RMC can be generated from peripheral blood mononuclear cells (PBMCs) (58). However, the signals required for differentiation into each cell type (Mreg vs. DCreg vs. MDSC) are different. For example, *in vitro* differentiation of human PBMC into Mregs is facilitated by interferon gamma and macrophage colony stimulating factor (M-CSF). In contrast, expansion of DCregs from human PBMC is thought to require granulocyte/monocyte (GM)-CSF in addition to IL-4, IL-10, and TGF-beta plus other potentially tolerogenic factors. Lastly, MDSCs differentiation from PBMCs is supported by G-CSF and GM-CSF, and activation of MDSC requires IL-1, IL-6, and other pro-inflammatory factors (58).

Regulatory myeloid cells have elicited significant interest from the transplantation tolerance community, and clinical studies involving the use of DCregs as well as Mregs have been undertaken.

Regulatory Macrophages

Regulatory macrophages are a uniquely characterized group of cells expressing a profile of distinct group of cellular markers. They possess a novel gene-expression profile that is different from monocytes, monocyte-derived DCs, resting macrophages, IFN-gamma stimulated macrophages, and M-1, M2a-, M2b-, and M2c-polarized macrophages (97). They are derived from peripherally isolated $CD14^+$ monocytes that are cultured for

7 days while exposed to M-CSF, 10% human serum, and a 24-h pulse of IFN-gamma (98). The mechanisms by which these cells work have been investigated in both mice and humans. Mouse Mregs have been shown to inhibit T cell activity *in vitro* via inducible nitric oxide synthase (iNOS). In addition, Mregs delete cocultured allogeneic T cells via phagocytosis. In small animal models, T cells that avoided phagocytosis developed an impaired ability to secrete IL-2 and IFN-gamma (99). Human Mregs have been found to be potently suppressive of T cell proliferation via IFN-gamma induced indoleamine 2,3-dioxygenase (IDO) activity and contact-dependent deletion of activated T cells (100). Riquelme and colleagues were able to demonstrate that a one-time intravenous dose of donor-derived Mregs given 8 days before cardiac transplantation in mice was able to significantly prolong allograft survival in immunocompetent recipients. The graft survival was antigen-specific as graft survival. Indeed, recipient Mreg infusions (and third party controls) yielded no survival prolongation (99). This mechanism appeared to be iNOS independent.

Regulatory macrophages are an attractive option for cell-based tolerance induction in human recipients. A number of clinical trials have begun investigating this approach. The TAIC-I clinical trial was a single center, open-label single-arm study to assess the safety and tolerability of administering Mreg cell preparations to renal transplant recipients. A total of 12 patients receiving their first renal transplant from a decreased donor were enrolled and infused with $0.9–5.0 \times 10^8$ cells via central venous access 5 days after transplantation. Mregs were isolated by culturing donor splenic mononuclear cells in M-CSF and stimulation with IFN-gamma. There were no acute or later observed adverse reactions, providing initial clinical evidence that this is a safe therapy (101). A subsequent trial, TAIC-II, assessed the safety and efficacy of administering Mreg cell preparations to recipients of living-donor renal transplants. A total of 5 living-related kidney transplant recipients were infused with $1.4–5.9 \times 10^8$ cells, received induction therapy with anti-thymocyte globulin, in addition to steroid and tacrolimus (trough levels of 8–12 ng/ml). Mregs were obtained by culturing donor pPBMCs in M-CSF and stimulation with IFN-gamma followed by coculture with recipient PBMCs. No acute reactions occurred. Steroids were weaned by 8 weeks posttransplant, and tacrolimus was decreased to 5–8 ng/ml. Four patients were successfully transferred to this dose of tacrolimus therapy, with no rejection occurring in two patients. Tacrolimus levels were further weaned to <2 ng/ml, and one patient experienced rejection at 36 weeks. Following cessation of immunosuppression, two patients experienced rejection at 2 and 34 weeks postcessation (102). Another patient that did not qualify for the TAIC-II trial because of measurable levels of anti-donor HLA antibodies was described by Hutchinson and colleagues. The patient received a presensitized living-related renal transplant. The patient was infused with 4.8×10^9 Mregs 17 days prior to transplant, which were isolated via the same protocol as the TAIC-II study. The patient was stable at 27 months posttransplant and interestingly was no longer positive for the anti-donor HLA antibodies. Serological screening determined that the patient remained hepatitis A virus positive (was positive before transplant) suggesting that this was a specific effect of Mreg treatment (103).

Since these two trials, Hutchinson and colleagues have refined their Mreg purification and treated two living-donor kidney transplant recipients. The first patient received a single HLA-B and DR mismatched-related kidney from her mother and 8×10^6 donor-derived Mregs via central venous infusion 6 days prior to transplant. Azathioprine, steroids, and tacrolimus were started at the time of transplantation and at 3 years posttransplant, and the patient was stable with no signs of rejection demonstrated via biopsy while maintaining tacrolimus trough levels of 4–5 ng/ml. The second patient received a fully mismatched kidney from a living unrelated donor and 7.1×10^6 Mregs 7 days prior to transplant. Azathioprine, steroids, and tacrolimus were started during transplantation. At 3 years posttransplant, the patient was stable with no signs of rejection via biopsy and was being maintained on tacrolimus with a trough level of 2.7 ng/ml (100). Taken together, preliminary evidence suggests that Mreg treatment preoperatively in renal transplant patients is safe, and further work needs to be done in humans to describe its effectiveness. The ONE Study is currently aiming to develop an array of cellular based therapies, one of which is Mregs, in order to achieve immunologic tolerance in transplant patients (104).

Dendritic Regulatory Cells

Dendritic regulatory cells have been reviewed in detail recently (51, 97, 105). In one early human study of DCregs, authors observed that in response to injection of 2×10^6 immature DCregs, antigen-specific Regulatory T cells were developed, and $CD8^+$ T cell effector function was inhibited (58, 106, 107). Additionally, a more recent study of DCregs was undertaken in type I diabetes, for the purposes of self-tolerance (overcome autoimmunity). Authors administered 10 million cells intra-abdominally every 2 weeks for a total of four injections. DCreg injections were not associated with adverse reactions. Perhaps important, investigators did observe an increase in the percentage of suppressive $B220^+$ B cells, which may help suppress autoimmunity in type 1 diabetes (108).

MDSCs

MDSCs are a heterogeneous, immature population of monocytic- (mMDSCs) and granulocytic (gMDSCs)-derived cells that work to negatively regulate the immune system. MDSCs are naturally occurring, and are expanded during times of stress and inflammation (109). Much of what we know about MDSCs comes from cancer biology and the mechanisms by which MDSC-mediated immunosuppression occurs are being investigated. MDSC-mediated immunosuppression occurs through several known mechanisms. Primarily MDSCs have been found to express high levels of arginase-1 (produces urea and L-ornithine from L-arginine) and iNOS (generates NO), which have a well-established role in the suppression of T cell function (110, 111). By expressing arginase-1, MDSCs deplete local L-arginine levels of arginine, which is required by lymphocytes. In addition, MDSCs increase NO production. Arginase-1 dependent L-arginine depletion and NO production diminish the ability of T cells to proliferate and express MHC

class II as well as inducing T cell apoptosis (112–116). MDSCs have also been shown to elicit immunosuppressive effects through the production of reactive oxygen species (ROS) and peroxynitrite (117–121). In the case of the latter, the peptide-MHC structure is altered, weakening the peptide's immunogenicity (109). Likely important for potential tolerance induction, MDSCs have been found (in the presence of IFN-gamma and IL-10) to induce *de novo* development of FoxP3+ Regulatory T cells (116, 122). MDSCs are capable of inducing the proliferation of existing Regulatory T cells and that depletion of Trges impairs the ability of MDSCs to accumulate (116, 123, 124). The mechanisms by which MDSCs contribute to immune tolerance is multifactorial, involves other cell types and is likely to be subset dependent as well (109, 125).

