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THE ROLE OF MICROBIOTA IN SOLID ORGAN TRANSPLANTATION

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DEDICATION

To my family and friends,

who have been extremely supportive along the way.

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LIST OF ABBREVIATIONS

Abx: Broad spectrum antibiotics
ANOVA: Analysis of variance
APC: Antigen-presenting cell
CFSE: Carboxyfluorescein succinimidyl ester
ChIP: Chromatin immunoprecipitation
DAMPs: Damage-associated molecular patterns
DC: Dendritic cell
dLN: Draining lymph node
DMEM: Dulbecco modified Eagle medium
DST: Donor splenocyte transfusion
F: Fecal material
GF: Germ-free
GVHD: Graft-versus-host disease
HBSS: Hanks balanced salt solution
HEPES: N-2-hydroxyethylpiperazine-N′-2- ethanesulfonic acid
HFD: High-fat diet
HSD: High-salt diet
H-Y: Male tissue specific antigen
IFN: Interferon
IFNα: Interferon alpha
IFNαR: Interferon alpha receptor
IFNγ: Interferon gamma
IL: Interleukin
ILC: Innate lymphoid cell
KO: Knock-out
LFD: Low-fat diet
MAMPs: Microbial-associated molecular patterns
MFI: Mean fluorescence intensity
MHC: Major Histocompatibility Complex
MNP: Mononuclear phagocyte
MST: Median survival time
MyD88: Myeloid differentiation primary response 88
n: Number in group
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
ns: Not significant
NSD: Normal salt diet
OVA: Ovalbumin
P: Probability
PAMPs: Pathogen-associated molecular patterns
PBS: Phosphate-buffered saline
pDC: Plasmacytoid dendritic cell
PD-L1: Programmed death ligand-1
PMA: Phorbol myristate acetate

PRR: Pattern recognition receptor
RAG: Recombination activating gene
SCFA: Short-chain fatty acid
SD: Standard deviation
SEM: Standard error of the mean
SPF: Specific pathogen free
TCR-Tg: T cell receptor transgenic
Tfh: T follicular helper
Th: T helper [cell]
TLR: Toll-like receptor
Tregs: Regulatory T cells

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ABSTRACT

Organ transplantation is the only treatment for end-stage organ failure. However, due to the genetic disparities between the donor and the recipient, transplanted organs are recognized by the recipient's immune system as foreign and rejected, with the exception of transplantation between identical twins. Therefore, a lifelong regimen of immunosuppression is required for patients to prevent graft rejection. Such level of immunosuppression significantly increases susceptibility to infections and malignancies, and can trigger additional adverse side effects, creating a substantial burden on the healthcare system and reducing the well-being of patients who have no alternative treatment options. In this thesis, we used mouse models of minor-mismatch heart or skin transplantation to determine the extent to which the microbiota, the collection of commensal bacteria residing at barrier surfaces, impacts the outcome of organ transplantation. We established that the microbiota is indeed an environmental factor that can fine-tune alloreactivity, with the diverse microbiota from specific pathogen-free mice accelerating graft rejection, an effect that is associated with an enhanced capacity of antigen-presenting cells (APCs) to activate alloreactive T cells. However, not all bacteria were capable of accelerating graft rejection, as residual microbes after antibiotic treatment did not enhance the kinetics of graft rejection. Moreover, colonization of either donor or recipient skin with a single commensal was sufficient to accelerate skin graft rejection, demonstrating a role for microbiota outside to the gut on allograft outcome. In all, this work demonstrates that the microbiota is an environmental factor that can impact alloreactivity, implying that prolongation of graft survival might be achieved by therapeutic interventions targeting the microbiota of different body location.

CHAPTERS

I. INTRODUCTION

A. Introduction

Transplantation is an accepted treatment for end-stage organ failure. However, with the exception of grafts between identical twins, transplanted organs are recognized by the recipient's immune system as foreign, which leads to graft rejection unless the transplant recipient is maintained on lifelong immunosuppression. Current therapies leave patients globally immunosuppressed, with increased susceptibility to infections and malignancies. Side effects and complications from global immunosuppressive drugs impose a hefty burden on the healthcare system and significantly threaten the lives of patients who do not have other treatment options. A better understanding of the mechanisms that drive solid organ transplant rejection is important for the development of new therapies to improve graft outcomes.

B. The role of the immune system in organ transplantation

Without immunosuppression, allograft rejection by the immune system is inevitable. Rejection, in turn, leads to allosensitization and predisposes to faster rejection of a second transplant if any alloantigen is shared with the first graft, making finding suitable subsequent donors increasingly difficult (Gibson and Medawar, 1943; Medawar, 1944). Both innate and adaptive immunity play crucial roles in rejection. In this section, I will cover some fundamental tenets of alloreactivity.

1. Mechanisms of allo-recognition

T cells are essential for allograft rejection and their activation is considered the rate-limiting step for rejection (Lin and Gill, 2016). For activation in general, T cells require cognate interaction with antigen-presenting cells (APCs) through the TCR-MHC/peptide interactions. In the context of transplantation, host T cells face two different sets of MHC complexes, those from the donor and those from the host. This may be counterintuitive because T cells are positively selected to be self-MHC-restricted, but it is becoming increasingly recognized that T cells can be poly-reactive and cross-reactive. Thus, a fraction of host T cells that should bind self-MHC+peptide from a foreign antigen can cross-react with donor MHC+donor self-peptide. How T cells interact with these MHCs of different origins could theoretically shape the mechanisms of allograft rejection.

Broadly speaking, allorecognition can be divided into two categories based on the origin of the APCs recognized by T cells. The first is termed direct recognition and refers to host T cells recognizing donor APCs displaying donor MHC molecules and donor self-peptides. The second is termed indirect recognition, and refers to host T cells being activated by recipient APCs that acquire, process and present donor-derived antigens. Because donor APCs bearing a different MHC from the host's MHC are recognized as non-self by the host's immune system and may be eliminated by the host T cells or NK cells (Garrod et al., 2010; Yu, 2006) it is thought that direct recognition is present early after transplantation, whereas indirect recognition can persist long-term (Brennan et al., 2009; Garrod et al., 2010).

In addition to these two major forms of allo-recognition, emerging studies are proposing a third mechanism of alloimmune recognition by T cells and have termed it semi-direct recognition. Herrera et al. showed that there is transfer of intact MHC molecules between donor cells and host dendritic cells (DCs) that can occur both *in vitro* and *in vivo* (Herrera et al., 2004), such that host APCs can display intact donor MHC on their surface in addition to their self-MHC and therefore activate both T cells with direct and with indirect specificities. Such acquisition of intact allogeneic MHC molecules is thought to be due to the transfer of cellular exosomes, micro-vesicles shed or secreted by many cell types, and is sometimes referred to as "cross-dressing" the immune system (Zeng and Morelli, 2018). Two independent studies recently showed that exosomes from the donor can drive alloreactive T cell responses and allograft rejection (Liu et al., 2016; Marino et al., 2016). Specifically, electron microscopy revealed DCs from B6 recipient mice (H-2^b) bearing intact H-$2K^d$ molecules on their surface following fully mismatched heart transplantation from BALB/c mice (Liu et al., 2016). The transfer of exosomes from mature donor DCs promoted the activation of recipient DCs as determined by an increase in CD86, CD40 and I-A^b expression. These cross-dressed, activated host APCs were shown to present donor MHC molecules to alloreactive T cells with direct specificity. The ability of host APCs to simultaneously activate T cells of direct and indirect specificities has challenged the conventional understanding of allorecognition but may explain how indirect alloreactive $CD4^+$ T cells can help direct alloreactive $CD8^+$ T cells if the APC is shared.

Lastly, beside the well-known T cell-dependent allorecognition, new studies have shown that innate mononuclear phagocytes are also capable of non-self recognition, a

feature that appears important for initiation of alloreactivity (Dai et al., 2017; Oberbarnscheidt et al., 2014). Using B6 $CX_3CR1^{gfp/+}$ mice, Oberbarnscheidt et al. showed an increased infiltration of monocyte-derived DCs in allogeneic than syngeneic heart grafts, prior to T cell recruitment, and their depletion prevented rejection. Such innate recognition of non-self was sufficient to drive some graft damage in the absence of T cells and NK cells, when allogeneic CB6F1-OVA hearts were transplanted to B6 $RAG^{-/-}$ $\gamma c^{-/-}$ $CX_3CR1^{gfp/+}$ recipients (Oberbarnscheidt et al., 2014). In addition, non-self recognition by mononuclear phagocytes allowed them to make more IL-12 and thus drive increased Th1 responses against the allograft. Innate non-self recognition was later attributed not to recognition of donor MHC, but rather of donor polymorphisms in signal regulatory protein α (SIRPα), the ligand of CD47 (Dai et al., 2017). Dai et al. showed that amino acid variations in SIRPα in different mouse strains contributed to differential binding to CD47. This SIRPα-CD47 interaction is critical to monocyte proliferation and innate alloresponses.

2. Structural basis of allorecognition

During development, T cells are positively and negatively selected to generate a repertoire of cells that recognize self-MHC molecules with moderate affinity at homeostasis. This creates an interesting paradox for alloreactive T cells of direct specificity, as these cells recognize donor MHC molecules that were not present during positive selection. The question of how these T cells can mount measurable alloimmune responses against an allograft has fueled significant research into differences and similarities between recognition of self-MHC+foreign peptide and recognition of foreign

MHC+donor self-peptide. It is thought that the flexibility within the TCR structure allows adaptation and cross-reactivity towards antigens to which they have not been exposed (Sherman and Chattopadhyay, 1993), and this could theoretically also allow recognition of a non-self MHC.

About 1-10% of T cells respond to allogeneic MHC molecules (Suchin et al., 2001). Within this population, the majority of the repertoire is accounted for by direct allospecificity (Whitelegg and Barber, 2004), although this may depend on the context of T cell stimulation. Numerous studies have compared the structures between TCR interaction with syngeneic and allogeneic MHC-peptide complexes (Garcia et al., 1997; Luz et al., 2002; Speir et al., 1998; Stewart-Jones et al., 2003). One study compared two complexes that interact with 2C TCR (H-2^b), i.e. the 2C-L^d/QL9 complex, an allogeneic complex, and the 2C-K^b/dEV8 complex, a syngeneic complex (Speir et al., 1998). While the binding register of the two peptides is the same, the 2C TCR was predicted to have more contact with the L^d peptide due to a prominent ridge at the floor of the binding groove that is absent in the interaction with the K^b peptide. Furthermore, structural changes in amino acid sequence drastically alter the interaction with the TCR. Mimicking the negative charge at Asp 77 in K^b, L^d possesses an Asp at position 8, which allows for closer interaction with the 2C HV4 loop by enhancing the electrostatic complementarity. The example of 2C TCR binding to both K^b/dEV8 and L^d/QL9 suggests that allorecognition by T cells may be achieved through TCR flexibility and molecular mimicry.

3. The impact of the local immune milieu

Whether the origin of the transplanted organ is cadaveric or from a living donor, there is an inevitable time delay between organ harvest and transplantation. This time gap results in various degrees of ischemia-reperfusion injury (IRI), which negatively impacts the survival of an allograft. For example, in a mouse model of vascularized orthotopic hindlimb transplantation, 6 hours of cold ischemia reduced the survival of the transplant and resulted in remote renal injury (Datta et al., 2017). In addition, IRI resulted in increased infiltration of macrophages and neutrophils to the graft muscle tissues. Treatment with Sotrastaurin, a modulator of protein kinase C, has been shown to reduce macrophage and neutrophil infiltration and decrease IRI in orthotropic liver transplantation (Kamo et al., 2011). Prolonged ischemia in liver transplantation has been shown to deplete Kupffer cells that are anti-inflammatory and protect the liver from IRI (Yue et al., 2017), which have been suggested to be tolerogenic (Horst et al., 2016; You et al., 2008).

The infiltration of innate immune cells is accompanied by their activation and upregulation of chemokines. Activation of peripheral macrophages (Datta et al., 2017) has been reported in IRI. In addition, high ischemic temperature has been shown to increase CXCL1/KC and CXCL2/MIP-2 levels and neutrophil infiltration and cause damage to transplanted kidneys (Fukuzawa et al., 2009). This damage has been ascribed to cathepsin-G (Shimoda et al., 2007), macrophage inflammatory protein (MIP)-2 and KC/Gro alpha (Miura et al., 2001). The increased cell infiltration and upregulation of cytokines and chemokines are reported to be type I interferon (IFN)-

dependent. Indeed, in a kidney transplantation model in which the organ was subjected to warm ischemia, IFNαR knock-out (KO) mice displayed reduced levels of serum creatinine and blood urea nitrogen compared to WT controls, as well as lower levels of TNFα, IL1, IL6 and CXCL2 transcripts in the graft (Freitas et al., 2011).

In addition to the inflammation caused by transplantation, the innate immune cells of donor origin that are present within a transplanted organ also contribute to the organ immunogenicity. Our laboratory has previously shown that the higher immunogenicity of skin when compared to heart allografts is in part dependent on the presence of donor Langerhans cells (LCs) in the skin. Genetic deficiency in LCs resulted in the prolongation of skin graft survival whereas systemic injection of donor LCs at the time of heart transplantation increased the immunogenicity of the heart allograft (Molinero et al., 2007). Also, passenger donor $CD4^{+}$ T cells have been shown to provide cognate help to host B cells, which augments alloantibody responses and impacts cardiac allograft survival (Harper et al., 2016; Win et al., 2009). NK cells activation by missing self-MHC on donor hematopoietic cells can limit these effects of donor passenger leukocytes (Ali et al., 2015a; Harper et al., 2016).

4. Quantitative aspects of alloreactivity

In addition to the qualitative factors mentioned above, the frequency of T cells reacting to donor antigens and the quantity of donor antigen load can also influence the magnitude of alloreactivity. Researchers have explored different ways to quantify the frequency of alloreactive T cells (Suchin et al., 2001). The initial T cell precursor frequency has been shown to be critical to dictate the lineage commitment of T cells. It

has been reported that, at low frequency, effector memory CD8$^+$ T cells failed differentiate into central memory T cells (Badovinac et al., 2007; Marzo et al., 2005). This was further confirmed in transplantation using TCR-Tg T cells, as that the initial frequency of naïve TCR-Tg cells determined the expansion, differentiation, memory generation and ultimately the kinetics of graft rejection (Ford et al., 2007). The induction of tolerance by co-stimulation blockade was has been shown to be affected by the precursor frequency of naïve and memory alloreactive T cells, becoming more difficult to achieve with higher precursor frequencies (Adams et al., 2003; Ford et al., 2007). Interestingly, intra-clonal competition has been suggested in which low precursor frequency favored the survival and activation of naïve CD4$^+$ T cells as well as the survival of their memory descendants (Hataye et al., 2006). This phenomenon may provide evolutionary advantage to the host, in which a wide range of TCR specificities with low precursor frequency for each antigen may better ensure host survival against a variety of infections.

In addition to T cell precursory frequency, the amount of antigen present in the graft is also thought to affect transplant outcomes. Perhaps counter-intuitively, more antigen does not necessarily lead to more rejection, when the initial T cell precursor frequency is fixed. For example, whereas female recipients could reject one syngeneic male skin graft, those receiving one heart graft or two skin grafts were unable to reject (He et al., 2004), suggesting that large tissue burden may survive attack by a fixed number of T cells. Indeed, heart rejection occurred if the transplanted donor heart mass was reduced by 50%. Together, these studies suggest that the precursor frequency of

alloreactive T cells, along with the size of the graft can modulate the outcome of a transplanted organ.

C. Mechanisms of transplant rejection

As covered in the previous section, the immune response against an allograft can be influenced by various factors. In this section, we will cover tissue-specific considerations in the context of skin and heart transplantation, the two transplant models utilized for this thesis project.

1. Skin transplantation

Skin is the largest tissue in human body with an area of approximately $2m^2$ (Eyerich et al., 2018). The skin can broadly be stratified into four different layers of barriers against environmental threats: microbiota, chemical, physical and immune barriers. The epidermis and dermis provide the main physical and immune barrier functions. They are also in close contact with alloantigen during skin transplantation. Following skin transplantation, cutaneous DCs, including both donor and recipient resident DCs, migrate from the skin to the draining lymph nodes (dLNs) and present alloantigen to alloreactive T cells. A study using topical application of a labeling agent on the skin showed that the labeling agent is carried by migrating cells (van Wilsem et al., 1994), and these cells were found in the dLNs (Richters et al., 1996, 1999), via the lymphatic system (Barker and Billingham, 1968), which was proven to be essential for the delivery of alloantigen by providing lymphatic drainage. Indeed, naïve aly/aly mice that congenically lack lymph node structures because of a defect in the enzyme Nik,

were shown to be incapable of rejecting skin grafts, unless they received memory T cells (Chalasani et al., 2002; Lakkis et al., 2000). These results suggest that naïve T cells need to be primed in the lymph nodes following skin transplantation, whereas memory T cells can traffic directly to the graft.

Skin DCs are abundant in the epidermis and dermis, and are proven to be critical to maintain homeostasis as well as to fight infections (Kashem et al., 2017). These DCs are comprised of two distinct subsets, Langerhans cells and dermal DCs that are either $CD103^+$ or $CD11b^+$. Batf3-dependent DCs, including $CD103^+$ DCs, have been shown to be required for the rejection of male skin grafts by female recipients (Atif et al., 2015). In contrast, using LC-DTA mice, Obhrai et al. showed that LCs were not required for major- or minor-mismatched skin graft rejection (Obhrai et al., 2008).

As mentioned in the previous section, T cells can recognize alloantigen in a direct or indirect manner, depending on the context and origin of the MHC molecules. While direct allorecognition drives strong measurable alloreactivity, indirect allorecognition is also critical for graft rejection. An early study that used MHC-II-deficient donor mice established that host $CD4^+$ T cells can recognize alloantigen through the indirect pathway on recipient's class II MHC molecules (Auchincloss et al., 1993). Further studies confirmed that both $CD4^+$ and $CD8^+$ T cells were capable of rejecting skin graft through indirect recognition (He and Heeger, 2004; Valujskikh and Heeger, 2000; Wise et al., 1999), while IFNγ from $CD4^+$ T cells appeared dispensable (Valujskikh and Heeger, 2000). Moreover, the transfer of $CD4^+$ transgenic (Tg) A1(M) cells, that are specific for the male H-Y antigen in the context of H2-E^k, was sufficient to reject male skin grafts transplanted onto female A1(M) $RAG1^{-/-}$ mice (Zelenika et al.,

1998) and transfer of $CD8^+$ T cells into $H\text{-}2^k$ SCID mice could mediate rejection of MHC class I-deficient major mismatched skin grafts (He and Heeger, 2004). Thus, both direct and indirect $CD4^+$ and $CD8^+$ T cells can participate in skin allograft rejection, though necessity and sufficiency of each pathway may depend on T cell frequency, TCR affinity and antigen load.

2. Heart transplantation

Heart transplant rejection can be categorized into four types: hyperacute rejection, acute cellular rejection (ACR), acute antibody-mediated rejection (AMR) and chronic rejection. Both innate and adaptive immune systems play a crucial role in all types of rejection. Hyperacute rejection depends on the presence of pre-formed antibodies, either to non-shared blood antigens (Worel, 2016) that are expressed not only on red blood cells, but also on donor endothelial cells, or to pre-existing conventional alloantigens. Alloantibodies also play a prominent role in antibody-mediated rejection and possibly in chronic rejection, although definitive proof is still lacking. Acute cellular rejection is mediated by T cells. Because the lymphatic vessels that drain the heart are not resutured during transplantation, it is thought that alloantigen circulates first via the blood. Indeed, initial proliferation of alloreactive T cells, as determined by CFSE dilution was first observed in the spleen of cardiac allograft recipients (Noorchashm et al., 2006) . Moreover, whereas aly/aly mice failed to reject skin grafts, heart allografts were still rejected unless the spleen was removed surgically or through the use of Hox11 mutant mice (Lakkis et al., 2000).

Chemokines are chemoattractant cytokines recognized by chemokine receptors on that direct immune cell migration. Using genetically-deficient mice or chemokine inhibitors, studies have shown that the receptors CCR5 (Gao et al., 2001) and CXCR3 (Hancock et al., 2000) are necessary to precipitate cardiac allograft rejection in mice. Combinatorial treatment with a CCR5 antagonist and cyclosporine A in non-human primates was associated with reduced cell infiltration of M2 macrophages and delayed alloantibody responses (Li et al., 2011).

In terms of innate immunity, as mentioned earlier, our group has previously shown that the transfer of donor LCs at the time of heart transplantation increased the immunogenicity of the heart allograft (Molinero et al., 2007). Oberbarnscheidt et al. showed that the depletion of monocyte-derived DCs using CD11b-DTR bone marrow chimeras reduced the graft damage and the rejection of allogeneic heart grafts in the absence of T cells and NK cells (Oberbarnscheidt et al., 2014). In addition, polymorphonuclear leukocytes have been found to infiltrate heart allografts very early following transplantation. Inhibiting the migration of these leukocytes by using CXCR2-antisera or CXCR2 KO mice prevented heart graft rejection (El-Sawy, 2005). In addition, the depletion of NK cells can improve heart allograft survival in CD28-deficient mice (Maier et al., 2001). However, the role of NKG2D, one of the key NK-cell activator receptor, in such rejection is controversial (Kim et al., 2007; McNerney et al., 2006).

Numerous studies have shown that $CD4^+$ and $CD8^+$ T cells are required for cardiac allograft rejection (Fischbein et al., 2002; Krieger et al., 1996), either via direct (Harper et al., 2015; Pietra et al., 2000) or indirect recognition (Ali et al., 2015b; Lee et al., 2001). In one study, the transfer of $CD4^+$ T cells into $RAG1^{-/-}$ mice was sufficient to

for acute cardiac graft rejection. This CD4 T cell-mediated rejection required MHC class II on the allograft, however, host MHC class II was not required (Pietra et al., 2000). In another study, $CD8^{-/-}$ mice displayed an attenuated heart rejection phenotype, compared control B10.BR mice that received heart transplanted from B10.A mice (Fischbein et al., 2002). Furthermore, passenger $CD4^{+}$ T cells provided help to host tissue-reactive B cells and augment alloantibody responses, which could affect cardiac allograft survival (Harper et al., 2016; Win et al., 2009).

In conclusion, innate and adaptive immune responses play major roles in shaping alloreactivity against skin and heart allografts. These allograft models, especially skin allografts, will be discussed in the Result section.

D. Determinants of allograft rejection

1. Genetic factors

Organ transplant rejection is mainly determined by the genetic disparities that exist between each donor and their recipient. Depending on the number of genetic differences between the donor and the recipient, the magnitude of the alloresponse may vary. Here, I will discuss the genetic factors that can affect transplant outcomes.

a. Major histocompatibility complex mismatch

Major histocompatibility complex mismatch refers to donor versus recipient differences in the MHC locus. As mentioned earlier, about 1-10% of T cells respond to allogeneic MHC molecules (Suchin et al., 2001). These T cells recognize allogeneic MHC through direct recognition, which is either governed by the structure of allogeneic

MHC molecule or by the wide array of peptides bound to allogeneic MHC molecules (Benichou et al., 2011). Allogeneic MHC molecule can also be recognized in the form of processed peptides presented by host MHC molecules through indirect recognition.

b. Minor histocompatibility antigen expression

In addition to mismatches within the MHC locus, the expression of minor antigens that are not within the MHC gene also impact graft survival, for instance, the male-specific H-Y antigen and ABO-blood antigen (Elizabeth Simpson et al., 1997; Worel, 2016).

c. Immuno-modulating polymorphisms

Studies have shown that polymorphisms that lie within genes that govern immune responses can also modulate graft survival. Polymorphisms in genes encoding cytokines, such as tumor necrosis factor α (TNFα) and IFNγ have been shown to impact graft rejection (Marshall and Welsh, 2001). Furthermore, single nucleotide polymorphisms in the Toll-like receptor 4 (TLR4) gene have been associated with graft failure in liver transplantation (Dhillon et al., 2010; Oetting et al., 2012). Interestingly, a new study has suggested that polymorphisms in SIRPα, which affect the strength of the interaction with its ligand, CD47, can modulate the innate recognition of non-self and downstream immune responses (Dai et al., 2017).

