Indoleamine 2,3-dioxygenase: a Potent Regulator of Graft versus Host Disease with Potential Therapeutic Applications

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Abstract

Hematopoietic stem cell transplant is the only curative therapy for a variety of malignant and non-malignant hematologic disorders and has the potential to treat a variety of other difficult diseases. Unfortunately, the procedure carries a high risk of graft versus host disease (GVHD), limiting its efficacy and wider application. GVHD occurs when antigen disparity between donor and host leads donor T cells to become activated and proliferate. They then migrate to the liver, skin, lung, and gut and mediate tissue damage. By uncovering the positive and negative regulatory pathways in GVHD biology, new therapeutic targets can be identified. Data presented here establishes indoleamine 2,3-dioxygenase (IDO), an immunosuppressive enzyme in the tryptophan metabolism pathway, as a major regulator of GVHD. IDO-deficient recipients of bone marrow transplant suffered accelerated GVHD lethality, with pathology concentrated in the colon. IFN-γ produced by donor T cells highly upregulated colon IDO; in its absence, donor T cells underwent more proliferation and less apoptosis, leading to increased disease severity and lethality. Tryptophan depletion was not central to these effects, as T cells lacking the sensor for tryptophan were suppressed by IDO. Accordingly, treatment of transplant recipients with kynurenines, the downstream metabolites of tryptophan breakdown, ameliorated disease. IDO expressed by antigen presenting cells (APCs) was responsible for its T cell suppressive effects. By targeting APCs using a TLR agonist, IDO could be upregulated in the colon before transplantation, and this upregulation was sufficient to diminish GVHD. This work identifies IDO as a potent modulator of GVHD. This finding is then used to develop two clinically relevant therapeutic strategies for GVHD treatment and prevention.

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List of Abbreviations

1-MT	1-methyl-tryptophan
3,4-DAA	N-(3,4-Dimethoxycinnamoyl) anthranilic acid
AML	acute myeloid leukemia
APC	antigen presenting cell
BM	bone marrow
BMT	bone marrow transplantation
BrdU	bromodeoxyuridine
CD40L	CD40 ligand
cDC	conventional dendritic cell
CFSE	carboxyfluorescein succinimidyl ester
CIA	collagen-induced arthritis
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DLI	donor lymphocyte infusion
EAE	experimental autoimmune encephalitis
EIF2α	eukaryotic initiation factor 2α
eGFP	enhanced green fluorescence protein
FasL	Fas ligand
FITC	fluorescein isothiocyanate
Flt3L	Flt3 ligand
GAS	gamma-activated sequence
GVHD	graft versus host disease
GVL	graft versus leukemia

H&E	hematoxylin and eosin
HCV	hepatitis C virus
HDAC	histone deacetylase
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HSCT	hematopoietic stem cell transplantation
iAPC	interstitial APC
IBD	inflammatory bowel disease
IDO	indoleamine 2,3-dioxygenase
IHC	immunohistochemistry
IFN	interferon
ISR	integrated stress response
ISRE	interferon-stimulated response element
KGF	keratinocyte growth factor
Kyn	kynurenine
LN	lymph node
LPS	lipopolysaccharide
MHC	major histocompatibility complex
mLN	mesenteric lymph node
mHAg	minor histocompatibility antigen
MLR	mixed lymphocyte reaction
NTCD	non-T cell depleted
ODN	oligodeoxynucleotides
pDC	plasmacytoid dendritic cell
PE	phycoerythrin

PP	Peyer's patch
qPCR	quantitative polymerase chain reaction
RIT	reduced intensity transplant
SAHA	suberoylanilide hydroxamic acid
SCID	severe combined immunodeficiency
SOD	superoxide dismutase
TBI	total body irradiation
TCD	T cell depleted
TCR	T cell receptor
TDLN	tumor-draining lymph node
TDO	tryptophan 2,3-dioxygenase
T_{CM}	central memory T cell
T_{EM}	effector memory T cell
TGF-β	transforming growth factor-β
TLR	toll-like receptor
TNF-α	tumor necrosis factor-α
TRAIL	TNF-related apoptosis-inducing ligand
Treg	T regulatory cell
tRNA	transfer RNA
Trp	tryptophan
UV	ultraviolet
Wt	wild-type

Chapter I: Introduction

Hematopoietic stem cell transplantation

Hematopoietic stem cell transplantation (HSCT) is a potentially curative therapy used most often for hematologic disorders and malignancies. It involves the infusion of stem cells capable of restoring bone marrow (BM) function, which is defective due to disease or because it has been damaged by radiation and/or chemotherapy. The first successful HSCT was performed as a leukemia treatment in 1956 by E. Donnall Thomas using BM from a patient's identical sibling[1]. About a decade later, Robert Good performed the first transplant for a non-cancer illness, severe combined immunodeficiency syndrome (SCID), using a matched sibling donor[2]. As research in the field progresses, the application of HSCT is expanding. Congenital anemias, including Fanconi anemia, sickle cell disease, and thalassemias[3,4], as well as immunodeficiencies such as SCID and Wiskott-Aldrich syndrome are now indications for transplant[5]. HSCT is also used to treat inborn errors of metabolism such as Hurler's disease[4]. Recently, HSCT has shown promise in treating a form of epidermolysis bullosa, a disease due to a defect in an extracellular structural protein[6]. It has also been proposed for treatment of solid malignancies and autoimmunity[7]. These more experimental applications will not be realized until research better defines transplant biology and the significant morbidity and mortality associated with HSCT can be reduced.

The transplant procedure differs with the patient's age, disease, and comorbidities. For young patients with aggressive leukemias, the conditioning regimen usually includes total body irradiation (TBI) and cyclophosphamide with or without busulfan. This combination is immunosuppressive and myeloablative and serves both to debulk the tumor and to create "space" for the incoming BM to engraft, eliminating host cells capable of rejecting the donor graft. Such conditioning is associated with considerable

toxicities, both acute and long-term, which is of concern considering the young age of many patients at the time of transplant. Patients undergoing transplant for nonmalignant disorders such as aplastic anemia or thalassemia have no tumor to kill and therefore require less conditioning. The advent of non-myeloablative conditioning, also called "mini" transplant or reduced intensity transplant (RIT), using lower doses of chemotherapy and radiation, has made transplant an option for those who otherwise could not tolerate the side effects, including older patients, patients with conditioning-sensitive diseases, and patients with comorbidities[8]. Rather than eradicating the tumor and host BM with conditioning, RIT relies on donor T cells to eliminate these cells, resulting in fewer treatment-related toxicities.

Following conditioning, stem cells are transfused intravenously. The source of the cells also depends on the patient. For older patients, autologous cells, mobilized through stem cell-inducing factors and harvested from peripheral blood, may be used following non-myeloablative conditioning. This essentially allows the use of higher doses of chemotherapy, which can kill more tumor but is toxic to the BM. Reconstitution with autologous cells rescues the patient from chemotherapy-induced BM failure. As will be discussed below, autologous cells do not react against the tumor, and do not themselves contribute to the cure of cancer. For patients who can tolerate more conditioning and transplant side effects, allogeneic transplant can be curative. In this procedure, the stem cell source can be BM, peripheral blood, or umbilical cord blood of a related or unrelated donor.

One of the functions of the immune system is to prevent the growth of transformed cells before they become malignant tumors. Therefore, the presence of a tumor indicates the

patient's immune system is not effectively recognizing and clearing malignant cells. The efficacy of HSCT in curing malignancy lies in the ability of the donor immune cells to kill tumor cells[9]. This is demonstrated by the fact that autologous and identical twin transplants have reduced efficacy, indicating the antigen mismatch between donor and host is crucial for eliminating the tumor. This principle underlies the use of donor lymphocyte infusion (DLI), in which transplant recipients whose disease has relapsed receive an infusion of lymphocytes from their original donor[10]. The infused lymphocytes are often capable of inducing remission[11]. The anti-tumor activity of donor cells is known as graft versus leukemia (GVL). Unfortunately, this antigenic difference also leads the donor cells to attack non-malignant host cells, causing graft versus host disease (GVHD). Balancing the life-saving effects of GVL with the life-threatening effects of GVHD is one of the main challenges facing transplant physicians.

Another major challenge is infection[12]. The initial conditioning leaves the patient without a functioning immune system until the transplanted cells engraft. This early time period is high-risk for opportunistic infections. Mature donor T cells transferred with the graft can contribute to antimicrobial immunity but also have the capacity to cause GVHD. Even after engraftment, the production of immune cells is defective, leaving patients immune-compromised for at least a year and often longer. This is likely due to the damage done by conditioning to the BM and thymus, the sites of immune cell generation. Complicating immune recovery is the frequent need for immunosuppressive drugs to counter GVHD. Transplant patients are therefore vulnerable to a wide range of pathogens. In fact, one of the reasons for increased HSCT survival rates over the last few decades has been due not to advances in understanding GVHD or GVL but simply to improved anti-bacterial, viral, and fungal medications.

GVHD

In the clinic

In 1950, Billingham postulated the requirements for GVHD: an immune-compromised host, immune competent donor cells, and antigenic disparity between donor and host. The antigen disparity can be divided into two classes, major and minor histocompatibility antigens. The first group comprises the human leukocyte antigen (HLA) alleles and is the basis for donor selection. The HLA genes are part of a larger major histocompatibility complex (MHC) that encodes proteins involved in antigen processing and presentation as well as other immune functions[13]. MHC class I molecules are present on antigen presenting cells (APCs) and most other cells and present peptides to $CD8^+$ T cells. MHC class II molecules are expressed primarily by professional APCs and present peptides to $CD4^+$ T cells. Three class I alleles, HLA-A, -B, and -C, and 2 class II alleles, HLA-DR and -DQ are considered when finding a donor for HSCT; the number of mismatches at these loci is significantly associated with overall survival[14]. HLA genes are codominantly expressed so that each person expresses a paternal and maternal allele for each locus. A "perfect" match would have identical alleles at all 10 loci. Because the HLA loci are close to each other in the MHC, the genes tend to be inherited in blocks known as haplotypes. Therefore a full sibling will usually either share all MHC alleles or half of them. Likewise a parent shares one haplotype with their child. Such donors are called haploidentical. MHC mismatches generate immune responses when T cells directly recognize foreign MHC on the surface of host cells, with little or no contribution of the presented peptide. The frequency of such directly alloreactive T cells is high, estimated at around 10%, and leads to a massive activation of the immune system, cytokine release, and tissue damage, resulting in GVHD.

GVHD can still occur even when HLA alleles are matched, although the risk is lowered. This is due to mismatch at non-HLA loci, termed minor histocompatibility antigens (mHAgs)[15]. Natural genetic diversity results in allelic polymorphisms in many genes; when these polymorphisms lead to the generation of immunogenic peptides that can be presented by MHC, they are called mHAgs. Therefore, in contrast to MHC antigens, donor T cells recognize mHAgs indirectly, presented by host or donor APCs in the context of MHC as processed peptides. The frequency of mHAg-reactive T cells is thought to be lower, partially explaining the reduced risk of GVHD in fully MHC-matched transplants. The identities of some mHAgs are known: some are encoded on the Y chromosome and termed H-Y antigens, some are derived from structural proteins such as myosin[16], and others are derived from genes with unknown function[17]. The full spectrum of possible mHAgs will likely be almost impossible to catalog. mHAgs are not considered during donor selection, but siblings will share at least half of their mHAgs while unrelated donors can be expected to have a greater degree of mismatch, contributing to the higher GVHD risk of unrelated donors.

The incidence of acute and chronic GVHD ranges from 20-90%. Factors known to increase the risk include increased age of donor or recipient, unrelated donor, multiparous female donor, and cytomegalovirus (CMV) positivity[18-20]. Certain HLA alleles and disease types also increase the risk. The source of the stem cells affects GVHD risk as well: umbilical cord blood has the lowest risk[21], likely due to an increased proportion of immature T cells, and peripheral blood has the highest[22], likely due to increased numbers of mature T cells. Acute GVHD usually occurs in the first three months after transplant and commonly presents with skin rash (ranging from slight

erythema to complete desquamation), liver disease (hyperbilirubinemia), and/or diarrhea, which can be bloody or nonbloody and may measure in multiple liters per day, causing malabsorption and dehydration. Lung complications can also occur. Patients receive GVHD prophylaxis, often methotrexate and cyclosporine[23], and can receive further immunosuppression, usually corticosteroids or T cell depletion, if needed[24]. Mortality rates can reach 75% in those who do not respond to therapy.

Chronic GVHD occurs after 3 months and is histologically distinct from acute GVHD, involving chronic inflammation and fibrosis. It targets a broader list of organs, including the classic acute GVHD organs and others such as the salivary and lacrimal glands and the eyes. Treatment is generally limited to steroids, and steroid-refractory GVHD has a poor prognosis. Acute GVHD is a risk factor for chronic GVHD[25], but the chronic form can occur without preceding acute disease. Chronic GVHD is associated with autoantibody production and has clinical features similar to autoimmune diseases such as scleroderma[26]. Recently, the B cell-directed anti-CD20 antibody (Rituximab) has shown promise for treating chronic GVHD[27], further supporting the involvement of autoreactive B cells. These autoimmune-like features of chronic GVHD suggested the hypothesis that thymic selection of T cells is dysfunctional post-transplant, leading to escape of autoreactive clones. The dysfunction could be due to thymic damage caused during acute GVHD[28], or it may be a function of donor BM-derived dendritic cells (DCs) in the thymus, which express different MHC than the host-derived epithelial cells and therefore fail to negatively select self-reactive T cells[29,30]. Chronic GVHD is a major clinical problem but is less well-understood than acute GVHD, partly due to its clinical heterogeneity and to the lack of suitable animal models.

In the lab

Mouse models of HSCT have provided extensive insight into the mechanism of acute GVHD. Seminal studies in the field established that gut microflora are crucial, as gnotobiotic (germ-free) mice are resistant to GVHD[31]. Korngold and Sprent then showed the disease is dependent on donor T cells[32]. Additionally, host antigen presenting cells (APCs) are essential, as their deletion prevents disease[33]. From these studies, we can propose a model where conditioning both creates space for the donor cells and causes inflammation. Rapidly dividing cells in the gut epithelium are damaged by the chemotherapy and radiation, allowing lumenal bacterial products access to the tissue. Bacteria and viruses contain components not found in mammalian cells, such as lipopolysaccharide (LPS), double-stranded RNA, and DNA containing unmethylated CpG motifs. These pathogen-derived molecules are ligands for a family of receptors called toll-like receptors (TLRs), which serve to detect infection. TLR ligands enter the tissue following gut damage and activate APCs to upregulate costimulatory molecules and secrete inflammatory cytokines. Damaged cells also release cytokines and contribute to inflammation. Infused donor T cells home to secondary lymphoid organs where the recognition of foreign antigen in the context of inflammation leads to their activation, proliferation, and migration to sites of disease, where more damage sustains more inflammation.

The timing of these events can be approximated from studies using donor T cells transgenically expressing firefly luciferase, which emit light and can be imaged in vivo. Negrin's group found signal from donor T cells in cervical lymph node (LN), mesenteric LN (mLN), spleen, and Peyer's patches (PPs) within 24 hours after transfer[34]. The signals increased in intensity over 2-3 days, reflecting proliferation, and by days 5 and 6,

T cells infiltrated the skin, liver and entire GI tract[34]. Similar studies from our lab using donor T cells expressing enhanced green fluorescence protein (eGFP) showed localization to LN as early as 6 hours after transfer[35]. Lymph node homing was observed within 24 hours regardless of recipient irradiation or allogenicity, but expansion and migration to target organs was only seen in irradiated recipients of allogeneic T cells[35]. Target organ infiltration began after 72 hours, consistent with the amount of time needed to respond to alloantigen, proliferate, and extravasate into tissues[35]. These studies support the notion that secondary lymphoid organs are the crucial location for activation and proliferation of T cells. Accordingly, effector memory T cells (T_{EM}), which do not express the lymphoid homing molecules CD62L or CCR7, did not cause GVHD[36], despite their ability to proliferate upon allostimulation in vitro[34]. This suggested that, because T_{EM} are relatively excluded from secondary lymphoid organs, the interactions that occur there must be critical for GVHD generation.

Recently, this assumption has come under question. Naïve cells deficient in CD62L or CCR7 were not defective in causing GVHD, and, more surprisingly, recipients that lacked LNs, PPs, and/or spleens were still susceptible to GVHD, although the severity and target organs varied with the priming site[37]. In addition, forced expression of CD62L on memory T cells did not endow them with GVHD-inducing capacity[37]. This evidence argues against T_{EM}'s inability to access lymphoid organs as the reason for their inability to cause GVHD. Beilhack *et al*. attempted to prevent GVHD in wild-type (wt) mice by impeding access to lymphoid organs using blocking antibodies to CD62L and its ligand[38]. This strategy did not abrogate GVHD unless the mice were splenectomized as well[38], suggesting redundancy among lymphoid organs and possible compensatory lymphoid tissue in mice engineered to lack LNs and PPs as in the previous study.

Together, this evidence indicates T cell priming ordinarily occurs in secondary lymphoid organs, but if access to lymphoid organs is blocked, priming may occur at other sites.

The inability of T_{EM} to cause GVHD may be due instead to their lower proliferative capacity[39]. Interestingly, a recent report demonstrated that central memory (T_{CM}) $CD8^+$ T cells were almost as potent as naïve $CD8^+$ T cells in causing GVHD[40]. Like naïve cells, T_{CM} express CD62L and CCR7, but the studies mentioned above imply these molecules are not crucial. However, while T_{EM} are faster to respond than T_{CM}, T_{CM} undergo more clonal expansion[39], possibly resulting in the higher cell numbers required to mediate GVHD and suggesting that T_{EM} simply cannot reach that threshold.

Characterizing the T cell response

Upon activation, naïve T cells differentiate along different paths depending on the cytokine milieu, resulting in effector T cells with different cytokine and effector profiles that are specialized for controlling different kinds of infections. The current paradigm for $CD4^+$ T cell differentiation includes Th1, Th2, and Th17 subsets. The Th1 response results in production of IL-2, IL-12, and interferon-γ (IFN-γ), expression of Tbet and Stat4 transcription factors, and is specialized for fighting intracellular pathogens. Th2 cells characteristically express Stat6 and Gata-3, secrete IL-4, IL-5 and IL-13, induce IgE production, and are associated with allergy and response to helminthes. The more recently described Th17 T cell subset, named for the production of IL-17, appears to have important functions in mucosal immunity and is implicated in many autoimmune disease models. High levels of Th1-type cytokines are found in GVHD and associated with more severe disease[41,42], which led to its categorization as a Th1-mediated disease. Th2 cells have been considered regulatory for GVHD due to their ability to downregulate

Th1 responses[43,44]. For example, neutralization of IL-12 led to decreased IFN-γ production and an increase in IL-5 and IL-10, favoring a Th2 profile and resulting in improved survival[45]. Likewise, pre-treatment of donors with IL-18 led to reduced IFN-γ and increased IL-4 production by donor T cells and was associated with decreased lethality in a Stat6-dependent process[46], suggesting Th2 responses were beneficial.

However, IL-4-deficient T cells caused delayed GVHD[47], and Stat4$^{-/-}$ T cells that show enhanced Th2 responses caused severe GVHD in the skin and liver[48], proving Th2 cells can contribute to GVHD. Stat6$^{-/-}$ T cells, which skew toward a Th1 phenotype, caused severe intestinal GVHD[48]. Interestingly, both Stat4$^{-/-}$ and Stat6$^{-/-}$ T cells each caused delayed GVHD compared to wild-type[48], suggesting both responses contribute to lethality by mediating disease in distinct organs. As for the Th17 response, while it's clear IL-17 plays an important role in colitis[49,50], so far most reports have identified only a minor role for IL-17 in GVHD[51,52], or possibly even a regulatory function via downregulation of Th1 activity[22]. Interestingly, in vitro polarized Th17 cells could mediate lethal GVHD characterized by severe cutaneous and pulmonary disease[53], consistent with different organs being susceptible to different immune responses. On the whole, while Th2 responses likely occur in some organs, GVHD, especially in the gut, remains categorized as a Th1 disease.

Paradoxically, a large body of evidence has demonstrated that the classic Th1 cytokine IFN-γ has a protective effect on GVHD lethality. Lack of IFN-γ via knockout donors or neutralizing antibody caused dramatically accelerated GVHD in lethally irradiated hosts[47]. One hypothesis was that because IFN-γ causes activation induced cell death, in its absence T cells continue to survive and mediate damage. However, IFN-γR$^{-/-}$ donor T

cells were impaired in causing GVHD[54], indicating IFN-γ, as expected, had pro-inflammatory effects on T cells. Conversely, IFN-γR$^{-/-}$ recipients succumbed to accelerated GVHD, suggesting a tissue-protective role for IFN-γ[54]. This effect seemed to be most prominent in the lung, where IFN-γ acted on pulmonary parenchyma to limit T cell infiltration and expansion[54]. In contrast, in sublethally irradiated hosts, IFN-γ caused accelerated lethality[55]. This was later attributed to the promotion of hematopoietic failure by IFN-γ, as inclusion of BM in the inoculum restored the protective effect of IFN-γ[56]. This study also demonstrated a tissue-protective role for IFN-γ, which inhibited epithelial injury in the lung and liver[56].

Effector mechanisms

Each T cell lineage has a variety of mechanisms for mediating damage. Consistent with the Th1 dominance of GVHD, most groups have implicated cell-mediated cytolytic pathways as the crucial effectors of the disease. Though CD8$^+$ T cells are traditionally thought of as cytotoxic T lymphocytes (CTLs), both CD4$^+$ and CD8$^+$ T cells can employ these methods. Cells expressing perforin are thought to produce pores in the membrane of target cells, into which they release Granzyme B, an enzyme that initiates apoptosis. A second pathway induces apoptosis by the ligation of Fas on the target cell surface by Fas ligand (FasL) expressed on the CTL. The use of these different pathways in GVHD seems to depend on the model, irradiation dose, and cell dose. One study found that class I-restricted GVHD, mediated by CD8$^+$ T cells, required perforin, while class II-restricted disease, mediated by CD4$^+$ T cells, depended more on FasL[57]. T cells lacking perforin or FasL or both were deficient in causing GVHD lethality in a sublethally irradiated MHC-mismatched model[58], but could still mediate GVHD in a lethally irradiated model, though they were less potent at lower irradiation doses[59]. In a similar model,

perforin-deficient donor T cells mediated less GVHD than wt, although at high T cell doses could still cause lethal disease[60]. In this study, both $CD4^+$ and $CD8^+$ T cells were less potent when they lacked perforin[60]. Effector mechanisms may be employed differentially in different tissues, as FasL blocking antibodies decreased weight loss and lethality without affecting skin or liver GVHD[61].