With regard to MDSCs and solid organ transplantation, Vanhove and colleagues have shown in a kidney transplant rat model that immune tolerance was induced via anti-CD28 and that MDSCs accumulated within the allograft (126, 127). *In vitro*, the MDSCs were able to induce contact-dependent apoptosis of T cells, which induced the expression of iNOS in the MDSCs. The MDSCs were also found to have a minimal effect on Regulatory T cells that failed to induce iNOS in the MDSCs. These results highlight the cross-talk between these two cell types in immune tolerance. Lu et al. demonstrated that transplantation of hepatic stellate cells into diabetic mice induced MDSCs. In addition, these MDSCs were associated with increased levels of iNOS and Arg-1 as well as $CD4^+$ and $CD8^+$ T cell suppression. The same group also demonstrated that with cotransplantation of 2.5×10^6 MDSCs and islet cells into diabetic mice, the survival of the islet cell allograft was significantly prolonged (128). *In vitro* and *in vivo* data both supported the necessity of the B7–H1 interaction for induction of Regulatory T cells involved in this process. Another study using repeated injections of LPS to induce MDSCs and evoke tolerance reported prolonged allograft survival through T cell suppression via a heme oxygenase-1 dependent pathway (129). This group was unable to reverse the T cell suppression by neutralizing iNOS or Arg-1, perhaps highlighting another immunomodulatory mechanism of MDSCs. Recently, Thomson and colleagues from the University of Pittsburgh showed that MDSCs can suppress T cell proliferation and cytokine secretion in non-NHP *in vivo* (130). This has raised the possibility of scaling these MDSC models to the NHP, and perhaps humans as well. In summary, much work is being done to uncover the mechanisms by which MDSCs contribute to establishing immune tolerance and the potential for use as a cellular based therapy is promising.

Regarding the potential for MDSCs in human transplantation, studies are lacking. Encouragingly, recent hematology data suggesting that MDCSs may control GVHD, and additional data demonstrating that MDSCs are upregulated after transplantation have highlighted MDSCs as a possible avenue to tolerance in humans (131). In a recent review, authors suggested that excitement for MDSCs in tolerance should be tempered until additional MDSC phenotyping can be performed. Indeed, it is not yet clear if the immunosuppressive effects of MDSCs are specific vs. non-specific, and it is not yet clear if MDSCs would need to be used synergistically with other therapies (127, 131).

B CELLS

While most studies have focused on the allo-reactive T cell in tolerance induction, the roles of allo-reactive B cells are largely unknown. However, a subset of B cells known as B regulatory cells (Bregs) has been identified as a potent factor in immune homeostasis and autoimmunity, and they have been found to be involved with maintaining immune tolerance associated with Regulatory T cells (132, 133). Recent work is uncovering a possible role in immunomodulation, which first gained attention when mice, deplete of B cells, were shown to develop a severe form of experimental autoimmune encephalomyelitis (EAE) (134). Further studies demonstrated similar findings in mouse models of autoimmune disorders such as collagen induced arthritis, ulcerative colitis, and allergy (135–138). In 2007, investigators at MGH (139) reported to achieve tolerance in a heart transplant mouse model. They first established B-cell dependent allo-reactive tolerance using anti-CD45RB antibody. The phenomenon required the interaction of costimulation molecules on B cells with T cells, which were $CD40^+$ and $CD80/86^+$. They also reported in islet allograft models that mice treated with anti-CD45RB antibody plus anti-T cells immunoglobulin domain and mucin domain-1 (anti-TIM-1) antibody were induced allo-reactive tolerance via an IL-10 dependent pathway (140). In addition, they recently showed that the Breg response was associated with Treg induction mediated by TGF-beta (141). A second group at University of Pittsburgh has indicated that TIM-1, which is an important marker for $IL\text{-}10^+$ Bregs (induced by TIM-1 ligation), plays a critical role in regulation the immune response (142). A third group in Wisconsin has shown in an acute EAE mouse model deficient in B cells led to a delay in the emergence of $FoxP3^+$ expression Regulatory T cells and the expression of IL-10 in the CNS. This was normalized by reconstitution with B cells, but was not normalized when reconstituted with B7 deficient B cells. The above work highlights a possible role for B cell dependent Treg expansion via B7 (143). Cell-to-cell contact has also been shown to contribute to B cell-dependent immunosuppression (144, 145). A recent study showed that coculture of purified Bregs was shown to suppress the proliferation of $CD4^+$ T cells. Furthermore, Bregs coculture with Regulatory T cells led to the upregulation of FoxP3 and CTLA4 in Regulatory T cells (144). This evidence has led to the suggestion that Breg therapy may have an indirect role in immune tolerance therapy via *ex vivo* Treg expansion (133).

An immunoregulatory role for B cells has also been suggested in human diseases based on findings in patients with autoimmune diseases such as multiple sclerosis, lupus, rheumatoid arthritis, and even cancer (146–149). Numerous studies have begun to suggest that B cells also play an integral part in inducing immune tolerance in transplant patients (141, 150–154). Although there are no studies to date regarding B cell therapy in humans, this technique has been quite successful in animal models of autoimmune diseases. Particularly exciting is

a model that has been developed in which polyclonal B cells are transduced with a retrovirus encoding specific antigens (155). Using this model, genetically modified B cells were able to inhibit autoimmune diseases such as uveitis, multiple sclerosis, type 1 diabetes, and rheumatoid arthritis in mouse models (19, 156–160). These genetically engineered B cells were also shown to be capable of inducing the proliferation of $FoxP3^+$ $CD4^+$ Regulatory T cells (161). Furthermore, another group was able to show that reconstitution with similarly engineered B cells *in vivo* protected against EAE in mice (162). Taken together, the success of B cell therapy for immunosuppression in animal models, and the established immunomodulatory role in humans suggests that the possibility of B cell-based cellular therapies for immune tolerance induction in humans is not out of the question.

OTHER CELL TYPES

The above discussion is by no means complete. There are additional cell types which not included here which may be worthy of mention, such as apoptotic cells (163). Apoptotic cell-based therapies may improve graft survival and inflammatory diseases. Perhaps most excitingly, apoptotic cells may also be effective for the treatment of GVHD (163–165).

SYNTHESIS OF THE DATA

Here, we have presented a number of different cell types, which contribute to tolerance induction. However, the presented data should be approached carefully. Indeed, mesenchymal stem cells or myeloid precursors (and/or MDSCs), which are present in the bone marrow may be involved in tolerance induction by cotransplantation of bone marrow and a solid organ. The same is true for facilitating cells. However, cell therapies based on regulatory T cells, B cells (Breg), dendritic cells, or macrophages emerge from their immunomodulatory properties rather than their sole presence in the bone marrow graft. Conversely, apoptotic cell-based therapies (i.e., administration of donor apoptotic cells) or facilitating cells may account for tolerance induction after cotransplantation of bone marrow and solid organ. As such, the notion of tolerance inducing versus tolerance facilitation may require further discussion.

AUTHOR CONTRIBUTIONS

JS – study design, analysis, literature review, wrote the paper, and edited the paper. YT – literature search, analysis, and wrote the paper. CL – literature search, analysis, and wrote the paper. WB – study design and wrote the paper.