2. Environmental factors

Beside genetic factors, environmental factors that are not linked to germline genetics of the donor or host may also affect transplant outcomes. Humans are constantly exposed to their surroundings, and parameters such as diet, pollutants, pathogens and microbial colonization have all been shown to impact the immune system. Therefore, it is conceivable that these factors may also affect alloimmunity and modulate transplant outcomes. In this section, I will discuss how environmental factors might impact transplant survival.

a. Diet

There has been renewed interest in modulating dietary habits and implementing dietary interventions to improve various clinical outcomes. In the context of organ transplantation, chronic metabolic diseases for which diet is held partly responsible such as hypertension, hyperlipidemia and obesity have been shown to significantly worsen the outcome of solid organ transplantation (James et al., 2002; Worel, 2016; Zelenika et al., 1998). This is due in part to increased surgical morbidity, but it is also possible that diet, or its metabolic consequences affect alloimmunity (Ganeshan and Chawla, 2014).

Indeed, a recent study from our laboratory showed that mice fed a high-fat diet (HFD) had enhanced alloreactivity and faster heart allograft rejection, compared to mice fed a control low-fat diet (LFD) (Molinero et al., 2016). This accelerated rejection kinetics was associated with increased T cell proliferation. Furthermore, splenic APCs from mice that were fed a HFD displayed an increased expression of CD80 and had a

greater ability to prime alloreactive T cells in vitro than APCs from mice fed a LFD (Yuan et al., 2015).

High salt diet has also been shown to accelerate the rejection of allogeneic heart grafts in mice, compared with normal salt diet (Safa et al., 2015). This was associated with a reduction in the proportion and proliferation of Tregs and was dependent on serum- and glucocorticoid-regulated kinase-1 (SGK1).

Further understanding of the impact of diet on transplant outcomes may allow incorporation of diet recommendations perhaps both before and after clinical transplantation (Marín et al., 2018; Romano et al., 2017)

b. Pollutants

Some studies have shown that pollutants could affect the immune response (Diaz-Sanchez, 2000), raising the possibility that they might affect alloimmunity. Aryl hydrocarbon receptor (AhR), a cytoplasmic receptor that senses environmental cues, can be activated by different ligands including environmental toxins or microbial products (Kiss et al., 2011; Lee et al., 2011a; Veldhoen et al., 2008). Depending on the context of exposure, AhR activation can lead to immune activation or regulation. AhR binding to its ligand, 6-Formylindolo[3,2-b]carbazole (FICZ), triggered Th17 cells and exacerbated mouse EAE through augmenting Th17 responses (Veldhoen et al., 2008). Interestingly, engagement of another AhR ligand, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), suppressed Th17 responses in a mouse EAE model (Quintana et al., 2008) and promoted type 1 regulatory cells in the human (Gandhi et al., 2010). In the context of alloimmunity, TCDD exposure prolonged both fully mismatched skin and heart

allograft survival (Cai et al., 2013; Pauly et al., 2012), whereas exposure to FICZ accelerated skin graft rejection (Pauly et al., 2012). In a murine islet cell transplant model, administration of VAG539, which is metabolized to VAF347 that binds to AhR, was associated with long-term graft acceptance (Hauben et al., 2008), correlating with an increase in Tregs. Adoptive transfer of CD11c^{+} cells from VAG539-treated mice allowed the induction of tolerance in recipients mice. These studies suggested that AhR may play an important role in integrating environmental signals and may influence transplant survival and tolerance induction.

c. Infection

Infection with pathogenic organisms could happen before, during or after organ transplantation. At each time point, various factors contribute to infection. For example, previous exposure to microbial organisms, existing infection on donor or recipient, end-stage disease manifestations, peri-operative infection or the use of immunosuppressive drugs are shown to affect the occurrence of infection. (Fishman, 2013).

In mice, the Alegre and Chong laboratories have previously reported that infections with *Listeria monocytogenes* or *Staphylococcus aureus* at the time of transplantation can increase alloreactivity and prevent the induction of transplantation tolerance by costimulation-blocking agents (Ahmed et al., 2011). Anti-microbial immune responses that are mounted following infection might theoretically affect alloimmunity by different mechanisms: i. Heterologous cross-reactivity between anti-microbial T and B cell responses with donor MHC molecules (cross-reactivity); ii. Pattern-recognition receptor (PRR)-induced activation of APCs that present alloantigen (adjuvant effect); iii.

Bystander activation by microbial-induced pro-inflammatory cytokines of alloreactive T cells (bystander effect) (Chong and Alegre, 2012).

In terms of cross-reactivity, viral infections have been shown in mice to induce the formation of memory T cells, some of which, by chance, cross-react with select allogeneic MHC molecules through direct TCR allorecognition (Adams et al., 2003; Yang and Welsh, 1986). In humans, memory alloreactive $CD4^+$ and $CD8^+$ T cells have been reported to react with viruses to which patients had not been previously exposed (Su et al., 2013), demonstrating that T cells can have more than one specificity through cross-reactivity. Further studies have demonstrated that pre-existing donor-reactive IFNγ-producing memory T cells are associated with higher risk of rejection episodes following renal transplantation in mice (Heeger et al., 1999) and humans (Valujskikh et al., 2002), and also in the setting of cardiac transplantation in non-human primates (Nadazdin et al., 2011).

Infections may also increase alloreactivity through adjuvant effects that augment the priming capacity of APCs. For instance, it has been reported that lymphocytic choriomeningitis virus (LCMV) infections at the time of transplantation can prevent the induction of transplantation tolerance and drive allograft rejection in mice (Williams et al., 2001). Such rejection was attributed to increased activation of DCs that primed and activated alloreactive T cells despite the use of CTLA-4-Ig and anti-CD154. Furthermore, exposure to TLR ligands at the time of transplantation has been shown to prevent the induction of tolerance in mice even in the presence of immunosuppression (Chen et al., 2006; Thornley et al., 2006)

Lastly, inflammatory responses that occur following infections may influence/enhance activation and differentiation of alloreactive T cells after transplantation through cytokine-dependent bystander effects (Richer et al., 2013). For instance, infections with *S. aureus* and *L. monocytogenes* in mice caused systemic production of IL-6 and type I IFN, which were sufficient to prevent the induction of tolerance (Ahmed et al., 2011; Wang et al., 2008), or even, in the case of *L. monocytogenes*, to break established tolerance and lead to cardiac allograft rejection (Wang et al., 2010). In vitro, IL-6 was found to increase proliferation of alloreactive T cells and type I IFN to enhance their differentiation into IFNγ-producing cells (Wang et al., 2010).

d. Microbiota

Since pathogenic and commensal bacteria express similar microbial-associated molecular patterns (MAMPs) and since pathogenic infections can enhance alloreactivity and accelerate transplant rejection, it is possible that the commensal microbiota may also play a role in transplant rejection. Humans exist as metaorganisms consisting of host cells and symbiotic microbes, including bacteria, eukaryotic viruses, phages, fungi, archae and others. By conventional wisdom, the bacteria alone approximate 100 trillion cells, outnumbering host cells by a factor of 10, with greater than 10-fold more microbial than eukaryotic genes being expressed (Ley et al., 2006). Recently, a group re-calculated the proportion of human and bacterial cells and revealed that the ratio is closer to 1:1. (Sender et al., 2016). Regardless of this new result, it is clear that the complex community of bacteria plays a fundamental role in modulating the physiology of

their host at homeostasis and in modulating disease manifestations (Belkaid and Hand, 2014). However, little is known about the effect of the intestinal microbiota on transplant outcomes. The relationship between the microbiota and transplant rejection has been suggested in clinical studies. *Lactobacillales* have been linked with better transplant survival and less severe graft-versus-host disease (GvHD) in clinical small bowel (Oh et al., 2012) and bone marrow (Jenq et al., 2012) transplantation, respectively. Protection from GvHD-associated intestinal inflammation was recapitulated following *Lactobacillales* reconstitution in mice, suggesting a causal role for this bacterial order in the protection against GvHD (Jenq et al., 2012). A decrease in *Lactobacillales* has been associated with acute rejection of small bowel transplants, compared to patients that are not rejecting the transplant (Oh et al., 2012). While microbial diversity did not change in the small bowel transplant study, structural shifts in bacterial composition and a reduced diversity of bacteria in the lung have been described in lung transplant patients (Charlson et al., 2012). Nonetheless, it is not yet clear whether such shifts are a cause or a consequence of acute rejection, or whether only the intestinal or also the transplanted organ-associated microbiota may be important. Indeed, the microbiota has been shown to have both local and distal effects on the immune system.

E. Local effect of the gut microbiota

1. Intestinal immune system

Host immune cells reside in the lamina propria of the intestine, below the epithelial layer. Despite the physical barrier between the lamina propria and luminal commensals, the host immune system is constantly exposed to commensal microbes

and their products. To avoid systemic inflammation, the mucosal immune system has evolved to localize its response. For instance, intestinal DCs activated by commensals are restricted to mesenteric lymph nodes (mLNs) and specifically induce the production of IgA by B cells (Macpherson, 2004). It has been well-documented that the gut microbiota plays a fundamental role in the integrity of the intestine as well as the development of a healthy intestinal immune network. The crosstalk between resident microbiota and immune cells of the lamina propria is the key to maintaining homeostasis in the intestine. In this section, we will cover some of the components that contributes to the health of the intestinal integrity and immune functions.

a. Mononuclear phagocytes

Mononuclear phagocytes (MNPs) in the intestine are a diverse population that includes DCs and macrophages (Cerovic et al., 2014). They participate in uptake of luminal antigens to promote downstream adaptive mucosal immune responses. However, the identity of the intestinal APCs that activate the adaptive mucosal immune system remains controversial. In 2001, it was shown that DCs can sample luminal content by expressing their own tight junction proteins and extending dendrites through these tight junctions into the lumen (Rescigno et al., 2001). When finer phenotypic characterization of intestinal MNPs became available, it was suggested that CX_3CR1^+ DCs were responsible for forming trans-epithelial dendrites and sampling antigen, leading to clearance of infectious bacteria (Niess, 2005). This idea was further supported by Diehl et al., who reported that signals from the microbiota through MyD88 in CX_3CR1^+ cells restricted trafficking of antigen to mLNs (Diehl et al., 2013). On the

other hand, $CD103^+$ DCs were also suggested to be the antigen-sampling cells that migrate to mLNs (Schulz et al., 2009). Evidence now suggests that division of labor exists between MNPs and that both subsets play a role. Indeed, it was recently demonstrated that CX_3CR1^+ cells uptake antigen and transfer it to $CD103^+$ DCs (Rossini et al., 2014) in a gap junction protein-dependent manner (Mazzini et al., 2014).

With the unique environment of the intestine where commensals and potential pathobionts are in close proximity to the mucosal immune system, it is important for MNPs not only to activate pro-inflammatory adaptive responses, but also anti-inflammatory ones, such as IL-10 (Kim et al., 2018) and TGFβ (Coombes et al., 2007). One of the critical roles of activated intestinal DCs is the generation of induced regulatory T cells (iTregs), in a TGFβ- and retinoic acid-dependent process (Coombes et al., 2007) and in a manner that involves TRAF6 in DCs (Han et al., 2013). Recently, Mortha et al. demonstrated that a cross-talk between GMCSF-producing ILC3s and IL-1β-producing macrophages was necessary to regulate the number and function of $CD103^+$ DCs and that this cross-talk was important for the generation of iTregs (Mortha et al., 2014). This anti-inflammatory function is microbiota-dependent and requires microbial association with the epithelium (Kim et al., 2018), suggesting the MNPs in the gut play a critical role in sampling and integrating signals from the microbiota.

b. Regulatory T cells

Intestinal homeostasis relies on the balance between pro-inflammatory and anti-inflammatory responses. Tregs in the intestine plays a central role in maintaining such balance. A few taxa of bacteria have been specifically implicated in mucosal tolerance

via iTreg induction. *Bacteroides fragilis* has been shown to enhance Treg function via the symbiosis factor polysaccharide A (PSA), as PSA^+ but not PSA-deficient *B. fragilis*-monocolonized animals had the ability to suppress Th17 inflammatory responses (Round and Mazmanian, 2010; Round et al., 2011). In addition, germ-free (GF) mice infected with the pathogenic bacterium *Helicobacter hepaticus* developed colitis, but when co-colonized with *B. fragilis*, PSA-induced IL-10 secretion by iTregs and allowed for suppression of colitis (Mazmanian et al., 2008). PSA-induced TLR2 signaling on $CD4^+$ T cells was shown to be necessary for iTreg differentiation and increased IL-10 secretion (Round et al., 2011). These results show that *B. fragilis* can mediate gut regulatory responses via a bacterium-associated molecule.

The *Clostridium* genus of commensals has also been shown to play a role in prevention of inflammatory bowel disease (IBD), particularly clusters IV and XIVa (Frank et al., 2007). Mice colonized with mixtures of commensal clostridial strains have been shown to have reduced symptoms of colitis. Whereas *B. fragilis* appears responsible for enhanced Treg activity, Clostridia have been shown to induce iTreg differentiation and frequency (Atarashi et al., 2011, 2013). When commensal Clostridia strains were isolated and identified and GF or SPF animals were colonized with the mixture of strains, they developed decreased Th17 responses and an increased frequency of IL-10-producing Tregs (Atarashi et al., 2013). Several mechanisms have been implicated in the ability of Clostridia strains to enhance iTreg differentiation, including induction of TGFβ in IELs (Atarashi et al., 2011), and production of the bacterial fermentation products short-chain fatty acids (SCFAs) (Smith et al., 2013). The effects of SCFAs, butyrate in particular, on iTreg differentiation was demonstrated both *in vitro* and *in vivo*

when butyrate was added to T cell tissue cultures (Furusawa et al., 2013), or mice received oral butyrate (Arpaia et al., 2013; Furusawa et al., 2013). The major mechanism of action of butyrate is thought to be its function as a histone deacetylase inhibitor (HDAC). Butyrate has been shown to increase acetylation at the *FoxP3* promoter and CNS elements, and its function as a promoter of Treg induction suggests that its activity as an HDAC inhibitor allows for greater accessibility of *FoxP3* and other regulatory transcription factors.

Tregs not only receive signals from non-specific receptors that respond to MAMPs or microbial metabolic products, but also from direct TCR recognition of microbial antigens (Lathrop et al., 2011). This, in the context of anti-inflammatory signals such as TGF-β, retinoic acid and SCFA may allow differentiation of conventional T cells into iTregs to provide tolerance to commensal microbiota and prevent immunopathology such as inflammatory bowel disease (Arpaia et al., 2013; Nutsch and Hsieh, 2012).

Mechanisms antagonistic to Tregs appear in place to ensure the mucosal immune system is not over-suppressed at the time of infection. DNA from the microbial community was found to be suppressive for Tregs conversion, instead facilitating the differentiation of IFNγ- and IL-17-producing effector T cells to fight against fungal infection (Hall et al., 2008). While this interaction was TLR9-dependent, the origin of the DNA remains unclear. Nevertheless, the microbiota contributes to the homeostasis of the Treg population.

c. Conventional T cells

The microbiota can also directly affect the differentiation of effector T cells. Segmented filamentous bacteria (SFB), non-culturable, gram-positive bacteria that are closely related to the genus *Clostridium* and tightly adhering to intestinal epithelial surfaces (Ivanov et al., 2009), are sufficient to promote the full development of the T helper cell response, including IFNγ-, IL-17- and IL10- producing $CD4^+$ T cells (Gaboriau-Routhiau et al., 2009). Th17 cells are particularly known to have a prominent role in intestinal immunity (Blaschitz and Raffatellu, 2010). In the intestine, where TGF-β is highly abundant, naïve T cells differentiate into Th17 cells in the presence of the pro-inflammatory cytokine IL-6 (Mucida et al., 2007). Colonization with SFB in mice previously devoid of it resulted in IL-17 and IL-22 expression by $CD4^+$ T cells in the lamina propria of the small intestine and promoted antimicrobial defense. In turn, these commensal-mediated responses restrain the growth of commensals. Indeed, the control of SFB colonization by SFB-induced Th17 expansion is through α-defensin, *Nox1* and *Pigr* in a IL-17R dependent manner (Kumar et al., 2016), while the depletion of IL-17A-mediated neutrophil recruitment significantly enhanced Th17 responses and SFB expansion (Flannigan et al., 2017).

The exact mechanisms by which SFB induces Th17 responses are still being investigated. As mentioned, intestinal MNPs are crucial in integrating microbial signals to maintain intestinal homeostasis. Indeed, CX_3CR1^+ macrophages, but not $CD103^+$ DCs are required for SFB-specific Th17 responses (Panea et al., 2015). Alternatively, recent studies also suggested that intestinal DCs can present SFB peptides directly to the TCR of T cells and induce SFB-specific Th17cells in an MHC class II-dependent

manner (Geem et al., 2014; Goto et al., 2014). However, whether the induction of Th17 cells by SFB requires secondary lymphoid organs is still controversial (Geem et al., 2014; Lécuyer et al., 2014). Interestingly, naïve T cells that bore a TCR transgene specific for SFB had a strong tendency to differentiate into Th17 cells even in the presence of the strong Th1 inducer *L. monocytogenes* (Yang et al., 2014). Thus, differentiation of T cells in the intestine may depend on direct TCR-dependent microbial antigen recognition and on the balance of pro- and anti-inflammatory molecules present at any given time providing equilibrium between effector T cells and Tregs.

d. B cells and IgA

B cell-produced IgA plays an important role in maintaining the microbiota-host relationship by neutralizing bacteria, reducing intestinal pro-inflammatory signals and balancing expression of bacterial epitopes (Boullier et al., 2009; Peterson et al., 2007). SFB also potently induces IgA production (Talham et al., 1999). Intestinal IgA can be produced at multiple sites, such as Peyer's patches, isolated lymphoid follicles, lamina propria and mesenteric lymph nodes. A recent study has shown that SFB can facilitate postnatal development of isolated lymphoid follicles with germinal centers, which are critical for IgA induction (Lécuyer et al., 2014).

The mechanism of intestinal IgA induction is not completely understood. In general, IgA can be induced by B cells following CD40L-CD40 interactions with activated T cells, but can also occur in a T cell-independent manner, whereby B cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL) secreted by IECs and DCs promote IgA induction (Pabst, 2012). A recent study suggested that

commensal bacteria are capable of eliciting T-independent IgA responses, which predominantly targets commensals in the small intestine (Bunker et al., 2015). For T cell-dependent induction of IgA, both Th17 (Hirota et al., 2013) and Tregs (Tsuji et al., 2009) cells are capable of being reprogrammed into T follicular helper (Tfh) cells to activate B cells to produce IgA. Additionally, soluble lymphotoxin from RORγt$^+$ ILCs was shown to facilitate T cell-dependent IgA production. In contrast, membrane lymphotoxin was important for T cell-independent IgA induction in the intestine (Kruglov et al., 2013).

Whereas it is known that the microbiota has a role in intestinal B cell activation and IgA production (Macpherson, 2004; Talham et al., 1999), how the microbiota contribute to the intestinal B cell repertoire is only beginning to unfold. Vossenkämper et al. showed that human transitional B cells responded to intestinal bacteria *in vitro (Vossenkamper et al., 2013)*, suggesting that B cells may be able to acquire a repertoire that recognizes commensals. Indeed, the microbiota drives early B cell development in the intestine, where receptor recombination and editing processes change the immunoglobulin repertoire. Colonization of GF mice increased Igλ-expression in intestinal B cells, an indication of receptor editing (Wesemann et al., 2013). Furthermore, production of TNFα and inducible nitric oxide synthase by IgA$^+$ plasma cells was dependent on signals from the microbiota (Fritz et al., 2011). Activation of immature B cells in gut-associated lymphoid tissue may also be important to remove autoreactive B cells and protect against autoimmunity. Indeed, B cells in patients with systemic lupus erythematosus where shown to exhibit a defect in expression of the gut-homing β7 integrin receptor (Vossenkamper et al., 2013).

While the production of a portion of the IgA repertoire is dependent on the microbiota, a new study has shown that a subset of the IgA repertoire is generated independently from the presence of microbiota or exogenous dietary antigens, despite the fact that this IgA repertoire is polyreactive to microbial components and to a subset of microbiota taxa (Bunker et al., 2017). Nonetheless, the function of these polyreactive IgA molecules remains to be elucidated. They may have wide of effects on intestinal homeostasis, such as promoting immune exclusion of pathogens, neutralization and establishment of the microbiota (Donaldson et al., 2018; Moor et al., 2017; Peterson et al., 2007; Stokes et al., 1975).

2. Intestinal architecture

In addition to the effect of the microbiota on the immune system, the microbiota can also impact non-immune functions that are crucial to the integrity of the intestinal barrier as well as the homeostasis of the micro-environment of the host intestine. The intestine is constantly facing challenges from food antigens, commensals, and pathogens and has to respond quickly and precisely to mount appropriate responses. According to Hooper et al., intestinal homeostasis can be achieved if the microbiota is stratified and compartmentalized (Hooper et al., 2012), with stratification referring to limiting contact between luminal antigens and the immune system, and compartmentalization to localizing the immune response to sections of the intestine to prevent systemic immunity. Such integrity and balance between the microbial community and the host provide evolutionary advantages to both entities through mutualism.

a. Mucus and goblet cells

The first line of defense in this barrier is the mucus layer produced by goblet cells. Mucus consists of heavily glycosylated mucins, which can be subdivided into secreted gel-forming and non-gel-forming, and cell-surface mucins, with gel-forming mucins, especially MUC2, as the major constituents (Linden et al., 2008). Due to the anatomy and absorptive requirements of the intestine, the mucus layer in the small intestine differs from that in the colon, going from a discontinuous distribution in the small intestine to continuous in the colon (Brown et al., 2013). Furthermore, the mucus in the colon can be subdivided into an outer and an inner layer, where the inner layer is denser and devoid of bacteria (Johansson et al., 2008). It is likely that this distribution of mucus is an evolutionary product of symbiosis, as the number of bacteria in the colon is higher than in the small intestine. Besides providing physical segregation, mucus also traps on its outer layer antimicrobial peptides (AMPs) and secretory immunoglobulin A (sIgA) that are at the first line of our immune defenses and help shape the microbial community. Disruption of the mucus layer by western diet has been shown to cause significant aberrations in the composition of the microbiota, and *Bifidobacteria* have been observed to be effective in protecting the host from the detrimental effects of western diet (Schroeder et al., 2018).

Interestingly, the intestinal mucus has been shown to contribute to promote oral tolerance (Shan et al., 2013). The porosity of the mucus layer in the small intestine allows antigen-MUC2 complexes to be taken up by DCs and the glycans associated with MUC2 have been found to deliver tolerogenic signals and induce anti-inflammatory

properties in DCs. The secretion of MUC2 was shown to be dependent on the NLRP6 inflammasome and autophagy in goblet cells (Wlodarska et al., 2014).