A third effector mechanism is the production of cytotoxic cytokines, especially tumor necrosis factor-α (TNF-α), an inflammatory cytokine produced in large amounts in GVHD and associated with more severe disease. This is of clinical interest due to the recent advances in anti-TNF-α therapies that have provided great relief for patients with rheumatoid arthritis and inflammatory bowel disease (IBD). TNF-α is released early after transplant by macrophages in response to LPS liberated by conditioning-related gut damage[62]. An early study showed blocking TNF-α significantly reduced intestinal and cutaneous damage due to GVHD[63]. This is in agreement with other studies suggesting TNF-α is especially critical in gut GVHD. Blocking TNF-α decreased apoptosis of intestinal cells early after transplant, while blocking FasL had no effect[64]. In addition, TNF-α inhibition led to fewer infiltrating T cells in the colon but had no effect on splenic T cells[65], and TNF-α-deficient T cells caused less gut, but not skin or liver, disease[66]. TNF-α acts on donor T cells to increase alloresponses[67] but can also directly signal epithelial cells, as recipient mice deficient in TNF-α receptor had diminished disease, although equivalent GVHD could be achieved with decreased irradiation and increased cell dose[68]. Anti-TNF-α therapy is being pursued as both prophylactic and therapeutic GVHD treatment, with mixed but generally promising results[69-73]. However, neutralization of TNF-α also significantly decreased GVL activity[66,74], suggesting timing and dose will be crucial to success of such treatments.

Overall, it's clear that donor T cells employ a variety of methods to damage target tissues, and blocking one or multiple pathways can only decrease, but not prevent, GVHD. This fact and the ability of increased cell doses to overcome the deficits of perforin- or FasL-deficient T cells underscore the cumulative nature of tissue injury and T cell activation in GVHD. No matter how the injury is caused or by what type of T cell, once a threshold is reached, the cycle of tissue damage and T cell activation culminates in disease and/or lethality.

APCs and inflammation

An area of intense focus has been APCs, as these cells were determined to be required for acute GVHD[33]. That study did not identify the subset of crucial APCs, which could include DCs, B cells, and/or macrophages. The authors used MHC class I-deficient BM to reconstitute wt mice, which were then used as transplant recipients, proving only that BM-derived APCs were required. They did note that class I-deficient macrophage reconstitution was not complete, with 3-30% of macrophages remaining wt and capable of antigen presentation[33]. GVHD was still prevented in these animals, suggesting macrophages were not important. Subsequently, it was found that reconstitution of MHC class II-deficient mice with DCs restored GVHD, while reconstitution with B cells could not, identifying DCs as the crucial GVHD-generating APC[75].

The subset(s) of DCs that are responsible for activating alloreactive T cells is not as clear. Host-derived Langerhans cells, a DC subset in the skin, were required for skin GVHD, and pre-transplant depletion of Langerhans cells using ultraviolet (UV) light prevented skin GVHD in a murine model[76], possibly explaining the efficacy of UV-

treatment for chronic skin GVHD[77]. This study exclusively focused on skin disease and did not evaluate the effects of Langerhans depletion in other organs. Another study used depleting antibodies to identify conventional DC (cDC), and not plasmacytoid DC (pDC) or macrophages, as the crucial alloantigen presenting cell[78]. $CD11c^+$ cDCs have thus generally been considered the APCs for GVHD generation. However, a recent study suggested pDCs may also play a role. The authors reconstituted irradiated, GVHD-resistant MHC class II-deficient mice with pDCs and restored GVHD[79], proving that pDCs are capable of priming T cells. This conclusion bears the caveat that the pDCs could restore GVHD in the artificial setting where they were only DCs present, and does not prove that they participate in GVHD in a normal host.

If DCs activated by inflammation present antigen and stimulate alloreactive T cells to initiate GVHD, then suppressive DCs might be able to induce tolerance to alloantigens, or at least weaken the response. Indeed, treating recipients with Flt3L pre-transplant to increase the number of suppressive $CD8\alpha^+$ DCs reduced GVHD[80]. Several investigators have also successfully diminished disease by transferring suppressive, recipient-derived DCs at or near the time of transplant. This was effective using immature DCs[81], DCs treated with the immunosuppressive molecules transforming growth factor-β (TGF-β)[82], vasoactive intestinal peptide[83], or IL-10 and TGF-β[84], or DCs transduced with TRAIL, a molecule that induced apoptosis in alloreactive T cells[85]. Interestingly, most of these approaches preserved GVL activity.

The central role of APCs and inflammation offered the hope that the non-myeloablative RITs being used to reduce conditioning toxicities might also reduce the risk of GVHD, as decreased tissue damage would lessen inflammation and APC activation. However,

reduced conditioning also delays the replacement of host APCs with those derived from the donor. Therefore, while reduced conditioning leads to fewer host APCs being activated to stimulate T cells, the host APCs survive for longer, allowing time to activate sufficient numbers of alloreactive T cells. While some studies did find lower rates of GVHD with reduced conditioning[86], others found development of acute GVHD was simply delayed[87,88]. This finding further supports the existence of a threshold of immune activation for GVHD that can be reached from cumulative activation of T cells and accrual of tissue injury.

Separating GVHD and GVL

Increased GVHD is associated with increased relapse-free survival, and, conversely, absence of GVHD is associated with relapse[89,90], suggesting the two are strongly linked. Whether the two processes are separable has not been firmly established but remains a major goal of transplant research. Intriguingly, the GVHD-defective T_{EM} mentioned previously retained GVL ability[91]. The authors showed that, on a per-cell basis, T_{EM} produced less IFN-γ and TNF-α than naïve T cells in vivo[91] and speculated that naïve T cells accumulate to a greater extent than T_{EM} and can produce the large amounts of inflammatory cytokines associated with GVHD, while smaller numbers of T_{EM} with ready access to the tumor—which is in the blood—can effectively mediate GVL without affecting host tissues. T_{EM} may also be enriched for anti-microbial, and therefore non-alloreactive, T cell receptors (TCRs). Accordingly, T_{EM} from donors primed with host alloantigen to increase the number of alloreactive cells were capable of mediating GVHD, albeit with slower kinetics and decreased lethality compared to naïve T cells[92]. The postulated requirement for fewer cells to mediate GVL than GVHD may explain the ability of the remaining alloreactive cells in the non-primed T_{EM} inoculum to clear tumor.

By TCR Specificity

One possible parameter for GVHD and GVL differentiation is antigen specificity. While it is thought that the TCR repertoire of GVHD- and GVL-causing cells is generally overlapping, there are likely some cells that cause only one or the other. Of note, mHAgs expressed selectively on hematopoietic cells may serve as GVL-inducing antigens[93,94]. If T cells that cause GVHD could be identified and removed pre-transplant, the infused T cells would contain only the beneficial anti-tumor and antimicrobial T cells. One separation strategy has been to culture donor T cells with allogeneic APCs in a mixed lymphocyte reaction (MLR) and identify the responding cells. These alloreactive cells can then be depleted by magnetic beads[95], cell-sorting[96], or immunotoxins[97]. The remaining cells have shown anti-viral and anti-leukemia activity and minimal GVHD in mouse models[95-97]. However, donor anti-host MLRs have proven to be unreliable in predicting clinical GVHD[98], and it is likely these approaches will suffer from the same issues. Another strategy is to use spectratyping to identify Vβ families used selectively by anti-tumor T cells or anti-tissue T cells[99]. Korngold's group demonstrated marked Vβ skewing in pre-transplant MLRs and in transplant patients, with 68% overlap, indicating certain Vβ families are associated with GVHD[100]. This data may be used in the future to create "designer" transplants. However, the significant risks of missing GVHD clones and the numerous Vβ families that showed only slight skewing suggest a more stringent identification method may be needed.

By T cell function

The Shlomchik group found that $CD4^+$ and $CD8^+$ T cells required direct contact with MHC on tumor cells for GVL, while $CD4^+$ T cells could mediate GVHD without

recognizing MHC on epithelial cells[101], suggesting they use different mechanisms depending on their target. This is in line with studies suggesting GVHD is dependent on gut damage and inflammatory cytokine production[62], while GVL requires direct cytolytic activity of the donor T cells. However, blockade of any single cytotoxic pathway, and even FasL and perforin in combination, did not abrogate GVL activity[91]. Other reports using a parent→F1 model found that T cells mediated GVHD largely through the FasL pathway, while GVL was dependent on the perforin pathway[102,103]. Conversely, in a transplant model targeting a solid tumor, FasL was required for graft-versus-tumor activity[104]. In addition, graft-versus-tumor, but not GVHD, capacity was reduced in T cells deficient in TNF-related apoptosis-inducing ligand (TRAIL)[105], another membrane-bound cytolytic effector molecule similar to FasL. These results suggest T cells likely employ a variety of cytotoxic pathways for GVL as they do for GVHD, depending on the cellular target, T cell number and phenotype, and inflammation in the environment.

By activation requirements

Roles for APCs and inflammation in GVL have also been explored. Ferrara's group demonstrated that host APCs were required for both GVHD and GVL, though donor APCs could contribute to GVL at low tumor burden[106]. In addition, expression of alloantigen on the tumor itself was required for GVL, while tumor associated antigens presented by the tumor, even in the presence of costimulation, were not sufficient to drive GVL[106]. DLIs effected better GVL in mixed chimeras (mice with both host and donor BM) than fully donor-derived mice, suggesting host APCs are important for GVL[107]. Together, these results indicate targeting host APCs to circumvent GVHD will likely reduce GVL as well. However, donor APCs contribute to more severe GVHD and

are not required for GVL[108], making donor APCs a potential treatment target, especially considering donor cells are easier to manipulate than host.

Delaying administration of donor T cells until after inflammation has subsided, as in DLI, could decrease GVHD while preserving GVL. Although donor T cells mediated better GVL when transferred to freshly irradiated recipients than to established mixed chimeras, GVL effects were observed in the latter setting without evidence of GVHD[109]. When inflammation was induced with TLR ligands, GVL was augmented but at the expense of causing severe GVHD[109]. Greater numbers of donor cells were required to effectively clear tumor when transferred to mixed chimeras in the absence of inflammation, as their effector function was suboptimal and they were more prone to apoptosis[109]. Therefore, increased numbers of donor T cells given as DLI could mediate GVL, albeit weakly, without GVHD. Likewise, LPS antagonists, which decreased early inflammation due to gut damage, reduced GVHD while preserving GVL[110]. However, DLI loses its efficacy over time[111]. This could be due to the turnover of host to donor APCs or the decrease in inflammation, the very parameters DLI is designed to exploit in order to avoid GVHD.

A number of other approaches are being explored. Noting that GVL occurs before GVHD, investigators have attempted to allow GVL activity to eradicate the tumor and then eliminate the alloreactive cells before they can mediate GVHD using cyclophosphamide[112] or by engineering the donor T cells to contain a suicide gene that kills the cells in response to ganciclovir[113,114]. The importance of gut damage for GVHD has been targeted by groups, including ours, using epithelial cell protectants such as keratinocyte growth factor (KGF) to minimize damage from conditioning[115,116].

One promising treatment possibility is the transfer of T regulatory cells (Tregs) at the time of transplant. These cells, characterized by FoxP3 and CD25 expression, are capable of suppressing a variety of immune responses through both contact-dependent and -independent mechanisms, including CTLA-4, IL-10 and TGF-β. Several groups have shown Tregs potently suppress GVHD[117,118] and do not appear to suppress GVL[119]. The Tregs required CD62L expression to be effective[120,121], suggesting a role for Tregs in suppressing T cell activation in lymphoid organs. However, the T_{EM} studies mentioned previously questioned the association between CD62L expression and LN homing as the mechanism for more efficient naïve T cell priming. The importance of CD62L and homing for Treg function in GVHD has not been further studied. Currently, Tregs are in Phase I clinical trials for GVHD prevention.

In summary, GVHD results from the accumulation of tissue damage and T cell activation, which promote each other and establish a feed-forward loop. The process is regulated at multiple levels, including conditioning-related inflammation, activation by APCs in secondary lymphoid organs, T cell effector mechanisms, cytokines, and target tissue environment. By better understanding this regulation, investigators aim to develop targeted treatments that restrain GVHD without resulting in general immunosuppression. One possible target is an intriguing immunomodulatory enzyme, indoleamine 2,3-dioxygenase (IDO), that has characteristics to suggest it may be involved in GVHD biology. IDO is emerging as a critical regulator of a wide range of immune responses. Relevant to GVHD, it is constitutively expressed in intestine and lung[122], two GVHD target organs; it can be induced by inflammation in APCs[123], the crucial cells for allopresentation; and it has been associated with the activation and

generation of Tregs[124,125], a cell with promising actions in suppressing GVHD while sparing GVL. The subject of this thesis is the suppressive role IDO plays in GVHD and the potential for IDO-based therapies.

IDO

Implication in immune function

IDO is an enzyme in the tryptophan catabolism pathway. It was discovered in 1967 as a heme-containing tryptophanase isolated from rabbit small intestine[126]. Originally believed to restrict the growth of tumors and microbes by starving them of tryptophan, in 1998 Munn and Mellor first reported its role as an immunosuppressant in the context of maternal-fetal tolerance. They found that inhibition of IDO, which is strongly expressed in trophoblastic cells of the placenta, by the competitive inhibitor 1-methyl-tryptophan (1-MT) caused spontaneous abortion of allogeneic, but not syngeneic, fetuses in mice[127]. The greater the antigenic disparity between mother and father, the greater the chance of fetal rejection[128]. 1-MT had no effect in $Rag^{-/-}$ mothers, indicating rejection was caused by the adaptive immune system[127]. Add-back of T cells specific for paternal antigens restored fetal rejection, identifying T cells as the mediators[128]. Munn and Mellor went on to show that human macrophages could be induced to express IDO by IFN-γ and CD40 ligand (CD40L), and the resulting tryptophan depletion suppressed T cell responses[129]. T cells activated in the presence of IDO-expressing cells or low-tryptophan conditions upregulated early activation markers but arrested at a mid-G1 point of the cell cycle. This arrest resembled classic anergy, in which T cells that receive TCR signals in the absence of costimulation do not proliferate and become unresponsive to further stimulation unless supplied with IL-2. However, cells arrested due to tryptophan depletion could be rescued by TCR restimulation in the presence of tryptophan, and,

unlike anergic cells, the cells underwent apoptosis if not restimulated in a short period of time[129]. Other groups have reported the toxic effects of tryptophan breakdown products, known collectively as kynurenines, on various T cell subsets[130,131]. Whether tryptophan depletion or kynurenine production is the primary mechanism of immunosuppression in vivo is still somewhat controversial, but since these initial studies, research has firmly established IDO as a major immune regulator, implicated in cancer immunity, chronic infection, prevention of tissue damage during immune responses, autoimmunity, and allotranplantation.

Enzymology

IDO is a monomeric, heme-containing enzyme that catalyzes the oxidative cleavage of L-tryptophan to N-formylkynurenine (Figure 1). The ferric heme must first be reduced to ferrous heme, allowing binding of O_2 which is then used to cleave the 2,3 double bond of the tryptophan indole moiety. The identity of the reductant is controversial, with most reports accepting superoxide anion ($O_2^{-\cdot}$) as the electron donor due to the inhibition of IDO by superoxide dismutase (SOD), an anti-oxidant enzyme[132]. However, a recent report notes the cellular concentration of superoxide anion may not reach the level needed to activate ferric IDO and provided evidence that cytochrome b5, the electron donor for the structurally related ferric myoglobin, is the physiological IDO reductant[133]. Many of the details of the IDO reaction remain obscure, including the order of binding of substrate and O_2, the redox properties of the heme moiety, the active site residues, and substrate-binding residues. The crystal structure of human IDO gave

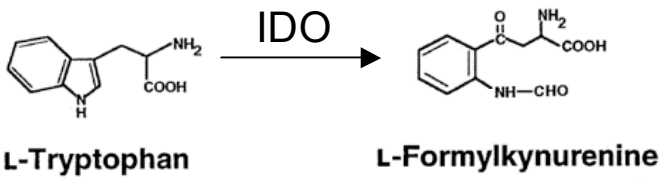


Fig. 1. IDO catalyzes the oxidative cleavage of L-tryptophan to N'-formylkynurenine.

insight into the dioxygenase mechanism and suggested the geometry of the heme and substrate binding was crucial for the reaction[134].

Tryptophan

Tryptophan is the least abundant of the eight essential amino acids. Dietary tryptophan is catabolized by tryptophan 2,3-dioxygenase (TDO), an enzyme that is expressed in the liver and, though structurally distinct from IDO, catalyzes the same reaction. The two enzymes differ in their substrate specificity, as TDO is specific for L-Trp while IDO can degrade a variety of indoleamines, including L-Trp, D-Trp, serotonin, melatonin, and tryptamine[135]. TDO expression is regulated only by availability of tryptophan, while IDO expression and activity are responsive to a wide range of inflammatory signals.

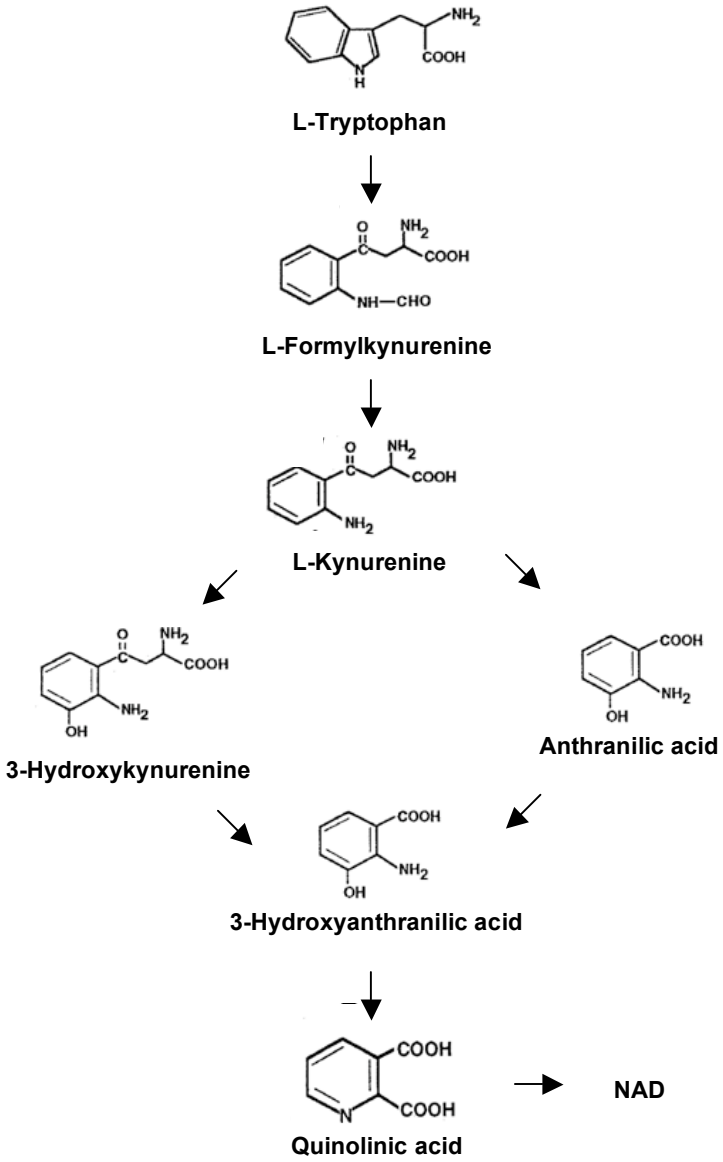


Fig. 2. Tryptophan metabolism along the kynurenine pathway.

IDO and TDO catalyze the first step in the kynurenine pathway that ultimately degrades tryptophan to niacin (Figure 2). Through alternate metabolic pathways, tryptophan is a precursor of the neurotransmitter serotonin and its relative melatonin, a neurohormone. Because IDO activity can have neurologic effects, including anxiety and depression, some have speculated that depletion of tryptophan leads to depletion of serotonin and melatonin. These effects now seem more likely due to direct neurotransmitter-like effects of kynurenines on neurons[136]. Regardless of the precise mechanism, these results provide an interesting link between chronic illness, inflammation and psychopathology.

Expression sites and induction

IDO is constitutively expressed in cells of the lung[137], placenta[138], intestine, and epididymis[139]. It can be induced in virtually any cell by IFN-γ, a type II IFN. Type I IFNs, IFN-α and IFN-β, can also induce IDO, but with much lower potency[140]. IFN-γ binding to its receptor activates Stat1, which induces IRF-1 transcription. Stat1 binds to gamma-activated sequences (GASs) in the IDO promoter, while IRF-1 binds to two interferon-stimulated response elements (ISREs). IFN-α induction of IDO also entails IRF-1 binding to the ISREs. IFN-γ-induced IDO transcription is enhanced by LPS, IL-1 and TNF-α[141]. All of these inducing signals are inflammatory mediators. By inducing IDO, they trigger a counteracting anti-inflammatory mechanism that limits the immune response, preventing excessive tissue damage and inappropriate T cell activation.

A wide variety of cells can express IDO and suppress T cells in response to inflammation, including keratinocytes[142], fibroblasts[143], endothelial cells[144], vascular smooth muscle cells[145], and colonic epithelial cells[146]. However, most data indicate the potent immunosuppressive properties of IDO are due to expression by APCs, which

activate naïve T cells directly and control T cell fate. Human macrophages express IDO in response to LPS[123] or IFN-γ and CD40L and can suppress T cell responses[129]. Langerhans cells, a DC subset in the skin, can also suppress T cells through IDO expression[147]. Puccetti's group had demonstrated previously that murine splenic $CD8\alpha^+$ DCs were tolerogenic and small numbers of them could negatively regulate the ability of immunogenic $CD8\alpha^-$ DCs to prime T cells[148]. A subsequent series of reports led to the identification of IDO as the mediator of this effect. The first clue was that suppression by $CD8\alpha^+$ DCs was potentiated by IFN-γ[149]. Pretreatment of the $CD8\alpha^-$ cells with IL-12[148] or of the $CD8\alpha^+$ cells with CD40L[150], which downregulated IDO activity, negated suppression. IL-6 treatment of $CD8\alpha^+$ DCs caused downregulation of IFN-γR and decreased tryptophan metabolism and abrogated tolerogenic function[151]. These studies made clear that the suppressive ability of $CD8\alpha^+$ DCs correlated with IDO expression and tryptophan breakdown. The group then found that, unlike macrophages, DCs constitutively expressed IDO protein. However, enzyme activation and tryptophan catabolism required IFN-γ, which they found to potently upregulate IDO activity in $CD8\alpha^+$ DCs[152]. Likewise, Munn and Mellor noted that an activation signal was required for IDO activity in human IDO-expressing monocyte-derived DCs and could be provided via B7 ligation, either directly by $CD4^+$ T cells or by crosslinking antibodies[153]. They later showed that a minor population of DCs in the spleen upregulated IDO in response to B7 ligation, which induced IFN-α production and subsequent Stat1 and IDO activation[154]. This DC population also expressed IDO in response to CTLA-4-Ig[155] and the TLR9 ligand CpG (which contains unmethylated CpG oligodeoxynucleotides (ODN))[156], and could be found expressing IDO constitutively in tumor-draining LN (TDLN)[157].