REFERENCES

1. Owen RD. Immunogenetic consequences of vascular anastomoses between bovine twins. *Science* (1945) **102**:400–1. doi:10.1126/science.102.2651.400
2. Brent L. The discovery of immunologic tolerance. *Hum Immunol* (1997) **52**:75–81. doi:10.1016/S0198-8859(96)00289-3
3. Billingham RE, Brent L, Medawar PB. Actively acquired tolerance of foreign cells. *Nature* (1953) **172**:603–6. doi:10.1038/172603a0
4. Sharabi Y, Sachs DH. Mixed chimerism and permanent specific transplantation tolerance induced by a nonlethal preparative regimen. *J Exp Med* (1989) **169**:493–502. doi:10.1084/jem.169.2.493
5. Strober S, Modry DL, Hoppe RT, Pennock JL, Bieber CP, Holm BI, et al. Induction of specific unresponsiveness to heart allografts in mongrel dogs treated with total lymphoid irradiation and antithymocyte globulin. *J Immunol* (1984) **132**:1013–8.
6. Sykes M. Mixed chimerism and transplant tolerance. *Immunity* (2001) **14**:417–24. doi:10.1016/S1074-7613(01)00122-4
7. Kawai T, Cosimi AB, Colvin RB, Powelson J, Eason J, Kozlowski T, et al. Mixed allogeneic chimerism and renal allograft tolerance in cynomolgus monkeys. *Transplantation* (1995) **59**:256–62. doi:10.1097/00007890-199501000-00018
8. Kawai T, Cosimi AB, Wee SL, Houser S, Andrews D, Sogawa H, et al. Effect of mixed hematopoietic chimerism on cardiac allograft survival in cynomolgus monkeys. *Transplantation* (2002) **73**:1757–64. doi:10.1097/00007890-200206150-00011
9. Kawai T, Sogawa H, Boskovic S, Abrahamian G, Smith RN, Wee SL, et al. CD154 blockade for induction of mixed chimerism and prolonged renal allograft survival in nonhuman primates. *Am J Transplant* (2004) **4**:1391–8. doi:10.1111/j.1600-6143.2004.00523.x
10. Kawai T, Cosimi AB, Spitzer TR, Tolkoff-Rubin N, Suthanthiran M, Saidman SL, et al. HLA-mismatched renal transplantation without maintenance immunosuppression. *N Engl J Med* (2008) **358**:353–61. doi:10.1056/NEJMoa071074
11. Spitzer TR, Delmonico F, Tolkoff-Rubin N, McAfee S, Sackstein R, Saidman S, et al. Combined histocompatibility leukocyte antigen-matched donor bone marrow and renal transplantation for multiple myeloma with end stage renal disease: the induction of allograft tolerance through mixed lymphohematopoietic chimerism. *Transplantation* (1999) **68**:480–4. doi:10.1097/00007890-199908270-00006
12. Kawai T, Sachs DH, Sykes M, Cosimi AB, Immune Tolerance N. HLA-mismatched renal transplantation without maintenance immunosuppression. *N Engl J Med* (2013) **368**:1850–2. doi:10.1056/NEJMc1213779
13. Scandling JD, Busque S, Dejbakhsh-Jones S, Benike C, Millan MT, Shizuru JA, et al. Tolerance and chimerism after renal and hematopoietic-cell transplantation. *N Engl J Med* (2008) **358**:362–8. doi:10.1056/NEJMoa074191
14. Scandling JD, Busque S, Dejbakhsh-Jones S, Benike C, Sarwal M, Millan MT, et al. Tolerance and withdrawal of immunosuppressive drugs in patients given kidney and hematopoietic cell transplants. *Am J Transplant* (2012) **12**:1133–45. doi:10.1111/j.1600-6143.2012.03992.x
15. Leventhal J, Abecassis M, Miller J, Gallon L, Ravindra K, Tollerud DJ, et al. Chimerism and tolerance without GVHD or engraftment syndrome in HLA-mismatched combined kidney and hematopoietic stem cell transplantation. *Sci Transl Med* (2012) **4**:124ra28. doi:10.1126/scitranslmed.3003509
16. Leventhal JR, Mathew JM, Salomon DR, Kurian SM, Friedewald JJ, Gallon L, et al. Nonchimeric HLA-identical renal transplant tolerance: regulatory immunophenotypic/genomic biomarkers. *Am J Transplant* (2016) **16**(1):221–34.
17. Fugier-Vivier IJ, Rezzoug F, Huang Y, Graul-Layman AJ, Schanie CL, Xu H, et al. Plasmacytoid precursor dendritic cells facilitate allogeneic hematopoietic stem cell engraftment. *J Exp Med* (2005) **201**:373–83. doi:10.1084/jem.20041399
18. Starzl TE, Demetris AJ. Transplantation tolerance, microchimerism, and the two-way paradigm. *Theor Med Bioeth* (1998) **19**:441–55. doi:10.1023/A:1009924907775
19. Agarwal RK, Kang Y, Zambidis E, Scott DW, Chan CC, Caspi RR. Retroviral gene therapy with an immunoglobulin-antigen fusion construct protects from experimental autoimmune uveitis. *J Clin Invest* (2000) **106**:245–52. doi:10.1172/JCI9168
20. Kinder JM, Jiang TT, Ertelt JM, Xin L, Strong BS, Shaaban AF, et al. Cross-generational Reproductive fitness enforced by microchimeric maternal cells. *Cell* (2015) **162**:505–15. doi:10.1016/j.cell.2015.07.006
21. D'Aveni M, Rossignol J, Coman T, Sivakumaran S, Henderson S, Manzo T, et al. G-CSF mobilizes CD34+ regulatory monocytes that inhibit graft-versus-host disease. *Sci Transl Med* (2015) **7**:281ra42. doi:10.1126/scitranslmed.3010435