Signals from the microbiota appear necessary for the normal function of goblet cells, as the engagement of TLRs within sentinel goblet cells enables protection of the colonic crypts from bacterial translocation (Birchenough et al., 2016). Underscoring the importance of microbiota signals, the function of goblet cells was shown to be impaired when the microbial community was perturbed during gastrointestinal infection or antibiotic treatment (Bergstrom et al., 2008; Wlodarska et al., 2011). In addition, goblet cell MyD88-dependent sensing of the luminal microbiota was necessary to reduce the formation of goblet cell-associated passage (GAP) formation, conduits known to increase luminal antigen delivery to the lamina propria (Knoop et al., 2015). However, the timing of the exposure to microbial signals is critical. A recent study showed that the encounter of bacterial antigen is associated with GAP formation and occurs at the preweaning stage of mouse development (Knoop et al., 2017). A disruption of such timing results in detrimental consequences with worsened DSS-induced colitis.

b. Anti-microbial peptides (AMPs)

AMPs and IgA, which are produced by Paneth cells and IgA^+ B cells respectively, adhere to the outer mucus layer and, in cooperation with the mucus, maintain microbial segregation in the intestinal lumen. AMPs include defensins and C-type lectins, and they are essential to contain the microbiota and minimize bacterial contact with epithelial cells. Secretion of AMPs by Paneth cells is regulated by IL-22 produced by innate lymphoid cells (ILCs) (Sonnenberg et al., 2011), the secretion of which is

modulated by the microbiota. Consistent with the microbiota regulating AMP secretion, expression by Paneth cells of RegIIIγ, a C-type lectin that preferentially binds to gram-positive bacteria, was decreased in GF mice (Cash, 2006). Signaling by the microbiota to Paneth cells is dependent on MyD88, as deleting MyD88 from the non-hematopoietic compartment of conventional mice (which includes Paneth cells) led to a significant reduction in RegIIIγ production and failure to fight *L. monocytogenes* infection (Brandl et al., 2007).

Like production of mucus by goblet cells, secretion of AMPs appears dependent on autophagy, as mutations in the autophagy gene ATG16L1 were shown to affect the normal function of Paneth cells in mice and humans (Cadwell et al., 2008).

c. Intestinal Epithelial Cells

Intestinal epithelial cells (IECs), an umbrella term that includes classical epithelial cells, M cells that sample luminal antigens, goblet cells and Paneth cells, serve as a major barrier that separates the intestinal luminal content from the mucosal immune system and are responsible for various functions, including the production of mucus and AMPs and the facilitation of secretory IgA transcytosis into the gut lumen. More than just a physical barrier, IECs signal in response to luminal microbial products to maintain homeostasis. For instance, IEC-intrinsic MyD88-dependent autophagy contributes to intestinal homeostasis upon bacterial invasion (Benjamin et al., 2013), and inflammasome activation is essential to control enteric bacterial colonization (Knodler et al., 2014; Nordlander et al., 2013; Song-Zhao et al., 2013). In addition to responding to gut microbiota, IECs were recently found to respond to systemic microbial products to

sustain host-commensal symbiosis during severe illness, such as GvHD (Mathewson et al., 2016). In addition, systemic LPS resulted in α(1,2)-fucosylation of IECs and fucosylated proteins shed into the intestinal lumen were shown to be metabolized by commensals and reduce expression of bacterial virulence genes thus improving tolerance to mild intestinal pathogens that could perhaps outgrow during sepsis-mediated anorexia. (Pickard et al., 2014).

Finally, IECs also interact with the underlying local host immune system to achieve mucosal integrity (Olszak et al., 2014; Peterson and Artis, 2014; Rescigno, 2014). For example, IECs were shown to be critical for the warding off *Salmonella* infection via cross-talk with localized $\gamma\delta$ IELs in a MyD88-dependent manner (Konijnenburg et al., 2017). Furthermore, additional studies show that specialized IECs, such as Paneth cells and Tuft cells, are capable of limiting bacterial infection via secretory autophagic lysozyme (Bel et al., 2017) and of orchestrating anti-helminth infection via the IL-25-type 2 ILC axis respectively (Gerbe et al., 2016; Howitt et al., 2016). These studies suggest that the intestinal barrier has evolved to adapt to the luminal and systemic environment to maintain intestinal homeostasis.

F. Systemic effect of the gut microbiota

1. Metabolic disorders

The relationship between the microbiota and the metabolism of both the commensals and host is deeply intertwined. The metabolic profile of commensals is distinct from but complementary to host metabolism. For instance, the microbiota is important for host metabolism such as conjugation and de-conjugation of bile acids, and

production of short chain fatty acids, essential vitamins and amino acids (Brestoff and Artis, 2013). One clinical study showed that patients supplemented with high fiber diet displayed an increase in *Bifidobacterium pseudocatenulatum* and a decreased in *Lachnospiraceae bacterium* CAG0409, which correlated with a change in carbohydrate fermentation in the microbial community and reduced body fat, fasting glucose and insulin resistance in patients. Thus, these microbiota-mediated metabolic functions are crucial for host immunity.

In addition to complementing host metabolism, commensal bacteria can control the virulence of pathogens through competition for similar energy sources and production of anti-microbial molecules (Kamada et al., 2012). A recent study showed that IL-22, a cytokine that is modulated by intestinal commensals, induces the hemoglobin scavenger haptoglobin and the heme scavenger hemopexin to sequester iron in order to limit iron availability to pathogens (Sakamoto et al., 2017). Thus, the microbiota provides tremendous value to the metabolic aptitude and overall health of the host.

The community composition of the intestinal microbiota's community composition has been associated with the occurrence of diabetes mellitus. In non-obese diabetes (NOD) mice, deficiency in MyD88 protected against the development of type 1 diabetes in conventional but not in GF mice, suggesting that the microbiota in MyD88-deficient mice may inhibit autoimmunity (Wen et al., 2008). Within the microbial community, SFB was shown to confer protection against insulitis in females but not males NOD mice (Kriegel et al., 2011). In addition to this bacterial lineage affecting males and females differently, 2 studies suggest that species differentially represented in males versus

females may be responsible for the higher prevalence of type 1 diabetes in female mice (Markle et al., 2013; Yurkovetskiy et al., 2013). Indeed, castration in male mice narrowed the differences in the microbiota, suggesting hormonal shaping of microbial constituents (Yurkovetskiy et al., 2013) or hormonal-dependent responsiveness to microbial products (Markle et al., 2013).

The gut microbiota also affects the amount of energy harvested from the diet and consequentially can play a role in obesity (Turnbaugh et al., 2006). A study in mice showed that lymphotoxin contributed to obesity by controlling the composition of the microbiota, perhaps in part by driving the expansion of SFB (Upadhyay et al., 2012). Conversely to lymphotoxin signaling, innate sensing through the NLRP6 and NLRP3 inflammasomes protected hosts from non-alcoholic fatty liver disease and obesity via reducing influx of microbial products to the liver and modulating microbial composition (Henao-Mejia et al., 2012). Accordingly, genetic defects in the inflammasome resulted in hepatic inflammation and obesity. These results suggest that a crosstalk between microbiota and immune system is essential to maintain a balanced metabolic state.

2. Autoimmune diseases

In addition to the local influence of the intestinal microbiota, some studies support a systemic role for gut commensals (Chervonsky, 2010). For instance, in a genetic mouse model of spontaneous arthritis, GF mice displayed a reduction in autoantibodies, autoantibody-secreting cells and Th17 cells, which was associated with an attenuation of arthritis (Wu et al., 2010). Reconstitution with SFB restored the pathological state of these mice. It was suggested that SFB promote autoimmunity by altering the activation

threshold of T cells, which makes them more susceptible to endogenous ligands (Chappert et al., 2013). SFB may not colonize humans, but intestinal *Prevotella copri* (*P.* copri) was associated with susceptibility to inflammatory arthritis in patients (Scher et al., 2013) and increased the risk for chemically-induced colitis in mice, suggesting that other pro-inflammatory bacterial species may drive distal clinical diseases.

While these studies showed that SFB colonization is sufficient to induce arthritis, a recent study showed that antibiotic (Abx) treatment to mice with established arthritis reduced intestinal Th17 and arthritis (Rogier et al., 2017). Also, GF and Abx-treated mice displayed a reduction in serum amyloid A (SSA) levels in both the ileum and the synovial tissue, consistent with previous studies in which SFB was shown capable of inducing SSA production from intestinal epithelial cells through the IL-23/IL-22 axis (Ivanov et al., 2009; Sano et al., 2015).

It is interesting that SFB, which is a gut-restricted commensal and requires attachment to the intestinal epithelium to be sustained, is capable of eliciting autoimmune responses at distal locations. How SFB elicits such responses is under intense investigation. Recent studies showed that colonization with SFB allows immune cells to migrate to systemic secondary lymphoid organs (Teng et al., 2016), and systemic tissues (Bradley et al., 2017). Teng et al. showed that SFB colonization increased germinal center formation, auto-antibody production, and T follicular helper (Tfh) cell responses in a DC-dependent manner. Furthermore, these Tfh cells were shown to migrate from the Peyer's patches to the spleen and peripheral LNs. Beside Tfh, Th17 cells from SFB-colonized mice were capable to migrate to the lungs via the CCR6-CCL20 axis to cause autoimmune lung pathology (Bradley et al., 2017).

Interestingly, these Th17 cells seem to possess dual TCRs composed of Vβ6 and Vβ14, suggesting that one TCR recognizes a microbial antigen while the other recognizes a self-antigen.

Other studies suggested that commensal bacteria may induce autoimmunity at distal locations through antigen receptor cross-reactivity between commensal- and auto-antigen epitopes. Viera et al. showed that *Enterococcus gallinarum* was sufficient to exacerbate lupus kidney disease following its translocation to the liver, enabled by reducing tight junction proteins in the intestine (Vieira et al., 2018). In this case, *E. gallinarum* appeared to induce hepatic production of lupus antigens that formed immune complexes with antibodies. Patients with systemic lupus erythematosus (SLE) had increased levels of anti-human RNA antibodies that positively correlated with antibodies to *E. gallinarum* RNA, suggesting a cross-reactivity between commensal- and auto-antigen-specific immune responses. In addition to SLE, retina-specific T cells were shown to be activated in the intestine by commensal-specific signals and migrate past the blood-retinal barrier to cause uveitis (Horai et al., 2015). Thus, gut commensals may induce distal autoimmune responses via migration of the bacteria themselves or of anti-bacterial T or B cells and cross-reactivity of the TCR or BCR between commensal antigens and auto-antigens.

3. Allergic responses

The hygiene hypothesis suggests that a shift in commensal flora over the past century may be responsible for the higher prevalence of allergic and autoimmune diseases, with the idea that modern humans may be missing protective microbes. In

support of microbes that can suppress allergic responses, the local lung microbiota has been shown to facilitate tolerance to allergens. In addition, it has been suggested that the intestinal microbiota can also reduce airway allergic responses by producing short-chain fatty acids that can drive generation of lung-seeding DC precursors less capable of inducing Th2 differentiation, a cell type implicated in allergic responses (Trompette et al., 2014). Similarly, a recent study reported that Clostridial groups could confer protection against food allergy in an IL-22 dependent manner (Stefka et al., 2014). In this study, GF or antibiotic-treated mice displayed elevated immune responses against an allergen and reconstituting GF mice with normal microbiota or a consortium of Clostridia restored protective immune responses. These studies suggest that the intestinal microbiota is important to regulate the development of allergic diseases.

4. Neurological disorders

Recent research has also revealed a relationship between the intestinal microbiota and the central nervous system (Wang and Kasper, 2014). Mice with an intact microbiota showed exacerbated spontaneous experimental autoimmune encephalomyelitis (EAE) compared to GF mice, which correlated with increased migration of autoreactive B cells to draining lymph nodes (Berer et al., 2011), greater proliferation of myelin-specific T cells and a decreased Treg population (Lee et al., 2011b). Interestingly, a recent study showed that the intestinal microbiota could also modulate psychological disorders that accompany neurodevelopmental defects, as treatment with *B. fragilis* mitigated defects in neurological behavior (Hsiao et al., 2013). In particular, PSA from *B. fragilis* provided protection against demyelination in EAE via

TLR2-mediated CD39 signaling in $CD4^+$ T cells (Wang et al., 2014), reinforcing the idea that bacterial components from the intestinal microbiota can have distal consequences though it is not known whether it is such components or the local effector cells they modulate that travel to distal regions to affect disease.

5. Immune defense

In healthy individuals, the microbiota may constantly calibrate and arm the immune system to be ready to fight potential infections. For example, the microbiota has been shown to confer protection against sepsis induced by systemic infection with *Escherichia coli*. Liu et al. (Deshmukh et al., 2014) demonstrated that antibiotic-induced dysbiosis resulted in reduced production of IL-17 and granulocyte-colony stimulating factor (G-CSF), and lethality from *E. coli* K1-mediated sepsis. Additionally, in a model of lung influenza infection, antibiotic-treated mice exhibited a reduction in influenza-specific $CD4^+$ and $CD8^+$ T cells, resulting in increased pulmonary viral titers (Ichinohe et al., 2011). This was associated with decreased migration of DCs after viral infection, an IL-1β- and inflammasome-dependent event, suggesting that the intestinal microbiota may participate in priming the distal immune system. Indeed, MNPs in GF mice were also shown to be less capable of producing type I IFNs (Ganal et al., 2012) and type II IFNs (Abt et al., 2012), compared to SPF mice, which resulted in defects in antiviral immunity, implicating microbiota signals in making the distal immune system competent. Whether dysbiosis from antibiotic treatment also reduces systemic innate immunity in human patients remains to be determined.

In immunocompromised patients, intestinal dysbiosis may occur as a result of antibiotic therapy and perhaps of reduced immune function, such that opportunistic pathogenic bacteria may bloom and possibly translocate and provoke systemic infections (Taur and Pamer, 2013). In general, administration of antibiotics in mice has been shown to impair immune response against West Nile Virus, Zika, and Dengue viral infections (Thackray et al., 2018), as well as lung bacterial infections by *Streptococcus pneumonia* and *Klebsiella pneumoniae* (Brown et al., 2017). Therefore, correction of microbial imbalances may help prevent outgrowth of pathogens and infectious complications.

The mechanisms by which the intestinal microbiota influences distal anti-bacterial or anti-viral responses are likely context-dependent. In the case of lung infections by *S. pneumoniae* and *K. pneumoniae*, the microbiota communities that are capable of stimulating Nod-like receptors protect the lung from infections by promoting GM-CSF signaling via the ERK pathway in alveolar macrophages in a IL17A-dependent manner (Brown et al., 2017). In addition, microbial metabolites have been shown to be protective of the immunopathology that accompanies influenza infections (Steed et al., 2017; Trompette et al., 2018). SCFAs have been shown to increase $Ly6C^-$ macrophages that enhance anti-viral $CD8^+$ T cells responses, and dampen lung immunopathology due to neutrophil infiltration to airways by reducing the production of CXCL1 in an free fatty acid receptor 3 (FFAR3)-dependent manner (Trompette et al., 2018). Furthermore, desaminotyrosine (DAT), a degradation product of flavonoids that is produced by *Clostridium orbiscindens*, has been shown to be protective in mice with influenza infections (Steed et al., 2017). Similar to a previous report (Ganal et al., 2012),

this protection was conferred by the induction of type I IFN signaling. These studies suggest that anti-microbial responses and the immunopathology that follows might be improved through the administration of microbial-derived metabolites.

6. Anti-tumor immunity

There is an increasing interest in the role of the microbiota on anti-tumor immune responses, including in the context of lymphoma (Yamamoto and Schiestl, 2014), colorectal (Dulal and Keku, 2014) and pancreatic cancer (Zambirinis et al., 2014), and melanoma (Sivan et al., 2015; Vétizou et al., 2015). In recent studies involving transplantable tumors, the microbiota has been shown to play a role in modulating responses to chemotherapy. GF or antibiotic-treated mice were found to be refractory to CpG-oligonucleotide immunotherapy and platinum (Iida et al., 2013) and cyclophosphamide (Viaud et al., 2013) chemotherapies. Iida et al. correlated the increased tumor growth with a reduction in TNFα and iNOS production in the myeloid cell compartment, whereas Viaud et al. found that cyclophosphamide promoted translocation of gram-positive bacteria, driving Th17 and memory Th1 responses, a phenomenon impaired in GF and antibiotic-treated mice.

In addition to chemotherapy, new studies have shown that the microbiota plays a critical role in facilitating responsiveness to immunotherapy in mice injected with melanoma (Sivan et al., 2015) and sarcoma (Vétizou et al., 2015) cell-lines, and in patients with metastatic melanoma (Gopalakrishnan et al., 2018; Matson et al., 2018; Routy et al., 2018). Sivan et al. discovered that there was a difference in tumor control by mice from different vendors with vendor mice enriched in *Bifidobacterium* having

enhanced responses to anti-PDL1 treatment. This suggested an essential role for specific microbiota communities in anti-tumor immunity (Sivan et al., 2015), which was supported by results showing that *Bacteroides thetaiotaomicron* and *Bacteroides fragilis* rescued the reduced anti-tumor immunity displayed by GF and antibiotic-treated mice upon anti-CTLA-4 immunotherapy (Vétizou et al., 2015). Furthermore, the 3 studies involving melanoma patients showed that responsiveness to anti-PD-1 therapy was associated with a fecal enrichment in certain bacterial species that may be protective, and reduction in other species that may be detrimental to tumor control. For instance, *Bifidobacterium longum* (Matson et al., 2018), *Ruminococcaceae* family (Gopalakrishnan et al., 2018) and *Akkermansia muciniphila* (Routy et al., 2018) were found to be associated with increased responsiveness to immunotherapy. Though some strains were in common between the different studies, some were different, perhaps reflecting different diets or geographical distribution of the study subjects, or pointing to beneficial metabolic processes shared by different microbial clades. Interestingly, fecal microbiota transplantation (FMT) from patients who were therapy responders into GF or antibiotic-treated mice recapitulated the better tumor control and responsiveness to immunotherapy compared with those following FMT from poor responders (Gopalakrishnan et al., 2018; Matson et al., 2018; Routy et al., 2018). *Akkermansia muciniphila* transfer into GF mice colonized with FMT from non-responder patients rescued defects in $CCR9^{+}$ $CXCR3^{+}$ $CD4^{+}$ T cells in an IL12-dependent manner (Routy et al., 2018), further suggesting a role for DCs in microbiota-dependent augmentation of anti-tumor responses and T cell activation (Sivan et al., 2015).

In conclusion, these studies imply that a specific microbiota is necessary for responsiveness to anti-tumor therapies. Further investigation into the relationship between the microbiota and anti-tumor immunity will allow better identification of responders versus non-responders ahead of therapy initiation, enabling a more personalized treatment plan for cancer.

G. Local effect of the skin microbiota

In addition to the local and systemic effects of the gut microbiota, emerging studies are beginning to elucidate the effects of the microbiota that resides in other tissues outside of the gut. These tissues include one that is known to be guarded by immune cells, the skin (Naik et al., 2012), and one that was previously considered an immune-privileged site, the eyes (Leger et al., 2017). Skin commensals have been shown to influence cutaneous immunity in the context of local infection and inflammation (Naik et al., 2012; Ridaura et al., 2018). For instance, mono-colonization of GF mice with *Staphylococcus epidermidis* (*S. epi*) resulted in increased IFNγ and IL17A production by skin T cells enabling better control of *Leishmania major* infection in an IL1-dependent manner (Naik et al., 2012), and suggesting that a skin commensal is sufficient to control local immunity. Furthermore, *S. epi* colonization of SPF mice imprinted a specific gene signature in skin CD11b$^+$ DCs to facilitate skin immunity against *Candida albicans* infection, including increased expression of *Myd88, Il18rap, Il1a, Il1b* and *Il6* (Naik et al., 2015). However, such control is likely to be context-dependent. Ridaura et al. showed that *Corynebacterium accolens* topical association at the skin increased the numbers and activation of dermal Vγ4$^+$ $\gamma\delta$ Th17 cells at steady

state, in an IL23-dependent manner, without resulting in inflammation (Ridaura et al., 2018). Interestingly, *C. accolens* colonization triggered dermal inflammation in mice fed a high fat diet but not normal chow, further confirming the contextual influence of skin commensals to local immunity and disease. This compartmentalized control of skin immunity is likely to be commensal-specific. Only association with *S. epi* but not *S. xylosus* or *S. aureus* enhanced IFNγ and IL17A production by dermal $CD8^+$ T cells (Naik et al., 2015). Similarly, *C. amycolatum* was less capable of helping topical Imiquomod (a TLR7 agonist) drive dermatitis than *C. accolens* (Ridaura et al., 2018). This type of commensal-specific responses is not limited to classical MHC-restricted T cells. Non-classical H2-M3-restricted $CD8^+$ T cells that recognize a *S. epi* epitope display unique gene signatures that correlate with immunoregulatory functions and an ability to enhance wound healing following punch biopsies (Linehan et al., 2018).

H. Summary

The rejection of solid organ transplants mostly depends on donor/recipient genetic disparities. However, a plethora of studies have now demonstrated that the composition of the microbiota can affect both local and distal immune responses, raising the possibility that it may modulate alloimmunity. Moreover, as transplanted organs differ in their microbial colonization, another intriguing question is whether organ colonization influence the half-life of allografts. Tools to address these questions include the use of antibiotic treatment as well as of GF mice, which have been utilized to demonstrate the local and distal roles of the microbiota in various health and disease models. Current clinical studies in organ transplantation have shown correlations

between changes in microbiota composition and transplant rejection. Therefore, important questions in the field are whether the microbiota plays a causal role in modulating solid organ transplant rejection and if so by which mechanisms, and whether effects are mediated by the intestinal microbiota or by the microbiota at other barrier surfaces. In Chapters II and III, I will cover experimental results that demonstrate that the microbiota is indeed an environmental factor that modulates alloreactivity and transplant outcomes in mice.

II. MATERIALS AND METHODS

Mice

Six-week-old SPF C57BL/6NHsd male and female mice were purchased from Envigo or bred at the University of CHicago. B6(C)-H2-Ab1^{bm12}/KhEgJ female mice were purchased from The Jackson Laboratory. CD45.1^{+} RAG2-knockout (RAG-KO) Marilyn TCR Tg B6 mice whose CD4^{+} T cells are specific for an H-Y antigen (Perez-Diez et al., 2007) were obtained from Dr. Charles Mainhart via Taconic Biosciences and bred in-house under SPF conditions. IFNαR-KO mice were maintained in our animal facility. B6 germ-free (GF) mice were kept in plastic isolators at the University of Chicago Gnotobiotic Research Animal Facility, and were fed autoclaved food and water. Fecal aerobic and anaerobic cultures were carried out weekly to ensure the absence of microbial colonization. All animals were used in agreement with Institutional Animal Care and Use Committee, according to the National Institutes of Health guidelines for animal use.

Antibiotic treatment

Eight-week-old B6 mice that had been cohoused to equilibrate their microbiota were divided into control and Abx treatment cages. Mice in Abx treatment cages received a daily gavage with 200 μl of Abx based on a previous report (Stefka et al., 2014) containing gentamycin (0.35mg/ml, Fresenius Kabi USA, LLC), kanamycin (5.25mg/ml, Gibco), colistin (8500U, RPI corp.), metronidazole (2.15mg/ml, Sigma) and vancomycin (0.5mg/ml, Hospira) diluted in autoclaved water. Abx were administered by

daily gavage rather than in the drinking water to ensure accurate dosing. Donor and recipient mice were treated for 10 days until the time of transplantation.

S. epi painting

S. epi bacterial stock was cultured as described previously (Naik et al., 2012). Briefly, *S. epi* was inoculated and cultured in Tryptic Soy Broth for 18 hours. The resulting culture was painted onto the skin of GF mice using cotton swabs every 2 days. Vancomycin was supplemented to the drinking water (500mg/L) starting a week before skin painting and continued until sacrifice to prevent intestinal colonization. Skin swabs and fecal samples were cultured before skin transplantation to confirm colonization status.