These IDO-competent DCs were further characterized and found to be a CD19-expressing subset of pDCs, a type of DC previously known to have tolerogenic functions. pDCs have a unique morphology and phenotype and appear to have a function that is distinct from that of cDCs. cDCs express high levels of CD11c, have a stellate (or "dendritic") appearance, and are specialized for presenting antigens to naïve T cells. They constantly sample their environment and present antigens on their surface. Upon activation, cDCs express high levels of MHC and costimulatory molecules, enhancing their ability to activate T cells. pDCs were discovered as a class of APCs that in the mouse express intermediate levels of CD11c along with B220[158]. They are round ("plasmacytoid") when unactivated and are specialized for secreting large amounts of type I IFNs in response to viruses via expression of TLRs 7 and 9, which recognize single-stranded RNA and CpG, respectively. Unlike other TLRs, TLR7 and TLR9 are expressed on endosomal membranes, rather than the cell surface, requiring endocytosis of their ligands. Though critical for anti-viral immunity through IFN production, pDCs present antigen poorly and have been implicated in immune suppression through the induction of oral tolerance[159] and the generation of Tregs[158,160,161], which, as will be described below, was found to require IDO expression. The $CD19^+$ IDO^+ pDCs discovered by Munn and Mellor represent a unique subset of pDCs that express high levels of CD11c and do not express the typical pDC marker 120G8[156], and, as about half of them express CD8α[157], they may overlap with the IDO-expressing splenic DCs isolated by Puccetti's group.

IFN-α produced by pDCs in response to TLR or B7 ligands can then signal in an autocrine manner to induce IDO in the pDC. However, some evidence suggests IDO induction requires signaling in addition to IFN-α, at least in pDCs. IDO can be induced in

pDCs via ligation of TNF-family members by CTLA-4-Ig[154], CD200-Ig[162], and GITR[163]. TNF receptors are traditionally thought of as costimulatory molecules expressed on APCs that provide T cells with positive or negative stimulation, but ligation of these molecules also activates signaling pathways in the APCs, which has been called "reverse" signaling. The Italian group has found several lines of evidence to indicate that, while ligation of all of these TNF receptors causes release of IFN-α, IFN-α alone is not sufficient to activate IDO. They first noted that a suboptimal concentration of CpG did not produce suppressive pDCs, even though it induced an equivalent amount of IFN-α as that produced in response to combined CpG and CD200-Ig, which did induce IDO[162]. Likewise, although IFN-α was required for GITR-mediated IDO induction, addition of IFN-α alone to pDCs did not induce IDO[163], uncovering an additional requirement. That requirement proved to be the non-canonical NF-κB pathway[163], which results in the production of the transcription factor p52-RelB. Interestingly, p52-RelB has a putative partial binding region in the IDO promoter[164].

Tregs

Tregs constitutively express CTLA-4, and when it was discovered that ligation of B7 molecules by CTLA-4 induced IDO, it was proposed that one mechanism for Treg suppression was the induction of IDO-expressing regulatory DC[155]. Conversely, IDO activity has been reported to activate Tregs. IDO^+ pDCs from TDLNs activated resting Tregs in an MHC-dependent manner and resulted in more potently suppressive Tregs than conventional activation by anti-CD3 crosslinking[125]. In contrast to such conventionally activated Tregs, which require IL-10 and TGF-β to suppress, IDO-activated Tregs mediated suppression by upregulating PD-L1 and PD-L2 on target DCs, converting them to suppressive DCs[125]. Therefore these Tregs were not intrinsically

suppressive but required DCs for their effect; when B cells were used as APCs in the readout assays, no suppression was observed[125]. A third suppressive mechanism involving Tregs is the conversion of naïve T cells into Tregs upon activation in the presence of IDO activity. This effect could be observed in vitro using low-tryptophan medium supplemented with kynurenines[165] or human pDCs stimulated to express IDO through activation of TLR9[166] or by human immunodeficiency virus (HIV)[167]. IDO-expressing acute myeloid leukemia (AML) tumor cells could generate Tregs in vitro, and increased IDO was associated with increased Treg numbers in patients[168]. Similarly, IDO was expressed in AML tumor cells and APCs at the invasive edge and metastatic lesions of uterine cervical cancer, the same regions where $FoxP3^+$ T cells were concentrated[169]. Tumors have therefore exploited the ability of Tregs to induce IDO, which can then activate resting Tregs and generate new Tregs, establishing a feed-forward loop to reinforce suppression.

Mechanism: Starvation vs. metabolites

Tryptophan depletion could lead to T cell proliferative arrest because the cell simply cannot make proteins without tryptophan. However, early studies by Munn and Mellor showed the block occurred specifically at mid-G1 phase and could not be restored with exogenous tryptophan[129]. T cells activated under low tryptophan conditions upregulated the activation markers CD25 and CD69 (indicating protein synthesis was still intact) but not cyclin D3 or cdk4, components of the cell cycle machinery. They routinely halted cell cycle progression 12 hours after activation, before beginning DNA synthesis, and showed an increased susceptibility to apoptosis[170]. These results suggested a specific program was being activated in response to low tryptophan concentration. Subsequently, the same group investigated the metabolic sensor GCN2 kinase, a

eukaryotic initiation factor 2α (EIF2α) kinase and a trigger for the integrated stress response (ISR) pathway. GCN2 binds to uncharged transfer RNA (tRNA) through a binding site that resembles tRNA synthetases[171]. Therefore, when IDO activity depletes tryptophan, a corresponding rise in uncharged trp-tRNA activates GCN2 kinase. The phosphorylation of EIF2α shuts down general protein translation and activates a smaller program of translation leading to cell cycle arrest. GCN2 was upregulated in T cells exposed to IDO^+ APCs in vitro and in vivo[172]. Proliferation of GCN2-deficient T cells was not inhibited by IDO in vitro or in vivo, nor was IDO able to mediate anergy induction in $GCN2^{-/-}$ cells[172]. GCN2 was also required for Treg activation[125] and conversion of naïve T cells to Tregs[165]. Interestingly, in myeloid but not lymphoid cells, IFN-γ upregulates tryptophanyl-tRNA-synthetase[173], the only tRNA synthetase regulated in such a manner, so that cells expressing IDO increase their ability to "capture" tryptophan and do not activate GCN2 themselves.

These studies suggested tryptophan depletion alone was responsible for the effect of IDO, with no requirement for kynurenine production. IDO-mediated suppression required GCN2, which was specifically activated in this system by tryptophan depletion; no mechanism for kynurenine-mediated GCN2 activation is known. In addition, low-tryptophan medium does not contain kynurenines but was sufficient to suppress T cell proliferation. Therefore the authors concluded only tryptophan starvation was needed for T cell suppression. However, kynurenines clearly have effects on T cells. Fallarino *et al*. treated various subsets of T cells with kynurenines and found the treatment caused thymocytes and Th1, but not Th2, cells to undergo apoptosis in vitro and in vivo[130]. Similarly, kynurenines caused apoptosis of rat T cells in an MLR and could prolong skin allograft survival[131]. In vitro, IDO activity led to downregulation of the TCR zeta chain in

$CD8^+$ T cells and induction of FoxP3 in $CD4^+$ T cells[165]. These effects were dependent on GCN2 kinase and could only be reproduced in low-tryptophan medium if kynurenines were added[165]. These last results suggest that both tryptophan depletion and kynurenine production may be required for full IDO effect. As tissue-level measurements of tryptophan and kynurenines are not possible, the contribution of each to IDO-mediated suppression in vivo may not be fully assessable.

Whatever the mechanism, IDO activity can suppress a variety of immune responses. Some groups have proposed that IDO preferentially suppresses Th1 responses and therefore promotes Th2 responses. This is supported by studies showing that kynurenines caused apoptosis of Th1 and not Th2 cells in vitro[130], human eosinophils constitutively expressed IDO and promoted Th2 responses[174], and the absence of IDO led to decreased airway disease in an allergy model[175]. However, IDO can also suppress Th2 responses, as induction of IDO via CpG inhibited experimental asthma[176], 1-MT administration impeded tolerance induction to allergens, and administration of kynurenines potentiated suboptimal allergen immunotherapy[177]. In addition, Tregs activated by IDO could suppress allergic inflammation following fungal infection[178]. One explanation for these conflicting findings is that IDO is more effective at suppressing Th1 than Th2 responses. At lower levels of activity, this manifests as suppression of Th1 and promotion of Th2, while higher IDO activity, perhaps in the context of weaker Th2-driving signals, can suppress both processes. Therefore, IDO may play a role in shaping the nature of the immune response, rather than simply suppressing it, based on its activity level and the environmental signals driving the response. For example, in the absence of IDO activity, Th17 cells caused exaggerated inflammation and tissue damage in response to pulmonary aspergillosis, impeding pathogen clearance[179].

Restoration of the IDO pathway dampened the Th17 response, allowing Th1-mediated pathogen clearance without excessive inflammation and tissue damage[179,180]. Importantly, IDO can suppress alloresponses as well. This was shown using a model that mimics some aspects of the graft versus host reaction in which alloreactive TCR-transgenic T cells proliferate after transfer into an allogeneic host. Accumulation of the alloreactive T cells was decreased in recipients transgenically expressing IDO under the MHC Class II promoter[181].

Multiple genes and inhibitors

Recent discovery of a second IDO gene, IDO-2, has caused some controversy[182,183]. The $IDO^{-/-}$ mouse used to implicate IDO in pregnancy and autoimmune models contained an intact IDO-2 locus, favoring IDO-1 as the more important enzyme. Other studies established IDO's role by inhibition with the D isomer of 1-MT. However, in cell-free systems, the L isomer is the more potent inhibitor of IDO-1[184], while the D isomer has little effect on IDO-1 but inhibits IDO-2[183,185]. In light of this evidence, it was proposed that the effects seen with 1-D-MT could not be attributed to inhibition of IDO-1 and possibly implicated IDO-2 as the true immunosuppressive enzyme. However, the in vivo anti-tumor effects of the D isomer of 1-MT were completely abrogated in $IDO\text{-}1^{-/-}$ mice[186], suggesting that 1-MT acts differently in vivo and in vitro. In addition, IDO-2 is less widely expressed, restricted mainly to the kidney, liver, and reproductive systems[182], though it can be found in APCs[183], and, unlike IDO-1, IDO-2 was not induced during malaria infection[182], indicating the two IDOs have distinct induction signals. Moreover, IDO-2 has a much lower efficiency, with a K_m value 500-1000-fold higher than that for IDO-1[187]. IDO-2 is widely expressed in lower vertebrates, while IDO-1 is only found in mammals and yeast, and the two genes are next to each other on chromosome 8[182],

consistent with gene duplication. It is therefore thought that IDO-2, or "proto-IDO" is a more primitive enzyme likely functioning in metabolism, while IDO-1 has evolved to play an important role in the immune response. Interestingly, up to half of some human populations have nonfunctional IDO-2 alleles[183], further questioning its importance.

Bystander Suppression

The presence of a small number of IDO^+ DCs can suppress T cell responses to more numerous and normally immunogenic IDO^- DCs. Clearly, direct contact between every responding T cell and an IDO-expressing DC cannot be required for the observed widespread suppression. This phenomenon, known as bystander suppression, can be accounted for in several ways. First, Tregs generated or activated by IDO^+ APCs could suppress the response of other T cells. These Tregs could also be capable of inducing IDO in other DCs, increasing the number of suppressor DCs. Second, tryptophan depletion or kynurenine production could be sufficient to affect the entire milieu, suppressing T cells being activated by otherwise stimulatory IDO^- APCs. Third, IDO could have effects on other cells, including macrophages and B cells, resulting in the release of regulatory cytokines. Whether one or all of these mechanisms is occurring, the expression of IDO in just a few cells can dramatically affect the immune response as a whole.

IDO and disease

Infection

In theory, depletion of tryptophan could starve any cell incapable of making its own, including pathogens. This was originally proposed as the mechanism of IDO. The anti-microbial properties of IFN-γ were attributed in part to its induction of IDO, which

depleted tryptophan and starved certain intracellular bacteria and parasites, as shown in vitro for Toxoplasma gondii[188] and Chlamydia species[189]. However, when it was observed that a lower, more physiological level of IFN-γ led to pathogen persistence[190], an alternative role for IDO as immunosuppressant was proposed.

IDO is now recognized to contribute to states of chronic infection. Individuals with HIV or chronic hepatitis C virus (HCV)[191] had high levels of IFNs leading to high IDO activity and low serum tryptophan/kynurenine ratios. These effects are compounded by the treatment of HCV with IFN-α, which induces IDO. The constitutive activation of IDO during these viral infections caused immune dysfunction and viral persistence and was associated with HIV dementia[192] and depression and anxiety in HCV patients[193,194]. Response to treatment was associated with decreased IDO activity[195], and inhibition of IDO by 1-MT resulted in increased immune activation[196] and enhanced clearance of infected cells[197]. As mentioned above, the observed psychopathology is likely due to release of neurotoxic tryptophan metabolites, rather than depletion of serotonin. Inhibition of IDO could be a useful tool, with the potential to increase anti-viral responses and decrease neurologic symptoms in these hard-to-treat conditions.

Cancer

IFN-γ also has anti-proliferative effects on malignant cells, and it was originally proposed that IFN-γ-induced IDO activity depleted tryptophan and starved the malignant cells[198,199]. Yet, IDO is constitutively expressed by a large number of human cancer cell lines and tumor samples[184,200] and generally associated with a worse prognosis. After the discovery of the immunosuppressive properties of IDO, it was proposed that IDO might facilitate tumor escape from immune surveillance. Intriguingly, inhibition of IDO

led to increased T cell responses to tumor in vitro and in vivo[200,201]. IDO was then identified as a major contributor to tumor growth when Muller *et al*. showed that loss of Bin1, a tumor suppressor commonly attenuated in human cancers, led to increased IDO expression and facilitated immune escape and that 1-MT was synergistic with cytotoxic agents in causing regression of resistant tumors[202].

In addition to autologous IDO upregulation, tumors can induce IDO expression in pDCs of TDLNs in mice[157]. In malignant melanoma patients, IDO^+ cells in the LN were associated with a worse prognosis, even in the absence of metastasis, suggesting a clinical correlation[157]. The IDO-inducing signal in TDLNs is unknown, but is likely related to accompanying inflammation. Recently, IDO activity in TDLN pDCs was shown to promote tumor growth by favoring Treg maintenance. When tumor-bearing mice were vaccinated against tumor antigen while IDO was blocked, Tregs converted to an inflammatory phenotype and could reduce tumor volume[203]. These findings identify IDO as a key tolerance-inducing mechanism exploited by tumors and have sparked intense investigation into IDO inhibition as adjuvant chemotherapy. The first Phase I clinical trials began in 2007.

Autoimmunity

As in other inflammatory states like infection and cancer, patients with various autoimmune diseases often show low serum tryptophan/kynurenine ratios, which was recognized as the effect of IFN-induced IDO. Increased IDO activity was detected in patients with lupus[204], rheumatoid arthritis[205], and Sjogren's syndrome[206]. Clearly, IDO is expressed and tends to increase with severity of disease, but whether IDO contributes to pathology or is a response to it cannot be deduced simply from this association. As

investigators began to define the role of IDO in autoimmunity, a picture emerged of IDO as an inflammation-induced anti-inflammatory mechanism that could suppress autoimmune responses but was insufficient to extinguish them. In human rheumatoid arthritis samples, IDO was detected in synovial pDCs but not the more prevalent myeloid DCs, perhaps accounting for the inability of IDO to suppress the inflammation[207]. In collagen-induced arthritis (CIA), a mouse model of rheumatoid arthritis, inhibition of IDO caused increased inflammation[208]. $IDO^{-/-}$ mice also suffered more severe CIA[209]. In experimental autoimmune encephalitis (EAE), a mouse model of multiple sclerosis and an example of a Th17-driven disease, IDO activity increased as the disease progressed and inhibition with 1-MT exacerbated symptoms[210,211]. IDO is strongly upregulated in human IBD[212], and 1-MT increases colitis severity in a murine IBD model[213]. Insulitis was accelerated in non-obese diabetic mice by loss of IDO-expressing pDCs[214], cells also implicated in chronic infection and tumor persistence. It therefore seems that IDO can dampen autoimmunity, but the physiological level of IDO activity is overwhelmed by the pathological immune response and cannot prevent disease. Whether late, insufficient, or ineffective IDO induction or activity is involved in the pathogenesis of autoimmunity is unknown. It is possible that increased IDO expression or activity will be effective in treating these diseases.

IDO as immunosuppressive therapy

Increased IDO expression

Like HSCT, solid organ transplantation requires titering immunosuppression in order to prevent alloresponses without impairing anti-microbial responses. Evidence suggests IDO is involved in establishing allograft tolerance. Acceptance of allogeneic heart transplants can be established in rats by blocking costimulation using CD40-Ig. This

acceptance was reversed by inhibition of IDO[215], implicating IDO in tolerance induction, though the cells responsible for IDO expression in that system were not identified. Many groups have used overexpression of IDO in the allografts themselves to effect local immunosuppression, protecting the graft while avoiding systemic suppression. Targeting the lung for IDO modulation was a logical first attempt, as lung interstitial APCs (iAPCs) constitutively express IDO, and 1-MT inhibits T cell proliferation stimulated by lung iAPCs, but not by those from spleen or liver[137]. In vivo, 1-MT caused mouse T cell proliferation and lung inflammation[137], identifying a physiological role for IDO in lung inflammation. Indeed, overexpression of IDO by adenovirus in rat lung allografts prevented rejection[137], and a similar study found IDO expression in rat lung allografts resulted in less fibrosis and near normal function[216]. This approach shows promise for other solid organ transplants, as adenovirus transduction of heart allografts led to prolonged survival[217], and overexpression of IDO in corneal allografts extended graft acceptance[218].

Transfer of IDO-expressing DCs has also been effective at suppressing unwanted immune responses. LPS treatment upregulated IDO in DCs, which, when injected into mice, led to induction of Tregs and amelioration of CIA[219]. Rats injected with IFN-γ-treated DCs had reduced numbers of autoantibody-secreting plasma cells and significant improvement of disease in a myasthenia gravis model, and the effect was reversed by pretreatment with 1-MT[220]. DCs transduced to overexpress IDO caused regression of CIA[221] and prolonged skin allograft survival[222]. While DC-based therapies hold promise, the practical difficulties of cellular immunotherapy remain a barrier to clinical application.

Kynurenines

Treatment with kynurenines is perhaps the most clinically pertinent application of IDO due to the ease of administration. When given during suboptimal immune therapy for allergy, kynurenines were able to reduce eosinophilia and Th2 cytokine levels[177], and kynurenine treatment ameliorated symptoms in CIA[209], suggesting kynurenines have efficacy against Th1/Th17 and Th2-mediated diseases. Both 3-hydroxyanthranilic acid and a synthetic tryptophan metabolite, N-(3,4-Dimethoxycinnamoyl) anthranilic acid (3,4-DAA), reversed paralysis in EAE[223], another Th17-driven process. This synthetic metabolite, used as an anti-allergy drug in Japan, also decreased inflammation and provided analgesia in CIA[224]. Chronic granulomatous disease, in which patients show exaggerated inflammatory responses due to defective NADPH oxidase in phagocytes, was shown to display a block in IDO activity and consequent decreased kynurenine production[179]. Restoration of metabolite generation by supplementation with kynurenines distal to the IDO block led to restoration of regulatory cells and resistance to invasive pulmonary aspergillosis[179]. These studies suggest tryptophan metabolites could be used to treat a range of inflammatory disorders. As 3,4-DAA has already been evaluated and approved for safety in Japan, clinical trials in this country are likely to occur.

IDO and GVHD

The biology of IDO could intersect with the mechanism of GVHD in several ways. First is the tissue level, as IDO is constitutively expressed in lung and intestine, two major GVHD target organs. IDO in the intestine is especially intriguing, as it is central to the amplification of inflammation and the immune response. The possibility that IDO could regulate GVHD locally in the tissue also suggests that suppressive effects on GVL or

antimicrobial immunity might be minimal. Second, IDO is expressed by APCs, the cells that initiate T cell responses in GVHD. Intriguing data has come out of studies using Flt3 ligand (Flt3L), which acts on hematopoietic cells to increase production of DCs. Treatment of mice with Flt3L can have stimulatory[225-227] or suppressive effects[228,229], and immune suppression due to Flt3L has been linked to increased CD8α^+ DCs and/or pDCs, two IDO-expressing DC subsets. Interestingly, Flt3L treatment of recipients pre-transplant decreased GVHD[80]; the contribution of IDO to this effect was not examined. The third possible link between IDO and GVHD is that IDO is involved in the generation and activation of Tregs. IDO activity during disease could contribute to Treg-mediated suppression of GVHD. In addition, IDO could be used to increase the number and/or potency of ex vivo cultured Tregs used for GVHD therapy.

The only previous study to examine IDO in the context of GVHD showed that monocytes and DCs from healthy volunteers upregulated IDO in response to IFN-γ, while cells from patients with high grade (III-IV) acute GVHD, but not low-grade (I-II), did not[230], suggesting higher IDO expression is associated with decreased GVHD. Following publication of the second chapter of this thesis, the group in Ann Arbor implicated IDO in the suppressive effect of histone deacetylase (HDAC) inhibitors. They found that suberoylanilide hydroxamic acid (SAHA), an HDAC inhibitor known to have anti-tumor function as well as the ability to suppress GVHD, reduced the stimulatory ability of DCs in vitro, and silencing of IDO via RNA interference or IDO-deficient DCs reduced the effect[231]. SAHA treatment early after transplant protected from GVHD unless the APCs of the recipient were IDO-deficient[231], demonstrating a suppressive role for IDO expressed by APCs during GVHD.