22. Ball LM, Bernardo ME, Roelofs H, Lankester A, Cometa A, Egeler RM, et al. Cotransplantation of ex vivo expanded mesenchymal stem cells accelerates lymphocyte recovery and may reduce the risk of graft failure in haploidentical hematopoietic stem-cell transplantation. *Blood* (2007) **110**:2764–7. doi:10.1182/blood-2007-04-087056
23. Ge W, Jiang J, Baroja ML, Arp J, Zassoko R, Liu W, et al. Infusion of mesenchymal stem cells and rapamycin synergize to attenuate alloimmune responses and promote cardiac allograft tolerance. *Am J Transplant* (2009) **9**:1760–72. doi:10.1111/j.1600-6143.2009.02721.x
24. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. *Science* (1999) **284**:143–7. doi:10.1126/science.284.5411.143
25. Mattar P, Bieback K. Comparing the immunomodulatory properties of bone marrow, adipose tissue, and birth-associated tissue mesenchymal stromal cells. *Front Immunol* (2015) **6**:560. doi:10.3389/fimmu.2015.00560
26. Franquesa M, Hoogduijn MJ, Bestard O, Grinyó JM. Immunomodulatory effect of mesenchymal stem cells on B cells. *Front Immunol* (2012) **3**:212. doi:10.3389/fimmu.2012.00212
27. Engela AU, Baan CC, Dor FJ, Weimar W, Hoogduijn MJ. On the interactions between mesenchymal stem cells and regulatory T cells for immunomodulation in transplantation. *Front Immunol* (2012) **3**:126. doi:10.3389/fimmu.2012.00126
28. Eggenhofer E, Luk F, Dahlke MH, Hoogduijn MJ. The life and fate of mesenchymal stem cells. *Front Immunol* (2014) **5**:148. doi:10.3389/fimmu.2014.00148
29. Bartholomew A, Sturgeon C, Siatskas M, Ferrer K, McIntosh K, Patil S, et al. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. *Exp Hematol* (2002) **30**:42–8. doi:10.1016/S0301-472X(01)00769-X
30. Casiraghi F, Azzollini N, Cassis P, Imberti B, Morigi M, Cugini D, et al. Pretransplant infusion of mesenchymal stem cells prolongs the survival of a semiallogeneic heart transplant through the generation of regulatory T cells. *J Immunol* (2008) **181**:3933–46. doi:10.4049/jimmunol.181.6.3933
31. Wang Y, Zhang A, Ye Z, Xie H, Zheng S. Bone marrow-derived mesenchymal stem cells inhibit acute rejection of rat liver allografts in association with regulatory T-cell expansion. *Transplant Proc* (2009) **41**:4352–6. doi:10.1016/j.transproceed.2009.08.072
32. Ge W, Jiang J, Arp J, Liu W, Garcia B, Wang H. Regulatory T-cell generation and kidney allograft tolerance induced by mesenchymal stem cells associated with indoleamine 2,3-dioxygenase expression. *Transplantation* (2010) **90**:1312–20. doi:10.1097/TP.0b013e3181fed001
33. Collins E, Gu F, Qi M, Molano I, Ruiz P, Sun L, et al. Differential efficacy of human mesenchymal stem cells based on source of origin. *J Immunol* (2014) **193**:4381–90. doi:10.4049/jimmunol.1401636
34. Qi H, Chen G, Huang Y, Si Z, Li J. Foxp3-modified bone marrow mesenchymal stem cells promotes liver allograft tolerance through the generation of regulatory T cells in rats. *J Transl Med* (2015) **13**:274. doi:10.1186/s12967-015-0638-2
35. Uccelli A, Moretta L, Pistoia V. Mesenchymal stem cells in health and disease. *Nat Rev Immunol* (2008) **8**:726–36. doi:10.1038/nri2395
36. Casiraghi F, Azzollini N, Todeschini M, Cavinato RA, Cassis P, Solini S, et al. Localization of mesenchymal stromal cells dictates their immune or proinflammatory effects in kidney transplantation. *Am J Transplant* (2012) **12**:2373–83. doi:10.1111/j.1600-6143.2012.04115.x
37. English K, Barry FP, Mahon BP. Murine mesenchymal stem cells suppress dendritic cell migration, maturation and antigen presentation. *Immunol Lett* (2008) **115**:50–8. doi:10.1016/j.imlet.2007.10.002
38. Le Blanc K, Rasmusson I, Sundberg B, Götherström C, Hassan M, Uzunel M, et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* (2004) **363**:1439–41. doi:10.1016/S0140-6736(04)16104-7
39. Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet* (2008) **371**:1579–86. doi:10.1016/S0140-6736(08)60690-X
40. Eggenhofer E, Benseler V, Kroemer A, Popp FC, Geissler EK, Schlitt HJ, et al. Mesenchymal stem cells are short-lived and do not migrate beyond the lungs after intravenous infusion. *Front Immunol* (2012) **3**:297. doi:10.3389/fimmu.2012.00297
41. Peng Y, Ke M, Xu L, Liu L, Chen X, Xia W, et al. Donor-derived mesenchymal stem cells combined with low-dose tacrolimus prevent acute rejection after renal transplantation: a clinical pilot study. *Transplantation* (2013) **95**:161–8. doi:10.1097/TP.0b013e3182754c53
42. Mudrabettu C, Kumar V, Rakha A, Yadav AK, Ramachandran R, Kanwar DB, et al. Safety and efficacy of autologous mesenchymal stromal cells transplantation in patients undergoing living donor kidney transplantation: a pilot study. *Nephrology (Carlton)* (2015) **20**:25–33. doi:10.1111/nep.12338
43. Tan J, Wu W, Xu X, Liao L, Zheng F, Messinger S, et al. Induction therapy with autologous mesenchymal stem cells in living-related kidney transplants: a randomized controlled trial. *JAMA* (2012) **307**:1169–77. doi:10.1001/jama.2012.316
44. Scalea JR, Okumi M, Villani V, Shimizu A, Nishimura H, Gillon BC, et al. Abrogation of renal allograft tolerance in MGH miniature swine: the role of intra-graft and peripheral factors in long-term tolerance. *Am J Transplant* (2014) **14**(9):2001–10. doi:10.1111/ajt.12816
45. Weiner J, Scalea J, Ishikawa Y, Okumi M, Griesemer A, Hirakata A, et al. Tolerogenicity of donor major histocompatibility complex-matched skin grafts in previously tolerant Massachusetts general hospital miniature swine. *Transplantation* (2012) **94**:1192–9. doi:10.1097/TP.0b013e31827254f5
46. Okumi M, Scalea JR, Gillon BC, Tasaki M, Villani V, Cormack T, et al. The induction of tolerance of renal allografts by adoptive transfer in miniature swine. *Am J Transplant* (2013) **13**:1193–202. doi:10.1111/ajt.12194
47. Sachs DH, Kawai T, Sykes M. Induction of tolerance through mixed chimerism. *Cold Spring Harb Perspect Med* (2014) **4**:a015529. doi:10.1101/cshperspect.a015529
48. Hongo D, Tang X, Baker J, Engleman EG, Strober S. Requirement for interactions of natural killer T cells and myeloid-derived suppressor cells for transplantation tolerance. *Am J Transplant* (2014) **14**:2467–77. doi:10.1111/ajt.12914
49. Sullivan JA, Adams AB, Burlingham WJ. The emerging role of TH17 cells in organ transplantation. *Transplantation* (2014) **97**:483–9. doi:10.1097/TP.0000000000000000
50. Dutta P, Burlingham WJ. Microchimerism: tolerance vs. sensitization. *Curr Opin Organ Transplant* (2011) **16**:359–65. doi:10.1097/MOT.0b013e3283484b57
51. Conde P, Rodriguez M, van der Touw W, Jimenez A, Burns M, Miller J, et al. DC-SIGN(+) macrophages control the induction of transplantation tolerance. *Immunity* (2015) **42**:1143–58. doi:10.1016/j.immuni.2015.05.009
52. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. *Nat Rev Immunol* (2012) **12**:253–68. doi:10.1038/nri3175
53. Lucas CL, Workman CJ, Beyaz S, LoCascio S, Zhao G, Vignali DA, et al. LAG-3, TGF-beta, and cell-intrinsic PD-1 inhibitory pathways contribute to CD8 but not CD4 T-cell tolerance induced by allogeneic BMT with anti-CD40L. *Blood* (2011) **117**:5532–40. doi:10.1182/blood-2010-11-318675
54. Hutchinson JA, Riquelme P, Geissler EK. Human regulatory macrophages as a cell-based medicinal product. *Curr Opin Organ Transplant* (2012) **17**:48–54. doi:10.1097/MOT.0b013e32834ee64a
55. Hutchinson JA. Somatic cell-based therapy. *Transplantation* (2015) **99**:1103–5. doi:10.1097/TP.0000000000000788
56. Broichhausen C, Riquelme P, Geissler EK, Hutchinson JA. Regulatory macrophages as therapeutic targets and therapeutic agents in solid organ transplantation. *Curr Opin Organ Transplant* (2012) **17**:332–42. doi:10.1097/MOT.0b013e328355a979
57. Ochando JC, Turnquist HR. Innate immune cell collaborations instigate transplant tolerance. *Am J Transplant* (2014) **14**:2441–3. doi:10.1111/ajt.12912
58. Rosborough BR, Raich-Regue D, Turnquist HR, Thomson AW. Regulatory myeloid cells in transplantation. *Transplantation* (2014) **97**:367–79. doi:10.1097/TP.0b013e3182a860de
59. Brunstein CG, Miller JS, Cao Q, McKenna DH, Hippen KL, Curtsinger J, et al. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood* (2011) **117**:1061–70. doi:10.1182/blood-2010-07-293795
60. Di Ianni M, Falzetti F, Carotti A, Terenzi A, Castellino F, Bonifacio E, et al. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood* (2011) **117**:3921–8. doi:10.1182/blood-2010-10-311894