Transplantation

Aseptic surgery for SPF mice: Tail skin from male B6 or from BALB/c donor mice was transplanted onto the flank of female recipients. Bandages were removed after 7 days. Graft survival was monitored every other day and the day at which less than 20% viable skin tissue was left was called the day of rejection. Sterile surgery for gnotobiotic and SPF mice was performed in a biological safety cabinet as previously described (Theriault et al., 2015). Bm12 hearts were transplanted into B6 mice as previously described (Corry et al., 1973). Because this is a chronic rejection model (Yuan et al., 2008), clinical graft functional scores adapted from Tanaka et al. (Tanaka et al., 2005) were calculated as follows: heart size (small=1, large=0), speed of heartbeat (too fast to count=3, 100-200 beats/min=2, <100beats/min=1, heartbeat cessation=0).

Leukocyte isolation

Spleen, or skin-dLN (inguinal, axillary and brachial) cells were isolated at the indicated times, resuspended in complete DMEM (Corning) with 10%FBS, 1% Penicillin/ Streptomycin, 1% L-glutamine, 1% NEAA, 1% HEPES, 0.028 mM β-mercaptoehtanol and counted using a Countess cell counter. For skin leukocyte isolation, skin grafts or shaved flank were harvested. The skin was cut into small pieces, resuspended in Roswell Park Memorial Institute (RPMI, Corning) medium supplemented with Liberase (0.4mg/ml, Roche) and DNAse (0.01%, MP Biomedicals) and incubated for 2 hours at 37°C. After incubation, the skin was homogenized using a 70μm cell strainer, a petri dish and a syringe plunger. Single cell suspensions were centrifuged and pellets were resuspended in complete RPMI medium before cells were counted.

In vitro stimulation of graft-infiltrating cells

Isolated graft-infiltrating cells (5×10^6) were plated in 6-well tissue culture plates. Phorbol myristate acetate (PMA, 50ng/ml), ionomycin (0.5μg/ml) and brefeldin A (5μg/ml) were added into each well and cells were incubated for 4 hours at 37°C in 8% CO_2. Stimulated cells were harvested for staining and flow cytometry analysis.

CFSE labeling

CD45.1$^+$ RAG-KO CD4$^+$ Marilyn T cells were collected from peripheral lymph nodes and spleen. Red blood cells in spleens were lysed with ACK lysis buffer for 5 minutes. Cells from lymph nodes and spleen were combined, counted and resuspended at a concentration of 20×10^6 cells/ml in serum-free DMEM. CFSE (C34554, Life

Technologies) was diluted to a final concentration of 5μM and incubated with cells for 10 minutes at 37°C. Labeling was stopped with an equal volume of fetal bovine serum. Cells were centrifuged and resuspended in complete DMEM. For adoptive transfer, the cells were washed and resuspended in 1x phosphate buffered saline (PBS).

Adoptive transfer of Marilyn T cells

CFSE-labeled $CD45.1^+$ RAG-KO $CD4^+$ Marilyn T cells (1 x 10^6) were transferred i.v. into C57BL/6 mice one day prior to skin transplantation. At day 4 or 6 post-transplantation, skin-draining lymph nodes (brachial and axillary) were harvested and isolated leukocytes stained to assess T cell proliferation.

Ex vivo stimulation of Marilyn T cells

APCs were isolated from skin-dLNs of male or female B6 mice following magnetic enrichment with biotinylated anti-Thy1.2 (53-2.1, eBioscience) and anti-NK1.1 (monoclonal antibody facility, University of Chicago) antibodies. Marilyn T cells were magnetically enriched from the peripheral lymph nodes and spleens of Marilyn mice using streptavidin magnetic beads (#88817, Pierce), anti-Ter119-biotin (TER-119, eBioscience), anti-CD11b-biotin (M1/70, eBioscience) and anti-NK1.1-biotin (PK136, Frank Fitch Monoclonal Antibody Facility at the University of Chicago) prior to CFSE labeling. APCs (1-2x10^5/well) were cultured in triplicate with Marilyn T cells (1x10^5/well) in complete DMEM for 3 days. T cells were restimulated with PMA (50ng/ml) and ionomycin (0.5μg/ml) in the presence of brefeldin A (5μg/ml) for 4 hours at 37°C and analyzed by flow cytometry. For stimulation with female APCs, the culture was

supplemented with 1-20pM of CD4 H-Y peptide (NAGFNSNRANSSRSS), synthesized by Dr. Joel Collier (University of Chicago).

Flow cytometry

All cells were stained with 1:1000 Live Aqua Live/Dead stain (Life Technologies) prior to phenotypic staining. For graft-infiltrating cell analyses, cells were stained with anti-CD45.2-APC (104, eBioscience), anti-TCRβ-PerCP Cy5.5 (H57597, eBioscience), anti-CD4-PE-Cy7 (RM4-5, eBioscience), anti-CD8α–eF450 (53-6.7, eBioscience) and anti-TCRγδ-PE (eBioGL3, eBioscience). For cell population analyses, cells were stained with anti-CD45.2-APC (104, eBioscience), anti-TCRβ-PerCP Cy5.5 (H57597, eBioscience), anti-CD19-FITC (eBio1D3, eBioscience), anti-CD4-BV605 (RM4-5, Biolegend), anti-CD8α-BV711 (53-6.7, Biolegend), anti-NK1.1-PE (PK136, BD Pharmingen), anti-CD11c-PECy7 (N418, eBioscience), anti-CD11b-APC-Cy7 (M1/70, eBioscience) SiglecH-PerCP-Cy5.5 (551, Biolegend), CD207-PE (eBioL31, eBioscience), CD103-FITC (2E7, eBioscience) and anti-Gr-1-AF700 (RB6-8C5, eBioscience). For adoptive transfer and in vitro stimulation analyses, cultured cells were stained with anti-CD45.1-APC-eF780 (A20, eBioscience), anti-CD45.2-APC (104, eBioscience), anti-TCRβ-PerCP-Cy5.5 (H57597, eBioscience), anti-CD4-PE-Cy7 (RM4-5, eBioscience) and anti-IFNγ-PE (XMG1.2, eBioscience). For intracellular cytokine staining, cells were fixed, permeabilized and stained with BD Fixation/Permeabilization Solution Kit (Cat No: 554714, BD Bioscience) as described in the manufacturer’s protocol. All samples were analyzed with the LSR Fortessa (BD Biosciences).

Fecal reconstitution

Fresh fecal pellets from untreated or antibiotic-treated female mice were resuspended in 1ml sterile PBS. GF mice were reconstituted by oral gavage with 200μl of such suspensions, 5-7 days before skin transplantation. The status of fecal and cutaneous colonization was analyzed at different time points using qRT-PCR for bacterial load and presence of select bacterial taxa.

DNA extraction

Fecal samples were homogenized with 0.1 mm zirconia/silica beads in 1.4 ml ASL buffer (51504, Qiagen) in a Mini-Beadbeater (Biospec). DNA was extracted with the QIAamp DNA Stool Mini Kit (51504, Qiagen). Tail skin samples were incubated in enzymatic lysis buffer (20mM Tris, 2mM EDTA, 1.2% Triton X-100, pH 8.0) and lysozyme (20mg/ml) for 3 hours at 37°C. Standard protocols for DNA purification of MasterPure Yeast DNA Purification Kit (MPY80200, Epicentre Biotechnologies) and QIAamp DNA Stool Mini Kit (51504, Qiagen) were followed for all subsequent steps.

16S rRNA gene sequencing and data analysis

16S rRNA genes were amplified from purified DNA using PCR primers (F515/R806) specific for the V4-V5 region of the 16S rRNA gene, and sequenced by Illumina MiSeq at the High-Throughput Genome Analysis Core in Argonne National Laboratory, using previously described sequencing primers (Caporaso et al., 2012). Quantitative Insights Into Microbial Ecology (QIIME) software (Caporaso et al., 2010) was used for *de novo* OTU selection (with 97% identity threshold), rarefaction, and

taxonomic classification. Microbial communities were compared using the unweighted UniFrac distance metric (Lozupone and Knight, 2005) and were displayed using principal coordinate analysis (PCA) plots. The data were uploaded to the European Nucleotide Archive with the study accession number PRJEB13722.

qRT-PCR for bacterial load and taxa assays

Bacterial load of extracted fecal DNA was determined by quantitative real-time PCR with the universal primers 8F and 338R for the 16S rRNA gene, using iQ SYBR green supermix (Bio-Rad Life Science) and 7300 Real Time PCR system (Applied Biosystems). DNA was quantified against a standard curve as described (Stefka et al., 2014) and the results were normalized to the weight of fecal samples. For taxa assays, forward and reverse primers of *Blautia coccoides* and *Lactobacillus* spp. were used: 5' ACTCCTACGGGAGGCAGC 3', 5' GCTTCTTAGTCAGGTACCGTCAT 3' and 5' AGCAGTAGGGAATCTTCCA 3', 5' CACCGCTACACATGGAG 3', respectively. Data acquisition and analysis were done using iQ SYBR green supermix (Bio-Rad Life Science) and 7300 Real Time PCR system (Applied Biosystems).

Gene expression profiling of DCs

The inguinal, brachial and axillary lymph nodes were minced with a razor blade, treated with 400U of Collagenase IV (Sigma) and 0.01% DNase (MP Biomedicals Inc.) and incubated at 37°C for 30 min, followed by homogenization with a syringe plunger and 40uM filters. Cells were stained with 1:1000 Live Aqua Live/Dead stain (Life Technologies), anti-NK1.1-BV421 (PK136, BioLegend), anti-CD19-BV421 (6D5,

Biolegend), anti-TCRβ-BV421 (H57-597, BD Horizon), anti-I-A^b-PE (AF6-120.1, BD Pharmingen) and sorted into RLT buffer (Qiagen) using the BD FACS Aria II (BD Biosciences). DCs were sorted in RLT buffer from the RNeasy Micro Kit (Qiagen). RNA was isolated according to the manufacturer's protocol and analyzed at the University of Chicago Genomics Core facility. RNA integrity and concentration were assessed using Agilent Bioanalyzer 2100. RNA with RNA Integrity Number greater than 9.0 was processed into cRNA and hybridized to the Illumina MouseRef8v2 array using the manufacturer's protocol and scanned in Illumina HiScan. Quantile normalized and background-subtracted values were analyzed using R. Genes with an expression value under 25 were removed from the analysis. Mean fold change in expression level between control and Abx DCs was calculated. Genes with a fold change of 1.5 were analyzed on the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 for pathway analysis. Genes that were significantly enriched in the pathway analysis ($p<0.05$) were chosen and plotted in a heat map using R. The data were uploaded onto GEO omnibus with study accession number GSE80487.

Statistical analysis

Graft survival curves were analyzed using Kaplan-Meier plots and log rank post hoc tests. Comparisons between control and Abx samples for Shannon Index, T cell populations, APC populations, costimulatory expression, Marilyn T cell division and cytokine production were analyzed with unpaired t-test. Analysis of DC subsets and costimulatory molecules was performed with two-way ANOVA. Multiple comparisons between control Abx, GF, GF-SPF.F and GF-Abx.F samples were done with one-way

ANOVA, together with Bonferroni's comparison. P values of less than 0.05 were considered to be statistically significant. Statistical analysis was performed using Graphpad Prism 6. 'ns' not significant, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

<u>Study approval</u>

Mouse studies were approved by the Animal Resources Center of the University of Chicago under IACUC guidelines (Protocol 71095).

III. RESULTS – THE COMPOSITION OF THE MICROBIOTA MODULATES ALLOGRAFT REJECTION

A. Abstract

Transplantation can cure end-stage organ failure but T cells rapidly reject allografts. While donor/recipient genetic disparities are major determinants of the kinetics of transplant rejection, little is known about the contribution of environmental factors. Because organs exposed to the outside world have worse transplant outcome than sterile organs, we tested the influence of host and donor microbiota on skin transplant rejection. Survival of minor antigen-mismatched skin grafts was prolonged in conventional mice when both donors and recipients were pre-treated with broad-spectrum antibiotics (Abx) prior to transplantation, as well as in germ-free (GF) mice, when compared to controls. This correlated with reduced ability of antigen-presenting cells (APCs) to activate the type I IFN pathway and to prime alloreactive T cells. Colonization of GF mice with fecal material from untreated, but not Abx-pre-treated, conventional mice restored the ability of APCs to prime alloreactive T cells and accelerated graft rejection, supporting a role for specific taxa rather than bacterial load in modulating alloimmunity. Abx-pre-treatment also delayed rejection of major-mismatched skin and MHC class II-mismatched cardiac allografts. This study suggests targeting microbial constituents as a potential therapeutic strategy for enhancing graft acceptance.

B. Introduction

Although solid organ transplantation is the only cure for end-stage organ failure, recognition of the transplanted tissue as foreign invariably leads, in the absence of immunosuppression, to its acute rejection in a T cell-dependent manner. The kinetics of graft rejection mostly depend on the extent of genetic disparities between the donor and the recipient, as allelic differences can result in protein polymorphisms recognized by alloreactive host T cells. Whether environmental factors modulate the intensity of the alloresponse is not clear. We have previously reported that infections with *Listeria monocytogenes* or *Staphylococcus aureus* at the time of transplantation can increase alloreactivity and prevent the induction of transplantation tolerance by costimulation-blocking agents (Ahmed et al., 2011; Wang et al., 2010). This led us to consider whether more ubiquitous bacteria such as the commensal communities that colonize donors and recipients might also modulate alloreactivity. Indeed, it is now well established that the microbiota can modify local and distal immune responses (Belkaid and Hand, 2014), thus raising the possibility that it might fine-tune alloreactivity. Several lines of evidence indirectly support this hypothesis. First, organs colonized with bacteria such as lung and intestine have poorer outcomes than organs considered sterile such as heart and kidney (OPTN/SRTR database, December 2012). Second, the survival of minor-mismatched skin grafts is prolonged when both donor and recipient lack MyD88-dependent TLR signaling (Goldstein et al., 2003), although the relative contribution of damage- versus microbe-associated molecular patterns has not been elucidated. Third, emerging clinical literature suggests that acute rejection of intestine and lung may be associated with shifts in bacterial composition (Charlson et al., 2012; Oh et al., 2012),

although it is not yet clear whether such shifts are a cause or a consequence of acute rejection. Therefore, direct assessment of the role of the microbiota on alloimmunity is needed.

C. Results

1. Minor-mismatched skin graft survival was prolonged in Abx-pre-treated mice

To determine the consequence of reduced bacterial diversity on allograft outcome, 6-8 week-old male and female purchased C57BL/6 (B6) mice were left untreated or received a daily gavage of Abx for 10 days. Skin from male donors was transplanted onto the flank of female mice 1 day later and recipients did not receive further Abx treatment. Abx-pre-treated recipients of skin grafts from Abx-pre-treated donors displayed prolonged skin allograft survival (Mean Survival Time, MST=53+/-23 days) compared to controls (MST=27+/-12 days) (Figure 1.1A). Similar results were obtained when comparing untreated and Abx-pre-treated in-house bred littermates derived from the same maternal lineage to minimize microbiota differences between groups prior to treatment (Figure 1.2A). In contrast, Abx pre-treatment of donors or recipients alone did not delay rejection (Figure 1.1A), indicating that Abx-induced changes in both the donor skin and the recipient mice are required for improved graft survival.

To understand the changes that had occurred in the microbiota prior to transplantation, bacterial DNA was extracted from tail skin and fecal samples of female hosts. This Abx regimen in adult mice did not reduce overall fecal bacterial burden as

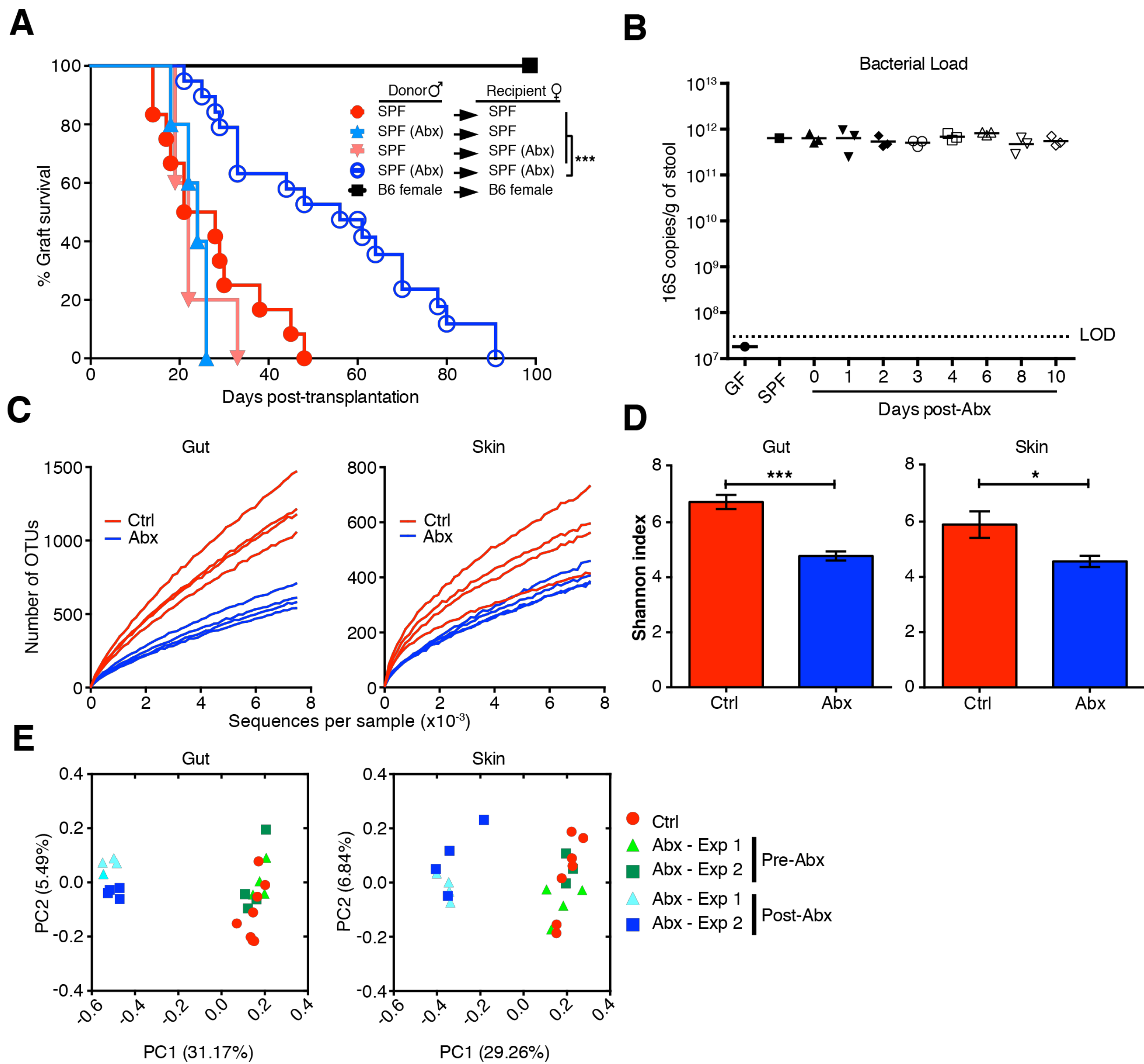


Figure 1.1. Abx pre-treatment results in prolonged skin graft survival and reduced bacterial diversity. (**A**) B6 females untreated or pre-treated for 10 days with Abx received a skin graft from B6 males untreated or pre-treated for 10 days with Abx. SPF → SPF, n=12; SPF (Abx) → SPF, n=5; SPF → SPF (Abx), n=5; Abx → Abx, n=19; Syngeneic, n=5. Log rank test. (**B**) Bacterial load by qPCR in fecal samples of GF, and SPF B6 females at the indicated time points after initiation of Abx treatment. n=3 (1 SPF and 1 GF samples shown for reference), LOD, level of detection (**C-E**) Bacterial DNA was isolated from gut and skin of female SPF controls and aged-matched mice on day 10 of Abx treatment and was analyzed by high throughput sequencing. Data are displayed as richness (**C**), diversity (**D**), principal component analysis (**E**). Each line (**C**) and dot (**B, E**) represents an individual mouse. Bars **(D)** represent the mean +/- SEM of 4 mice per group; student t-test. (**C, D, E**) Results are representative of 4 experiments with n = 3-4.

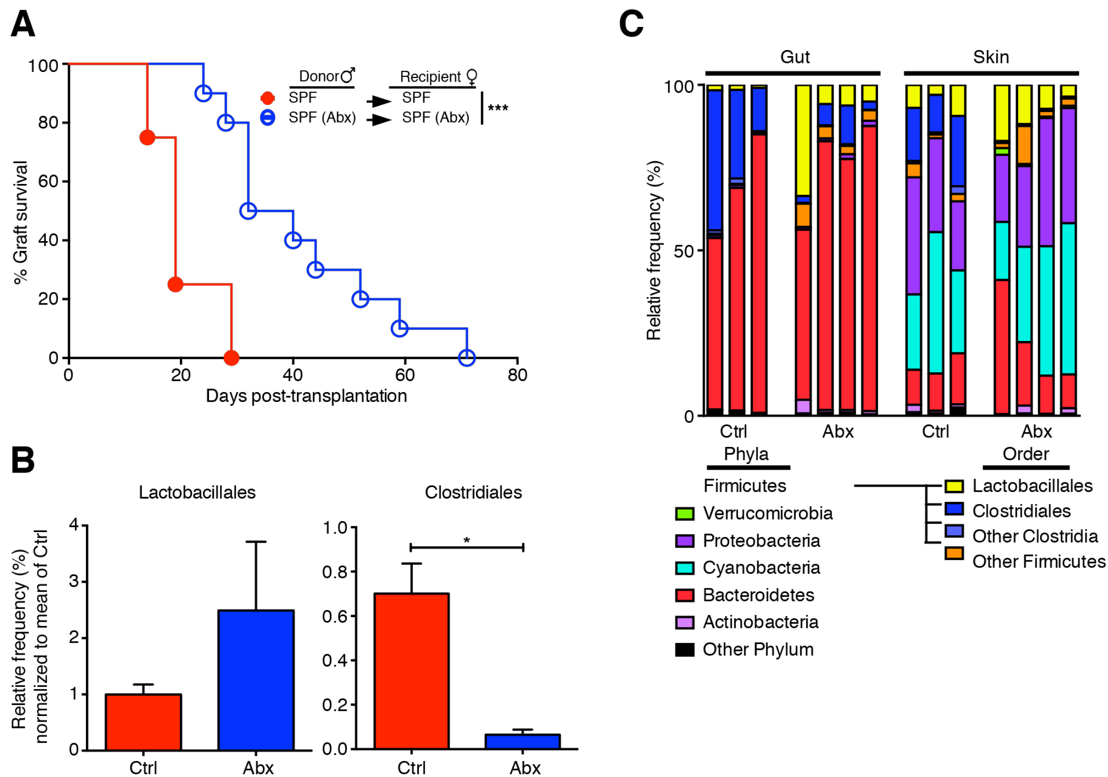


Figure 1.2. Abx treatment prolonged minor-mismatched skin graft survival and altered bacterial composition in both gut and skin. (**A**) In-house bred B6 littermates were used as male donors of skin grafts and female recipients. Untreated, n = 4, Abx-pre-treated n = 10. Log rank test. (**B,C**) Fecal and skin samples were harvested and sequenced using the 16S MiSeq platform. (**B**) Relative frequency of bacterial phyla and order in Ctrl mice and mice on day 10 of Abx treatment. Each bar represents an individual mouse. Representative of 4 independent experiments. **(C)** Normalized relative frequencies of *Lactobacilliale* and *Clostridiales.* Data were pooled from 5 individual experiments with n = 3-4 for Ctrl and n = 4-5 for Abx groups. Student t-test. Data represent the mean +/- SEM.

determined by real time PCR using universal primers for the 16S rRNA gene (Figure 1.1B), in contrast with the reduced bacterial load that occurs if a similar regimen is started in 2 week-old mice prior to weaning (Stefka et al., 2014). However, 10-day Abx pre-treatment resulted in significantly reduced bacterial richness and diversity on the day of transplantation (Figure 1.1C,D) in both fecal and skin samples, as determined by 16S rRNA sequencing using the MiSeq platform. Furthermore, principal component

analysis (PCA) showed that microbial communities of fecal and skin samples on day 10 of Abx-pre-treatment clustered separately from those of the control and the pre-treatment groups (Figure 1.1E). In particular, Abx pre-treated mice had a relative expansion of *Lactobacillales* in their feces and a reduction of *Clostridiales* in both feces and skin (Figure 1.2B,C). Thus, Abx pre-treatment changed the composition of the microbiota, but not the overall bacterial load.