Using a lethally irradiated, fully MHC-mismatched murine model, this thesis presents data identifying IDO as a crucial regulator of GVHD. The first chapter shows that IDO is highly upregulated during GVHD. This upregulation is limited to the colon, where pathology is significantly worse and T cell infiltration is significantly increased in $IDO^{-/-}$ recipients. Despite the capability of Tregs to induce IDO and the ability of IDO to mediate suppression through Tregs, IDO upregulation and suppressive activity in GVHD are independent of donor Tregs. IDO activity does not affect early proliferation or activation events, including acquisition of GVHD effector molecules like FasL and Granzyme B, nor does it affect the cytokine profile. Though, as a heme-containing protein, IDO may have cytoprotective effects as a free-radical scavenger, IDO deficiency does not cause radiation sensitivity. Instead, the capacity of IDO to suppress GVHD was determined to be a local phenomenon. In the colon, where it is highly expressed, IDO acts to dampen donor T cell proliferation and increase apoptosis, leading to decreased colon damage and decreased lethality.

The second chapter will then present data that IDO upregulation is dependent on donor T cell production of IFN-γ. In the absence of IFN-γ, the differences in survival and colon T cell apoptosis and proliferation between wt and $IDO^{-/-}$ recipients are abrogated, possibly explaining the previously observed GVHD-protective effects of IFN-γ. IDO-based suppression is mediated through BM-derived APCs, which are capable of suppressing proliferation and increasing apoptosis of colon T cells regardless of the expression of IDO by epithelial cells. This reiterates the central role of APCs in GVHD and complements the HDAC study demonstrating GVHD suppression by SAHA, which induced IDO in APCs[231]. In the same vein, experiments presented here employ a TLR7/8 agonist to upregulate IDO, most likely in pDCs, which express TLR7[232], are IDO-

competent[157], and can effectively present antigen in GVHD[79]. Agonist treatment induces IDO in the colon pre-transplant and is effective in ameliorating GVHD, identifying one possible therapeutic strategy based on IDO. In addition to pre-transplant IDO induction, treatment with IDO metabolites is a viable treatment possibility. Here, IDO is found to act independently of donor GCN2 kinase in GVHD, suggesting tryptophan depletion is not the crucial mechanism for suppression. Accordingly, administration of a cocktail of three kynurenines significantly increases survival. Therefore, IDO plays a crucial role in GVHD biology, induced by inflammation and acting at the site of its expression to decrease the proliferation and survival of donor T cells. This suppressive ability can be harnessed by pre-transplant IDO induction or administration of kynurenines, resulting in improved survival. As TLR7/8 agonists and some kynurenines are already approved for use in the clinic, data reported here present the possibility that these interventions could be translated into clinical use with relative ease.

Chapter II: Indoleamine 2,3-dioxygenase is a critical regulator of acute graft-versus-host disease lethality

Jasperson LK, Bucher C, Panoskaltsis-Mortari A, Taylor PA, Mellor AL, Munn DH, Blazar BR. *Blood.* 2008 Mar 15;111(6):3257-65.

Graft-versus-host disease (GVHD) is initiated after activation of donor T cells by host antigen-presenting cells (APCs). The immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO) is expressed by APCs and parenchymal cells and is further inducible by inflammation. We investigated whether lethal conditioning and GVHD induce IDO and if IDO prevents tissue injury by suppressing immune responses at the induction site. We determined that IDO is a critical regulator of GVHD, most strikingly in the colon, where epithelial cells dramatically up-regulated IDO expression during GVHD. $IDO^{-/-}$ mice died more quickly from GVHD, displaying increased colonic inflammation and T-cell infiltration. GVHD protection was not mediated by control of T-cell proliferation, apoptosis, or effector mechanisms in lymphoid organs, nor did it require donor T regulatory cells. Instead, T cells in $IDO^{-/-}$ colons underwent increased proliferation and decreased apoptosis compared with their wild-type counterparts. This evidence suggests that IDO can act at the site of expression to decrease T-cell proliferation and survival, diminishing colonic inflammation and reducing disease severity. These studies are the first to identify a function for IDO in GVHD lethality and indicate that modulation of the IDO pathway may be an effective strategy for treatment of this disease.

Introduction

Wider application of allogeneic bone marrow transplantation (BMT) is restricted due to graft-versus-host disease (GVHD), the most significant cause of morbidity and mortality after transplantation. Despite extensive investigations of GVHD pathophysiology, the environmental effects that influence GVHD injury and the natural regulatory mechanisms by which GVHD is controlled at the tissue level are incompletely understood. Indoleamine 2,3-dioxygenase (IDO) catalyzes the first and rate-limiting step of tryptophan catabolism. Decreased tryptophan and/or increased metabolite

concentrations elicit a stress response in nearby responding T cells, leading to anergy or apoptosis[130,172]. Several aspects of IDO biology make it an intriguing target for transplantation. IDO expression has been shown to be critical for allogeneic fetal tolerance[127], tumor tolerance[202], and downregulation of autoimmunity[233]. The lung and intestine, which are GVHD target organs, constitutively express IDO and can upregulate expression during inflammation. Ectopic IDO expression in lung allografts prolongs acceptance[137,216]. In the gut, inhibition of functional IDO activity during immune-mediated colitis markedly worsens disease[213]. APCs can be induced to express IDO by interferons[156,234], LPS[235], and ligation of costimulatory molecules[153,162], all of which are prevalent during the intense inflammation of GVHD. High IDO expression in APCs in tumor draining lymph nodes is associated with poor tumor immune responses. In addition, IDO activity has been linked to the generation of T regulatory cells[236]. Given the expression pattern of IDO and the function of IDO in the local environment to control the immune response and reduce tissue injury in the colon, we sought to determine whether IDO expression in the host would regulate GVHD-induced tissue injury and lethality.

To date, IDO often has been studied in vivo by transfer of IDO-expressing cells. Whereas these studies have highlighted the immunosuppressive and tolerance-promoting effects of IDO, the physiologic role of IDO in disease processes has been less well explored. The small molecule 1-methyltryptophan (1-MT) competitively inhibits IDO. This compound has been administered through slow-release capsules implanted subcutaneously or by supplementation of drinking water. Both of these delivery approaches pose particular problems for models of GVHD because GVHD colitis prevents reliable absorption of orally administered agents, whereas implantation of pumps or pellets causes significant inflammation, which can accelerate the lethality

process. To circumvent these issues, we have investigated the role of IDO in GVHD using genetically IDO-deficient mice as BMT recipients.

We show here that IDO deficiency accelerates GVHD lethality. Allogeneic bone marrow (BM) plus supplemental donor T-cell infusion, but not BM alone, transferred into lethally irradiated recipients up-regulates IDO in the colon. $IDO^{-/-}$ recipients suffered significantly worse inflammation and T-cell infiltration in the colon. GVHD suppression was not dependent on donor T-regulatory cells (Tregs) and did not affect donor T-cell proliferation or apoptosis in secondary lymphoid organs or the acquisition of effector molecules. Instead, T cells in colons of $IDO^{-/-}$ recipients underwent increased rates of proliferation and decreased rates of apoptosis. These results suggest IDO induced by GVHD is capable of reducing ongoing donor T-cell proliferation and survival at the site of IDO expression and suggest that IDO up-regulation may be useful for preventing or treating GVHD.

Materials and Methods

Mice

Recipient C57BL/6 ($H2^b$) (termed B6) and BALB/C ($H2^d$) were purchased from The Jackson Laboratory (Bar Harbor, ME). Donor BALB/C and B6 mice were purchased from the National Institutes of Health (Bethesda, MD). B6 and BALB/C $IDO^{-/-}$ mice were generated as previously described[237] and bred at the University of Minnesota. Mice were housed in a specific pathogen-free facility in microisolator cages and used at 6-16 weeks of age.

GVHD induction

Transplants were performed according to protocols approved by the University of Minnesota IACUC. B6 or B6.IDO$^{-/-}$ mice were irradiated with 8.0-9.0 Gy TBI by x-ray on day -1. Ten million BALB/C BM cells with or without purified T cells were infused on day 0. T cells were isolated from lymph nodes (LNs) and purified by incubation with PE-labeled antibodies to CD19, γδ TCR, and DX5 (BD PharMingen, San Diego, CA) followed by incubation with anti-PE beads and magnetic column depletion (Miltenyi Biotec, Bergisch-Gladbach, Germany). Experiments using CD4^{+} T cells included anti-CD8 antibody in the depletion, and Treg-depleted cells were obtained with the addition of anti-CD25 antibody. Cell preparations were verified by flow cytometric phenotyping as more than 99% of the desired phenotype. In experiments using T cell-depleted BM (TCD BM), BM was incubated with antibodies to CD4 and CD8 on ice for 20 minutes followed by addition of rabbit complement at 37^{o} C for 30 minutes. Mice were monitored daily for survival and weighed twice weekly for the first month, then once weekly thereafter. In some experiments, recipients were electively killed and hematoxylin and eosin-stained cryosections of liver, lung, colon, small intestine, thymus, skin, and spleen were histologically assessed using a semiquantitative GVHD scoring system (0 to 4.0 grades in 0.5 increments) as published[238]. Coded sections were graded by one of us (A.P.-M.) without knowledge of the treatment.

Frozen tissue preparation

Tissues, including colon, small intestine, liver, lung, thymus, mesenteric LN, skin, and spleen were taken at indicated days after transplantation, embedded in Optimal Cutting Temperature (OCT) compound (Miles, Elkhart, IN), snap-frozen in liquid nitrogen, and stored at -80°C.

Immunohistochemistry

Following acetone fixation, cryosections (6 μm) were immunoperoxidase-stained using avidin-biotin blocking reagents, ABC-peroxidase conjugate, and DAB chromogenic substrate, all purchased from Vector Laboratories (Burlingame, CA). Antibodies used were biotinylated mAbs to CD4 (clone GK1.5) and CD8 (clone 2.43), both purchased from BD PharMingen. Sections were counterstained with methyl green.

For IDO staining, tissues were fixed in formalin and paraffin embedded. Sections underwent high-temperature antigen retrieval using Target Retrieval Solution (Dako Corporation, Carpinteria, CA) and stained using rabbit anti-IDO polyclonal antibody (prepared by Southern Biotech, Birmingham, AL based on the peptide sequence previously described[239]). Biotinylated anti-rabbit secondary antibody was incubated for one hour, followed by ABC-peroxidase conjugate and AEC chromogenic substrate, all from Biogenex (San Ramon, CA). Sections were counterstained with hematoxylin.

Images were obtained on an Olympus BX51 microscope using 20x/0.70 or 10x/0.40 objective lens and acquired using a Spot CCD digital camera and Spot software (Diagnostic Instruments, Inc., Sterling Heights, MI) and then processed with Adobe Photoshop CS2 v9.0.2 (Adobe Systems, San Jose, CA).

CFSE experiments

Splenocytes were labeled ten minutes with 2.5μM CFSE (Molecular Probes, Eugene, OR) at room temperature followed by quenching with fetal calf serum. $60x10^6$ labeled splenocytes were infused into lethally irradiated recipients. Spleens and LNs were harvested on indicated days, and single cell suspensions were made. Cells were stained

with fluorochrome-labeled antibodies to CD4, CD8, and H-2^b (BD PharMingen) and assessed for apoptosis using Annexin V staining kit (BD PharMingen) and acquired on a FACSCalibur flow cytometer (Becton Dickenson). Analysis was performed using FlowJo software (TreeStar, Inc, Ashland, OR).

BrdU incorporation assays

GVHD was induced as described and drinking water was supplemented with 0.8mg/mL BrdU (Sigma) for indicated periods. BrdU was made fresh daily. Recipients were sacrificed on indicated days. Spleens and mesenteric LN were harvested and single cell suspensions were stained with fluorochrome-labeled antibodies to CD4, CD8, and H-2^b (BD PharMingen). BrdU incorporation was assessed using FITC BrdU Flow Kit (BD PharMingen) and analyzed using FlowJo software (TreeStar, Inc, Ashland, OR).

T cell isolation

Livers were harvested and mashed into a single-cell suspension. Colons were harvested, cut into 5mm pieces, and incubated three times with 5mM EDTA in RPMI with 5% serum for 15 min. at 37^{o}C. Supernatants containing intraepithelial lymphocytes were discarded. Colons were then incubated twice with 0.5 mg/mL and once with 1mg/mL Collagenase D (Roche, Indianapolis, IN) for 1 hour at 37^{o}C with shaking. Remaining colon pieces were mashed, and cells from livers and colons were isolated on a 40/80 Percoll (Sigma) gradient. Some cells were stained for with antibodies for CD4 and CD8 (eBioscience, San Diego, CA) and Annexin V (BD Pharmingen) according to manufacturer's instructions.

Intracellular cytokine staining

Isolated cells were incubated four hours on αCD3-coated plates at 37°C. Cells were stained for CD4 and CD8 (eBioscience) and stained intracellularly with antibodies to IL-10, IL-4, IFN-γ, and Granzyme B using a fixation/permeabilization kit (BD Pharmingen).

Immunofluorescence

Following acetone fixations, cryosections (6μm) were incubated with monoclonal rat anti-CD4 and polyclonal rabbit anti-Ki-67 (Abcam, Cambridge, MA) antibodies for one hour at room temperature. Fluorochrome-labeled secondary antibodies to rat and rabbit IgG (Jackson Immunoresearch) were incubated for 30 minutes. Slides were mounted with DAPI slow fade gold (Invitrogen, Carlsbad, CA). Confocal images were acquired on an Olympus FluoView 500 Confocal Laser Scanning Microscope under 40x/0.9 oil-immersion objective lens using Fluoview 3.2 software (Olympus) and processed with Adobe Photoshop CS2 v9.0.2 (Adobe Systems, San Jose, CA).

Statistics

Survival data were analyzed by life-table methods and actuarial survival rates are shown. Group comparisons were made by log-rank test statistics. Group comparisons of cell counts and flow cytometry data were analyzed by Student t test. P values below .05 were considered significant in all tests.

Results

Host IDO expression is upregulated during GVHD and is associated with decreased lethality.

To determine if host IDO expression would regulate GVHD lethality, we transplanted lethally irradiated wild-type (wt) and $IDO^{-/-}$ B6 mice with BM from allogeneic BALB/C

donors with or without purified donor $CD4^+$ T cells to induce GVHD. $CD4^+$ T cell-mediated GVHD lethality was markedly accelerated in $IDO^{-/-}$ vs wt recipients (Figure 1A). The absence of host IDO expression did not result in an increase in post-BMT lethality in recipients given BM without supplemental $CD4^+$ T cells, although $IDO^{-/-}$ recipients of BM alone that were long-term survivors were not GVHD-free by clinical appearance, in contrast to wt recipients of BM alone. Nonetheless, together, these results indicated that host IDO expression was needed to slow the GVHD lethality process in this model system.

Subsequent studies were performed to determine whether GVHD lethality would be augmented in $IDO^{-/-}$ vs wt recipients of combined $CD4^+$ and $CD8^+$ T cells (Figure 1B). As seen with donor $CD4^+$ T cell supplementation, $IDO^{-/-}$ recipients of $CD4^+$ and $CD8^+$ T cells had markedly accelerated GVHD lethality when compared to wt recipients. In addition, lethality was not increased in $IDO^{-/-}$ vs wt recipients of BM alone and long-term survivors in both groups had regained their pre-BMT body weight (not shown). To determine whether these findings were unique to the strain combination used, a second GVHD model was used. Again, GVHD lethality was accelerated in BALB/C $IDO^{-/-}$ versus wt recipients of B6 BM and combined $CD4^+$ and $CD8^+$ T cells (Figure 1C).

To determine if IDO expression was upregulated by GVHD, lymphoid and GVHD target tissues of mice were analyzed on day 7 after transplantation. Figure 2A shows expression of IDO in colonic and small intestinal cells with a morphology and location consistent with epithelial cells. IDO expression was dramatically upregulated in colons of GVHD mice. Interestingly, mice given BM alone expressed far less IDO in the small intestine and colon, similar to nontransplanted controls (see Figure 8A), indicating that

gut toxicity caused by irradiation was not sufficient for IDO induction, and that donor T cells were required for IDO upregulation. IDO was not significantly expressed or upregulated by GVHD in liver or skin (Figure 3), nor was IDO upregulation significant in lung, mesenteric LN, or thymus (not shown). IDO expression in the spleen was also upregulated by GVHD (Figure 4), although the origin of these cells (host or donor) cannot be derived by this assessment.

Histopathologic examination of tissues taken 27 days after transplantation showed increased inflammation and tissue destruction in colon and spleen of $IDO^{-/-}$ recipients (Figure 2B). Liver, lung, and skin inflammation and destruction were not significantly different (Figure 5). Quantitative grading of Day 27 tissues confirmed significantly worse GVHD of the colon and spleen in $IDO^{-/-}$ recipients, with a mean colon score for $IDO^{-/-}$ vs. wt of 3.88 ± 0.25 vs. 2.50 ± 0.79 ($p<.02$) and a mean spleen score of 3.88 ± 0.25 vs. 3.00 ± 0.41 ($p<.02$) on a 4-point scale (n=3-5/group). No significant differences were found in GVHD scores of liver, lung, skin, or small intestine (not shown). Quantification of T cell infiltration showed increased numbers of $CD4^+$ (Figure 2Ci-iv) and $CD8^+$ (Figure 2Di-iv) cells in $IDO^{-/-}$ colons, which were associated with increased GVHD scores in this organ. Numbers of infiltrating cells were not significantly different in small intestine or liver, and few infiltrating cells were seen in skin (not shown). IHC for CD4 and CD8 in spleens demonstrated the disorganization in $IDO^{-/-}$ spleens undergoing GVHD, with smaller and disrupted follicles and T cell zones (Figure 2Cv-viii and Dv-viii). Total spleen cellularity was significantly lower in $IDO^{-/-}$ vs. wt GVHD mice at day 20 (not shown).

Host IDO upregulation and protection from GVHD lethality occur independently of donor Tregs.

Evidence suggests that IDO may have a role in activating $CD4^+25^+$Tregs[169]. To determine if IDO-mediated reduction of GVHD lethality required the presence of donor Tregs in our system, we compared survival of wt and $IDO^{-/-}$ mice receiving grafts containing CD25-depleted T cells to eliminate donor Tregs. Both donor $CD4^+$ alone (Figure 6A) and $CD4^+$ and $CD8^+$ T cells (Figure 6B) depleted of $CD25^+$ cells caused accelerated GVHD in $IDO^{-/-}$ hosts as compared to wt. In both experiments, there were comparable (0-2 grams difference) mean body weights between wt and $IDO^{-/-}$ recipients of BM alone (not shown). Therefore, the acceleration of GVHD lethality in $IDO^{-/-}$ mice is not dependent on donor Tregs given at the time of BMT. Conversely, in some experimental systems, Tregs have been shown to mediate suppression by upregulating IDO through CTLA-4 ligation of B7 molecules[155]. Although 3 of 8 lethally irradiated $IDO^{-/-}$ recipients of BM alone failed to survive long-term, supplemental donor $CD4^+$ or $CD4^+25^-$ T cells clearly accelerated GVHD lethality as compared to BM alone. Moreover, in $IDO^{-/-}$ hosts, CD25-depleted $CD4^+$ T cells accelerated GVHD over that caused by non-depleted (i.e. Treg replete) $CD4^+$ T cells (Figure 6C), indicating Tregs in the donor graft were still capable of slowing the course of GVHD lethality even in the absence of IDO. In addition, $CD4^+CD25^-$ T cells were able to induce IDO expression in colon and small intestine (Figure 7A), suggesting a suppressive mechanism other than induction of IDO upregulation by naturally occurring donor $CD4^+25^+$ Tregs in these GVHD target organs. Consistent with the pathology seen in recipients of $CD4^+$ and $CD8^+$ T cells, $CD4^+CD25^-$ T cells caused worse colon and spleen pathology (Figure 7B) and showed greater colon T cell infiltration (Figure 7Ci-iv) in $IDO^{-/-}$ hosts, and $IDO^{-/-}$ spleens again had sparser T cells and disrupted architecture (Figure 7Cv-viii). Increased colon inflammation and T cell infiltration was also observed in a transplant using a 2-fold lower dose of donor

$CD4^+CD25^-$ T cells (not shown). Collectively, these results indicate the GVHD-sparing effects of IDO are not dependent on the presence of Tregs in the donor graft.

$IDO^{-/-}$ hosts do not have increased radiation sensitivity

As a heme-containing protein, IDO might function as a free-radical scavenger. We therefore wanted to exclude the possibility that radiation injury was more severe in $IDO^{-/-}$ mice. Sublethally irradiated (5.0 Gy) $IDO^{-/-}$ mice showed no morbidity or mortality, and maintained weight similarly to sublethally irradiated wild-type mice (Figure 8A). To prove that $IDO^{-/-}$ mice could recover after lethal irradiation in the absence of the confounding effect of GVHD on survival, we transplanted lethally irradiated (8.0 Gy) wt and $IDO^{-/-}$ recipients with TCD or non-T cell depleted (NTCD) syngeneic BM cells (10^7). All mice survived and maintained weight (Figure 8B). This was also true using a higher radiation dose (9.0 Gy) along with a lower syngeneic BM dose ($5x10^6$) (not shown). Therefore, the absence of host IDO did not adversely affect the survival of lethally irradiated, BM-rescued recipients, consistent with the fact that BM transplantation alone, in the absence of supplemental donor T cells, did not induce IDO in allogeneic recipients (Fig 2A). Taken together, these results indicate the increased mortality in $IDO^{-/-}$ recipients requires allogeneic T cell infusion.

Host IDO deficiency does not affect proliferation, apoptosis or effector mechanisms of donor T cells in spleen or LN

Naïve donor T cells infused into irradiated recipients travel to LN and spleen, where they are activated by host APC and proliferate for 3-4 days before leaving for the periphery[34,35]. In other models, reduced numbers of alloreactive T cells were recovered after transfer into mice in which DCs were systematically induced to express IDO[181]. We

therefore reasoned transplanted T cells first may encounter IDO^+ APC in the LN and spleen, halting their proliferation and/or leading to apoptosis. As the colon is the first and most severely affected target organ in this model, T cell proliferation or survival effects of IDO deficiency may result in increased colon infiltration, where the absence of IDO amplifies damage and recruits more T cells, leading to increased GVHD. To investigate the proliferation and survival of donor T cells, allogeneic splenocytes were labeled with the dye CFSE to track cell division and then transferred into lethally irradiated wt or $IDO^{-/-}$ hosts. Spleen and LN were analyzed on day 4 for CFSE dilution as a measure of proliferation and for Annexin V staining as a marker of apoptosis. Figure 9A shows representative CFSE/Annexin V profiles for gated donor $CD4^+$ and $CD8^+$ spleen cells in wt and $IDO^{-/-}$ recipients four days after transplantation. Neither responder frequency (percent of original cells that divided) nor proliferation index (average number of divisions of divided cells) was significantly increased in $IDO^{-/-}$ recipients compared to wt. Percentages of Annexin V positive cells in both groups were similar as well. Similar results were obtained in identical experiments evaluated at days 2 and 3 (not shown). Additionally, while donor T cells in mesenteric LN proliferated significantly more than in spleen or peripheral (inguinal) LN, no differences were observed between wt and $IDO^{-/-}$ recipients (not shown).