61. Safinia N, Scotta C, Vaikunthanathan T, Lechler RI, Lombardi G. Regulatory T cells: serious contenders in the promise for immunological tolerance in transplantation. *Front Immunol* (2015) **6**:438. doi:10.3389/fimmu.2015.00438
62. Dummer CD, Carpio VN, Goncalves LF, Manfro RC, Veronese FV. FOXP3+ regulatory T cells: from suppression of rejection to induction of renal allograft tolerance. *Transpl Immunol* (2012) **26**:1–10. doi:10.1016/j.trim.2011.08.009
63. Veronese F, Rotman S, Smith RN, Pelle TD, Farrell ML, Kawai T, et al. Pathological and clinical correlates of FOXP3+ cells in renal allografts during acute rejection. *Am J Transplant* (2007) **7**:914–22. doi:10.1111/j.1600-6143.2006.01704.x
64. Thornton AM, Korty PE, Tran DQ, Wohlfert EA, Murray PE, Belkaid Y, et al. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *J Immunol* (2010) **184**:3433–41. doi:10.4049/jimmunol.0904028
65. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. *Nat Rev Immunol* (2008) **8**:523–32. doi:10.1038/nri2343
66. Hara M, Kingsley CI, Niimi M, Read S, Turvey SE, Bushell AR, et al. IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo. *J Immunol* (2001) **166**:3789–96. doi:10.4049/jimmunol.166.6.3789
67. Fu S, Zhang N, Yopp AC, Chen D, Mao M, Chen D, et al. TGF-beta induces Foxp3 + T-regulatory cells from CD4 + CD25 – precursors. *Am J Transplant* (2004) **4**:1614–27. doi:10.1111/j.1600-6143.2004.00566.x
68. Shevach EM. From vanilla to 28 flavors: multiple varieties of T regulatory cells. *Immunity* (2006) **25**:195–201. doi:10.1016/j.immuni.2006.08.003
69. Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM, et al. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* (2007) **450**:566–9. doi:10.1038/nature06306
70. Velaga S, Ukena SN, Dringenberg U, Alter C, Pardo J, Kershaw O, et al. Granzyme A is required for regulatory T-cell mediated prevention of gastrointestinal graft-versus-host disease. *PLoS One* (2015) **10**:e0124927. doi:10.1371/journal.pone.0124927
71. Geis AL, Fan H, Wu X, Wu S, Huso DL, Wolfe JL, et al. Regulatory T-cell response to enterotoxigenic bacteroides fragilis colonization triggers IL17-dependent colon carcinogenesis. *Cancer Discov* (2015) **5**:1098–109. doi:10.1158/2159-8290.CD-15-0447
72. Garcia-Martinez K, Leon K. Modeling the role of IL2 in the interplay between CD4+ helper and regulatory T cells: studying the impact of IL2 modulation therapies. *Int Immunol* (2012) **24**:427–46. doi:10.1093/intimm/dxr120
73. Kleijwegt FS, Laban S, Duinkerken G, Joosten AM, Koeleman BP, Nikolic T, et al. Transfer of regulatory properties from tolerogenic to proinflammatory dendritic cells via induced autoreactive regulatory T cells. *J Immunol* (2011) **187**:6357–64. doi:10.4049/jimmunol.1101638
74. Golovina TN, Mikheeva T, Suhoski MM, Aqui NA, Tai VC, Shan X, et al. CD28 costimulation is essential for human T regulatory expansion and function. *J Immunol* (2008) **181**:2855–68. doi:10.4049/jimmunol.181.4.2855
75. Hippen KL, Merkel SC, Schirm DK, Sieben CM, Sumstad D, Kadidlo DM, et al. Massive ex vivo expansion of human natural regulatory T cells (T(regs)) with minimal loss of in vivo functional activity. *Sci Transl Med* (2011) **3**:83ra41. doi:10.1126/scitranslmed.3001809
76. Longhi MS, Meda F, Wang P, Samyn M, Mieli-Vergani G, Vergani D, et al. Expansion and de novo generation of potentially therapeutic regulatory T cells in patients with autoimmune hepatitis. *Hepatology* (2008) **47**:581–91. doi:10.1002/hep.22071
77. Lapierre P, Lamarre A. Regulatory T cells in autoimmune and viral chronic hepatitis. *J Immunol Res* (2015) **2015**:479703. doi:10.1155/2015/479703
78. Golshayan D, Jiang S, Tsang J, Garin MI, Mottet C, Lechler RI. In vitro-expanded donor alloantigen-specific CD4+CD25+ regulatory T cells promote experimental transplantation tolerance. *Blood* (2007) **109**:827–35. doi:10.1182/blood-2006-05-025460
79. Jiang S, Camara N, Lombardi G, Lechler RI. Induction of allopeptide-specific human CD4+CD25+ regulatory T cells ex vivo. *Blood* (2003) **102**:2180–6. doi:10.1182/blood-2003-04-1164
80. Sagoo P, Ali N, Garg G, Nestle FO, Lechler RI, Lombardi G. Human regulatory T cells with alloantigen specificity are more potent inhibitors of alloimmune skin graft damage than polyclonal regulatory T cells. *Sci Transl Med* (2011) **3**:83ra42. doi:10.1126/scitranslmed.3002076
81. Bettini M, Vignali DA. Regulatory T cells and inhibitory cytokines in autoimmunity. *Curr Opin Immunol* (2009) **21**:612–8. doi:10.1016/j.coi.2009.09.011
82. Rosengard BR, Fishbein JM, Gianello P, Ojikutu CA, Guzzetta PC, Smith CV, et al. Retransplantation in miniature swine. Lack of a requirement for graft adaptation for maintenance of specific renal allograft tolerance. *Transplantation* (1994) **57**:794–9. doi:10.1097/00007890-199403270-00003
83. Utsugi R, Barth RN, Lee RS, Kitamura H, LaMattina JC, Ambroz J, et al. Induction of transplantation tolerance with a short course of tacrolimus (FK506): I. Rapid and stable tolerance to two-haplotype fully mhc-mismatched kidney allografts in miniature swine. *Transplantation* (2001) **71**:1368–79. doi:10.1097/00007890-200105270-00003
84. Scalea JR, Bromberg J, Bartlett ST, Scalea TM. Mechanistic similarities between trauma, atherosclerosis, and other inflammatory processes. *J Crit Care* (2015) **30**(6):1344–8. doi:10.1016/j.jcrc.2015.07.024
85. Pillai AB, George TI, Dutt S, Strober S. Host natural killer T cells induce an interleukin-4-dependent expansion of donor CD4+CD25+Foxp3+ T regulatory cells that protects against graft-versus-host disease. *Blood* (2009) **113**:4458–67. doi:10.1182/blood-2008-06-165506
86. Griesemer AD, Lamattina JC, Okumi M, Etter JD, Shimizu A, Sachs DH, et al. Linked suppression across an MHC-mismatched barrier in a miniature swine kidney transplantation model. *J Immunol* (2008) **181**:4027–36. doi:10.4049/jimmunol.181.6.4027
87. Rosengard BR, Ojikutu CA, Guzzetta PC, Smith CV, Sundt TM III, Nakajima K, et al. Induction of specific tolerance to class I-disparate renal allografts in miniature swine with cyclosporine. *Transplantation* (1992) **54**:490–7. doi:10.1097/00007890-199209000-00020
88. Gianello PR, Lorf T, Yamada K, Fishbein JM, Nickeleit V, Vitiello DM, et al. Induction of tolerance to renal allografts across single-haplotype MHC disparities in miniature swine. *Transplantation* (1995) **59**:884–90. doi:10.1097/00007890-199503150-00023
89. Gianello PR, Fishbein JM, Rosengard BR, Lorf T, Vitiello DM, Arn JS, et al. Tolerance to class I-disparate renal allografts in miniature swine. Maintenance of tolerance despite induction of specific antidonor CTL responses. *Transplantation* (1995) **59**:772–7. doi:10.1097/00007890-199503150-00023
90. Okumi M, Fishbein JM, Griesemer AD, Gianello PR, Hirakata A, Nobori S, et al. Role of persistence of antigen and indirect recognition in the maintenance of tolerance to renal allografts. *Transplantation* (2008) **85**:270–80. doi:10.1097/TP.0b013e31815e8eed
91. Hamano K, Rawsthorne MA, Bushell AR, Morris PJ, Wood KJ. Evidence that the continued presence of the organ graft and not peripheral donor microchimerism is essential for maintenance of tolerance to alloantigen in vivo in anti-CD4 treated recipients. *Transplantation* (1996) **62**:856–60. doi:10.1097/00007890-199609270-00026
92. Scalea JR, Villani V, Gillon BC, Weiner J, Gianello P, Turcotte N, et al. Development of antidonor antibody directed toward non-major histocompatibility complex antigens in tolerant animals. *Transplantation* (2014) **98**:514–9. doi:10.1097/TP.0000000000000249
93. Graca L. Transplantation tolerance: context matters. *Eur J Immunol* (2015) **45**:1921–5. doi:10.1002/eji.201545762
94. Chai JG, Ratnasothy K, Bucy RP, Noelle RJ, Lechler R, Lombardi G. Allospecific CD4(+) T cells retain effector function and are actively regulated by Treg cells in the context of transplantation tolerance. *Eur J Immunol* (2015) **45**:2017–27. doi:10.1002/eji.201545455
95. Rosenblum MD, Gratz IK, Paw JS, Lee K, Marshak-Rothstein A, Abbas AK. Response to self antigen imprints regulatory memory in tissues. *Nature* (2011) **480**:538–42. doi:10.1038/nature10664
96. Warren KJ, Iwami D, Harris DG, Bromberg JS, Burrell BE. Laminins affect T cell trafficking and allograft fate. *J Clin Invest* (2014) **124**:2204–18. doi:10.1172/JCI73683
97. Riquelme P, Geissler EK, Hutchinson JA. Alternative approaches to myeloid suppressor cell therapy in transplantation: comparing regulatory macrophages to tolerogenic DCs and MDSCs. *Transplant Res* (2012) **1**:17. doi:10.1186/2047-1440-1-17
98. Hutchinson JA, Riquelme P, Geissler EK, Fändrich F. Human regulatory macrophages. *Methods Mol Biol* (2011) **677**:181–92. doi:10.1007/978-1-60761-869-0_13
99. Riquelme P, Tomiuk S, Kammler A, Fändrich F, Schlitt HJ, Geissler EK, et al. IFN-γ-induced iNOS expression in mouse regulatory macrophages prolongs allograft survival in fully immunocompetent recipients. *Mol Ther* (2013) **21**:409–22. doi:10.1038/mt.2012.168