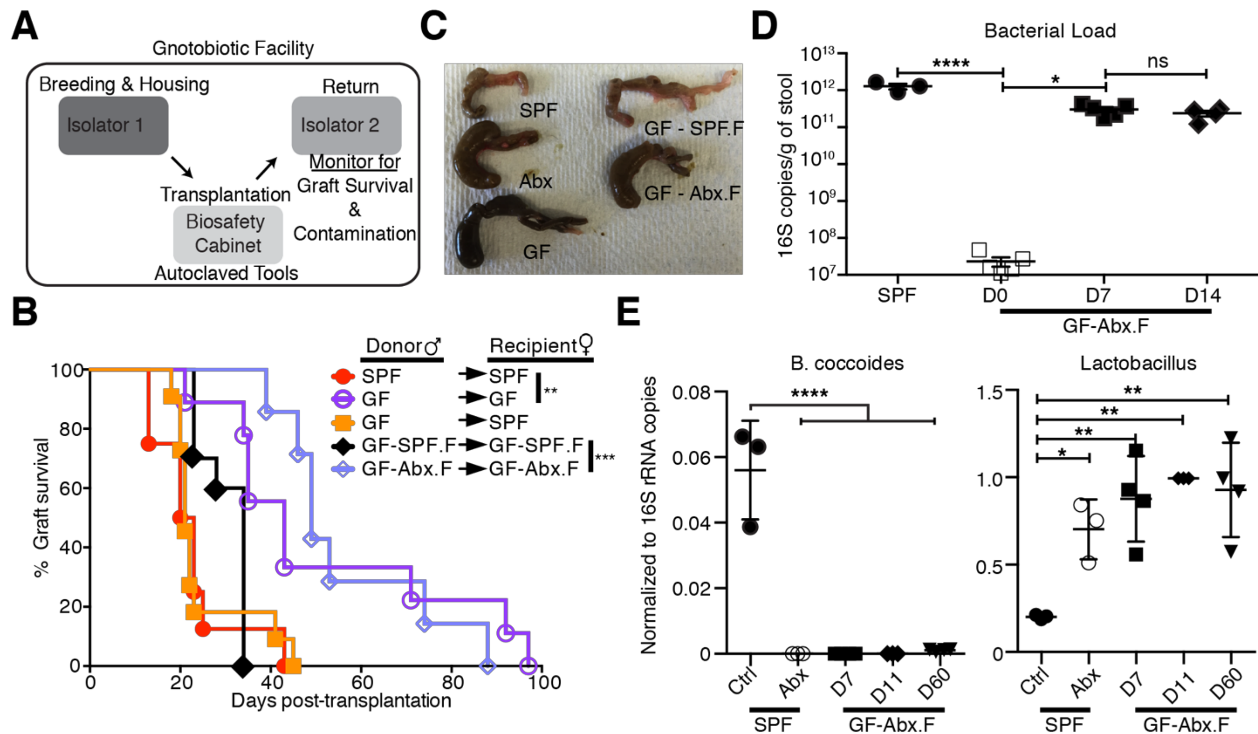


Figure 1.3. GF mice display prolonged skin graft survival. (**A**) Schematics of transplantation in the gnotobiotic facility. (**B**) B6 female SPF, GF and GF mice gavaged with PBS-diluted fecal material from control SPF (GF-SPF.F) or Abx-pre-treated SPF mice (GF-Abx.F) 5-7 days prior were transplanted with skin grafts from the indicated male donors. SPF → SPF, n=8; GF → GF, n=8; GF → SPF, n=11; GF-SPF.F → GF-SPF.F, n=10; GF-Abx.F → GF-Abx.F, n=7. Log rank test. (**C**) Representative cecum appearance at day 7 post-gavage. (**D**) qPCR of 16S rRNA gene to assess bacterial load in SPF, GF and GF-Abx.F mice before and after gavage. n=3-5. One-way ANOVA. (**E**) Normalized abundance of *B. coccoides* and *Lactobacillus* spp. as determined by qPCR in feces of SPF, 10- day Abx-treated SPF mice and GF-Abx.F mice post-fecal gavage. n=3-5. One-way ANOVA. Data are representative of 2 experiments (**D, E, F**) or combined from 2-3 experiments (**B**). (**B, E**) Data represent the mean +/- SEM.

2. Minor-mismatched skin allograft survival was prolonged in GF mice

As a second approach to test the role of the microbiota on graft outcome, we used GF mice, devoid of live bacteria, and transplanted them in a biological safety cabinet in the gnotobiotic facility using sterile techniques (Figure 1.3A) (Theriault et al., 2015). GF female recipients of GF male skin transplants also displayed prolonged graft survival when compared to specific pathogen-free (SPF) mice transplanted sterilely (Figure 1.3B). This was not due to a reduced susceptibility of GF skin to rejection, as GF grafts were rejected as promptly as SPF grafts by SPF mice (Figure 1.3B). To test the causal role of the microbiota, GF mice received a gavage with feces from SPF mice 5-7 days prior to transplantation (GF-SPF.F). Although the microbial communities that established in the intestine of GF-SPF.F mice differed from those in the donor fecal samples (Figure 1.4A,B), restoration of the microbiota was sufficient to accelerate rejection (Figure 1.3B), indicating that prolonged graft survival in sterile GF mice is due at least in part to their lack of microbiota.

To determine if all microbial communities accelerated graft rejection, GF mice were gavaged with feces from Abx-pre-treated SPF mice (GF-Abx.F) prior to transplantation. In contrast to GF-SPF.F mice, GF-Abx.F mice did not display faster rejection than GF mice (Figure 1.3B) and their cecal appearance remained enlarged as is typical of GF mice (Itoh and Mitsuoka, 1985) (Figure 1.3C), despite a bacterial load close to that in SPF mice (Figure 1.3D). The dysbiosis induced by Abx pre-treatment in the fecal donors was transferred and remained stably established in GF-Abx.F recipients that were not exposed to other environmental bacteria, as supported by the

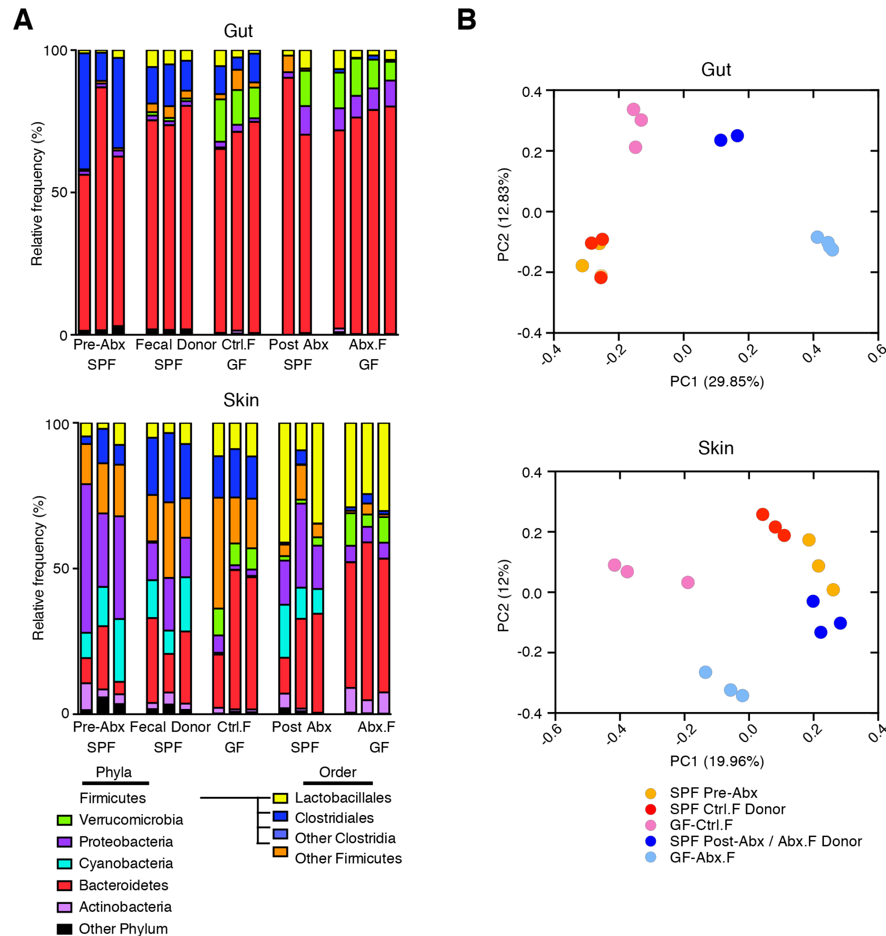


Figure 1.4. Reconstituted GF mice had different microbial communities than their corresponding fecal donor. Fecal and skin bacterial DNA was sequenced using the 16S MiSeq platform. (**A**) Relative frequency of bacterial phyla and order in SPF mice prior to Abx treatment (pre-Abx), in SPF mice on day 10 of Abx treatment (post-Abx), or in GF mice 7 days post oral gavage with fecal material from untreated SPF mice (GF-Ctrl.F) or from 10-day Abx-treated SPF mice (GF-Abx.F). (**B)** Principal component analysis. Data were pooled from 2 individual experiments with n = 2-4.

persistent contraction of *Blautia coccoides* [formerly classified as *Clostridium coccoides* (Liu et al., 2008)] and expansion of *Lactobacillus* spp. (Figure 1.3E), although their total bacterial communities were still distinct from those in the donor fecal samples (Figure 1.4A,B). Interestingly, fecal transfer into GF mice not only resulted in intestinal but also

skin colonization (Figure 1.4A,B), such that whether it is the gut and/or skin microbiota that influences skin graft outcome remains to be elucidated.

3. Abx-pre-treatment resulted in reduced alloimmunity

To investigate if improved graft survival in Abx-pre-treated mice was due to reduced alloimmunity, we analyzed graft-infiltrating leukocytes isolated on day 10 post-transplantation. Abx pre-treatment was associated with a reduced percentage of $CD4^+$ T cells and fewer IFNγ-producing cells among them (Figure 1.5A-C). To test if the reduced percentage of intra-graft effector $CD4^+$ T cells was due to diminished expansion of alloreactive T cells, H-Y-specific $CD45.1^+$ congenic $CD4^+$ TCR/RAG-KO-Tg (Marilyn) T cells were CFSE-labeled and adoptively transferred into 10-day-Abx-pre-treated female recipients 1 day prior to transplantation with skin grafts from Abx-pretreated male donors. Marilyn T cells isolated 4 days later from the skin graft-draining lymph nodes (dLNs), the site of initial T cell priming after skin transplantation (Reed et al., 2003), displayed reduced proliferation in Abx pre-treated mice compared with untreated hosts (Figure 1.5D,E). Thus, Abx pre-treatment triggered reduced priming of graft-reactive T cells, supporting the conclusion that the microbiota that associates with SPF B6 mice in our colony promotes the activation of alloreactive T cells and/or that Abx-pre-treatment promotes a microbial community that reduces alloreactivity.

In order to modulate T cell priming, microbial signals might directly or indirectly affect T cells, APCs, or both. To determine if the Abx pre-treatment affected APCs, we isolated T and NK cell-depleted APCs from the skin-dLNs of Abx pre-treated or control SPF male mice and used them to stimulate CFSE-labeled Marilyn T cells from naïve mice in vitro. APCs from Abx-treated mice stimulated Marilyn T cells less vigorously

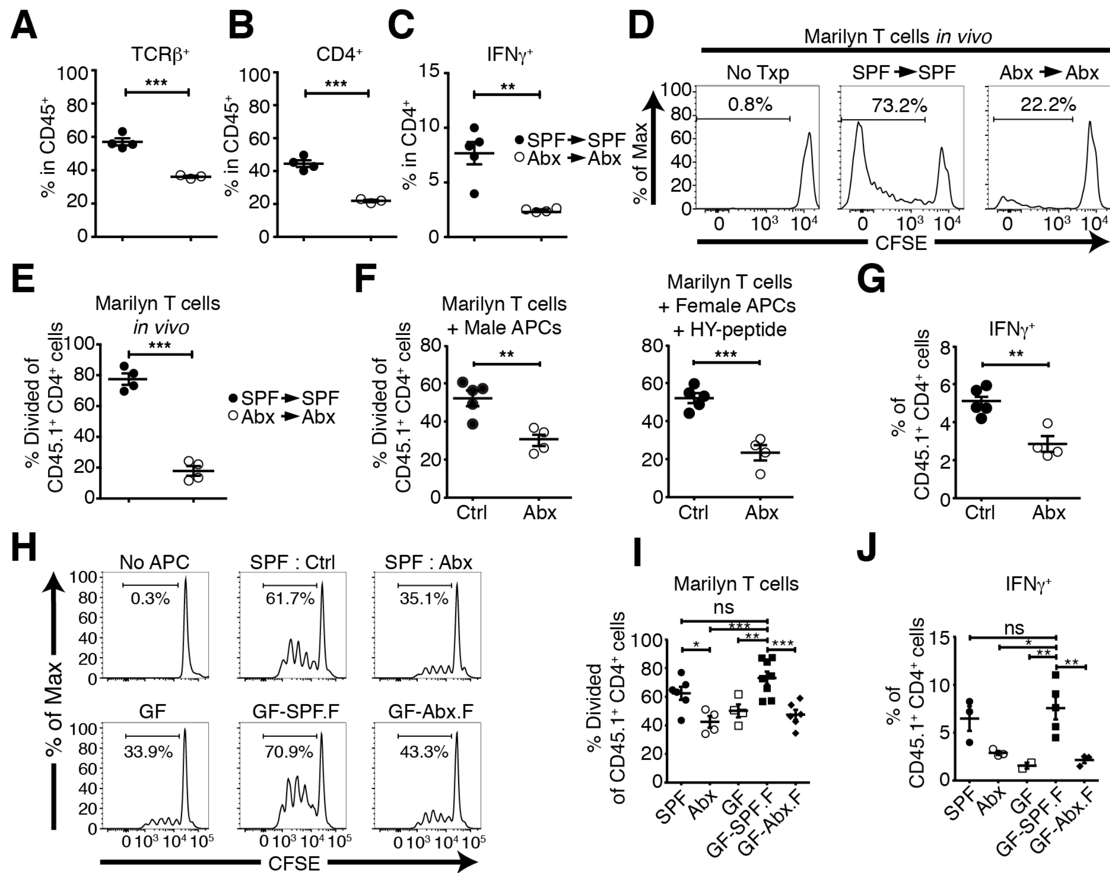


Figure 1.5. Abx pre-treatment and GF status result in reduced allogeneic T cell priming. (**A-C**) Graft-infiltrating cells were isolated from control and Abx-pre-treated SPF mice on day 10 post-transplantation for flow cytometric analysis to determine the percent TCRβ⁺ (**A**) or CD4⁺ (**B**) cells among CD45⁺ cells, and the percent IFNγ-producing cells (**C**) among PMA/ionomycin-stimulated CD4⁺ cells. (**D, E**) Congenic Marilyn T cells were labeled with CFSE and transferred (10^6 cells/mouse) into SPF female recipients 1 day prior to transplantation with male skin grafts; donors and recipients were both untreated or both Abx-pre-treated. Mice were sacrificed 4 days post-transplantation and cells were isolated from the graft-dLNs for analysis of CFSE dilution. Representative plots (**D**) and quantitation (**E**) of divided Marilyn T cells. (**F, G**) APCs from skin-dLN cells were isolated from control or 10-day Abx-pre-treated SPF B6 males or females, and cultured with CFSE-labeled T cells from naïve Marilyn females. (**F**) Quantitation of CFSE dilution in Marilyn-gated T cells on day 4 of the culture. (**G**) Percent of IFNγ⁺ cells among Marilyn T cells after a 3-day culture and following restimulation with PMA/ionomycin for 4 hours. (**A-**G) n=3-5 mice per group. Experiments were repeated 3-4 times. No Txp, no transplant. Student t-test. (**H**) Marilyn T cell-CFSE dilution after culture as in (**F**) with male APCs from different groups. APCs were harvested 7 days post-gavage of GF mice. Quantitation of divided Marilyn T cells (**I**) and IFNγ production after restimulation (**J**). n= 2-8. One-way ANOVA. Data represent the mean +/- SEM.

than control APCs (Figure 1.5F). Marilyn T cell proliferation was also reduced when stimulated with H-Y peptide-pulsed APCs from Abx-pre-treated females (Figure 1.5F), when compared to peptide-pulsed APCs from untreated females, suggesting that the APC defect is unlikely to be at the antigen-processing level. Marilyn T cells stimulated with APCs from Abx-treated mice also produced less IFNγ (Figure 1.5G). Differences in T cell-priming ability were not due to an alteration in the composition of skin-dLN APCs after Abx treatment, as the proportion of B cells, CD11b$^+$ cells, CD11c$^+$ dendritic cells (DCs), as well as of subsets of CD103$^+$, CD8α^+ or Siglec-H$^+$ DCs was comparable in

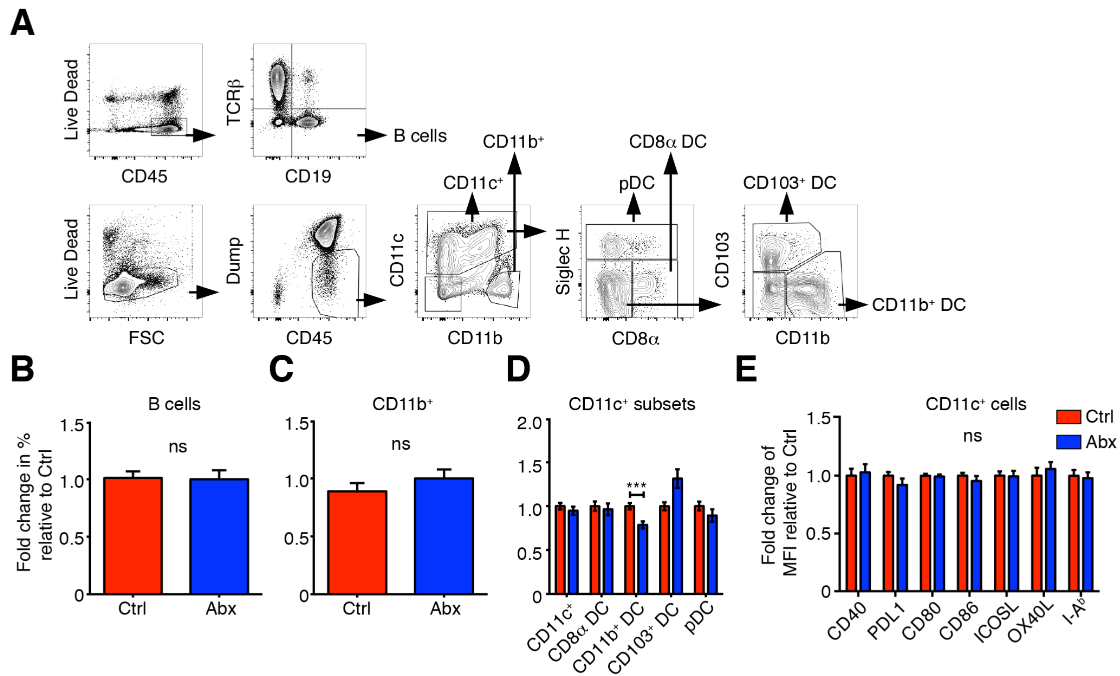


Figure 1.6. Abx pre-treatment did not alter the composition of APCs in dLN. APCs from the peripheral LNs of Abx mice were analyzed by flow cytometry **(A)** Gating strategy for B cells, CD11b$^+$ cells and DCs. **(B-D)**. Fold change relative to controls in percentage of B (**B**), CD11b$^+$ (**C**), and CD11c$^+$ cells (**D**). Student t test. **(E).** Fold change in mean fluorescence intensity (MFI) relative to that in controls for costimulatory molecules on CD11c$^+$-gated cells. One-way ANOVA. Data were normalized from 3 individual experiments with n = 5 per group. Data represent the mean +/- SEM.

control and Abx-treated mice (Figure 1.6A-D), with the exception of a reduction in $CD11b^+$ DCs. Moreover, expression levels of CD40, PDL1, CD80, CD86, ICOSL, OX40L and $I\text{-}A^b$ were similar on Abx- and control DCs (Figure 1.6E). Thus, Abx treatment reduced the capacity of APCs to prime alloreactive T cells. In the gnotobiotic setting, skin-dLN APCs from male GF and GF-Abx.F mice also induced less proliferation and IFNγ production by Marilyn T cells than did APCs from SPF or GF-SPF.F mice (Figure 1.5H-J). Together, these data argue against a direct effect of oral Abx on immune cells or skin grafts and suggest instead that Abx-sensitive taxa play a critical role in poising skin-dLN APCs for alloreactive T cell priming.

4. Abx treatment downregulates the type I IFN pathway in DCs

Since DCs are the main APC subset that activates alloreactive H-Y-specific T cells (Atif et al., 2015), skin-dLN $CD11c^+$ DCs were sorted from control and 10 day-Abx-treated mice for gene expression profiling. DCs from Abx-treated mice displayed reduced expression of genes associated with the type I IFN pathway, such as IRF3, IRF7, OAS2, OAS1G, OASL1 and OASL2, as well as of genes related to activation of the NF-κB pathway, such as RSAD2, SPHK1, IRAK2. In addition, genes involved in cytokine regulation and production were also significantly less expressed in DCs from Abx-treated than control mice (Figure 1.7A,B). In keeping with this gene profiling analysis, survival of type I IFNαR-deficient male skin grafts was significantly prolonged in type I IFNαR-deficient females when compared to control mice (Figure 1.7C), supporting a role for type I IFN signaling in the response to $H\text{-}Y^+$ skin grafts. IFNαR

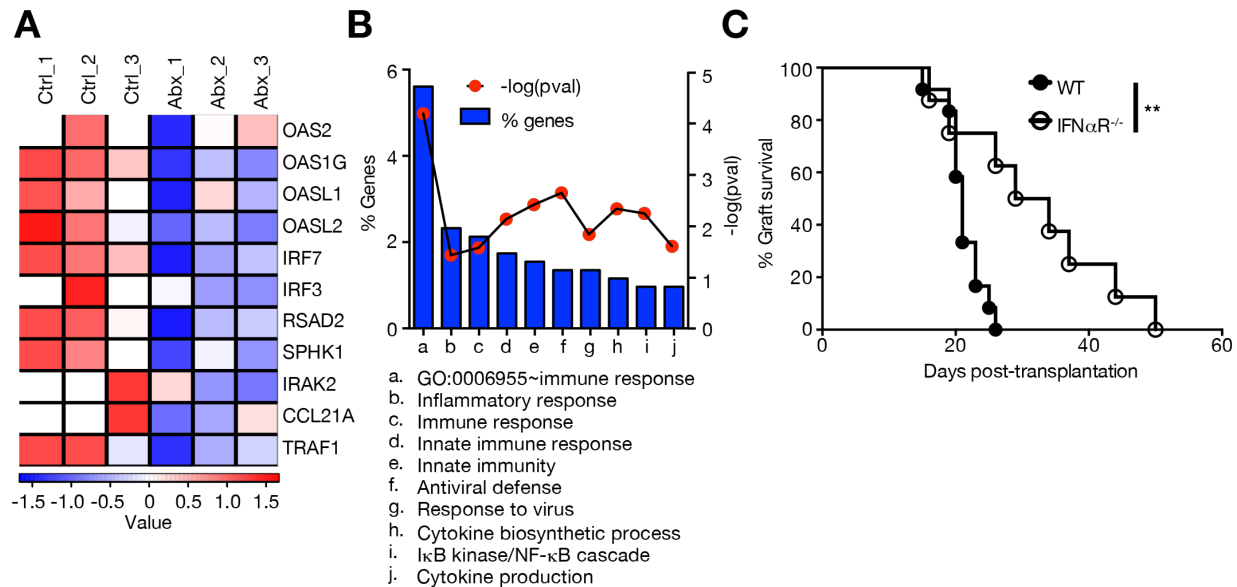


Figure 1.7. Abx pre-treatment results in downregulation of the type I IFN pathway. (**A, B**) DCs from skin-dLN of control and 10-day-Abx-pre-treated SPF mice were sorted and cDNA was analyzed for gene expression profiling. (**A**) Examples of genes differentially expressed (n=3 per group). (**B**) DAVID analysis of enriched pathways in DCs from control relative to Abx-treated groups. (**C**) Female B6 control and IFNαR$^{-/-}$ SPF mice were transplanted with skin grafts from males of the host genotype. Results are combined from 2 experiments. WT, n=12; IFNαR$^{-/-}$, n=8. Log rank test.

expression had to be absent in both donor and recipient mice for graft survival to be prolonged (not shown). Notably, Abx pre-treatment of IFNαR-deficient mice did not prolong survival compared to untreated counterparts as they all rejected within 50 days (not shown), further suggesting that the effects of Abx pre-treatment were dependent at least in part on a reduction of the type I IFN pathway.