Because weight loss curves begin to show a divergence between wt and $IDO^{-/-}$ recipients starting two weeks after transplantation (data not shown), we sought to determine if later stages of T cell proliferation were affected by host IDO expression. Mice were given drinking water supplemented with the thymidine analogue BrdU, which is incorporated into DNA in replicating cells. T cells labeled from days 5-9 after transplantation showed very low levels of proliferation in splenic T cells and increased

but equivalent proliferation in wt and $IDO^{-/-}$ mesenteric LN (not shown). The percentages of donor $CD4^+$ and $CD8^+$ T cells were equivalent in both spleen and LNs in wt vs $IDO^{-/-}$ hosts, and BrdU labeling from day 15-20 (Figure 9B) and 20-25 (not shown) also showed no significant differences in $BrdU^+$ $CD4^+$ or $CD8^+$ T cells in mesenteric LN or spleen.

An alternate possibility was that IDO might limit the ability of T cells to become fully activated. Therefore donor T cells present in secondary lymphoid organs were analyzed seven days after transplantation for the expression of FasL, IFN-γ, and Granzyme B, which are effector molecules known to be employed in GVHD. Lethally irradiated B6 or $IDO^{-/-}$ mice were infused with BALB/C TCD BM and $4x10^6$ $CD4^+$ and $CD8^+$ T cells. Spleens (Figure 9C) and mesenteric LN (not shown) were harvested on day 7 and analyzed by flow cytometry for FasL, Granzyme B, and IFN-γ expression. Cells obtained from the spleen seven days after transplantation showed little FasL expression. Granzyme B expression was in fact slightly higher in T cells from wild-type recipients. IFN-γ expression was similar in wt and $IDO^{-/-}$. Mesenteric LN cells revealed similar findings (not shown). These results indicated that host IDO was not decreasing proliferation, survival, or acquisition of effector molecules during this initial phase of donor T cell activation under the conditions tested.

These data are consistent with the fact that maximal IDO expression in this GVHD model is seen in the colon and not in lymphoid tissues. Therefore, IDO effects on donor T cell responses may not be measurable until these cells reach GVHD tissues, especially the gastrointestinal tract.

T cells in IDO$^{-/-}$ vs wt recipients proliferate more and survive better in the colon

It was possible that IDO was acting on T cells not in secondary lymphoid organs but in GVHD target tissues. To assess the phenotype of T cells in target tissues, we transplanted lethally irradiated B6 or IDO$^{-/-}$ mice with CD4^{+} and CD8^{+} T cells and isolated cells from the colon and liver 21 days later. Intracellular staining for IFN-γ, IL-4, IL-10, and Granzyme B revealed no differences between wt and IDO$^{-/-}$ hosts in either colon or liver (Figure 10). However, IDO$^{-/-}$ colons contained significantly more T cells than their wt counterparts, with mean CD4^{+} and CD8^{+} counts 50-fold higher in IDO$^{-/-}$ than wt, while cell counts in the livers were similar (Figure 11A). Moreover, significantly fewer T cells in IDO$^{-/-}$ colons were Annexin V positive, suggesting a decrease in the rate of local apoptosis in the colon in the absence of IDO (Figure 11B). No differences in Annexin V staining were seen in the liver. These data suggest IDO decreases T cell apoptosis without affecting their effector phenotype. Consistent with this, day 7 sera showed no differences in IL-6, IL-12, TNFα, or IFN-γ levels (not shown).

We next examined the tissues for proliferating T cells. IDO$^{-/-}$ and wt hosts were infused with BM with or without 3 million CD4^{+} and CD8^{+} T cells. On day 27, tissue sections were stained for Ki-67 (a marker for proliferating cells) and CD4. Double-positive cells, representing proliferating CD4^{+} cells, were detected in spleen, LN and thymus, but few double-positive cells were evident in liver, lung, small intestine, or skin (not shown). In contrast, colons of IDO$^{-/-}$ mice with GVHD were filled with CD4 and Ki-67 double positive cells (Figure 11C). These proliferating CD4^{+} cells were not prevalent in colons of wt mice under the same conditions, even in the most densely infiltrated and inflamed regions. Isotype controls were negative, ruling out nonspecific staining due to tissue

destruction and inflammation. Colons of IDO$^{-/-}$ given BM alone contained more CD4^{+} cells than their wt counterparts, and occasional cells were also positive for Ki-67.

Collectively, these findings reveal a role for IDO induction by allogeneic T cells in the colon. In the absence of IDO expression, T cells continue to proliferate and survive late into disease, accumulating in the colon. Increased colonic infiltration is correlated with accelerated GVHD lethality.

Discussion

These findings uncover an important function for IDO during GVHD. Without host IDO expression, mice experience markedly more severe disease. Infused donor T cells cause striking upregulation in gut epithelial cells, and the absence of IDO results in increased gut GVHD, as evidenced by histopathology and CD4^{+} and CD8^{+} T cell infiltration. In line with other studies investigating GVHD colitis[240,241], mice with worse colon GVHD had higher lethality. As compared to wt recipients, the increased severity of colonic GVHD in IDO$^{-/-}$ mice correlated with prolonged or increased proliferation and survival of T cells later into disease. CD4^{+} T cells in IDO$^{-/-}$ GVHD colons expressed Ki-67 several weeks after transplantation, while wt colons had few, if any, of these cells. Colons of IDO$^{-/-}$ recipients also contained fewer Annexin V positive T cells than wt colons. These two effects resulted in massively increased numbers of T cells in IDO$^{-/-}$ colons. No differences in T cell number, proliferation, or survival could be detected in spleen or LN, nor were any differences detected in effector function or cytokine profiles in any site, suggesting T cell effects are confined to control of proliferation and survival in the colon, the site of maximal host IDO expression.

An immunologic role for IDO was first suggested by the finding that acute inhibition of IDO by 1-MT caused rejection of allogeneic fetuses[127]. The likelihood of fetal rejection in 1-MT-treated pregnant mothers increased with greater histocompatibility disparity between mother and fetus, although $IDO^{-/-}$ animals do not reject allogeneic fetuses. These data suggest that, while 1-MT acutely disrupts IDO activity and leads to allogeneic fetal rejection in wt animals, chronic genetic deficiency of IDO leads to compensatory suppressive mechanisms that allow an allogeneic fetus to survive despite the absence of IDO. Likewise, $IDO^{-/-}$ mice do not develop spontaneous autoimmunity, even though 1-MT administration worsens existing autoimmunity in a number of models[176,211,213]. Despite these potential compensatory immunological changes in $IDO^{-/-}$ mice, we see an effect of host IDO deficiency in GVHD. It is therefore possible that IDO is even more potent in suppressing GVHD than demonstrated here. This reasoning predicts that 1-MT would result in even more dramatic GVHD acceleration, if it could be reliably absorbed at sufficient levels to block IDO activity. Consistent with this hypothesis and despite likely malabsorption defects that may have limited the amount of 1-MT present in the recipient, we did observe that wt mice given 1-MT in drinking water suffered significantly accelerated GVHD lethality (not shown).

Most immunological studies have focused on the role of IDO-expressing APCs, usually DC, in modulating T cell responses. Our results instead point to expression of IDO in gut epithelial cells as the main regulator of lethality in our GVHD model. It is possible, however, that IDO-expressing APCs in spleen or other organs contributed to the suppressive effect as well. Although IDO expression was difficult to accurately quantify in the spleen of mice undergoing a GVHD reaction due to tissue necrosis, we did observe markedly increased GVHD in the spleen in $IDO^{-/-}$ hosts suggesting that IDO^{+}

APCs contained within the spleen may have contributed to the inhibition of donor T cell mediated destruction of the spleen. In support of these possibilities, ongoing preliminary chimera experiments, in which BMT recipients contain wt tissue and $IDO^{-/-}$ BM-derived cells, or vice versa, have indicated that both APC and tissue expression of IDO contribute to GVHD suppression such that maximum GVHD lethality is observed only when both host APCs and host tissues lack IDO expression.

Although the frequency of Ki-67^{+} CD4 cells was increased in the colon of $IDO^{-/-}$ vs wt mice at four weeks post-transplant, staining with BrdU, which is incorporated into the DNA of dividing cells, was not increased in T cells in mesenteric LN in $IDO^{-/-}$ vs. wt recipients. Since Ki-67 marks proliferating cells in any phase of the cell cycle, including those that have either proliferated in situ or that have proliferated just before reaching the gut, taken together, these data could indicate that increased T cell proliferation occurs only upon arrival of T cells in $IDO^{-/-}$ vs wt colon and not in the LN compartment prior to their migration to the colon. Alternatively, increased proliferation may be occurring but not be detectable in $IDO^{-/-}$ LNs, as proliferating T cells may downmodulate LN homing receptors and upregulate gut homing receptors, resulting in increased Ki-67^{+} T cells in the colon but no accumulation of proliferating T cells in the LN. Increased T cell infiltration leading to more severe tissue damage may cause an increase in inflammatory chemokines, further amplifying the response and increasing T cell infiltration into the colon from the LN. Despite the clear requirement for allogeneic donor T cells in upregulating IDO expression in the gastrointestinal tract and the lack of such induction in wt recipients of BM alone, in some experiments, lethally irradiated, BM-rescued $IDO^{-/-}$ recipients had significantly more weight loss than wt recipients, suggesting a potential role of host IDO expression in the gastrointestinal tract as

modifying T cell responses or tissue recruitment post-transplant. The cause of these findings is unknown and will be the subject of future investigations.

The gut is a complex immunological milieu. Epithelial cells are in constant contact with foreign dietary antigens and with commensal bacteria expressing immunostimulatory toll-like receptor ligands. Suppressive mechanisms, including TGF-β and Tregs, are prevalent in the gut to counteract the stimulatory environment. The constitutive expression of IDO fits with this strategy of protecting epithelial cells against environmental triggers that might set off an overly aggressive and destructive immune response. Induction of IDO by inflammatory stimuli may be a mechanism for quenching immune responses that otherwise may cause tissue damage. GVHD creates much stronger inflammation than the gut has evolved to control, and the IDO that is induced can only delay, but not prevent, disease mortality. IDO is induced and suppresses injury in the gut in other colitis models. A murine model of trinitrobenzene sulfonic acid colitis showed upregulation of IDO in the colon, and inhibition with 1-MT significantly increased mortality[213], and human inflammatory bowel disease patients showed highly upregulated IDO in monocytes in colonic lesions[212]. GVHD of the intestine is a particularly important therapeutic target. Not only is the gut a frequent and severely affected organ, dramatically decreasing the quality of patients' lives, but it is also an amplifier of systemic disease. Gut injury allows for translocation of endotoxin and other immunostimulatory molecules from commensal gut flora into the bloodstream, leading to the cytokine storm responsible for tissue injury and subsequently more endotoxin translocation. Strategies aimed at gut protection, such as epithelial cytoprotection or blockade of gut-homing molecules on T cells, are being pursued as therapies. Similarly, IDO may be a useful tool for gut protection alone or in combination with such other

therapies. Strategies to upregulate IDO may be specifically useful for GVHD, as a clinical study showed PBMCs from patients with severe acute GVHD were less able to upregulate IDO on exposure to IFN-γ than healthy donors or those with milder GVHD[230]. Therefore, suboptimal IDO upregulation may predispose patients to more severe GVHD, similar to our findings that $IDO^{-/-}$ mice have more severe GVHD than wt mice.

In summary, IDO is a critical suppressor of GVHD. It is induced in the colon and exerts its effects there, regulating T cell proliferation and survival. This local control of the immune response results in gut protection and a marked delay of GVHD lethality. Enhanced IDO activity or induction of IDO before transplantation may suppress disease, and we speculate the administration of tryptophan catabolites may mimic IDO activity and dampen disease. Our studies show IDO does not require donor Tregs for its effect, suggesting Treg infusional therapy may be additive with IDO therapy. Future studies will examine methods for utilizing the IDO pathway for GVHD prevention or treatment.

Figure Legends

Figure 1. Donor T cells cause accelerated GVHD in $IDO^{-/-}$ versus wt recipients. B6 or $IDO^{-/-}$ mice were lethally irradiated (9.0 Gy) and infused with 10^7 MHC-mismatched BALB/C NTCD BM with or without $3x10^6$ BALB/C $CD4^+$ T cells. Survival plots of B6 vs. $IDO^{-/-}$ recipients are shown. ●, BM→B6; ■, $CD4^+$ T cells→B6; ○, BM→$IDO^{-/-}$; □, $CD4^+$ T cells→$IDO^{-/-}$. $p<.03$. Data are from one experiment, n=8 mice/group. B. B6 or $IDO^{-/-}$ mice were lethally irradiated and given BALB/C TCD BM with or without $3x10^6$ donor $CD4^+$ and $CD8^+$ T cells. Survival plots of B6 vs. $IDO^{-/-}$ recipients are shown. ●, BM→B6; ■, $CD4^+$ and $CD8^+$ T cells→B6; ○, BM→$IDO^{-/-}$; □, $CD4^+$ and $CD8^+$ T cells→$IDO^{-/-}$. $p<.04$, data are from one experiment, n=8 mice/group. C. BALB/C or IDO^-

$^{/-}$ mice were lethally irradiated (7.0 Gy) and infused with 10^7 B6 TCD BM with or without $2x10^6$ $CD4^+$ and $CD8^+$ T cells. Survival plots of BALB/C vs. $IDO^{-/-}$ recipients are shown. ●, BM→BALB/C; ■, $CD4^+$ and $CD8^+$ T cells→BALB/C; ○, BM→$IDO^{-/-}$; □, $CD4^+$ and $CD8^+$ T cells→$IDO^{-/-}$. Data are pooled from two experiments. n=16 mice/group. p<.001.

Figure 2. Donor T cells upregulate host IDO expression and worsen GVHD in $IDO^{-/-}$ versus wt recipients. A. Wt or $IDO^{-/-}$ mice were lethally irradiated and infused with $3x10^6$ $CD4^+$ and $CD8^+$ T cells and sacrificed on day 7. Cryosections of colon (left) and small intestine (right) were stained by immunohistochemistry for IDO. i, v, wild-type BM only; ii, vi, wild-type GVHD; iii, vii, $IDO^{-/-}$ BM only; iv, viii, $IDO^{-/-}$ GVHD. B. H&E staining of wt and $IDO^{-/-}$ colons (i-iv) and spleens (v-viii) 27 days after transplantation with $3x10^6$ $CD4^+$ and $CD8^+$ BALB/C T cells. i, v, wild-type BM only; ii, vi, wild-type GVHD; iii, vii, $IDO^{-/-}$ BM only; iv, viii, $IDO^{-/-}$ GVHD. C. IHC staining of tissues in B for CD4. Cell numbers in colon are quantitated below. *=p<.002. D. IHC staining of tissues in B for CD8. Cell numbers in colon are shown below. *=p<.02. Original magnification is x200, except spleens, which were taken at x100. Pictures are representative of 3-5 mice per group.

Figure 3. GVHD does not upregulate IDO in liver or skin. B6 or $IDO^{-/-}$ mice were lethally irradiated and infused with BALB/C TCD BM with or without 3 million $CD4^+$ and $CD8^+$ T cells. Seven days later, tissues were harvested, fixed, paraffin embedded, and stained by IHC for IDO. Pictures are representative of 3-5 mice/group.

Figure 4. IDO is increased in GVHD spleens. B6 mice were lethally irradiated and infused with BALB/C TCD BM with or without 3 million $CD4^+$ and $CD8^+$ T cells. Seven

days later, spleens were harvested, fixed, paraffin embedded, and stained by IHC for IDO. Pictures are representative of 3-5 mice/group.

Figure 5. GVHD histopathology is equivalent in small intestine, liver, and skin. B6 or $IDO^{-/-}$ mice were lethally irradiated and infused with BALB/C TCD BM with or without 3 million $CD4^+$ and $CD8^+$ T cells. 27 days later, tissues were harvested and cryosections were stained with H&E. Pictures are representative of 3-5 mice/group.

Figure 6. IDO-mediated Inhibition of GVHD lethality occurs independently of the presence of donor Tregs. A. BALB/C TCD BM with or without $3x10^6$ BALB/C CD25-depleted $CD4^+$ and $CD8^+$ T were infused into lethally irradiated B6 or $IDO^{-/-}$ recipients. Survival plots are shown. ●, BM→B6; ■, CD25-depleted T cells→B6; ○, BM→$IDO^{-/-}$; □, CD25-depleted T cells→$IDO^{-/-}$.For T cell groups of B6 vs. $IDO^{-/-}$, $p<.005$, data are from one experiment, n=8 per group. B. BALB/C TCD BM with or without $2x10^6$ BALB/C $CD4^+CD25^-$ T cells were infused into lethally irradiated B6 or $IDO^{-/-}$ recipients. Survival plots of B6 vs. $IDO^{-/-}$ are shown. ●, BM→B6; ■, $CD4^+CD25^-$ T cells→B6; ○, BM→$IDO^{-/-}$; □, $CD4^+CD25^-$ T cells→$IDO^{-/-}$.For T cell groups, $p<.02$, data are from one experiment, n=8 mice/group. C. BALB/C TCD BM with or without $1x10^6$ BALB/C $CD4^+$ T cells or $CD4^+CD25^-$ T cells were infused into $IDO^{-/-}$ recipients. Survival plots of CD25-depleted vs. non-depleted are shown. ○, BM→$IDO^{-/-}$; □, $CD4^+$ T cells→$IDO^{-/-}$; △, $CD4^+CD25^-$ T cells→$IDO^{-/-}$. For T cell groups $p<.02$, data are from one experiment, n=8 mice/group.

Figure 7. Host IDO induction and IDO-mediated spleen and colon protection do not require donor Tregs. A. IHC for IDO in colon seven days after transplantation with BALB/C TCD BM with or without $2x10^6$ $CD4^+CD25^-$ T cells. B. H&E staining of

colon (i-iv) and spleen (v-viii) 7 days after transplantation with Balb'c TCD BM with or without $2x10^6$ $CD4^+CD25^-$ T cells. i, v, wild-type BM only; ii, vi, wild-type GVHD; iii, vii, $IDO^{-/-}$ BM only; iv, viii, $IDO^{-/-}$ GVHD. C. Tissues from B stained for CD4 by IHC. Original magnification x200 for colons, x100 for spleens.

Figure 8. Host IDO deficiency does not increase radiation sensitivity. A. B6 or $IDO^{-/-}$ mice were sublethally irradiated (5.0 Gy), and weights were monitored twice weekly. Weights are shown as percent of weight on day -1. No mice died. n=4 mice/group. B. Mice were lethally irradiated (8.0 Gy) and infused with $10x10^6$ B6 (syngeneic) TCD or NTCD BM, and weights were monitored twice weekly. Weights are shown as percent of weight on day -1. No mice died. ●, B6 NTCD BM→B6; ■, B6 TCD BM→B6; ○, B6 NTCD BM→$IDO^{-/-}$; □, B6 TCD BM→$IDO^{-/-}$; n=5 mice/group.

Figure 9. Proliferation, survival, and effector function of allogeneic T cells in secondary lymphoid organs are not increased in $IDO^{-/-}$ mice. A. $60x10^6$ CFSE-labeled BALB/C splenocytes were transferred into lethally irradiated B6 or $IDO^{-/-}$ recipients. Mice were sacrificed on day 4 and spleen was examined by flow cytometry for CFSE dilution and Annexin V positivity. Spleen data shown are representative of 4 mice/group/day. Cells were gated on CD4 or CD8 positive, H-$2K^b$ negative events. Numbers indicate percentage of Annexin V positive cells. B. Lethally irradiated B6 or $IDO^{-/-}$ mice were infused with BALB/C TCD BM and $3x10^6$ $CD4^+$ and $CD8^+$ T cells. Drinking water was supplemented with BrdU at days 15-20 after transplantation. Spleen and mesenteric LN were harvested and analyzed by flow cytometry for BrdU incorporation. Data are pooled from two identical experiments, n=3-4 mice/group. No significant differences were observed. Cells were gated on CD4 or CD8 positive, H-$2K^b$

negative events. C. Lethally irradiated B6 or IDO$^{-/-}$ mice were infused with BALB/C TCD BM and 4x10^6 CD4$^+$ and CD8$^+$ T cells. Seven days after transplantation, spleens were harvested and analyzed by flow cytometry for FasL, Granzyme B, and IFN-γ expression. n=4 mice/group. Cells were gated on CD4 or CD8 positive, H-2K^b negative events. *=p<05.

Figure 10. Effector phenotype of donor T cells in target organs is not changed by IDO. B6 or IDO$^{-/-}$ mice were lethally irradiated and infused with BALB/C TCD BM and 3x10^6 CD4$^+$ and CD8$^+$ T cells. 21 days after transplantation, T cells were isolated from colons and livers, stimulated with plate-bound anti-CD3 for four hours, and stained for IFN-γ, IL-4, Granzyme B, and IL-10. n=4 mice/group. No significant differences were observed.

Figure 11. Proliferation and survival are increased in colons of IDO$^{-/-}$ recipients. A. B6 or IDO$^{-/-}$ mice were lethally irradiated and infused with BALB/C TCD BM and 3x10^6 CD4+ and CD8+ T cells. 21 days after transplantation, T cells were isolated from colons and livers and counted. n=4 mice/group. *=p<.04. B. T cells isolated from colon and liver were stained for Annexin V. n=4 mice/group, *=p<.01. C. Day 27 tissues from the transplant in Figure 2 were stained for CD4 in Cy5 (shown in red) and Ki-67 in FITC (shown in green), a nuclear marker of proliferation. Proliferating CD4$^+$ cells are yellow. Original magnification x400. Pictures are representative of two similar experiments, 2-5 fields/mouse, 3-5 mice/group.