100. Hutchinson JA, Riquelme P, Sawitzki B, Tomiuk S, Miqueu P, Zuhayra M, et al. Cutting edge: immunological consequences and trafficking of human regulatory macrophages administered to renal transplant recipients. *J Immunol* (2011) **187**:2072–8. doi:10.4049/jimmunol.1100762
101. Hutchinson JA, Riquelme P, Brem-Exner BG, Schulze M, Matthäi M, Renders L, et al. Transplant acceptance-inducing cells as an immune-conditioning therapy in renal transplantation. *Transpl Int* (2008) **21**:728–41. doi:10.1111/j.1432-2277.2008.00680.x
102. Hutchinson JA, Brem-Exner BG, Riquelme P, Roelen D, Schulze M, Ivens K, et al. A cell-based approach to the minimization of immunosuppression in renal transplantation. *Transpl Int* (2008) **21**:742–54. doi:10.1111/j.1432-2277.2008.00692.x
103. Hutchinson JA, Roelen D, Riquelme P, Brem-Exner BG, Witzke O, Philipp T, et al. Preoperative treatment of a presensitized kidney transplant recipient with donor-derived transplant acceptance-inducing cells. *Transpl Int* (2008) **21**:808–13. doi:10.1111/j.1432-2277.2008.00712.x
104. Geissler EK. The ONE Study compares cell therapy products in organ transplantation: introduction to a review series on suppressive monocyte-derived cells. *Transplant Res* (2012) **1**:11. doi:10.1186/2047-1440-1-10
105. Ezzelarab MB, Zahorchak AF, Lu L, Morelli AE, Chalasani G, Demetris AJ, et al. Regulatory dendritic cell infusion prolongs kidney allograft survival in nonhuman primates. *Am J Transplant* (2013) **13**:1989–2005. doi:10.1111/ajt.12310
106. Dhodapkar MV, Steinman RM, Krasovsky J, Munz C, Bhardwaj N. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. *J Exp Med* (2001) **193**:233–8. doi:10.1084/jem.193.2.233
107. Dhodapkar MV, Steinman RM. Antigen-bearing immature dendritic cells induce peptide-specific CD8(+) regulatory T cells in vivo in humans. *Blood* (2002) **100**:174–7. doi:10.1182/blood.V100.1.174
108. Giannoukakis N, Phillips B, Finegold D, Harnaha J, Trucco M. Phase I (safety) study of autologous tolerogenic dendritic cells in type 1 diabetic patients. *Diabetes Care* (2011) **34**:2026–32. doi:10.2337/dc11-0472
109. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* (2009) **9**:162–74. doi:10.1038/nri2506
110. Bronte V, Zanovello P. Regulation of immune responses by L-arginine metabolism. *Nat Rev Immunol* (2005) **5**:641–54. doi:10.1038/nri1668
111. Rodríguez PC, Ochoa AC. Arginine regulation by myeloid derived suppressor cells and tolerance in cancer: mechanisms and therapeutic perspectives. *Immunol Rev* (2008) **222**:180–91. doi:10.1111/j.1600-065X.2008.00608.x
112. Rodriguez PC, Zea AH, Culotta KS, Zabaleta J, Ochoa JB, Ochoa AC. Regulation of T cell receptor CD3zeta chain expression by L-arginine. *J Biol Chem* (2002) **277**:21123–9. doi:10.1074/jbc.M110675200
113. Rodriguez PC, Quiceno DG, Ochoa AC. L-arginine availability regulates T-lymphocyte cell-cycle progression. *Blood* (2007) **109**:1568–73. doi:10.1182/blood-2006-06-031856
114. Harari O, Liao JK. Inhibition of MHC II gene transcription by nitric oxide and antioxidants. *Curr Pharm Des* (2004) **10**:893–8. doi:10.2174/1381612043452893
115. Rivoltini L, Carrabba M, Huber V, Castelli C, Novellino L, Dalerba P, et al. Immunity to cancer: attack and escape in T lymphocyte-tumor cell interaction. *Immunol Rev* (2002) **188**:97–113. doi:10.1034/j.1600-065X.2002.18809.x
116. Huang B, Pan PY, Li Q, Sato AI, Levy DE, Bromberg J, et al. Gr-1+CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. *Cancer Res* (2006) **66**:1123–31. doi:10.1158/0008-5472.CAN-05-1299
117. Hiramoto K, Satoh H, Suzuki T, Moriguchi T, Pi J, Shimosegawa T, et al. Myeloid lineage-specific deletion of antioxidant system enhances tumor metastasis. *Cancer Prev Res (Phila)* (2014) **7**:835–44. doi:10.1158/1940-6207.CAPR-14-0094
118. Corzo CA, Cotter MJ, Cheng P, Cheng F, Kusmartsev S, Sotomayor E, et al. Mechanism regulating reactive oxygen species in tumor-induced myeloid-derived suppressor cells. *J Immunol* (2009) **182**:5693–701. doi:10.4049/jimmunol.0900092
119. Vickers SM, MacMillan-Crow LA, Green M, Ellis C, Thompson JA. Association of increased immunostaining for inducible nitric oxide synthase and nitrotyrosine with fibroblast growth factor transformation in pancreatic cancer. *Arch Surg* (1999) **134**:245–51. doi:10.1001/archsurg.134.3.245