5. Abx pre-treatment also prolongs survival of major-mismatched skin and MHC class II-mismatched cardiac allografts

It was conceivable that the effects of the microbiota on graft outcome were limited to the H-Y skin graft mouse model, or applicable only to transplantation of

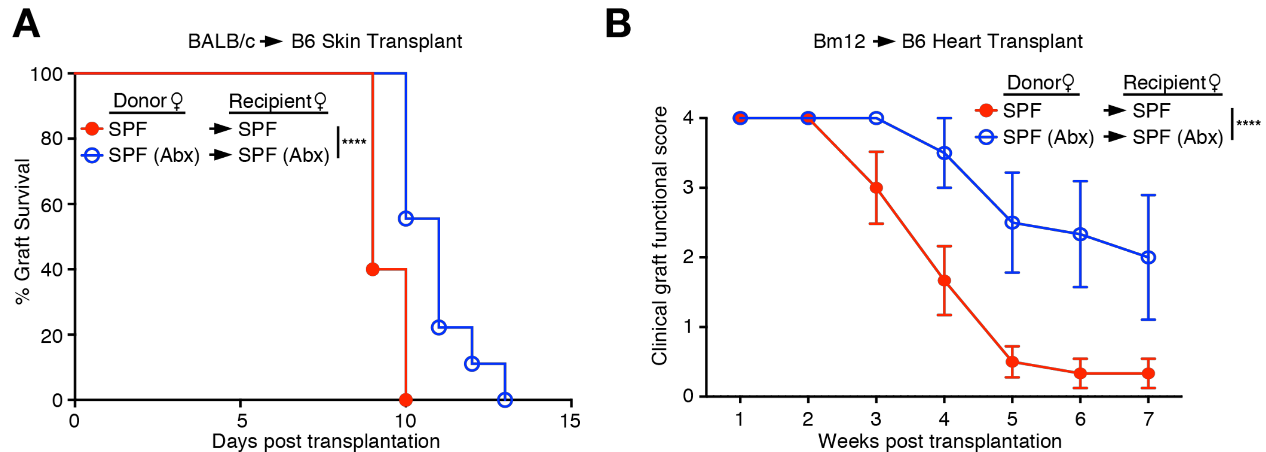


Figure 1.8. Abx pre-treatment delays rejection of major-mismatched skin and Class II-mismatched cardiac allografts. (**A**) Graft survival of BALB/c skin transplanted onto B6 recipients. Untreated, n = 5; Abx-pre-treatment, n = 9. Log rank test. (**B**) Clinical graft functional score of Bm12 heart transplanted into B6 recipients. n = 6 per group. Two-way ANOVA. Data represent the mean +/- SEM.

colonized organs. To this end, donor and recipient mice were pre-treated with Abx prior to transplantation of BALB/c skin or Bm12 heart into B6 animals. Abx pre-treatment resulted in a significant delay in graft rejection in both models (Figure 1.8A,B), arguing for a more generalizable effect of the microbiota on allograft rejection.

D. Discussion

Collectively, our results suggest that Abx pre-treatment reduces the priming capacity of alloreactive T cells by skin-dLN APCs, in part by impairing activation of the type I IFN pathway.

Our study demonstrates that bacterial communities existing in the fecal samples of untreated, but not Abx-pre-treated, SPF mice can accelerate skin graft rejection after colonization of GF mice. This result underscores remarkable differences in the physiological effects of these bacterial communities based on their composition rather

than their load, and suggests that the antibiotics have eliminated species with key microbial products or metabolic functions for the priming of alloreactive T cells, or enabled growth of inhibitory species. It is important to note that fecal gavage not only resulted in established intestinal species in the reconstituted GF mice, but also in colonization of their skin (Figure 1.4A), presumably because the fur is in contact with soiled bedding in the gnotobiotic cages. Thus, it is yet unclear whether it is the microbial constituents of the intestine or of the skin that can promote alloreactivity. Indeed, defined skin commensals have recently been shown to modify local cutaneous immunity, affecting skin dendritic cells and resident T cells and enhancing barrier immunity (Naik et al., 2015). The intestinal microbiota has also been shown to affect immunity as distal as the peripheral joints or the central nervous system in models of autoimmune arthritis or autoimmune encephalomyelitis (Lee et al., 2011b; Wu et al., 2010).

Gene expression profiling of dendritic cells isolated from the lymph nodes of Abx-pre-treated mice revealed reduced expression of genes in the NF-κB and type I IFN pathways. These pathways have been previously associated with transplant rejection, as NF-κB-inhibition in DCs with decoy oligodeoxyribonucleotides has been shown to promote allograft survival (Giannoukakis, 2000). Similarly, type I IFN therapy has been associated with liver rejection in the clinic (Stravitz et al., 2004), and viral and bacterial infections that induce a type I IFN response were reported to prevent the induction of transplantation tolerance (Cook et al., 2008; Stapler et al., 2008; Wang et al., 2008). Peritoneal and splenic mononuclear phagocytes have been shown to require signals from the microbiota to elicit NK cell priming and antiviral immunity, including expression

of type I IFN genes, and to promote an activating epigenetic transcriptional landscape for cytokine production (Ganal et al., 2012). Recent work from our group has identified select bacterial species within the Bifidobacterium genus capable of inducing the type I IFN pathway in dendritic cells, resulting in improved tumor control (Sivan et al., 2015). Our results indicate that the microbiota and type I IFNR signaling promote rejection of H-Y$^+$ skin grafts, and that Abx may eliminate bacterial species that normally poise skin-dLN APCs for stimulation of alloreactive T cells, or favor outgrowth of species that do not activate the type I IFN pathway in skin-dLN APCs. Finally, the APC-poising effects are rapidly reversible suggesting a continuous dialogue between the microbiota and the immune system, as APCs from SPF mice can lose their priming function upon a 10-day Abx treatment, and APCs from GF mice can regain it upon a 5-7-day exposure to fecal microbiota from control SPF but not Abx-pre-treated mice.

These studies raise the question of which microbial-associated molecular patterns may be responsible for signaling to the skin-dLN APCs to prepare them to stimulate T cells. The more extensive survival of male skin grafts by female hosts observed when both male donors and female recipients are deficient in MyD88-KO (Goldstein et al., 2003) compared to the effects of Abx-pre-treatment in our study suggests the possibility that both microbial and sterile inflammation ligands additively induce MyD88-dependent signaling following H-Y skin transplantation. Indeed, damage-associated molecules such as haptoglobin have been shown to contribute to the rejection of male skin grafts in a MyD88-dependent manner, with skin grafts from haptoglobin-deficient mice eliciting slower acute rejection than control skin grafts (Shen et al., 2012). In contrast, whether the pro-rejection effects of the microbiota are MyD88-

dependent or not remains to be investigated. The fact that Abx-pre-treatment resulted in prolonged survival of fully MHC mismatched allogeneic skin grafts in our study whereas rejection kinetics of major mismatched skin grafts were unmodified compared to controls when both donors and recipients lacked MyD88 (Goldstein et al., 2003) suggests that the microbiota may exert at least some of its effects in a MyD88-independent manner. Many pattern-recognition receptors can recognize microbial molecular patterns and signal in a MyD88-independent manner (Brubaker et al., 2015), including TLR3 and TLR4, Nod-like receptors, C-type lectin receptors and sensors in intracellular DNA and RNA. Moreover, the microbiota can elaborate metabolic products that may have local or distal immune effects independently of the expression of pattern-recognition receptors. For instance, Clostridia can generate short chain fatty acids that have been shown to promote intestinal Treg differentiation (Arpaia et al., 2013; Atarashi et al., 2013; Furusawa et al., 2013; Smith et al., 2013). Thus, the microbial products capable of promoting allograft rejection and the sensors that recognize them need to be identified. In addition, whether immune cells are directly targeted by such microbial products, or whether dendritic cells are poised by signals elicited by intermediary cells such as mucosal or epidermal cells will need to be established. Our observation that Abx-pre-treatment can prolong survival of cardiac allografts, which are considered sterile, demonstrates distal effects of the microbiota, further suggesting that microbes, their products or the local cells they instruct presumably circulate to sites of priming or effector function of alloreactive T cells. It is conceivable that incisions to the skin for any kind of surgery, including cardiac transplantation, may allow some cutaneous bacteria to enter the bloodstream and signal at distant sites, and/or that intestinal bacterial

translocation during open abdominal surgery, as is the case in our model of heterotopic cardiac transplantation, may contribute to the enhanced alloimmunity observed in colonized compared with GF animals. Indeed, portal endotoxin has been detected after abdominal surgery that did not involve intestinal sectioning (Abdala et al., 2007).

The demonstration that the microbiota is an important environmental factor that continuously modulates the activation state of skin-dLN APCs and their ability to prime alloreactive T cells points to the control of the composition of the microbiota as a potential therapeutic target to improve transplant outcomes.

IV. RESULTS – A SINGLE SKIN BACTERIAL SPECIES IS SUFFICIENT TO ACCELEREATE SKIN ALLOGRAFT REJECTION

A. Abstract

Solid organ transplantation can treat end-stage organ failure, but the half-life of transplanted organs colonized with commensals is much shorter than that of sterile organs, suggesting that the extent of microbial colonization may impact organ graft survival. However, whether organ colonization by bacteria plays a causal role in this shorter half-life is not known. We have previously shown that an intact microbiota can accelerate the kinetics of minor-mismatch solid organ allograft rejection in untreated colonized mice when compared to germ-free (GF) or antibiotic-pre-treated colonized mice, and that this is associated the capacity of antigen-presenting cells (APCs) to activate alloreactive T cells. However, the contribution of intestinal versus skin microbiota to these effects was not clear. Here, we demonstrate that colonization of the skin either the donor or the recipient with a single commensal, *Staphylococcus epidermidis* (*S. epi*), while preventing intestinal colonization with oral vancomycin, was sufficient to accelerate skin graft rejection. Notably, unlike the mechanism by which whole-body microbiota accelerates skin graft rejection, *S. epi* colonization on both donor and recipient skin did not enhance the priming of alloreactive T cells in the skin-draining lymph nodes (LNs). Rather, cutaneous *S. epi* augmented the ability of skin APCs to drive alloreactive T cell proliferation and IFNγ production. This study reveals that extra-intestinal microbiota can affect transplant outcome.

B. Introduction

Solid organ transplantation is an accepted therapy for end-stage organ failure. Depending on the type and location of the transplanted organ, the level of commensal colonization varies. Sterile organs, such as heart and kidney, have a longer half-life following transplantation than barrier organs that are colonized with commensal bacteria, such as intestine and lungs (Organ Procurement and Transplantation Network (OPTN), https://optn.transplant.hrsa.gov/data/; Scientific Registry of Transplant Recipients (SRTR), http://www.srtr.org/, December 2012). This clinical observation prompted us to investigate whether the microbiota in barrier organs could promote graft rejection.

Numerous studies have established that the gut microbiota plays a critical role in local immune development and function (Belkaid and Hand, 2014), for instance inducing lamina propria regulatory T cells (Atarashi et al., 2011, 2013) and Th17 cells (Goto et al., 2014; Ivanov et al., 2009). The gut microbiota can also affect immune responses at distal locations (Lee et al., 2011b; Sivan et al., 2015; Teng et al., 2016; Wilmore et al., 2018), such that, in theory, the gut microbiota might affect alloimmunity to any transplanted organ, whether sterile or colonized. Indeed, we previously showed that oral administration of broad-spectrum antibiotics reduced the diversity of the gut microbiota and resulted in prolonged survival of both heart and skin allografts (Lei et al., 2016). However, whether the microbiota within the barrier organ itself can affect alloimmunity or graft outcome after it is transplanted had not yet been determined. In fact, our mice gavaged with broad-spectrum antibiotics displayed reduced microbial diversity not only of the intestine, but also of the skin (Lei et al., 2016), making it important to understand

whether target organ-restricted microbiota can affect the outcome of that transplanted organ.

S. epi, a skin commensal, has been shown to calibrate cutaneous T cell responses and local inflammation to fight local *Leishmania major* infection (Naik et al., 2012). This tissue-specific barrier immunity was attributed to interactions between *S. epi* and dermal $CD103^+$ and $CD11b^+$ DCs (Naik et al., 2015). Given the influence of a skin commensal to a local infectious immune response, we investigated the impact of skin *S. epi* on skin allograft outcome.

C. Results

1. *S. epi* colonization on the skin accelerates skin allograft rejection

To investigate the impact of a skin commensal on graft rejection, *S. epi* was painted on the skin of male and female C57BL/6 GF mice prior to transplantation of male skin onto female recipients (Figure 2.1A). To prevent gut colonization, vancomycin was supplemented continuously to the drinking water starting before *S. epi* painting.

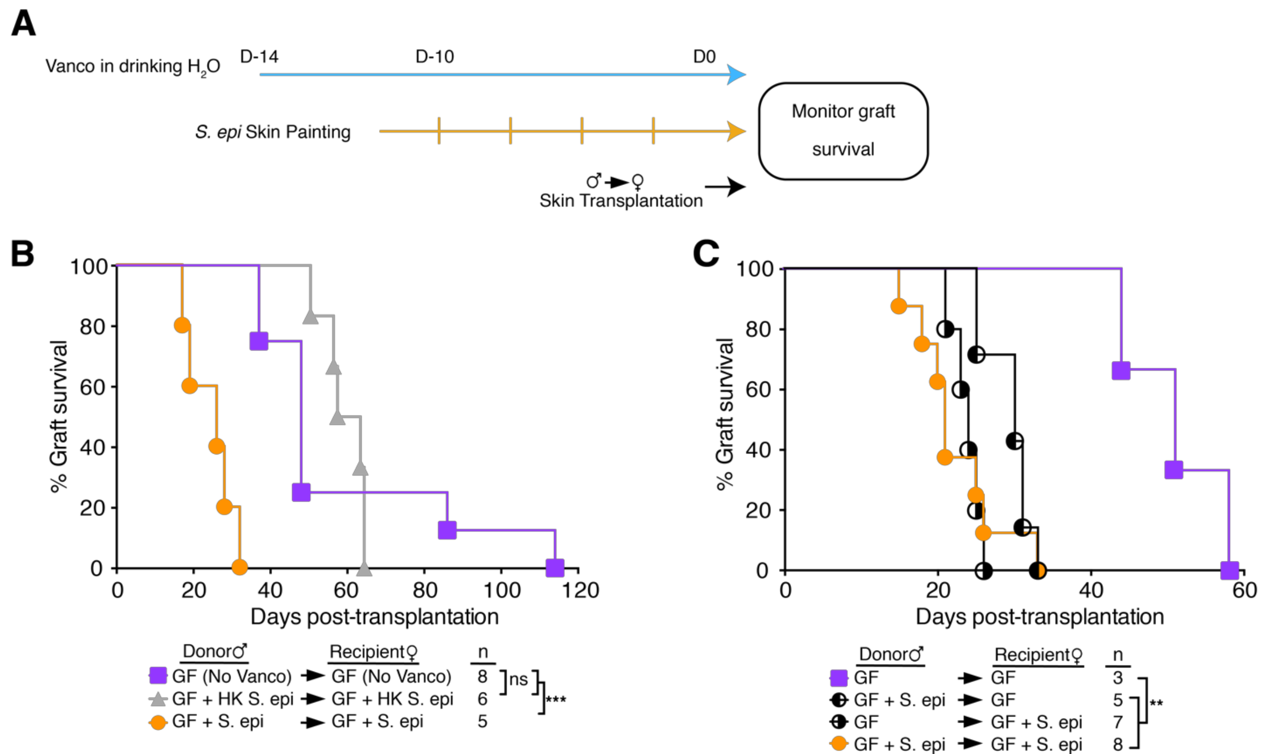


Figure 2.1. Cutaneous *S. epi* colonization is sufficient to accelerate skin allograft rejection. (**A**) Schematics of the transplant logistics in the gnotobiotic facility. (**B**) Both GF male donor and female recipients were colonized with live or heat-killed (HK) *S. epi* for 10 days before male to female skin transplantation. (**C**) GF male donor and/or female recipients were left uncolonized or colonized with *S. epi* for 10 days before skin transplantation. (**B**,**C**) All mice were treated with vancomycin throughout the experiment except the group labeled “No Vanco”.

Cultures of skin swabs and fecal samples prior to transplantation confirmed skin but not gut colonization of the *S. epi*-painted gnotobiotic mice (Figure 2.2).

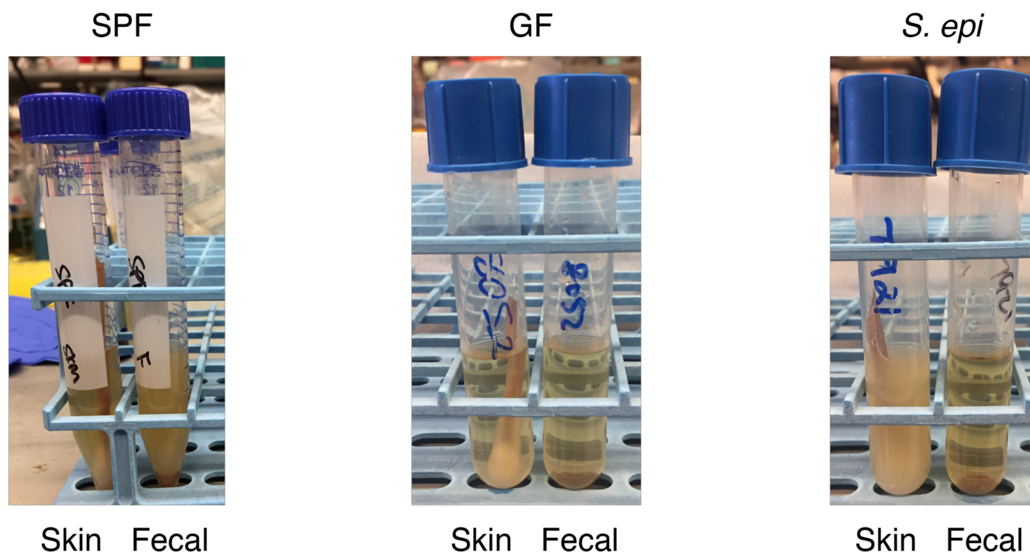


Figure 2.2. *S. epi* colonized the skin but not the gut of oral vancomycin-treated gnotobiotic mice. GF mice were painted with *S. epi*. After 10 days, skin swabs and fecal samples were cultured in aerobic conditions overnight.

S. epi skin colonization of both donor and recipient GF mice resulted in marked acceleration of skin graft rejection over that in uncolonized GF controls (Figure 2.1B). Skin colonization did not drive inflammation or infection as grafts looked healed in and healthy at the time of bandage removal. Importantly, *S. epi* needed to be alive to promote rejection, as colonization with heat-killed *S. epi* did not accelerate rejection (Figure 2.1B), suggesting that pattern recognition alone of molecular components in *S. epi* were not sufficient to accelerate graft rejection.

To determine whether accelerated rejection was driven by donor or recipient skin microbiota, only male donors or female recipients were painted with *S. epi* prior to transplantation. Either donor or recipient skin colonization was sufficient to accelerate rejection (Figure 2.1C). This is in keeping with our previous finding (Lei et al., 2016) that

skin graft survival was prolonged only when global microbial diversity was reduced by antibiotic pre-treatment from both donor and recipient mice, suggesting that colonization of either was sufficient to accelerate skin graft rejection, although those experiments did not distinguish which microbial compartment (intestine or skin) impacted graft outcome. Our current results demonstrate that skin microbiota in either the donor or the recipient is sufficient to accelerate skin graft rejection. We acknowledge that a GF graft will be rapidly colonized when transplanted into a *S. epi*-colonized host, and, vice-versa, that a host GF skin will be rapidly colonized following transplantation of a *S. epi*-painted skin graft, but such cross-colonization will occur post-transplantation. Thus, if donor colonization requires the donor commensals to spill over onto the host to accelerate graft rejection, our results still suggest an extremely rapid impact on the host.