Figures

Figure 1

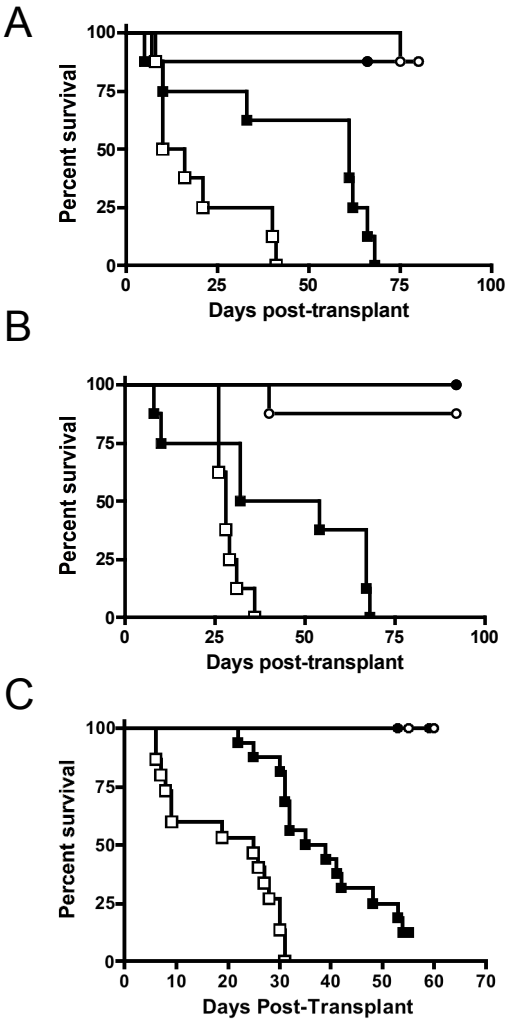
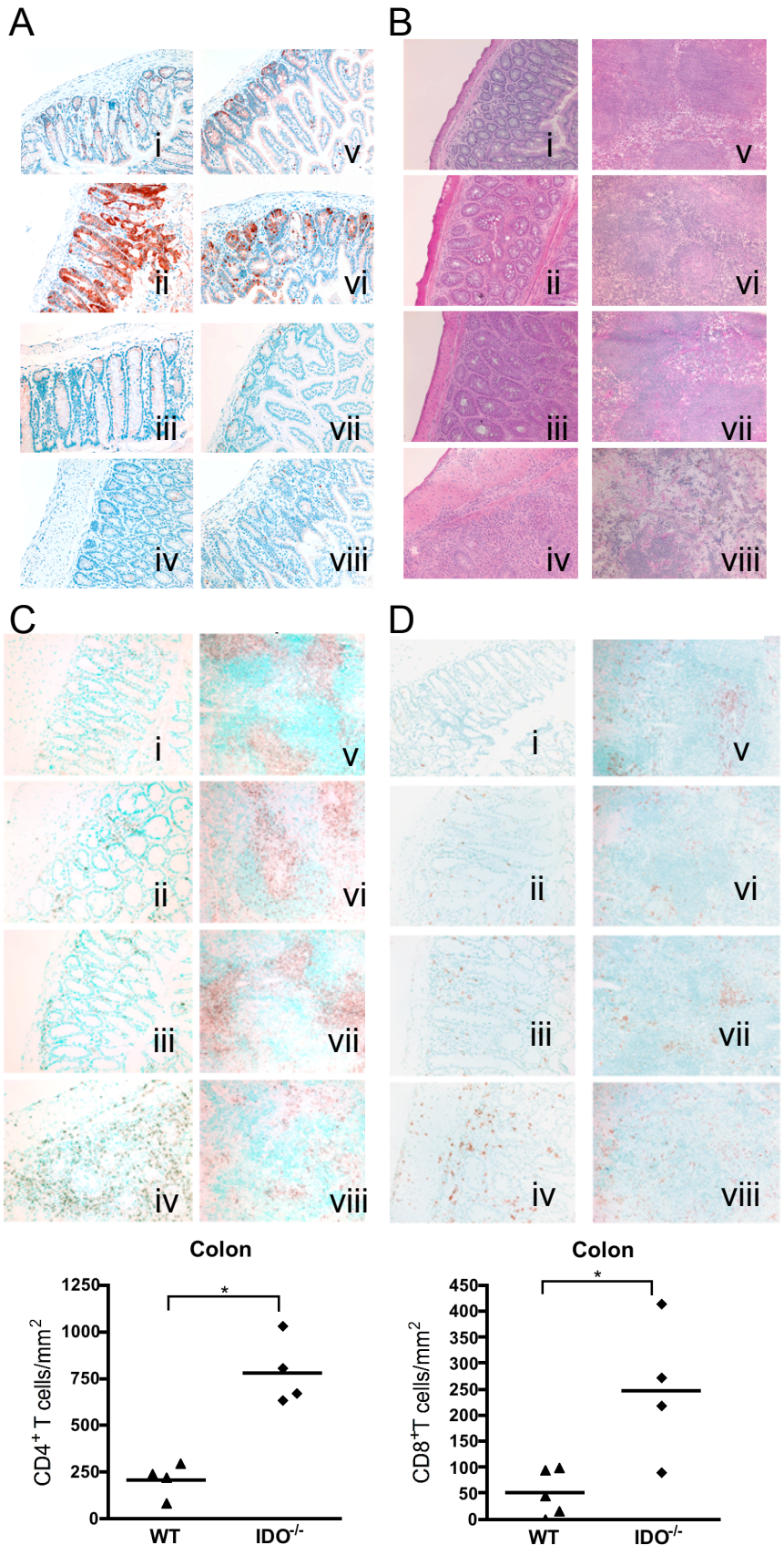


Figure 2


A
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vii
iv
viii
B
i
v
ii
vi
iii
vii
iv
viii
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v
ii
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iii
vii
iv
viii
D
i
v
ii
vi
iii
vii
iv
viii
Colon
CD4+ T cells/mm2
1250
1000
750
500
250
0
*
WT
IDO-/-
Colon
CD8+T cells/mm2
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350
300
250
200
150
100
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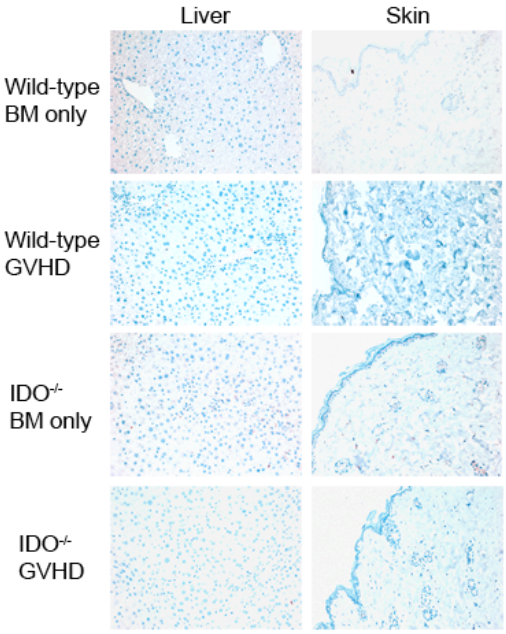
WT
IDO-/-

Figure 3



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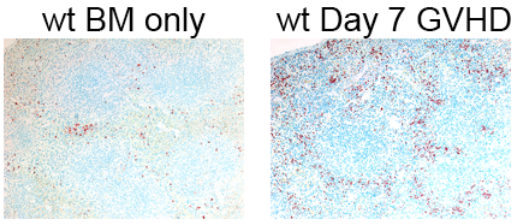

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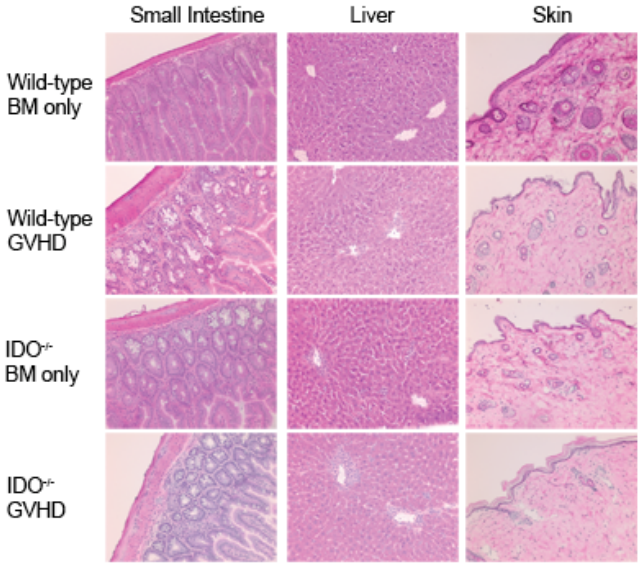

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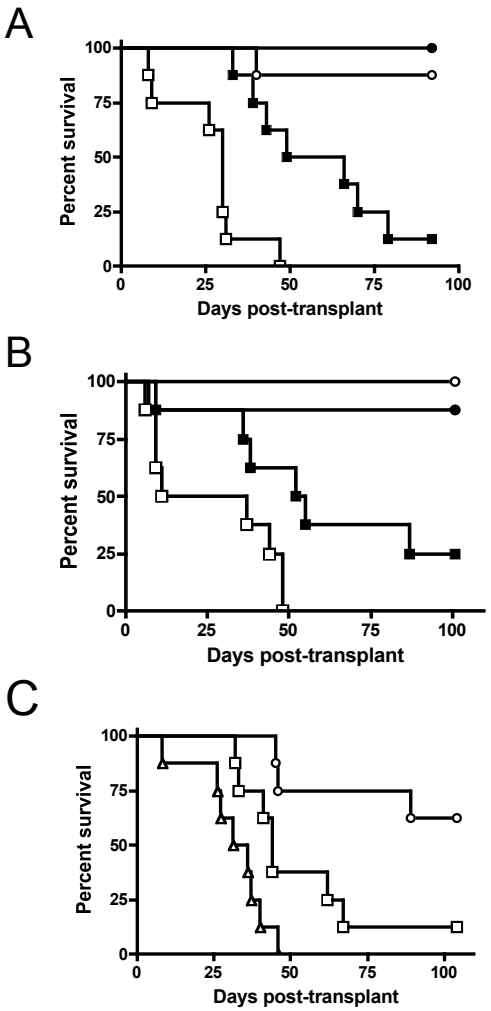
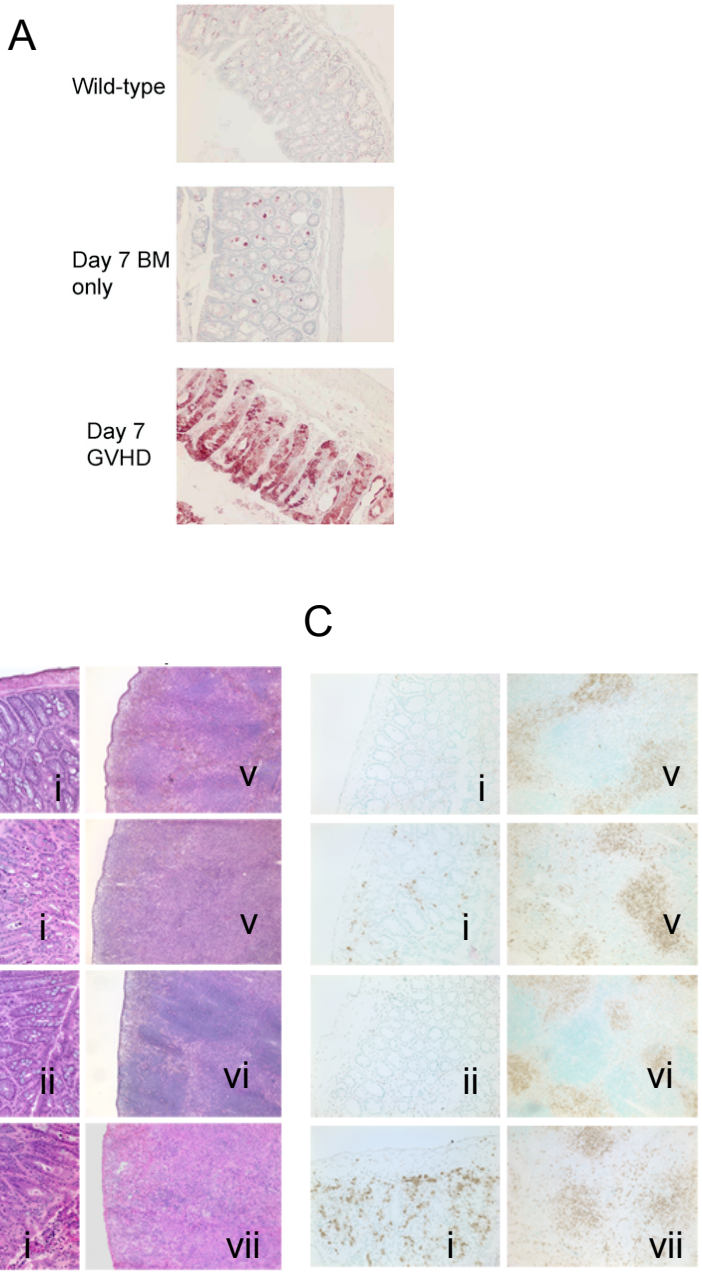

A
Percent survival
Days post-transplant
B
Percent survival
Days post-transplant
C
Percent survival
Days post-transplant

Figure 7



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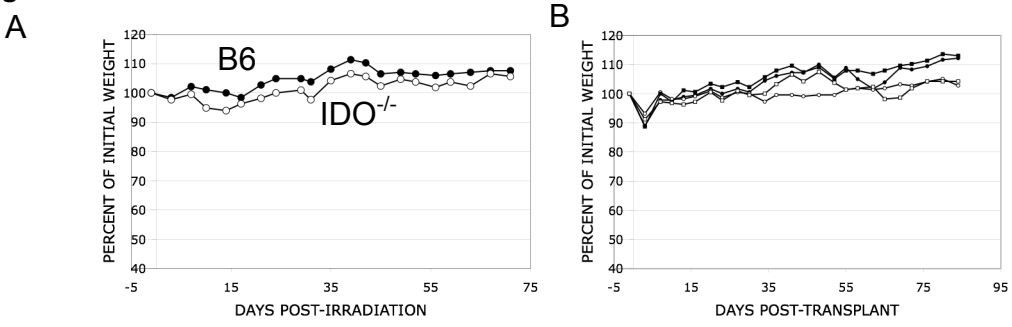

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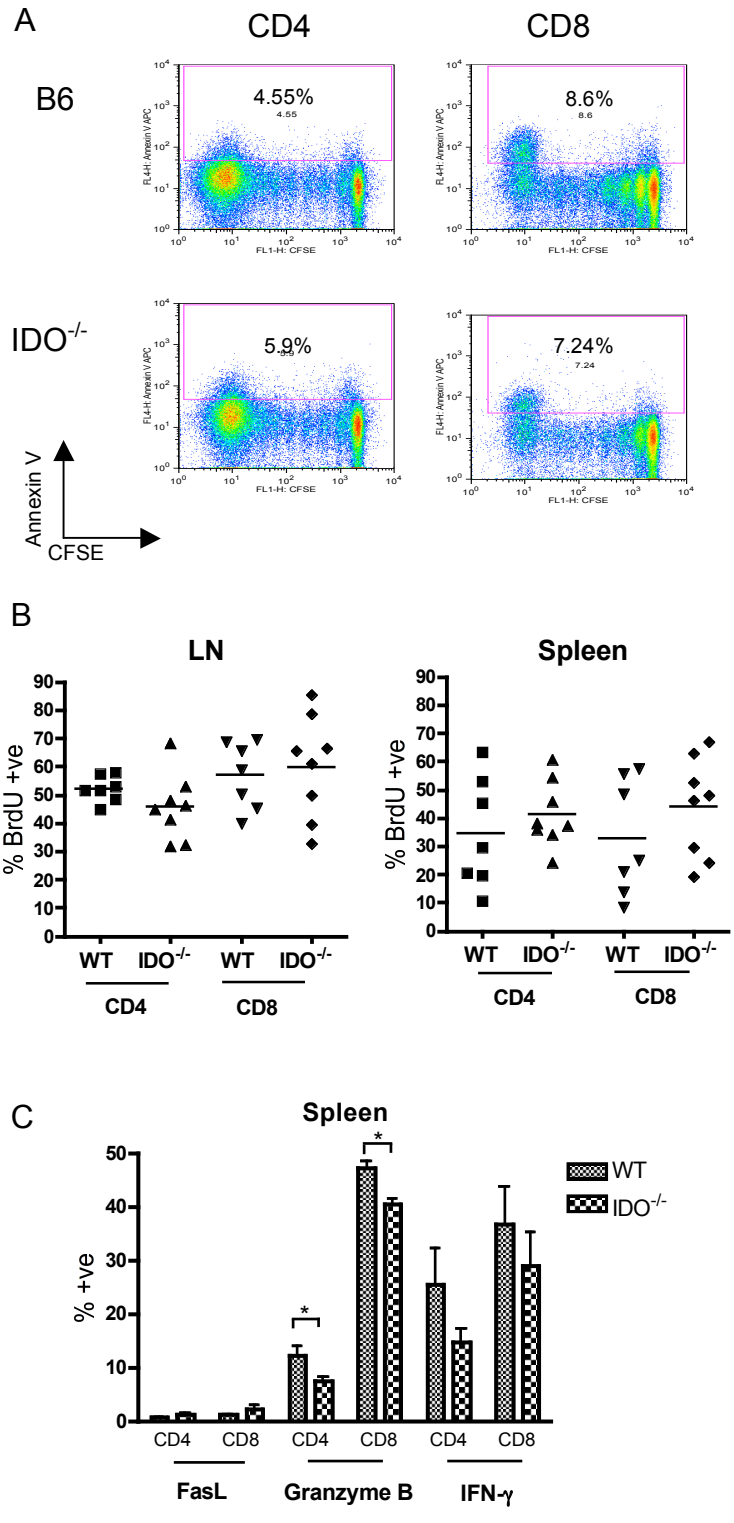
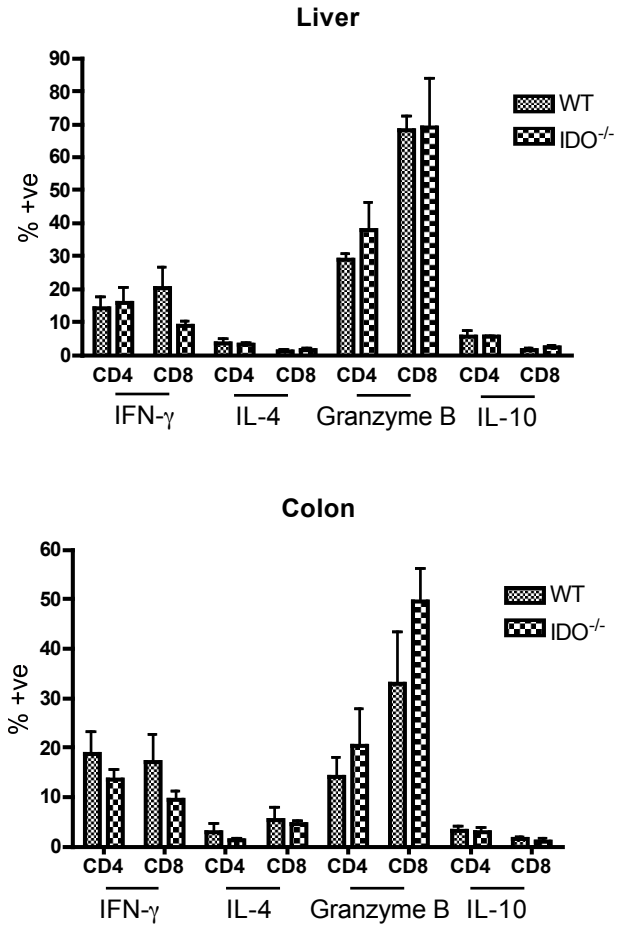

A
CD4
CD8
B6
4.55%
8.6%
IDO-/-
5.9%
7.24%
Annexin V
CFSE
B
LN
Spleen
% BrdU +ve
WT
IDO-/-
CD4
CD8
C
Spleen
% +ve
WT
IDO-/-
FasL
Granzyme B
IFN-γ

Figure 10


Liver
% +ve
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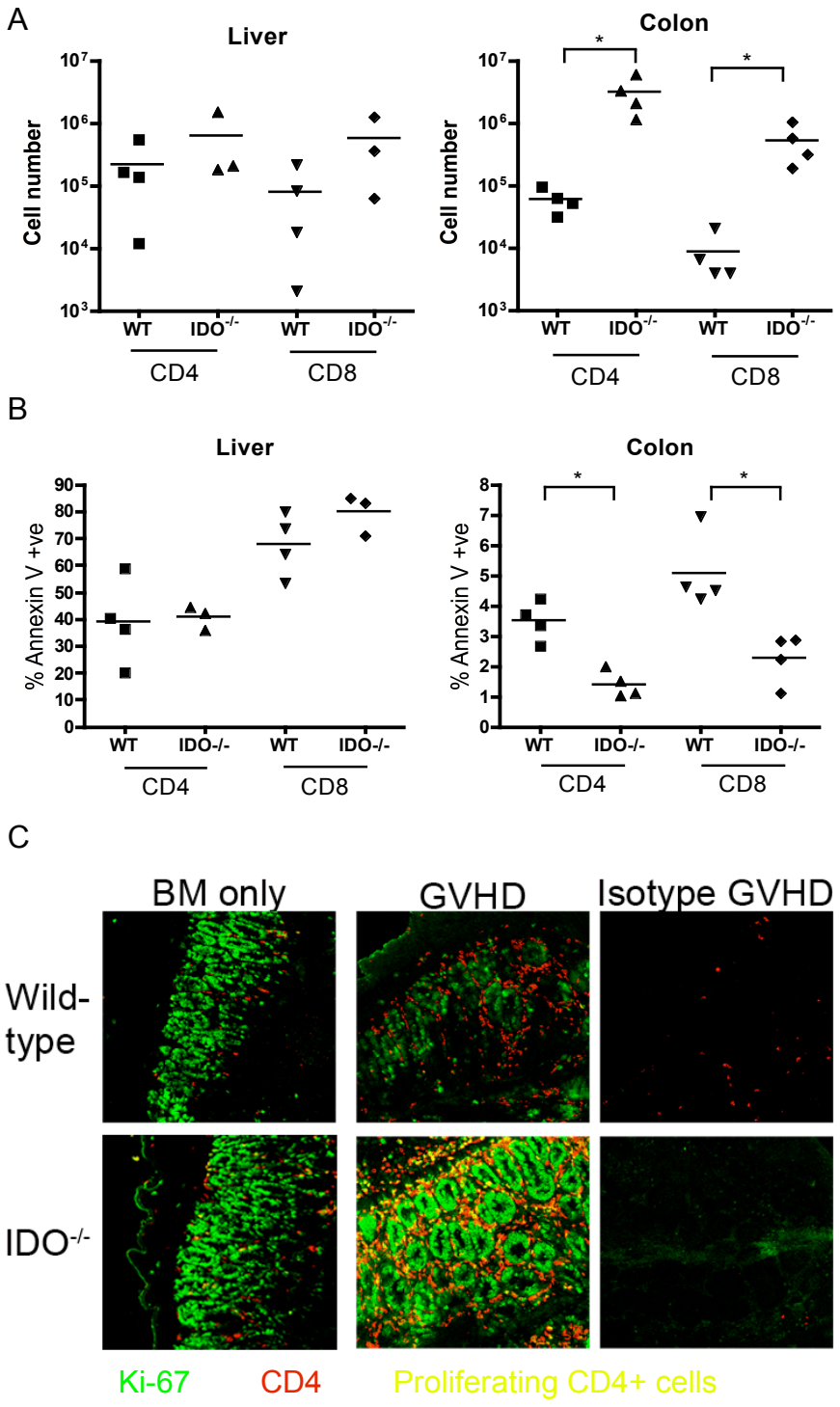
CD4 CD8 CD4 CD8 CD4 CD8 CD4 CD8
IFN-γ
IL-4
Granzyme B
IL-10
Colon
% +ve
WT
IDO-/-
CD4 CD8 CD4 CD8 CD4 CD8 CD4 CD8
IFN-γ
IL-4
Granzyme B
IL-10

Figure 11


A
Liver
Colon
Cell number
WT
IDO-/-
CD4
CD8
B
% Annexin V +ve
C
BM only
GVHD
Isotype GVHD
Wild-type
IDO-/-
Ki-67
CD4
Proliferating CD4+ cells

Chapter III: Using the IDO pathway to treat and prevent GVHD

During graft-versus-host disease (GVHD), donor T cells become activated and migrate to tissue sites. Previously, we demonstrated a crucial role for the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO) in GVHD regulation. Here, we show that upon arrival in the colon, activated donor T cells produced IFN-γ that upregulated IDO, causing T cell anergy and apoptosis. IDO induces GCN2 kinase, upregulating a T cell stress response implicated in IDO immunosuppression. Donor T cells did not require GCN2 kinase to respond to IDO, suggesting toxic IDO metabolites, and not tryptophan depletion, were responsible for suppression. When exogenous metabolites were administered, GVHD lethality was reduced. To determine if IDO could be induced pre-transplantation for enhanced GVHD suppression, we first determined whether antigen presenting cells (APCs) or epithelial cells were primarily responsible for IDO expression and subsequent GVHD suppression. Recipients with wild-type vs. $IDO^{-/-}$ APCs had increased survival, regardless of epithelial cell expression of IDO, suggesting that APCs were suitable targets for inducing IDO. Administration of an agonist to toll-like receptor (TLR)-7/8, a receptor expressed primarily on APCs, induced IDO in the colon, which was sufficient for GVHD amelioration. We conclude that IDO upregulation may have therapeutic potential for preventing GVHD in the clinic.