120. Cobbs CS, Whisenhunt TR, Wesemann DR, Harkins LE, Van Meir EG, Samanta M. Inactivation of wild-type p53 protein function by reactive oxygen and nitrogen species in malignant glioma cells. *Cancer Res* (2003) **63**:8670–3.
121. Nakamura Y, Yasuoka H, Tsujimoto M, Yoshidome K, Nakahara M, Nakao K, et al. Nitric oxide in breast cancer: induction of vascular endothelial growth factor-C and correlation with metastasis and poor prognosis. *Clin Cancer Res* (2006) **12**:1201–7. doi:10.1158/1078-0432.CCR-05-1269
122. Yang R, Cai Z, Zhang Y, Yutzy WH, Roby KF, Roden RB. CD80 in immune suppression by mouse ovarian carcinoma-associated Gr-1+CD11b+ myeloid cells. *Cancer Res* (2006) **66**:6807–15. doi:10.1158/0008-5472.CAN-05-3755
123. Serafini P, Mgebroff S, Noonan K, Borrello I. Myeloid-derived suppressor cells promote cross-tolerance in B-cell lymphoma by expanding regulatory T cells. *Cancer Res* (2008) **68**:5439–49. doi:10.1158/0008-5472.CAN-07-6621
124. Ambrosino E, Spadaro M, Iezzi M, Curcio C, Forni G, Musiani P, et al. Immunosurveillance of Erbb2 carcinogenesis in transgenic mice is concealed by a dominant regulatory T-cell self-tolerance. *Cancer Res* (2006) **66**:7734–40. doi:10.1158/0008-5472.CAN-06-1432
125. Nagaraj S, Youn JI, Gabrilovich DI. Reciprocal relationship between myeloid-derived suppressor cells and T cells. *J Immunol* (2013) **191**:17–23. doi:10.4049/jimmunol.1300654
126. Dugast AS, Haudebourg T, Coulon F, Heslan M, Haspot F, Poirier N, et al. Myeloid-derived suppressor cells accumulate in kidney allograft tolerance and specifically suppress effector T cell expansion. *J Immunol* (2008) **180**:7898–906. doi:10.4049/jimmunol.180.12.7898
127. Dilek N, Poirier N, Usal C, Martinet B, Blancho G, Vanhove B. Control of transplant tolerance and intragraft regulatory T cell localization by myeloid-derived suppressor cells and CCL5. *J Immunol* (2012) **188**:4209–16. doi:10.4049/jimmunol.1101512
128. Chou HS, Hsieh CC, Charles R, Wang L, Wagner T, Fung JJ, et al. Myeloid-derived suppressor cells protect islet transplants by B7-H1 mediated enhancement of T regulatory cells. *Transplantation* (2012) **93**:272–82. doi:10.1097/TP.0b013e31823ffd39
129. De Wilde V, Van Rompaey N, Hill M, Lebrun JF, Lemaître P, Lhommé F, et al. Endotoxin-induced myeloid-derived suppressor cells inhibit alloimmune responses via heme oxygenase-1. *Am J Transplant* (2009) **9**:2034–47. doi:10.1111/j.1600-6143.2009.02757.x
130. Zahorchak AF, Ezzelarab MB, Lu L, Turnquist HR, Thomson AW. In vivo mobilization and functional characterization of nonhuman primate monocytic myeloid-derived suppressor cells. *Am J Transplant* (2016) **16**(2):661–71.
131. Dilek N, Vuillefroy de Silly R, Blancho G, Vanhove B. Myeloid-derived suppressor cells: mechanisms of action and recent advances in their role in transplant tolerance. *Front Immunol* (2012) **3**:208. doi:10.3389/fimmu.2012.00208
132. Kim JI, Rothstein DM, Markmann JF. Role of B cells in tolerance induction. *Curr Opin Organ Transplant* (2015) **20**:369–75. doi:10.1097/MOT.0000000000000204
133. Sicard A, Koenig A, Morelon E, Defrance T, Thaunat O. Cell therapy to induce allograft tolerance: time to switch to plan B? *Front Immunol* (2015) **6**:149. doi:10.3389/fimmu.2015.00149
134. Wolf SD, Dittel BN, Hardardottir F, Janeway CA. Experimental autoimmune encephalomyelitis induction in genetically B cell-deficient mice. *J Exp Med* (1996) **184**:2271–8. doi:10.1084/jem.184.6.2271
135. Mauri C, Gray D, Mushtaq N, Londei M. Prevention of arthritis by interleukin 10-producing B cells. *J Exp Med* (2003) **197**:489–501. doi:10.1084/jem.20021293
136. Mizoguchi A, Mizoguchi E, Smith RN, Preffer FI, Bhan AK. Suppressive role of B cells in chronic colitis of T cell receptor alpha mutant mice. *J Exp Med* (1997) **186**:1749–56. doi:10.1084/jem.186.10.1749
137. Amu S, Saunders SP, Kronenberg M, Mangan NE, Atzberger A, Fallon PG. Regulatory B cells prevent and reverse allergic airway inflammation via FoxP3-positive T regulatory cells in a murine model. *J Allergy Clin Immunol* (2010) **125**:1114.e–24.e. doi:10.1016/j.jaci.2010.01.018
138. Braza F, Chesne J, Castagnet S, Magnan A, Brouard S. Regulatory functions of B cells in allergic diseases. *Allergy* (2014) **69**:1454–63. doi:10.1111/all.12490
139. Deng S, Moore DJ, Huang X, Lian MM, Mohiuddin M, Velededeoglu E, et al. Cutting edge: transplant tolerance induced by anti-CD45RB