2. Cutaneous *S. epi* colonization does not augment alloreactive T cell proliferation in skin-dLN

Skin colonization-dependent acceleration of skin graft rejection may be due to increased host alloreactivity at the priming phase of the anti-skin graft response. Previously, we found that the normal global microbiota enhances the ability of APCs in the skin-draining LNs to prime alloreactive T cells when compared to APCs from antibiotic pre-treated or GF mice, without changing the composition and the co-stimulatory profile of APCs (Lei et al., 2016). In keeping with these data, *S. epi* skin colonization did not

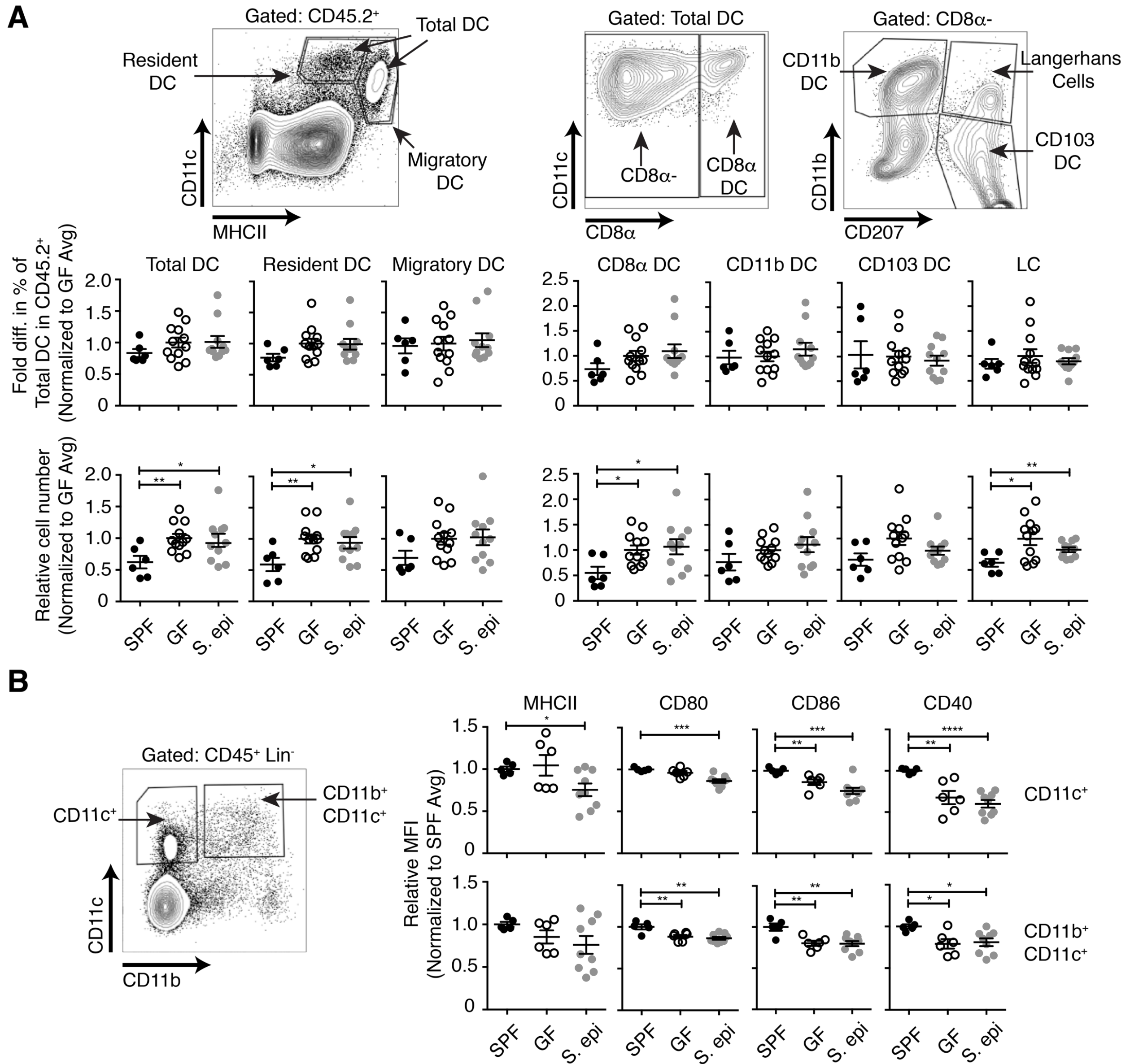


Figure 2.3. *S. epi* skin colonization does not change the composition or co-stimulatory molecule expression of LN APCs relative to GF APCs. APCs from the peripheral LNs of SPF, GF and *S. epi*-painted GF mice were analyzed by flow cytometry (**A)** Gating strategy for different DC populations and fold change relative to the average in percentage of DC subsets observed in GF controls within each experiment. (**B**) Fold change in mean fluorescence intensity (MFI) relative to that in GF mice for costimulatory molecules on CD11c$^+$-gated cells. Data were normalized from 3 individual experiments with n = 2-5 per group. Data represent the mean +/- SEM.

change either the composition of LN CD8α^+, CD11b$^+$, CD103$^+$ dendritic cells (DCs) and CD207$^+$ Langerhans cells (Figure 2.3A), nor their expression of MHCII, CD80, CD86 or CD40 when compared to GF DCs (Figure 2.3B). To determine whether LN APCs from *S. epi* skin-colonized mice acquire better T cell priming capacity, we cultured CFSE-labeled male-specific CD4$^+$ TCR transgenic T cells (Marilyn T cells) with LN APCs from *S. epi*-painted GF male mice *in vitro*. In contrast to the ability of LN APCs from globally colonized specific pathogen-free (SPF) mice to augment Marilyn T cell proliferation over that by APCs from GF mice, LN APCs from *S. epi*-painted mice failed to significantly enhance Marilyn T cell division over APCs from GF mice (Figure 2.4A and B). To investigate the impact of skin *S. epi* painting on the priming of T cells *in vivo*, CFSE-labeled Marilyn T cells were transferred into *S. epi*-colonized GF mice on the day of skin transplantation and T cells were harvested from skin-draining LNs at day 4 or 6 post-transplantation. Surprisingly, Marilyn T cell proliferation was not enhanced in *S. epi*-colonized mice, suggesting that the accelerated rejection kinetics resulting from *S. epi* colonization were not due to an increased priming phase of the alloresponse (Figure 2.4C and D).

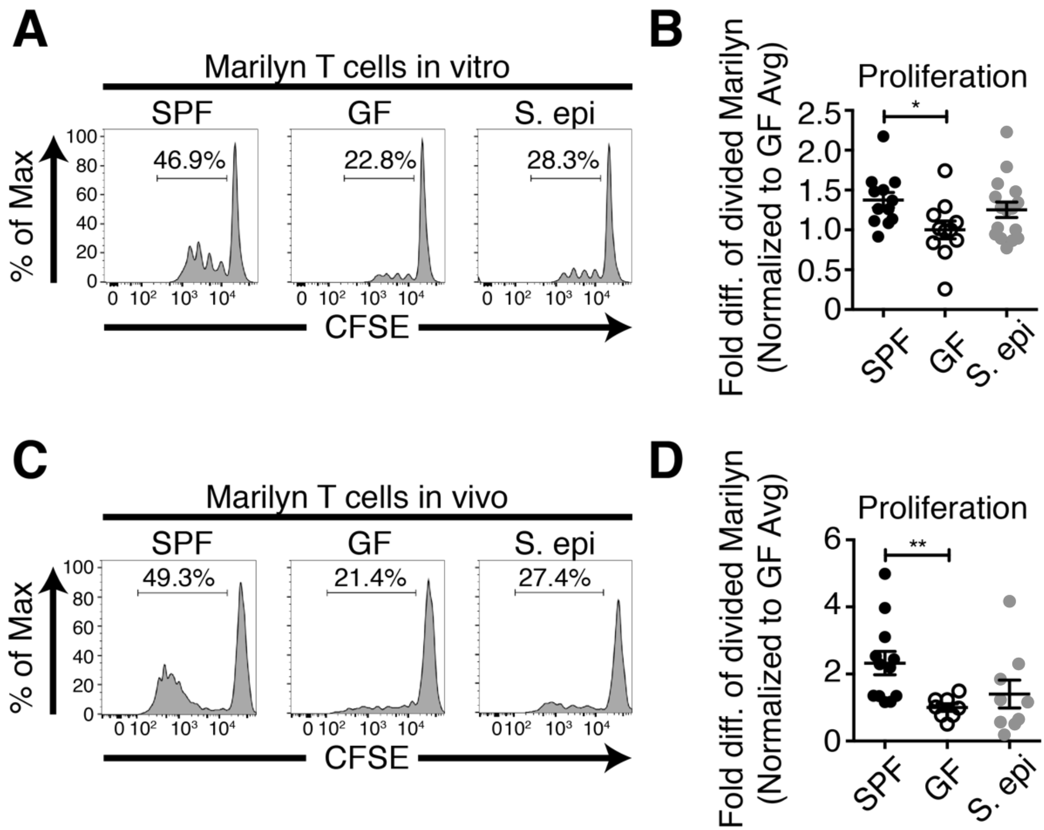


Figure 2.4. Skin *S. epi* colonization does not enhance the proliferation of alloreactive T cells in the skin-dLNs. (**A**, **B**) APCs from skin-dLNs were isolated from male SPF, GF or S. epi-painted GF *mice*, and cultured with CFSE-labeled T cells from Marilyn females for 3 days, followed by flow cytometric analysis. (**C, D**) Congenic Marilyn T cells were labeled with CFSE and transferred into GF and *S. epi*-painted GF recipients on the day of transplantation with male GF or GF+*S. epi* skin grafts. Mice were sacrificed 4 or 6 days post-transplantation and cells were isolated from the graft-dLNs for analysis of CFSE dilution. Representative plots (**A, C**) and quantitation of CFSE dilution (**B, D**) of divided Marilyn T cells are shown. (**B, D**) Quantitation represents normalized data with normalization to the average (Avg) of % divided Marilyn T cells in GF mice within each experiment from 3-5 experiments with n=2-4 mice per group. Data represent the mean +/- SEM.

3. Skin *S. epi* colonization enhances skin APC activation of alloreactive T cells within the graft

S. epi colonization can modulate local cutaneous immune responses (Naik et al., 2012, 2015). To determine whether the accelerated skin graft rejection mediated by *S. epi* colonization was due to enhanced alloimmunity at the effector phase of anti-transplant immunity, we isolated APCs from the skin of *S. epi*-colonized GF mice and cultured them with CFSE-labeled Marilyn T cells. In contrast with skin APCs from GF mice that elicited modest T cell proliferation, APCs from *S. epi*-colonized mice induced as robust T cell proliferation as APCs from the skin of SPF mice (Figure 2.5A). APCs from *S.epi*-colonized

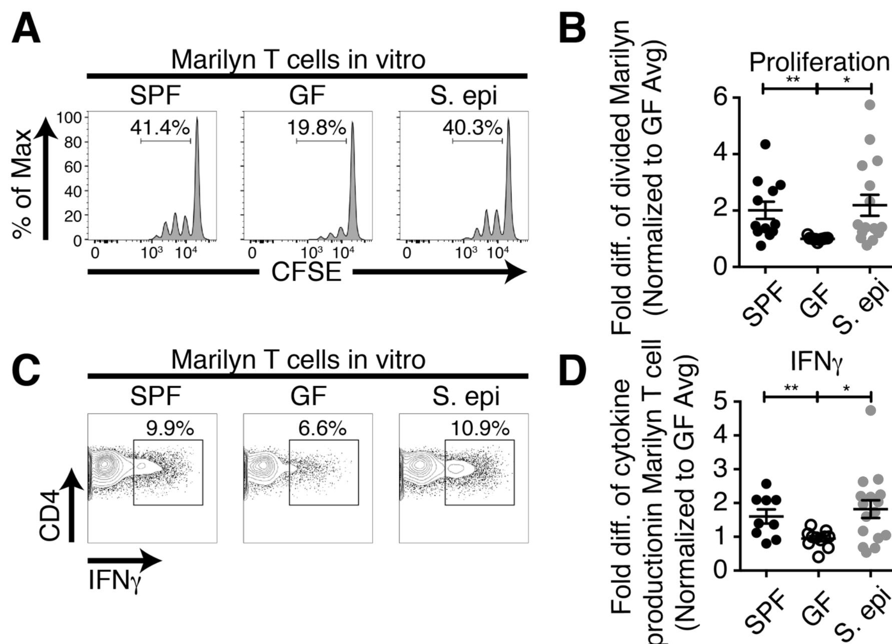


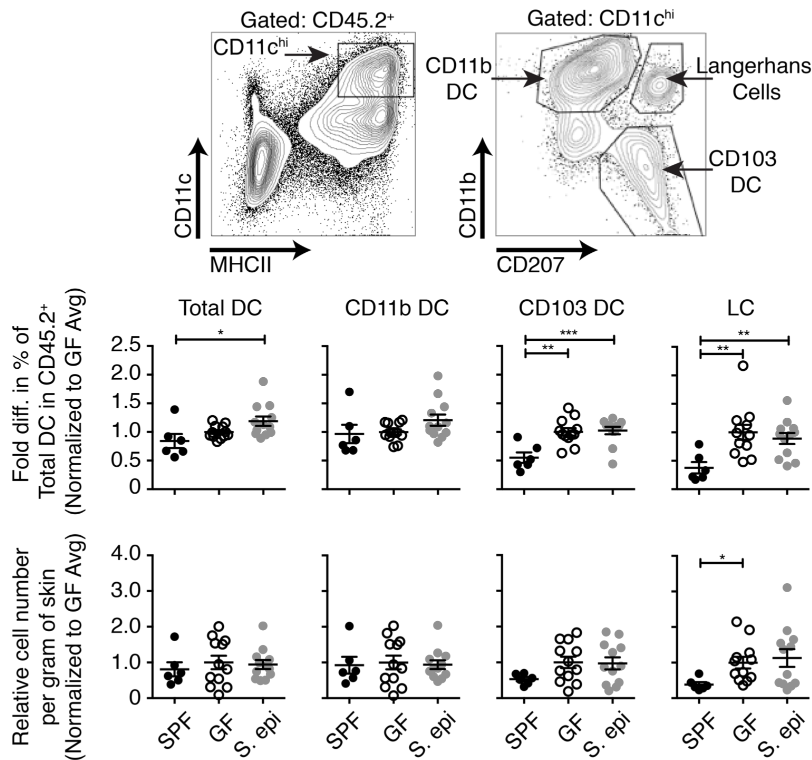
Figure 2.5. Skin *S. epi* colonization enhances skin APC activation of alloreactive T cells. (**A**-**D**) Skin APCs were isolated from male SPF, GF or *S. epi*-painted GF mice and cultured with CFSE-labeled T cells from Marilyn females for 3 days, followed by flow cytometric analysis. (**C-D**) For IFNγ detection, cells were stimulated with PMA and ionomycin before staining. Representative plots (**A, C**) and quantitation of CFSE dilution of Marilyn T cells (**B**) and IFNγ production by Marilyn T cells (**D**). Quantitation represents data normalized to the average of % divided Marilyn T cells (**B**) or IFNγ production (**D**) in GF mice within each experiment from 2-3 experiments with n=2-4 mice per group. Data represent the mean +/- SEM.

mice also triggered enhanced IFNγ production by Marilyn T cells following PMA and ionomycin re-stimulation (Figure 2.5B). The proportion of skin DCs and expression of co-stimulatory molecules in skin DCs were unchanged in *S. epi*-colonized mice, compared to uncolonized GF mice (Figure 2.6A and B). Together, these results suggest that skin colonization with *S. epi* enhances the differentiation/effector function of, rather than the priming of, the alloreactive T cell response.

D. Discussion

The kinetics of skin graft rejection in skin only-colonized mice was similar to that of globally colonized SPF mice or of GF mice that became globally colonized following SPF fecal transfer by gavage (Lei et al., 2016). However, it is important to note that not all skin microbiota accelerates skin graft rejection. Indeed, we previously showed that GF mice that received fecal material from antibiotic pre-treated mice, which resulted in both gut and skin colonization, did not display hastened skin graft rejection (Lei et al., 2016), suggesting that different skin microbiota communities have different consequences on alloimmunity. Similarly, pulsing of DCs with *S. epi* but not *S. xylosus* or *S. aureus* has been reported to enhance IFNγ and IL17A production by dermal $CD8^+$ T cells (Naik et al., 2015), indicating that even single commensals of the same genus can have a different immune impacts.

A



B

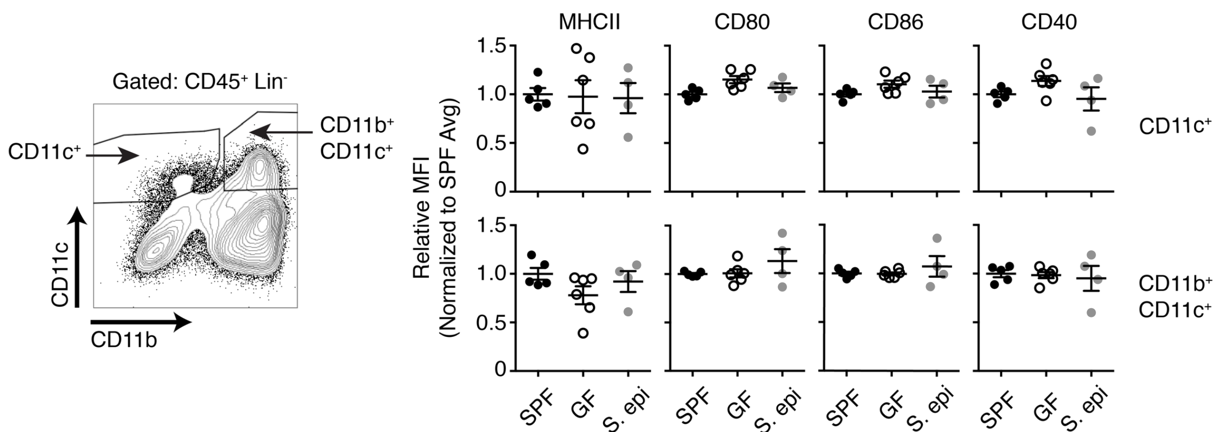


Figure 2.6. *S. epi* skin colonization does not change the composition or co-stimulatory molecule expression of SPF skin APCs relative to GF APCs. APCs from the flank skin of SPF, GF and *S. epi*-painted GF mice were analyzed by flow cytometry (**A)** Gating strategy for different DC populations and fold change relative to the average percentage of DC subsets within GF controls in each experiment. (**B**) Fold change in MFI relative to that in GF mice for costimulatory molecules on CD11c^{+}-gated cells. Data were normalized from 3 individual experiments with n = 2-5 per group. Data represent the mean +/- SEM.

The exact mechanism by which skin *S. epi* accelerates skin graft rejection is not fully elucidated. The observation that painting with heat-killed *S. epi* did not accelerate graft rejection suggests that PRR signaling is not sufficient. In addition to engaging PRRs, live *S. epi* may produce metabolites that enable skin DCs to better activate alloreactive T cells when they reach the skin. This is consistent with our data that skin APCs from *S. epi*-colonized mice enhanced T cell proliferation and IFNγ production *in vitro*. *S. epi* has been shown to elicit local commensal-specific immune responses, such as the accumulation of Tregs in the skin (Scharschmidt et al., 2015) and the activation of cutaneous non-classical MHC class I-restricted *S. epi*-specific CD8$^+$ T cells (Linehan et al., 2018). The impact of these local changes in skin immune homeostasis on skin graft rejection remains to be elucidated. Skin *S. epi* has also been linked to faster skin wound closure (Linehan et al., 2018), which might allow faster infiltration of alloreactive T cells following skin transplantation.

Although our data demonstrate that a skin commensal can modulate alloresponses locally, it remains possible that the gut microbiota affects alloreactivity distally, such that both commensal body locations, skin and intestine, may modulate alloreactivity to skin grafts. Future studies will need to investigate if colonization of only the intestine impacts skin graft outcomes. This will require a commensal that colonizes only the intestine but not the anaerobic hair follicle despite skin contact with fecal material in the cage.

The discovery that a graft-restricted commensal enhances the effector phase of alloimmunity suggests that modulation of the graft microbiota before transplantation may improve graft outcomes.

V. DISCUSSION (30 pages max)

A. Introduction

Our work demonstrates that the microbiota is indeed an environmental factor that influences the outcome of allografts. A diverse “normal” SPF microbiota was shown to accelerate graft rejection of both colonized and sterile organs. Importantly, the effect of the microbiota on allograft survival is taxon-specific and potentially location-specific as well. Overall, we have begun to understand that the microbiota can affect solid organ transplant survival, which may lead to future therapeutic interventions to improve graft outcome. Manipulating the microbiota of both the donor and the recipient has the potential to improve graft survival.

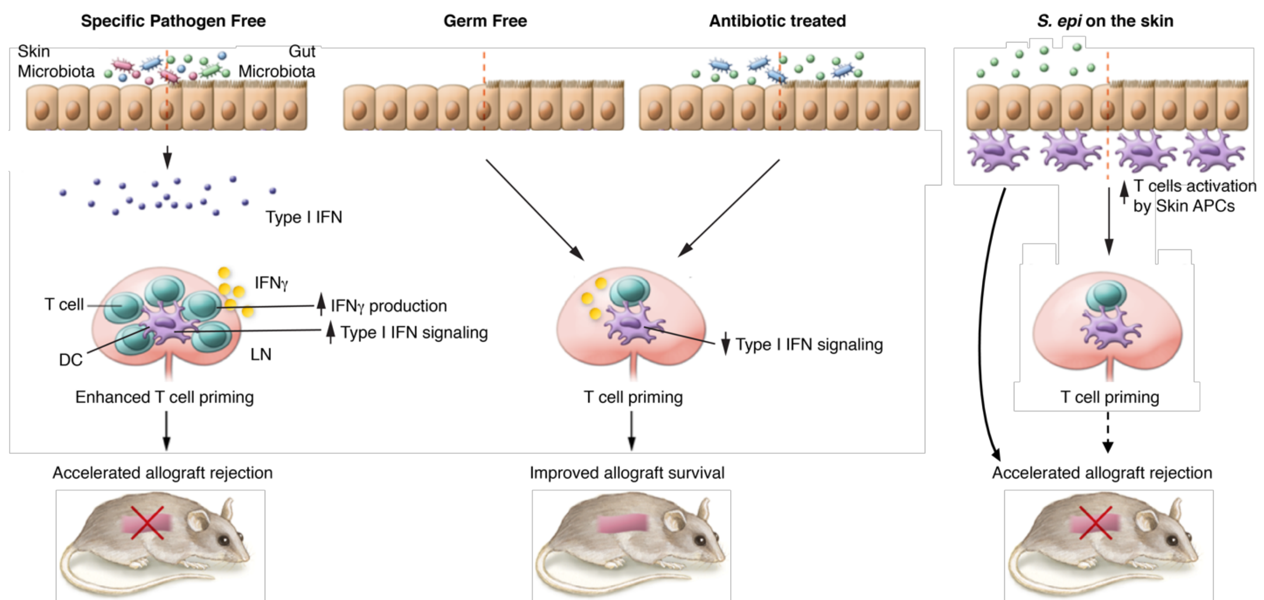


Figure 3.1. Working Model on how the microbiota modulates allograft survival. In an unperturbed state, the normal microbiota accelerates allograft survival through increasing the priming of alloreactive T cells, dependent on type I IFN signaling in LN DCs. Eliminating bacteria with antibiotics or using GF mice results in less T cell priming and therefore improved graft survival. Colonizing only the skin with *S. epi* accelerates skin graft rejection without enhancing alloreactive T cell priming, perhaps by augmenting the effector phase of the alloresponse. Figure adapted from Ford et al. *JCI.* 2016

B. Working Model

Based on the work presented in this thesis, we propose a model in which the normal microbiota that comprises many diverse microbial constituents accelerates graft rejection (Figure 3.1). Perturbing the microbiota with Abx pre-treatment in SPF mice or completely removing commensal bacteria in GF mice prolongs skin graft survival significantly. While full reconstitution with normal commensal flora (GF-SPF.F) or topical colonization with *S. epi* accelerate graft rejection in GF mice, not all commensals are sufficient to drive faster skin graft rejection, as shown by slow skin graft rejection in GF mice colonized with fecal material from Abx-pre-treated mice (GF-Abx.F), in which both gut and skin are colonized with commensal bacteria from the donor stool (Table 1).

Mice	Microbiota	Graft rejection	Marilyn T cell activation
SPF	Normal	+++	+++ (LN APC)
SPF	Abx-pre-treated	+	+ (LN APC)
GF	None	+	+ (LN APC)
GF	Normal SPF	+++	+++ (LN APC)
GF	Abx-pre-treated SPF	+	+ (LN APC)
GF	*S. epi*	+++	+ (LN APC) ++ (Skin APC)

Table 1: Summary of skin graft rejection outcome and *in vitro* Marilyn T cell activation by APCs from mice with different microbiota.