Introduction

Graft-versus-host disease (GVHD) is a complication of hematopoietic stem cell transplantation that occurs due to recognition of host antigens as foreign by donor T cells. Treatment is limited to immunosuppression, further endangering patients with already weakened immune systems. GVHD is initiated when conditioning damages gut epithelial cells, allowing translocation of bacterial products from the lumen into the tissue. These products induce innate immune cells to produce inflammatory cytokines

and chemokines, attracting alloreactive donor T cells that arrive and mediate more tissue damage. The primacy of the gut in GVHD is demonstrated by the fact that gnotobiotic (germ-free) mice are resistant to GVHD[31] and that interventions to spare the gut lead to increased survival[240]. We recently published that indoleamine 2,3-dioxygenase (IDO) is highly upregulated in the colon after allogeneic transplantation and that lack of IDO leads to increased colon GVHD and accelerated lethality[242]. The seemingly localized action of IDO makes it an intriguing target for GVHD therapies. We sought to better understand IDO's induction, location, and mechanism to identify ways in which it could be manipulated to suppress GVHD.

IDO is an intracellular enzyme that catalyzes the first and rate-limiting step in tryptophan catabolism. It has immunosuppressive properties that have been implicated in maternal-fetal acceptance[127], tumor immunity[202], chronic infection[197], and autoimmunity[208,213]. IDO's mechanism of action is still somewhat controversial, but is thought to result from tryptophan depletion and/or accumulation of the tryptophan breakdown products, called kynurenines. Tryptophan depletion leads to an increase in the intracellular concentration of uncharged tryptophanyl-tRNA. This accumulation activates GCN2 kinase, a member of a family of kinases that phosphorylate the translation initiation factor EIF2α and depress general protein translation while activating a stress pathway that leads to anergy and/or apoptosis. GCN2$^{-/-}$ T cells cannot respond to IDO as shown in vitro[125,165] and some in vivo studies[172]. On the other hand, kynurenines cause T cell apoptosis in vitro[130,243] and suppress inflammation in vivo[131,177,223]. One study found both tryptophan depletion and kynurenine production in vitro were required for immunosuppression[165]. Which of these mechanisms is operative during GVHD is unknown.

IDO is induced by inflammatory signals including Type I and Type II interferons and TLR signaling and therefore serves to downregulate immune responses. This ability of IFN-γ, the signature cytokine of highly inflammatory Th1 responses, to also trigger suppressive mechanisms may explain its contradictory role in GVHD. Though Th1 cytokines are highly expressed during GVHD and associated with increased disease[42,45], lack of IFN-γ through knockout donors[47], neutralizing antibody[47], or IFN-γR$^{-/-}$ recipients[54] leads to dramatically accelerated GVHD in lethally irradiated hosts. The actions of IFN-γ in GVHD are complex, causing different effects on different organs and varying with conditioning[54,55,244]. Understanding how IDO and IFN-γ interact during GVHD may help to parse out these effects. In addition, identifying the signals for IDO induction and the cell types capable of expressing IDO and modulating GVHD will suggest strategies for increasing IDO production as a means of prevention or therapy.

A recent report indicated pDCs, a prominent IDO-expressing APC subtype, are capable of antigen presentation in GVHD[79]. Endowed with the capacity to produce enormous amounts of Type I interferons in response to pathogens, plasmacytoid dendritic cells (pDCs) are considered a crucial part of the innate anti-viral immune response. Interferon production is triggered by activation of pattern recognition receptors, which include toll-like receptors (TLRs). The anti-viral activity of pDCs depends in part upon their expression of TLR7 and 9, which recognize single-stranded RNA and unmethylated-CpG-containing oligonucleotides, respectively. Although secretion of inflammatory cytokines aids the immune response, ligation of TLRs on pDCs also upregulates the immunosuppressive enzyme IDO[156,176]. Therefore, pDCs have suppressive properties under some conditions. As GVHD is critically dependent on host APCs[33], it is likely the propensity of pDCs to produce IDO could be manipulated to increase suppression.

Here we show that donor T cells upregulate colon IDO by IFN-γ production, contributing to the known suppressive effects of IFN-γ. IDO does not mediate suppression through GCN2 kinase, suggesting its effects are not due to tryptophan depletion but to the production of tryptophan catabolites, i.e. kynurenines. Consistent with this, we found exogenous kynurenines were capable of suppressing GVHD. We further determined APC0s are the crucial IDO-expressing cell, and therefore we targeted APCs for IDO upregulation using a TLR7/8 agonist. This intervention led to decreased GVHD lethality. Therefore, our results have increased our understanding of the mechanism of GVHD suppression by IDO and led to two therapeutic approaches with clinical relevance both for the treatment and prevention of GVHD.

Materials and Methods

Mice

IFN-$\gamma^{-/-}$, IFN-$\gamma R^{-/-}$ and recipient C57BL/6 ($H2^b$) (termed B6) and BALB/C ($H2^d$) were purchased from The Jackson Laboratory (Bar Harbor, ME). Donor B6 and BALB/C mice were purchased from the National Institutes of Health (Bethesda, MD). B6 $IDO^{-/-}$, BALB/C $IDO^{-/-}$, and $GCN2^{-/-}$ mice were generated as previously described[172,237] and bred at the University of Minnesota. Mice were housed in a specific pathogen-free facility in microisolator cages and used at 6-16 weeks of age.

BM transplantation

B6 or B6 $IDO^{-/-}$ mice, or B6.Ly5.1 for chimeras, were irradiated with 8.0-9.0 Gray (Gy) total body irradiation (TBI) and BALB/C or BALB/C $IDO^{-/-}$ mice with 6.0-7.0 Gy by an x-ray or cesium source on day -1. Ten million bone marrow (BM) cells with or without

purified T cells were infused on day 0. To deplete BM of T cells, BM was incubated with anti-CD4 and -CD8 on ice for 20 minutes followed by addition of rabbit complement at 37^{o} C for 30 minutes. For GVHD induction, T cells were isolated from lymph nodes (LNs) and purified by incubation with PE-labeled antibodies to CD19, γδ TCR, and DX5 or NK1.1 (eBioscience, San Diego, CA) followed by incubation with anti-PE beads and depletion on a magnetic column (Miltenyi Biotec, Bergisch-Gladbach, Germany). Cell preparations were verified by flow cytometric phenotyping to be more than 99% of the desired phenotype. Mice were monitored daily for survival and weighed twice weekly for the first month, then once weekly thereafter as well as examined for the clinical appearance of GVHD. In some experiments, recipients were sacrificed and hematoxylin and eosin-stained cryosections of liver, lung, colon, small intestine, and spleen were histologically assessed using a semiquantitative GVHD scoring system (0 to 4.0 grades in 0.5 increments) as published[238]. Coded sections were graded by one of us (A.P.-M.) without knowledge of the treatment.

Frozen tissue preparation

Tissues, including colon, small intestine, liver, lung, and spleen were taken at indicated days after transplantation, embedded in Optimal Cutting Temperature (OCT) compound (Miles, Elkhart, IN), snap-frozen in liquid nitrogen, and stored at -80°C.

Immunohistochemistry (IHC)

Following acetone fixation, cryosections (6 μm) were immunoperoxidase-stained using avidin-biotin blocking reagents, ABC-peroxidase conjugate, and DAB chromogenic substrate, all purchased from Vector Laboratories (Burlingame, CA). Antibodies used

were biotinylated mAbs to CD4 (clone GK1.5) and CD8 (clone 2.43), both purchased from BD Biosciences, San Jose, CA. Sections were counterstained with methyl green.

For IDO staining, tissues were fixed in formalin and paraffin embedded. Sections underwent high-temperature antigen retrieval using Target Retrieval Solution (Dako Corporation, Carpinteria, CA) and stained using rabbit anti-IDO polyclonal antibody (prepared by Southern Biotech, Birmingham, AL based on the peptide sequence previously described[239]). Biotinylated anti-rabbit secondary antibody was incubated for one hour, followed by ABC-peroxidase conjugate and AEC chromogenic substrate, all from Biogenex (San Ramon, CA). Sections were counterstained with hematoxylin.

Images were obtained on an Olympus BX51 microscope using 20x/0.70 objective lens and acquired using a Spot CCD digital camera and Spot software (Diagnostic Instruments, Inc., Sterling Heights, MI) and processed with Adobe Photoshop CS2 v9.0.2 (Adobe Systems, San Jose, CA).

T cell isolation

Livers were harvested and mashed into a single-cell suspension. Colons from cecum to rectum were harvested, cut into pieces, and incubated 3 times with 5 mM EDTA in RPMI with 10% serum for 15 minutes at 37°C. Supernatants containing intraepithelial lymphocytes were discarded. Colons were then incubated twice with 0.5 mg/mL and once with 1 mg/mL Collagenase D (Roche, Indianapolis, IN) for 1 hour at 37°C with shaking. Colon pieces were mashed, and liver and colon cells were isolated on a 40/80 Percoll (Sigma-Aldrich) gradient. Some cells were stained with antibodies for CD4 and

CD8 (eBioscience) and Annexin V or Ki-67 (BD Biosciences) according to manufacturer's instructions.

Reagents

L-Kynurenine, 3-hydroxyanthranilic acid, and 3-hydroxykynurenine (Sigma-Aldrich) were dissolved in 1 M NaOH and brought to a final concentration of 1.5mg/mL in PBS after adjusting pH to 7.2-7.4. 3M-011 was obtained from 3M Corp (St. Paul, MN), dissolved in DMSO, and diluted in PBS to 400μg/mL.

Quantitative PCR (qPCR)

cDNA was synthesized from TRIzol-isolated total RNA using the SuperScript® III First Strand Synthesis SuperMix for qRT-PCR (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. For quantitative real-time reverse transcription–PCR experiments, reactions containing the TaqMan Universal PCR Master Mix and probes for IDO (Mm00492586_m1) or glyceraldehyde-3-phosphate dehydrogenase (Mm99999915_g1) as the control gene were run on the Applied Biosystems 7300 Real Time PCR System and analyzed with ABI Relative Quantification Study software (Applied Biosystems, Foster City, CA). The $2^{(-\Delta\Delta C_t)}$ method was used to generate RQ for quantifying IDO expression in samples in comparison with unmanipulated wild-type (wt) mice. The formula used was RQ = {9i}.

Statistics

Survival data were analyzed by life-table methods and actuarial survival rates are shown. Group comparisons were made by log-rank test statistics. Group comparisons

of cell counts, pathology scores, and flow cytometry data were analyzed by Student t test. P values below .05 were considered significant in all tests.

Results

IFN-γ response is required for IDO induction during GVHD

We have published that IDO is highly upregulated in the colon following allogeneic transplantation, but we did not identify the signal for IDO induction[242]. IFN-γ was a reasonable candidate, as it is a potent stimulus for IDO induction. We examined the involvement of IFN-γ in IDO's role in GVHD by transplanting wt and IFN-γR$^{-/-}$ mice, which are incapable of receiving IFN-γ signals from donor T cells, with allogeneic BM with or without T cells. Fig. 1A shows recipients that cannot respond to IFN-γ were far more susceptible to GVHD-induced lethality. We asked whether this protective effect of IFN-γ was due to its ability to induce IDO. Colons from wt and IFN-γR$^{-/-}$ recipients were harvested 7 days after transplantation and IDO expression was assessed by qPCR and IHC. As expected, wt mice receiving allogeneic T cells highly upregulated IDO mRNA and protein (Fig. 1B). In contrast IFN-γR$^{-/-}$ recipients expressed less IDO than even untransplanted wt mice, indicating IFN-γ is required for IDO upregulation in the colon. To exclude the possibility that fewer T cells traffic to IFN-γR$^{-/-}$ colons, leading to less production of the IDO-inducing factor, we performed qPCR for CD4 and found equivalent levels, indicating similar numbers of T cells were available in both colons for inducing IDO.

Though it was possible lethal irradiation and infusion of allogeneic BM could produce sufficient inflammation, and therefore IFN-γ, to induce IDO, we did not detect IDO induction in colons unless the mice also received allogeneic T cells, leading us to

hypothesize that donor T cells were the source of IFN-γ. Therefore, we transplanted BM with or without wt or IFN-$\gamma^{-/-}$ T cells into lethally irradiated allogeneic recipients and seven days later harvested colons to determine IDO induction by IHC and qPCR. Figure 1C confirms that donor T cells were required for IDO upregulation and shows that, while wt allogeneic donor T cells induced substantial IDO mRNA and protein, donor T cells unable to make IFN-γ could not induce IDO above that seen in BM only controls.

Having shown that IFN-γ is an IDO-inducing factor, we hypothesized that GVHD acceleration in the absence of IFN-γ was due, at least in part, to the lack of IDO upregulation. Therefore, we transplanted wt or $IDO^{-/-}$ mice with wt or IFN-$\gamma^{-/-}$ T cells. As has been shown before, IFN-$\gamma^{-/-}$ T cells caused accelerated GVHD in wt hosts (Fig. 1D). Whereas wt T cells caused accelerated lethality in $IDO^{-/-}$ vs wt recipients, there was no difference in survival between wt and $IDO^{-/-}$ recipients of IFN-$\gamma^{-/-}$ T cells. Thus, in the absence of IFN-γ and upregulation of IDO, the survival advantage of wt over $IDO^{-/-}$ recipients was abrogated.

Our previous work showed IDO-based suppression of GVHD correlated with decreased proliferation and survival of donor T cells in the colon. We now examined these IDO effects in mice receiving wt or IFN-$\gamma^{-/-}$ T cells. As shown in Figure 1E and Figure 2, a higher proportion of wt colon $CD4^+$ and $CD8^+$ T cells from $IDO^{-/-}$ recipients were positive for Ki-67 and negative for Annexin V as compared to wt recipients. When donor T cells were IFN-$\gamma^{-/-}$, there was no difference in Annexin V staining in either $CD4^+$ or $CD8^+$ donor T cells and only a slight, albeit significant, increase in Ki-67 staining in $CD4^+$ T cells in wt vs. $IDO^{-/-}$ recipients of IFN-$\gamma^{-/-}$ T cells. These latter data suggest that basal levels of IDO

in wt mice are capable of restraining T cell proliferation, but to a lesser degree than when IDO is strongly upregulated.

BM-derived rather than epithelial cell expression of IDO is more critically important for GVHD suppression

Both epithelial cells and bone marrow-derived APCs are capable of IDO expression. To determine which cell type is critical for IDO-mediated GVHD suppression, we created BM chimeras in which the tissue cells, including epithelial cells, are $IDO^{-/-}$, and BM-derived cells, including most APCs, are $IDO^{+/+}$. These mice ($wt \rightarrow IDO^{-/-}$) could then be used to determine if replacing $IDO^{-/-}$ with wt APCs could ameliorate disease. Conversely, we reconstituted wt mice with $IDO^{-/-}$ BM, creating $IDO^{-/-} \rightarrow wt$ chimeras that cannot express IDO in BM-derived cells but do express IDO in epithelial cells. We also created two groups of control chimeras ($wt \rightarrow wt$ and $IDO^{-/-} \rightarrow IDO^{-/-}$). After waiting three months to ensure turnover of host BM-derived cells, we analyzed peripheral blood for engraftment. Nearly 100% of $CD19^{+}$ cells were of BM donor origin (not shown). We then re-irradiated the chimeras and infused BM and allogeneic T cells to induce GVHD. As expected, $IDO^{-/-} \rightarrow IDO^{-/-}$ chimeras died significantly faster than $wt \rightarrow wt$ chimeras (Fig. 3A). As compared to $IDO^{-/-} \rightarrow IDO^{-/-}$ chimeras, survival was significantly prolonged in $wt \rightarrow IDO^{-/-}$ chimeras (IDO only expressed in APCs), supporting an important role of IDO expression in host APCs and GVHD prevention. Survival was also prolonged in $wt \rightarrow wt$ vs. $IDO^{-/-} \rightarrow wt$ chimeras (IDO only expressed in tissues), although this did not quite reach statistical significance, suggesting that in the presence of epithelial cell IDO expression, IDO expression in donor APCs contributes to GVHD downregulation albeit to a lesser extent than when IDO is absent in host epithelial cells. Epithelial cell expression of IDO had a smaller effect, as survival was modestly reduced in $IDO^{-/-} \rightarrow IDO^{-/-}$ vs. $IDO^{-/-} \rightarrow wt$

chimeras and even less so in wt→IDO$^{-/-}$ vs. wt→wt chimeras. Taken together, these data suggest that wt APCs provide a physiologically important source of IDO that contributes to GVHD prevention.

We next used these chimeras to examine the effect of IDO expression limited to APCs or host non-hematopoietic cells on colon CD4^{+} and CD8^{+} T cells. In line with the survival data, Figure 3B shows replacing IDO$^{-/-}$ with wt APCs resulted in significantly less proliferation and more apoptosis in colon CD4^{+} T cells. Conversely, replacing wt APCs with IDO$^{-/-}$ APCs led to less apoptosis and increased proliferation in colon CD4^{+} T cells. Therefore, IDO^{+} APCs mediate the suppressive effects on donor T cells in the colon. Because there was no difference in Ki-67 expression or Annexin V staining in colon T cells between wt→IDO$^{-/-}$ chimeras and wt→wt chimeras or between IDO$^{-/-}$→IDO$^{-/-}$ chimeras and IDO$^{-/-}$→wt chimeras, these data suggest that epithelial cell expression of IDO does not control T cell proliferation or apoptosis. Given the link we have previously established between the IDO and another regulatory pathway, the PD-1/PD-L1, -L2 system[125], we examined colon T cells for PD-1, a marker of "exhausted" T cells. Intriguingly, CD4^{+} T cells in IDO$^{-/-}$→IDO$^{-/-}$ colons expressed lower levels of PD-1 than those from wt→wt, and replacing either the IDO$^{-/-}$ APCs or epithelial cells with wt cells increased PD-1 expression (Fig. 3B). These data suggest that in the complete absence of host IDO expression, despite higher proliferation, colonic T cells are less prone to an exhausted phenotype than when IDO is expressed. However, replacing host wt APCs or tissue with IDO$^{-/-}$ cells did not change PD-1 expression compared to wt→wt chimeras, suggesting PD-1 is upregulated as long as some IDO is present, regardless of which cell expresses it. Notably, no differences were seen in Ki-67, Annexin V, or PD-1 in T cells from spleen or liver, confirming the colon-specific actions of IDO (not shown).

Kynurenines and not donor T cell GCN2 kinase influence GVHD suppression by the IDO pathway

IDO metabolizes tryptophan, resulting in two effects: decreased tryptophan and increased tryptophan metabolites. Both have been cited as the mechanism of IDO-based immunosuppression. T cells require GCN2 kinase to respond to low tryptophan. To determine if this pathway is operative during GVHD, we compared survival in wt hosts receiving wt or $GCN2^{-/-}$ T cells. In the context of GVHD, if GCN2 activation leads to cell cycle arrest and apoptosis, then $GCN2^{-/-}$ donor T cells should cause increased GVHD. However, this hypothesis proved incorrect, as $GCN2^{-/-}$ vs. wt T cells did not accelerate GVHD in wt hosts (Figure 4). If donor T cells require GCN2 to respond to IDO, then the survival advantage of wt vs. $IDO^{-/-}$ hosts should disappear if donor T cells cannot express 6GCN2. However, $IDO^{-/-}$ recipients of $GCN2^{-/-}$ T cells died more quickly than wt recipients of $GCN2^{-/-}$ T cells. Together, these data indicated that IDO suppresses GVHD through a GCN2-independent mechanism.

Because survival is not a sensitive measure of GVHD, we also examined the effects of GCN2 deficiency on T cell phenotype and organ pathology. $GCN2^{-/-}$ vs wt T cells underwent similar rates of proliferation and apoptosis and expressed similar amounts of IFN-γ early after transplantation. Colons of mice receiving wt or $GCN2^{-/-}$ T cells showed similar $CD4^{+}$ T cell infiltration three weeks after transplantation (not shown). Therefore, the lack of GCN2 did not affect the proliferative or IFN-γ-secreting behavior of donor T cells during the priming or effector phases of GVHD.