requires B lymphocytes. *J Immunol* (2007) **178**:6028–32. doi:10.4049/jimmunol.178.10.6028
140. Lee KM, Kim JI, Stott R, Soohoo J, O'Connor MR, Yeh H, et al. Anti-CD45RB/anti-TIM-1-induced tolerance requires regulatory B cells. *Am J Transplant* (2012) **12**:2072–8. doi:10.1111/j.1600-6143.2012.04055.x
141. Lee KM, Stott RT, Zhao G, SooHoo J, Xiong W, Lian MM, et al. TGF-β-producing regulatory B cells induce regulatory T cells and promote transplantation tolerance. *Eur J Immunol* (2014) **44**:1728–36. doi:10.1002/eji.201344062
142. Ding Q, Yeung M, Camirand G, Zeng Q, Akiba H, Yagita H, et al. Regulatory B cells are identified by expression of TIM-1 and can be induced through TIM-1 ligation to promote tolerance in mice. *J Clin Invest* (2011) **121**:3645–56. doi:10.1172/JCI46274
143. Mann MK, Maresz K, Shriver LP, Tan Y, Dittel BN. B cell regulation of CD4+CD25+ T regulatory cells and IL-10 via B7 is essential for recovery from experimental autoimmune encephalomyelitis. *J Immunol* (2007) **178**:3447–56. doi:10.4049/jimmunol.178.6.3447
144. Kessel A, Haj T, Peri R, Snir A, Melamed D, Sabo E, et al. Human CD19(+) CD25(high) B regulatory cells suppress proliferation of CD4(+) T cells and enhance Foxp3 and CTLA-4 expression in T-regulatory cells. *Autoimmun Rev* (2012) **11**:670–7. doi:10.1016/j.autrev.2011.11.018
145. Tretter T, Venigalla RK, Eckstein V, Saffrich R, Sertel S, Ho AD, et al. Induction of CD4+ T-cell anergy and apoptosis by activated human B cells. *Blood* (2008) **112**:4555–64. doi:10.1182/blood-2008-02-140087
146. Duddy M, Niino M, Adatia F, Hebert S, Freedman M, Atkins H, et al. Distinct effector cytokine profiles of memory and naive human B cell subsets and implication in multiple sclerosis. *J Immunol* (2007) **178**:6092–9. doi:10.4049/jimmunol.178.10.6092
147. Blair PA, Noreña LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR, et al. CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. *Immunity* (2010) **32**:129–40. doi:10.1016/j.immuni.2009.11.009
148. Flores-Borja F, Bosma A, Ng D, Reddy V, Ehrenstein MR, Isenberg DA, et al. CD19+CD24hiCD38hi B cells maintain regulatory T cells while limiting TH1 and TH17 differentiation. *Sci Transl Med* (2013) **5**:173ra23. doi:10.1126/scitranslmed.3005407
149. Dong HP, Elstrand MB, Holth A, Silins I, Berner A, Trope CG, et al. NK- and B-cell infiltration correlates with worse outcome in metastatic ovarian carcinoma. *Am J Clin Pathol* (2006) **125**:451–8. doi:10.1309/15B66DQMFYYM78CJ
150. Silva HM, Takenaka MC, Moraes-Vieira PM, Monteiro SM, Hernandez MO, Chaara W, et al. Preserving the B-cell compartment favors operational tolerance in human renal transplantation. *Mol Med* (2012) **18**:733–43. doi:10.2119/molmed.2011.00281
151. Brouard S, Mansfield E, Braud C, Li L, Giral M, Hsieh SC, et al. Identification of a peripheral blood transcriptional biomarker panel associated with operational renal allograft tolerance. *Proc Natl Acad Sci USA* (2007) **104**:15448–53. doi:10.1073/pnas.0705834104
152. Newell KA, Asare A, Kirk AD, Gisler TD, Bourcier K, Suthanthiran M, et al. Identification of a B cell signature associated with renal transplant tolerance in humans. *J Clin Invest* (2010) **120**:1836–47. doi:10.1172/JCI39933
153. Sagoo P, Perucha E, Sawitzki B, Tomiuk S, Stephens DA, Miqueu P, et al. Development of a cross-platform biomarker signature to detect renal transplant tolerance in humans. *J Clin Invest* (2010) **120**:1848–61. doi:10.1172/JCI39922
154. Thaunat O. Pathophysiologic significance of B-cell clusters in chronically rejected grafts. *Transplantation* (2011) **92**:121–6. doi:10.1097/TP.0b013e31821f74fe
155. Scott DW, Zhang AH, Su Y. B-cell based gene therapy for autoimmune diseases. *Infect Disord Drug Targets* (2012) **12**:241–7. doi:10.2174/187152612800564383
156. Liang W, Karabekian Z, Mattapallil M, Xu Q, Viley AM, Caspi R, et al. B-cell delivered gene transfer of human S-Ag-Ig fusion protein protects from experimental autoimmune uveitis. *Clin Immunol* (2006) **118**:35–41. doi:10.1016/j.clim.2005.08.007
157. Melo ME, Qian J, El-Amine M, Agarwal RK, Soukhareva N, Kang Y, et al. Gene transfer of Ig-fusion proteins into B cells prevents and treats autoimmune diseases. *J Immunol* (2002) **168**:4788–95. doi:10.4049/jimmunol.168.9.4788
158. Xu B, Scott DW. A novel retroviral gene therapy approach to inhibit specific antibody production and suppress experimental autoimmune encephalomyelitis induced by MOG and MBP. *Clin Immunol* (2004) **111**:47–52. doi:10.1016/j.clim.2003.12.013
159. Soukhareva N, Jiang Y, Scott DW. Treatment of diabetes in NOD mice by gene transfer of Ig-fusion proteins into B cells: role of T regulatory cells. *Cell Immunol* (2006) **240**:41–6. doi:10.1016/j.cellimm.2006.06.004
160. Satpute SR, Soukhareva N, Scott DW, Moudgil KD. Mycobacterial Hsp65-IgG-expressing tolerogenic B cells confer protection against adjuvant-induced arthritis in Lewis rats. *Arthritis Rheum* (2007) **56**:1490–6. doi:10.1002/art.22566
161. Skupsky J, Zhang AH, Su Y, Scott DW. B-cell-delivered gene therapy induces functional T regulatory cells and leads to a loss of antigen-specific effector cells. *Mol Ther* (2010) **18**:1527–35. doi:10.1038/mt.2010.95
162. Calderón-Gómez E, Lampropoulou V, Shen P, Neves P, Roch T, Stervbo U, et al. Reprogrammed quiescent B cells provide an effective cellular therapy against chronic experimental autoimmune encephalomyelitis. *Eur J Immunol* (2011) **41**:1696–708. doi:10.1002/eji.201041041
163. Poon IK, Lucas CD, Rossi AG, Ravichandran KS. Apoptotic cell clearance: basic biology and therapeutic potential. *Nat Rev Immunol* (2014) **14**:166–80. doi:10.1038/nri3607
164. Saas P, Kaminski S, Perruche S. Prospects of apoptotic cell-based therapies for transplantation and inflammatory diseases. *Immunotherapy* (2013) **5**:1055–73. doi:10.2217/imt.13.103
165. Bonnefoy F, Perruche S, Couturier M, Sedrati A, Sun Y, Tiberghien P, et al. Plasmacytoid dendritic cells play a major role in apoptotic leukocyte-induced immune modulation. *J Immunol* (2011) **186**:5696–705. doi:10.4049/jimmunol.1001523

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read, for greatest visibility

COLLABORATIVE PEER-REVIEW
Designed to be rigorous – yet also collaborative, fair and constructive

FAST PUBLICATION
Average 85 days from submission to publication (across all journals)

COPYRIGHT TO AUTHORS
No limit to article distribution and re-use

TRANSPARENT
Editors and reviewers acknowledged by name on published articles

SUPPORT
By our Swiss-based editorial team

IMPACT METRICS
Advanced metrics track your article's impact

GLOBAL SPREAD
5'100'000+ monthly article views and downloads

LOOP RESEARCH NETWORK
Our network increases readership for your article

Frontiers
EPFL Innovation Park, Building I • 1015 Lausanne • Switzerland
Tel +41 21 510 17 00 • Fax +41 21 510 17 01 • info@frontiersin.org
www.frontiersin.org

Find us on