In Abx-pre-treated SPF mice, two possibilities might explain how graft survival is prolonged: 1. Elimination of pro-rejection bacteria or 2. Bloom of pro-survival bacteria. First, Abx treatment could eliminate bacteria that are capable of accelerating graft rejection. This could explain the lack of accelerated rejection in GF-Abx.F mice. Alternatively, the Abx treatment could liberate the limited niche for pro-graft survival bacteria to thrive. Although there was no further prolongation of graft survival in GF-Abx.F compared with uncolonized GF mice, the idea of pro-survival bacteria cannot be ruled out. A recent publication from our laboratory suggests that some bacterial taxa may have pro-graft survival capability (McIntosh et al., 2018). In this study, genetically similar mice obtained from two vendors and harboring different fecal microbiota were shown to display different rejection kinetics. Co-housing, or fecal transfer from the slow rejecting mice into the fast rejecting mice before skin transplantation was sufficient to promote slower skin graft rejection, whereas the reciprocal fecal transfer had no impact on rejection kinetics. Slower graft rejection was associated with the presence of a bacterial genus, *Alistipes,* in the feces of the donor and recipient mice. Further characterization is needed to identify the bacterial strain that modulates graft survival, but the dominant transfer of a slow rejecting phenotype suggests the existence of microbiota with a favorable profile for transplant outcome.

In mice that were pre-treated with Abx, the survival of not only of skin allografts, but also of heart allografts was prolonged, despite skin grafts representing colonized organs and heart grafts representing sterile organs. In addition, the immune response to a skin graft (non-vascularized) is thought to be primed in the draining lymph nodes, whereas an immune response to a heart allograft (vascularized but lymphatic vessels

not re-anastomosed) appears to be initiated in the spleen. As discussed, the gut microbiota is capable of affecting distal immune functions away from the gut (Belkaid and Hand, 2014). The prolongation of heart graft survival in Abx-pre-treated mice may be due to a systemic reduction of selected immune functions, such as type I interferon signaling (Abt et al., 2012).

Alternatively, the skin microbiota has been showed to influence local immunity within the tissue itself (Naik et al., 2012; Ridaura et al., 2018). Instead of being influenced by the gut microbiota distally, skin graft rejection may be modulated by the local cutaneous microbiota. Indeed, while investigating which microbiota compartment was responsible for modulating skin graft rejection, we discovered that cutaneous colonization by *S. epi* in mice with a sterile gut was sufficient to accelerate skin graft rejection and that skin DCs from colonized mice could enhance the activation of alloreactive T cells. Besides the effect of the skin microbiota on immune cells illustrated by our work and that from others (Naik et al., 2012, 2015), the skin microbiota may also affect non-immune functions. When gene expression profiles were compared between GF and SPF mice (Meisel et al., 2018), in addition to genes that are associated with innate immune responses such as TLRs, antimicrobial peptides and complement cascade, SPF mice also displayed increased expression of genes linked to epidermal differentiation and development processes. Therefore, understanding the impact of the local microbiota on immune and non-immune functions could potentially benefit the development of therapeutic interventions to prolong the survival of colonized organs, such as skin and the intestine.

C. Discussion and Future Directions

1. Identification of bacterial species or microbial components that modulate graft survival

The potential identification of bacterial species that could be used in a clinical setting as probiotics has generated intense interest. As we now established that the microbiota is an environmental factor that can modulate graft rejection, it is logical to ask which bacterial species or communities are involved in such modulation. Several bacterial species have been associated with immune-modulating functions in different contexts, for example, *Clostridium spp.,* SFB and *Bifidobacterium*. (Atarashi et al., 2011, 2013; Ivanov et al., 2009; Sivan et al., 2015). In transplantation, a recent study showed that a decrease of the SCFA butyrate in intestinal epithelial cells was observed after bone marrow transplantation. Supplementing butyrate-producing *Clostridia* or butyrate improved intestinal epithelial cell junctional integrity and mitigated GVHD (Mathewson et al., 2016). Furthermore, in mice, SCFA administration was associated with a decrease in DC maturation and T cell proliferation, resulting in a reduction in ischemia/reperfusion injury in the kidney (Andrade-Oliveira et al., 2015). In addition, a decrease in the order *Lactobacillales* was associated in intestinal transplant patients with episodes of rejection (Oh et al., 2012). *Lactobacillus* administration in rats reduced liver injury after ischemia-reperfusion (Xing et al., 2006) or liver transplantation (Xie et al., 2014), and the reduced injury was associated with an increased proportion of intestinal Tregs (Xie et al., 2014).

To investigate whether bacteria reported to have immunosuppressive properties could delay graft rejection, we colonized GF mice orally with *L. murinus* or with a consortium of *Clostridia* for 10-14 days, prior to male into female skin transplantation. In contrast to our expectations, both colonization regimens resulted in accelerated skin graft rejection when compared to GF mice despite an increased percentage of intestinal Tregs in *Clostridia*-colonized mice, while reconstitution with *Clostridia* provided minimal graft survival benefits when compared with SPF mice (Figure 3.2). These data highlight the complexity of modulating downstream readouts such as alloimmunity and kinetics of graft rejection and how regulatory properties of individual taxa may not translate into prolongation of graft survival, as shown by the reconstitution of GF mice with a consortium of *Clostridia*, which is known for its ability on the induction of regulatory immune response.

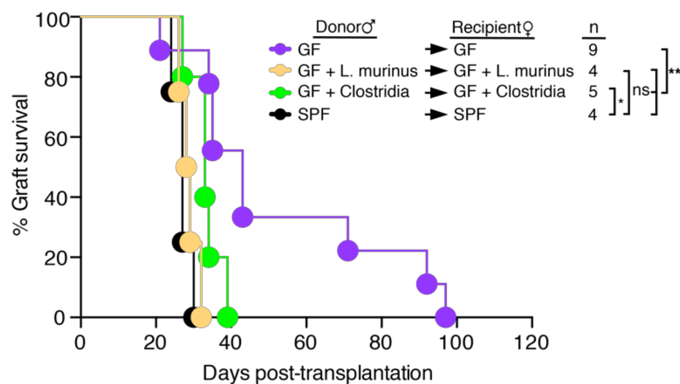


Figure 3.2. Colonization of *L. murinus* or *Clostridia* accelerates skin graft rejection in GF mice. GF mice were colonized with bacteria for 10-14 days, followed by HY skin transplantation.

In our experimental model, prolongation of graft survival following Abx pre-treatment starting in adulthood was associated with a slight increase in *Lactobacillales* and a decrease in *Clostridiales*. A separate study showed that Abx treatment (ampicillin, vancomycin, metronidazole and neomycin), when given for the first 3 weeks of life, predisposed to exacerbated vascular rejection in mice (Rey et al., 2018). This was

associated with an increase in *Clostridia* and a reduction in *Bacteroides,* suggesting that *Clostridia* are not always immunosuppressive and that characterization at the genus level is not sufficient to determine which *Clostridia* strain is immunosuppressive. Alternatively, the immunosuppressive properties of some strains may be outweighed by the pro-inflammatory properties of other microbiota.

Although we found that mono-colonization with the 2 bacterial species we tried or with the *Clostridia* consortium accelerated skin graft rejection, not all bacteria are capable of accelerating rejection. As shown in Figure 1.3 and 1.4, both the gut and skin of GF mice reconstituted with fecal material from Abx pre-treated mice were colonized with commensal bacteria, but GF-Abx.F mice did not display acceleration of skin graft rejection when compared with uncolonized GF mice. This confirms that not all bacteria are pro-rejection. In fact, some species might even be pro-survival, as suggested by McIntosh et al., where *Alistipes* was associated with prolonged skin graft survival (McIntosh et al., 2018). It will be important to establish whether there are dominant species of bacteria that are pro-rejection or pro-survival for future development of therapeutics for transplant patients, and also important to study how the immunomodulatory properties of individual bacteria are modified when confronted to other microbes in a more diverse microbial community.

2. Mechanisms by which the microbiota may impact and shape alloimmunity

Although we established that the microbiota plays a causal role in modulating alloresponses, how the microbiota signals the immune system in molecular terms remains unclear. Previously, Goldstein et al. established that MyD88 plays a critical role

in H-Y minor mismatched skin transplant rejection (Goldstein et al., 2003). In their study, when both donor and recipient were MyD88 KO, male skin grafts were not acutely rejected. Although MyD88 is downstream of both the IL-1/IL-18 and the TLR pathways, caspase-1-KO mice displayed normal rejection kinetics, suggesting that it is TLR signals and not IL-1/IL-18 signals that are necessary for H-Y skin allograft rejection by female mice. However, both damage-associated molecular patterns (DAMPs) induced after transplantation because of ischemia/reperfusion injury of the organ, and microbial-associated molecular patterns (MAMPs) can activate TLRs. A link between the signals of the microbiota and the alloresponse post-transplantation has yet to be established and further investigation is required to understand whether this link exists. To start

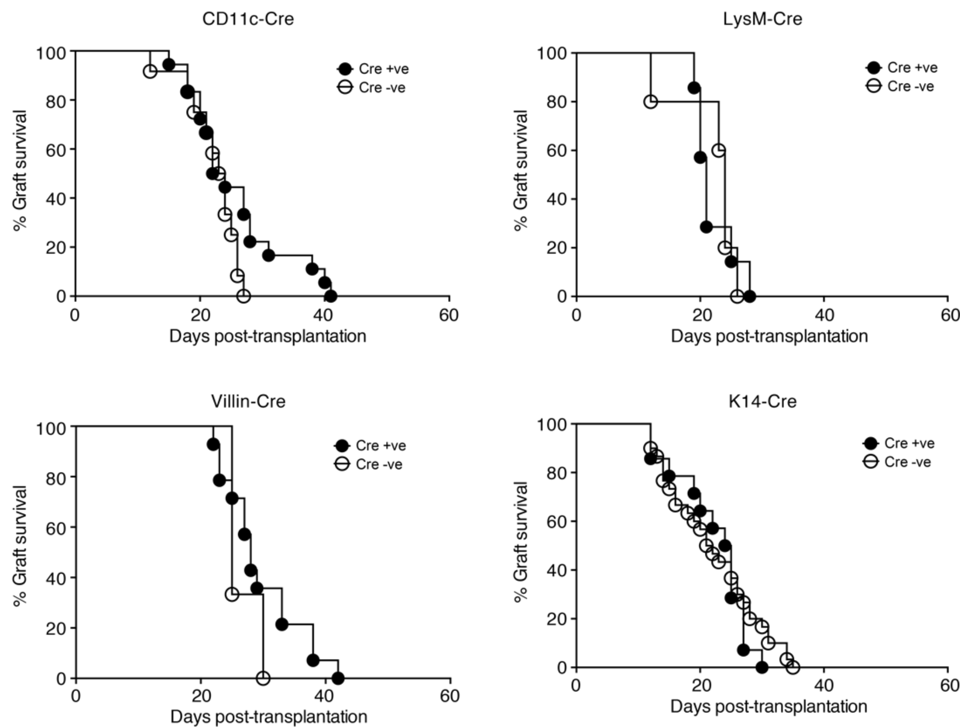


Figure 3.3 Conditional KO of MyD88 in a single cell type does not prolong graft survival. Male HY skin was transplanted on to female recipient with the same genotype (CD11c-cre, LysM-cre, Villin-cre, K14-cre)

addressing this, future experiments should use GF MyD88 KO mice together with fecal microbiota transfer from either untreated SPF of Abx pre-treated mice to help to understand whether the SPF microbiota needs to signal in a MyD88-dependent manner to precipitate graft rejection.

In addition, the microbiota has been shown to influence the function of different immune cells. In one study, the SPF microbiota was shown to drive progression of type 1 diabetes whereas the microbiota in MyD88 KO mice was protective, as MyD88 KO non-obese diabetic mice developed diabetes in GF but not SPF conditions (Wen et al., 2008). Thus, the wild-type microbiota capable of eliciting MyD88-dependent signals was stimulatory for diabetes development whereas the MyD88-KO microbiota was able to induce protection independent of MyD88. As female mice globally deficient in MyD88 could not reject male MyD88 KO skin grafts, we hypothesized that the microbiota that signals in a MyD88-dependent manner may be pro-graft rejection. To investigate which cell type needs to receive signals via MyD88, we have begun to use a cre/loxP system. As shown in Figure 3.3, we found that conditional ablation of MyD88 in any single cell type (T cells, B cells, DCs, macrophages, intestinal or skin epithelial cells) was not sufficient to result in prolonged graft survival. However, it is possible that there is redundancy of MyD88-dependent effects in two or more cells types. To more thoroughly investigate the cellular requirements for MyD88 in the context of skin transplantation, future experiments should include delineation of the MyD88 requirements in either hematopoietic or non-hematopoietic cells using MyD8 KO bone marrow chimeras. Furthermore, it is possible that MyD88 plays no role in orchestrating microbial signals that could influence alloreactivity regardless of the critical role of MyD88 in skin graft

rejection observed by Goldstein et al. (Goldstein et al., 2003) and in our own studies (data not shown). In a new study following the work of Wen et al., Burrows et al. showed that TIR-domain-containing adapter-inducing interferon-β (TRIF), a signaling adaptor downstream of TLR4, has a role in microbiota-dependent protection from type 1 diabetes (Burrows et al., 2015). Further investigations are required to understand the signaling pathways by which the microbiota communicates with the immune system to drive or reduce alloreactivity.

Finally, the work described in this thesis shows that the priming capacity of APCs is reduced after Abx treatment. A recent study demonstrated that Batf3-dependent DCs are critical for the rejection of H-Y minor mismatched skin transplants (Atif et al., 2015). Therefore, understand the types of APCs involved in influencing alloreactivity in a microbiota-dependent manner would be useful. It is also interesting to consider that the microbiota may reduce the priming capacity of APCs to alloreactive T cells through modulating the epigenetic profile of APCs. Ganal et al. showed that the NF-κB and IRF3 chromatin-binding sites are less assessible for the binding of these transcription factors after antibiotics (Ganal et al., 2012). Thus, studying the epigenetic landscape of APCs under conditions that support or reduce graft rejection may allow a better understanding of how the microbiota affects alloreactivity.

3. Impact of microbiota from different body locations on skin graft rejection

In this thesis work, we discovered that cutaneous colonization with *S. epi* was found to be sufficient to accelerate skin graft rejection, not via augmenting the priming of alloreactive T cells but correlating with enhanced differentiation/effector function of

alloreactive T cells promoted by skin APCs. Besides directly impacting allospecific responses, *S. epi* may also elicit commensal-specific responses that might influence indirectly, through the production of cytokines that promote the differentiation/effector function of alloreactive T cells. In addition, commensal-specific responses might impact on graft rejection, in parallel to alloreactive T cells. Tools such as the peptide-MHC multimers described by Scharschmidt et al. (Scharschmidt et al., 2015) that can identify T cells specific for a given commensal, could be used to track such commensal-specific responses. *S. epi* has also been shown to elicit non-classical commensal specific T cells responses (Linehan et al., 2018) which accelerate wound healing processes. This might allow faster infiltration of alloreactive T cells into a tissue that is potentially neo-vascularized with faster kinetics. In addition, the colonization by skin commensals could potentially sensitize epithelial stem cells in the skin, which is critical to recovery from tissue damage (Naik et al., 2017). Overall, our data and the work of others support the conclusion that the skin microbiota is capable of locally modulating immunity, including alloimmunity against a transplanted skin. Because the intestinal microbiota can affect immune responses distally from the gut, it may be of interest to investigate if the skin microbiota can not only modulate local immunity but also immune responses away from the skin. This could be achieved theoretically by the migration of activated cells from the skin to other locations, the secretion of soluble factors from the skin commensals that could traverse through skin barrier to distal locations or the activation of skin cells by commensal-associated soluble factors, leading to the release of secondary soluble factors that then travel systemically.

In addition to the skin microbiota, the gut microbiota could contribute to skin graft rejection distally. Commensals in the intestine could theoretically produce metabolites that circulate and impact the local immune milieu at distal locations. For example, SCFAs have been shown to impact host health and disease progression distally in several settings (Makki et al., 2018). Interestingly, whereas SCFAs have largely been associated with immune regulation, in one study, SCFAs were shown to have detrimental effect on host health by modulating microglia and augmenting Parkinson's disease pathology, suggesting that bacterial metabolites can be either anti- or pro-inflammatory. Additionally, gut commensals might educate gut immune cells, which could then travel to distal sites and modulate graft rejection. As reported by Horai et al., the gut microbiota is responsible for activating retina-specific T cells, which migrate through the circulation and trigger uveitis in a spontaneous uveitis mouse model (Horai et al., 2015). The activation of retina-specific T cells is dependent on microbial signals from intestinal contents. Therefore, it is possible that gut commensals may impact distal skin graft survival.

In our work, treatment with Abx affected both the gut and the skin microbiota. To study whether the gut microbiota in isolation can affect skin graft survival, a gut-tropic commensal must be used to avoid skin colonization. SFB is known to require mucosal adhesion to thrive, and it elicits Th17 responses (Ivanov et al., 2009). Also, *Bacteroides thetaiotamicron,* a gram negative obligate anaerobe, is known to provide signals to host macrophage through sulfatase-dependent outer membrane vesicles (Hickey et al., 2015). Obligate anaerobes with immunogenic capacity could be good candidates to test

the exclusive role of the gut microbiota in graft survival via mono-association of GF mice followed by skin transplantation.

4. The relationship between the microbiota and immunosuppression

Immunosuppression is indispensable to prevent graft rejection. Transplants without immunosuppression are inevitably rejected, unless between syngeneic animals. As we have established a causal link between the microbiota and transplant rejection, it may be possible to develop microbiota-based therapies in conjunction with standard-of-care immunosuppressive drugs for transplant patients. To achieve this goal, one needs to understand the relationship between immunosuppression and the microbiota.

Several studies have shown that immunosuppressive drugs perturb the composition of the microbiota. In cynomolgus monkeys, one dose of alemtuzumab, the lymphocyte-depleting agent, altered the microbial composition of the intestine. Increased microbial richness associated with alemtuzumab administration correlated with a decrease in intestinal T cell numbers, which then slowly repopulated as the pre-treatment flora returned (Li et al., 2013). In mice, 14 days of prednisolone treatment reduced *Bacteroidetes* and increased *Firmicutes* in the gut. Interestingly, either prednisolone or combinatorial therapy (prednisolone, mycophenolate mofetil and tacrolimus) reduced *Clostridium* correlating with altered ileal expression of C-type lectins Reg3γ and Reg3β as well as IL-22. With the altered expression of antimicrobial peptides, the combinatorial therapy allowed *E. coli* to bloom in the gut (Tourret et al., 2017).

These studies showed that administration of immunosuppressive drugs can alter the composition of the microbiota; however, these results two additional questions. Firstly, is the change of microbiota caused by the immunosuppression or by the selective force exerted from the host that is affected by the immunosuppressive drug? It is reasonable to speculate that the microbiota can be shaped by the immunosuppressive drugs, as much as by antibiotics. A recent study showed that the human gut microbiota metabolizes non-antibiotic drugs, which leads to inhibition of growth in some bacterial strains, further highlighting the impact of drug-microbiome interactions (Maier et al., 2018). Alternatively, it is known that the gut immune system is capable of shaping the microbial landscape via the secretion of antimicrobial peptides and the production of IgA. Secondly, how does the immunosuppression-altered microbial profile affect transplant outcomes? A recent study may have shed light in answering this question. Zhang et al. showed that administration of high-dose tacrolimus altered the microbiota in mice and prolonged major-mismatched skin allograft survival. Transfer of microbiota from mice treated with high-dose tacrolimus into skin graft recipients treated with a low dose of tacrolimus that could not prolong skin graft survival resulted in similar graft survival benefit as treatment of the host with a high dose of tacrolimus, an effect associated with increased colonic and systemic Tregs (Zhang et al., 2018).

These studies demonstrate the impact of immunosuppressive drugs on the microbiota. Better characterization of drug-microbiome interactions should enable further development of therapies aimed at combining immunosuppression with pre- or probiotics.

5. The microbiota as a predictive biomarker for organ transplantation

As sequencing technology advances, which allows the field to move forward with high-resolution and accurate sequencing of the microbiome, is it possible to use the microbiota as a marker to predict allograft outcome and an individual's responsiveness to immunosuppression? A clinical study in small bowel transplantation demonstrated that there could be specific microbiome signatures in patients experiencing different transplant outcomes (Oh et al., 2012). Sampling gut microbiome from patients who were at either non-rejecting, pre-rejecting or actively rejecting stages showed that intestinal transplant rejection episodes were associated with a decrease in Firmicutes and *Lactobacillales* and an increase in *Enterobacteriaceae*, suggesting that inter-individual microbiome data can potentially be used to identify universal microbial taxa as predictive biomarkers for transplant outcome. However, another clinical study counters this argument. Fricke et al. monitored longitudinally the microbiota in blood, urine, and oral and rectal swabs in renal transplant patients before and after transplantation (Fricke et al., 2014). Although a drastic change in their microbiota was observed in all cases as transplantation is accompanied by prophylactic antimicrobials, immunosuppression, surgical inflammation, fasting etc., the high inter-personal variations observed in the microbiota suggested that finding universal predictive microbiome markers (the same species or same few species across all patients) is unlikely to be possible.

However, despite inter-personal variation in the microbiota, several pilot studies have shown that the microbiota composition might be useful as a predictor for the responsiveness to anti-PD-1 immunotherapy in melanoma patients (Gopalakrishnan et

al., 2018; Matson et al., 2018; Routy et al., 2018). For example, Matson et al. showed that patients who responded to anti-PD-1 immunotherapy had a distinct profile of microbiota before treatment compared to non-responders. Transfer of fecal microbiota from a responder into GF mice led to better tumor control and greater efficacy of anti-PD-1 than transfer of fecal microbiota from a non-responder. This example prompts the question of whether the microbiota might affect the efficacy or dosage of immunosuppressive drugs. A recent study demonstrated that kidney transplant patients who required higher dose of tacrolimus had greater abundance of fecal *Faecalibacterium prausnitzii*, compared to patients who only required a stable dosage (Lee et al., 2015). These studies suggest that it might be possible to use the microbiota as a predictive biomarker for both transplant outcomes and to adjust the dose of immunosuppression in individual patients.

6. The microbiota as probiotics in transplantation

As discussed earlier, the identification of bacterial species that modulate graft survival could enable the development of probiotics to improve transplant outcome. In a murine melanoma model, oral administration of *Bifidobacterium spp*. in mice that lacked this genus was shown to improve anti-tumor immunity (Sivan et al., 2015). In transplantation, supplementing *Bifidobacterium* and *Lactobacillus* reduced liver damage in rats following ischemia/reperfusion injury (Xing et al., 2006). In a meta-study review of probiotic clinical trials, administration of probiotics before or on the day of liver transplantation was associated with better graft outcome in patients (Sawas et al., 2015). Surprisingly, in rats that were malnourished, administration of probiotics weeks after transplantation improved intestinal barrier function and animal health (Ren et al.,

2011), suggesting beneficial effects in transplanted hosts even if not administered around the time of transplantation. Nevertheless, competition with endogenous microbiota may not allow establishment and long-term effects of probiotics in all patients. To overcome this limitation, a recent study has shown that genetic engineering of bacteria, adding loci encoding genes that can utilize rare nutrients such as marine porphyran, can allow establishment of intestinal bacteria when the nutrient/prebiotic is supplemented (Shepherd et al., 2018). This approach could enable treatment with probiotics that have immune suppressive or immune potentiating effects during the duration of the prebiotic supplementation and could be a fantastic tool to fine-tune alloreactivity.

D. Conclusion

In summary, the work discussed in this thesis has established that the microbiota is an environmental factor that modulates transplant survival. Although the microbiota in mice is different than the ones in human, which is one of the limitations of conducting microbiome research in mice, it is important to note that manipulating the composition of the microbiota is sufficient to impact the survival of both colonized and uncolonized organs. The mechanisms by which the microbiota shapes alloreactivity are likely to be contextual, depending on the type of organ and colonization status of the organ prior to, during, and after transplantation. Lastly, future studies will be necessary for a deeper molecular understanding of how the microbiota communicates with the host immune system in the context alloreactivity, as well as the interaction between the microbiota

and immunosuppression, to enable the development of microbiota modulators as a new therapeutic approach to help transplant patients in the clinic.

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