The donor T cell GCN2-independence of the influence of IDO expression on GVHD suggested that IDO suppression of GVHD occurred via kynurenine production rather than tryptophan depletion. To determine if exogenous kynurenines could suppress GVHD, we treated recipients with PBS or 75mg/kg/day of a mixture of L-kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranillic acid by intraperitoneal injection starting 17 days after transplantation. As shown in Figure 5A, kynurenine injection significantly ameliorated GVHD. When kynurenine administration was stopped at day 60, previously treated mice succumbed to GVHD, indicating that continuous exposure to kynurenines was required for GVHD inhibition even at this later time post-BMT (Fig. 5B).

Pre-BMT IDO upregulation suppresses GVHD

A second strategy for suppressing GVHD through IDO is to induce IDO expression prior to transplantation, rather than waiting for activated T cells to produce IFN-γ and induce IDO after the alloresponse has started. Figure 3 highlights the importance of APCs for IDO-based suppression; these data suggested targeting APCs for IDO upregulation may be successful for GVHD suppression. Studies have determined pDCs are crucial IDO-expressers[157,162,245]. In response to TLR ligation, pDCs release large amounts of type I IFNs, leading to IDO upregulation. Therefore, we examined whether TLR ligation could induce IDO in recipient mice. Four to five hours after lethal irradiation on day -2, recipient mice were injected intravenously with 200μg of a TLR7/8 agonist, 3M-011. BM and T cells were transferred on day 0, thereby minimizing any direct effects that this small molecule agonist might have on transferred cells. On day 0, prior to donor T cell infusion, we observed significant upregulation of IDO in colons but not spleens of mice injected with agonist (Fig. 6A). On day 2, IDO expression had fallen to baseline. On day 4, IDO expression in the colon and spleen began to rise in PBS-treated animals,

consistent with increased inflammation and IFN-γ production, while IDO remained low in the agonist-treated animals, suggestive of a reduced GVHD reaction.

We then tested whether this treatment could delay GVHD. Figure 6B shows that mice injected with TLR7/8 agonist prior to transplantation had significantly delayed GVHD lethality. We then examined the colons 37 days after transplantation. Figure 6C shows mice treated with 3M-011 had significantly lower colon pathology scores compared to those treated with PBS (average score of 2.7 vs. 3.6, $p<0.05$). In addition, IHC for CD4 revealed that 3M-011-treated recipients had fewer colon-infiltrating $CD4^+$ cells than mice treated with PBS (Figure 6D), consistent with the protective effect of increased colonic IDO expression. $IDO^{-/-}$ recipients did not benefit from TLR7/8 agonist injection, indicating its effects were dependent on IDO (Fig. 7).

Discussion

Several major findings were uncovered by the present study. First, IFN-γ proved critical for host IDO induction during the GVHD response. Second, host APCs were a biologically more important source of IDO for suppressing GVHD than epithelial cells. Third, GCN2 kinase, which senses tryptophan depletion, was not involved in regulating GVHD lethality in wt or $IDO^{-/-}$ hosts. Fourth, kynurenines, tryptophan catabolic products, reduced GVHD lethality but only while administration continued. Fifth and lastly, the purposeful induction of IDO pre-BMT by a TLR7/8 agonist reduced subsequent GVHD lethality.

This work further characterizes the biology of IDO during GVHD. The complete lack of IDO upregulation in the absence of donor IFN-γ is somewhat surprising, given the ability

of multiple inflammatory signals, including Type I IFNs, to induce IDO. Gut damage due to conditioning leads to translocation of bacterial products from the lumen to the tissue, ligating TLRs and causing the release of cytokines including IFNs. It is possible these signals do not reach the threshold required for IDO upregulation in this setting.

Though IFN-γ produced by donor T cells leads to massive IDO upregulation in epithelial cells as seen by IHC, our chimera studies indicate APCs are the more important cells for IDO expression and control of GVHD. Examination of the phenotype of colon T cells implicates APC expression of IDO as the regulator of T cell survival and proliferation. Most of the parameters examined were more prominently affected in the $CD4^+$ T cell subset. As $CD4^+$ T cells are the predominant infiltrating cell in the colon, this may simply be due to low numbers of $CD8^+$ T cells recovered. In support of the importance of APC-derived IDO in GVHD, a recent study found that the amelioration of GVHD by histone deacetylase (HDAC) inhibitors was due to the induction of IDO in APCs[231]. Injection of wt but not $IDO^{-/-}$ DCs treated with HDAC inhibitor delayed GVHD, and treatment of recipients with the inhibitor was only efficacious in mice with wt APCs[231]. In these studies, chimeras were used but no survival difference between wt→wt and $IDO^{-/-}$→wt chimeras was found[231]. Our chimera experiments using larger group sizes uncovered a trend toward decreased survival in $IDO^{-/-}$→wt chimeras. At the tissue level, the consequence of $IDO^{-/-}$ APCs was more evident, with significant effects on T cell survival and proliferation. These conclusions beg the question of the importance of IDO expression in epithelial cells. It is possible epithelial cell expression of IDO dampens GVHD through T cell-independent means or by affecting T cells in ways not related to survival and proliferation. This contribution may have a beneficial effect not detectable by the survival rate, a relatively crude measurement.

In fact, our data suggest epithelial cell, as well as APC-derived, IDO is associated with PD-1 expression, though the differences determined by FACS analysis did not perfectly match the survival differences. The PD-1 effect is especially relevant in our system, as blockade of the PD-1 pathway dramatically accelerates GVHD[246]. Thus, higher PD-1 expression in wt vs. IDO$^{-/-}$ mice may contribute to the reduced GVHD lethality seen in the former. Only a few links between IDO and PD-1 have been reported. Increased PD-1 expression was observed in association with increased IDO expression and decreased viral clearance in an HCV model[247], and IDO and PD-1 were upregulated in tumors that did not respond to cytokine treatment, along with several other negative immune regulators[248]. Neither of these studies looked specifically at T cell expression of PD-1. IDO may regulate PD-1 expression or the two pathways may be simply activated by the same immune modulatory signals. It is interesting that cells stimulated by alloantigen for weeks and continuing to proliferate are also less "exhausted" due to IDO expression. Whether this is a direct effect of IDO exposure and regulators of the PD-1 pathway on donor T cells or is an indirect effect is not known. A stronger association exists between IDO and the PD-1 ligands, PD-L1 and PD-L2. Expression of these ligands in tolerogenic environments, such as placenta[249] and tumor microenvironments[250], sites that express IDO, as well as their upregulation by IFN-γ[250], the main IDO inducer, suggests a possible link between the two. In addition, we have previously reported that the function of IDO-activated Tregs required upregulation of PD-L1 and PD-L2 on DCs, rendering such DCs suppressive[125]. The exact mechanistic link between IDO and the PD-1 pathway requires further study.

Though GCN2 is not the exclusive sensor for amino acid deprivation, it is the only mechanism implicated in IDO-based immunosuppression. The GCN2-independence of GVHD suppression suggests tryptophan metabolites are the primary mechanism for IDO in this system. In addition, the ability of IDO to act as a free-radical scavenger could contribute to its anti-inflammatory properties and gut protection, and this action would be GCN2-independent as well.

The in vitro and in vivo suppressive effects of exogenous kynurenines are in accord with other murine studies on autoimmunity, allergen immunotherapy and alloresponses[131,177,223]. Furthermore, in a murine model of chronic granulomatous disease, Romani and colleagues showed defective IDO activation led to the inability to produce tryptophan metabolites[179]. By providing kynurenine, the first breakdown product, they could bypass the IDO block and reestablish immune suppression. This strategy required co-injection of IFN-γ to induce the tryptophan metabolism enzymes downstream of IDO, including kynurenine 3-monooxygenase. Presumably the high levels of IFN-γ seen in GVHD would ensure the expression of these enzymes in the tryptophan catabolic pathway, so that even in the absence of IDO, providing kynurenine would lead to production of each of these potentially suppressive metabolites. Differing reports have found 3-hydroxykynurenine[177] or 3-hydroxyanthranilic acid[130] (and not the other) or both[223,243] to be suppressive. Whether they are further metabolized in vivo to other more or less suppressive compounds and what equilibrium is reached between concentrations of these compounds is not known. Future studies are needed to determine whether GVHD inhibition by kynurenines can override host IDO deficiency. Lastly, it is notable that discontinuing kynurenines was followed by GVHD lethality. These data suggest that kynurenines held donor T cell number below a threshold

needed to generate lethality; discontinuing kynurenines released this brake, indicating that kynurenines did not induce tolerance in donor T cells but rather controlled the size of the alloreactive T cell population. Whether kynurenine treatment affects antimicrobial immunity will require further study.

The striking ability of TLR ligation to lessen GVHD is even more interesting considering the early and transient upregulation of IDO. The 5-10-fold induction two days following TLR agonist injection is far less than the hundreds-to-thousands-fold induction seen on day 7 post-BMT due to donor T cell IFN-γ production, and yet it controls GVHD better than the later, more intense IDO upregulation. Because donor T cells expand 50-100 fold and are highly activated by day 5 post-BMT[251], early heightened IDO expression may be particularly effective at reducing the overall T cell burden during the most intense inflammatory phase of pre-BMT conditioning. The chimera experiments highlighted the importance of APCs in suppressing GVHD through IDO, despite strong IDO expression in colonic epithelial cells. Thus, the efficacy of lower levels of TLR-induced IDO could be due in part to the greater importance of IDO expression in APCs vs. epithelial cells. Since TLR7/8 agonists target APCs and colons contain fewer APCs than epithelial cells, the upregulation of IDO in this small population of cells likely results in a physiologically more effective mechanism to suppress GVHD, perhaps due to the special interactions between T cells and APCs. Early IDO expression in APCs may be capable of suppressing the first T cells that arrive in the colon, stopping one of the primary events in the cycle of inflammation and destruction. Moreover, early IDO induction may have tissue-protective effects as mentioned above, and it's possible that decreasing epithelial cell damage dampens another step in the destructive cycle. Given the minor component of APCs in the colon, it is not surprising that a massive increase in IDO expression in the

colon was not seen following TLR agonist administration, as colonic APCs and epithelial cells were not separately analyzed.

GVHD inhibition was achieved by administering TLR7/8 agonist pre-BMT, in contrast to the GVHD acceleration we observed with post-BMT TLR agonist administration, the latter likely due to overwhelming proinflammatory cytokine release with inadequate control by IDO[252]. These results are especially encouraging given the local and temporary nature of IDO induction, suggesting immunity to pathogens in the GI tract would not be affected by such a treatment. Since regulation of the immune response by IDO during GVHD appears confined to colon T cells[242], and because TLR7 agonists given to patients have anti-tumor effects[253], this approach may prove to be especially efficacious in preserving a graft-versus-malignancy effect as will be addressed in future studies.

In conclusion, these data increase our understanding of the mechanism of IDO induction and action during a relevant disease process and, most importantly, have led to the development of two novel and clinically applicable therapeutic strategies to prevent and treat GVHD. Because TLR7/8 agonists have been, and kynurenines are being, developed for use in the clinic, we believe these results are an exciting first step toward potent new GVHD therapies.

Figure Legends

Fig. 1. Donor T cell-produced IFN-γ is required for colon IDO induction and effects on T cell proliferation and survival. A. Wt (filled symbols) or IFN-γR$^{-/-}$ (open symbols) B6 mice were lethally irradiated and infused with allogeneic BM with (squares) or without

(circles) $3x10^6$ whole T cells. n=8 mice/group. Survival is shown. p<0.0001. B. Mice from transplant in A were sacrificed 7 days after transplantation and colons were examined for IDO protein by IHC and IDO and CD4 mrNA by qPCR. For IDO in wt vs. IFN-$\gamma R^{-/-}$ GVHD groups, n=4 mice/group, p=0.0006. C. Wt B6 mice were lethally irradiated and received BM with or without $3x10^6$ wt or IFN-$\gamma^{-/-}$ T cells. Seven days later, colons were harvested and assessed for IDO protein and mRNA. n=4 mice/group, p=0.03. D. Wt (filled symbols) or $IDO^{-/-}$ (open symbols) B6 mice were transplanted with wt BM with (squares) or without (circles) $3x10^6$ wt (left) or IFN-$\gamma^{-/-}$ (right) T cells. Survival is shown. n=8 mice/group. For wt donors, p=0.04, for IFN-$\gamma^{-/-}$ donors, p=0.78. E. Wt or $IDO^{-/-}$ BALB/C mice were lethally irradiated and received $1x10^6$ wt or IFN-$\gamma^{-/-}$ T cells. Sixteen days after transplantation T cells were isolated from colons and assessed by flow cytometry for Annexin V and Ki-67. Data are pooled from two identical experiments with n=3-4 mice/group. *p<0.05, **p<0.005, ***p<0.0005. Only statistically significant differences are noted.

Figure 2. The difference in proliferation and apoptosis in colon Tcells in wt vs. $IDO^{-/-}$ hosts is abrogated in the absence of IFN-γ. Wt or $IDO^{-/-}$ mice were irradiated and infused with allogeneic BM and wt or IFN-$\gamma^{-/-}$ T cells. 16 days later, T cells were harvested from the colon and assessed by flow cytometry for Ki-67 (A) and Annexin V (B).

Figure 3. BM-derived host cells are more critical than epithelial cells in suppressing donor T cells via the IDO pathway. A. WT or $IDO^{-/-}$ B6 mice were lethally irradiated and reconstituted with wt or $IDO^{-/-}$ B6 BM. After three months, the chimeras were re-irradiated and infused with allogeneic BALB/C BM and $3x10^6$ T cells. Survival is shown. For wt→wt (■) vs. $IDO^{-/-}$→$IDO^{-/-}$ (□) chimeras, p=0.02. For wt→$IDO^{-/-}$

(▲) vs. $IDO^{-/-}$→$IDO^{-/-}$ chimeras, p=0.04. For wt→wt vs. $IDO^{-/-}$→wt (△), p=0.07. Representative of two experiments, n=8-10 mice/group. B. Chimeras were generated and transplanted as above. Eighteen to twenty days later, T cells were isolated from colons and assessed by flow cytometry for Ki-67, Annexin V, and PD-1. Data is pooled from two similar experiments. n=3-5 mice/group, *p<0.05, **p<0.005, ***p<0.0005.

Fig. 4. IDO-based GVHD suppression is GCN2-independent. Wt (filled symbols) or $IDO^{-/-}$ (open symbols) BALB/C mice were lethally irradiated and infused with B6 BM with or without (circles) $2x10^6$ wt (squares) or $GCN2^{-/-}$ (triangles) T cells. Survival is shown, n=8 mice/group. For wt vs. $IDO^{-/-}$ given wt cells, p<0.0001; for wt vs. $IDO^{-/-}$ given $GCN2^{-/-}$ T cells, p=0.0013; for wt vs. $GCN2^{-/-}$ in wt recipients, p=0.38; for wt vs. $GCN2^{-/-}$ in $IDO^{-/-}$ recipients, p=0.53. Data are representative of 4 independent experiments.

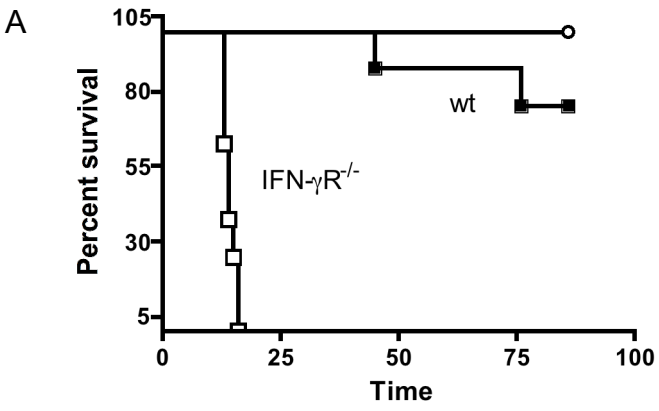
Fig. 5. Exogenous kynurenines inhibit GVHD lethality. A. Wt mice were lethally irradiated and infused with BM with or without $3x10^6$ T cells. On day 17, when weight loss was apparent, recipients were started on daily i.p. injections of PBS (■) or 75mg/kg of a mixture of L-kynurenine, 3-hydroxyanthranilic acid, and 3-hydroxykynurenine (□). Survival is shown, n=5-6 mice/group. p=0.03. B. Treatment of mice from A was discontinued after 60 days. Survival of mice surviving at day 60 is shown.

Fig. 6. Ligation of TLR7/8 induces transient expression of IDO in the colon and reduces GVHD lethality. A. Mice were lethally irradiated and 5 hours later injected i.v. with PBS or 200μg 3M-011. On day 0, 48 hours later, some mice were sacrificed and colons and spleens were assessed for IDO mRNA. The remaining mice were infused with BM and $3x10^6$ T cells. On days 2 and 4 post-BMT, IDO mRNA was again assessed

from colons and spleens. For colon IDO on D0, p=0.05, for spleen on D4, p=0.02. n=4 mice/group. D0 colon data are representative of two identical experiments. B. Mice were pre-treated with PBS (■) or 3M-011 (□) and transplanted 48 hours later as in A. BM only was untreated (●). Survival is shown. Data are pooled from two identical experiments, n=7-8 mice/group. p=0.03. C. Mice were treated and transplanted as in A and B. 37 days after transplantation, colons were harvested, sectioned and stained by H&E. Sections were scored for pathology, n=5 mice/group. *p<0.05. D. Sections taken from day 37 tissues in C were stained for CD4 by IHC. Representative sections are on the left, quantification of $CD4^+$ cells is shown on the right. n=5 mice/group, p=0.01.

Figure 7. IDO is required for protective effect of TLR7/8 ligation. $IDO^{-/-}$ mice were lethally irradiated and then injected i.v. with PBS or 200ug 3M-011. 48 hours later, recipients were infused with BM and $2x10^6$ T cells. Untreated mice received BM alone as a survival control. No significant difference was detected between GVHD groups. n=7-8 mice/group.

Fig. 1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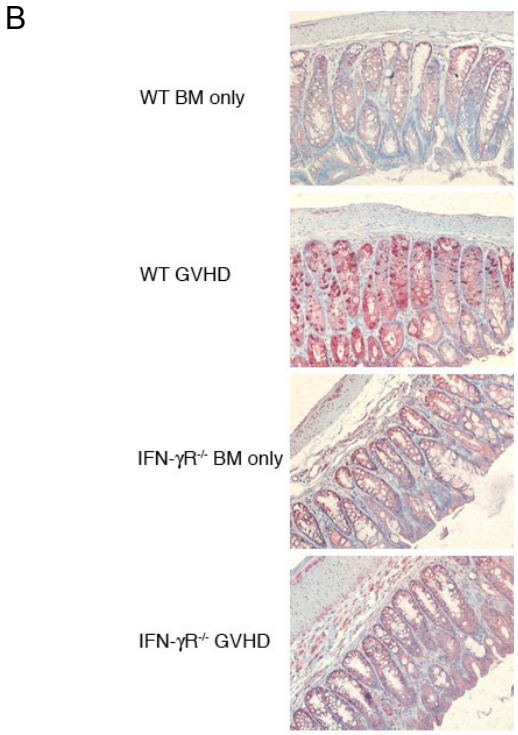
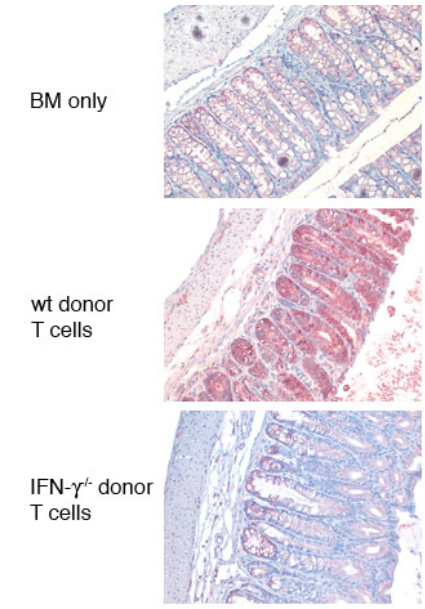
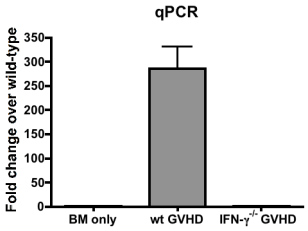
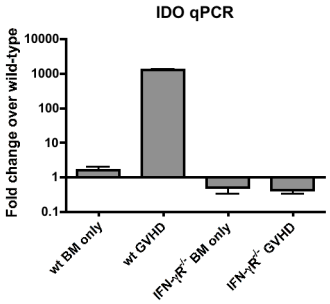
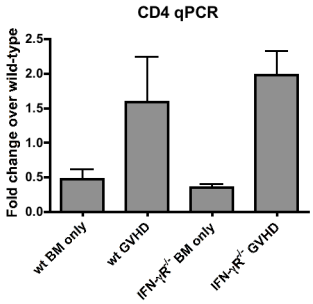
75
100
Time
wt
IFN-γR-/-


B
WT BM only
WT GVHD
IFN-γR-/- BM only
IFN-γR-/- GVHD

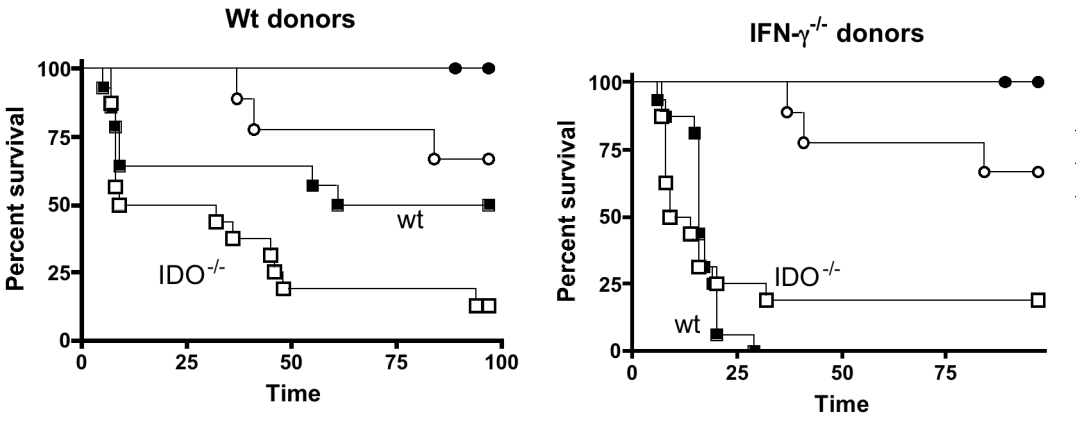

C
BM only
wt donor
T cells
IFN-γ-/- donor
T cells


qPCR
Fold change over wild-type
350
300
250
200
150
100
50
0
BM only
wt GVHD
IFN-γ-/- GVHD


IDO qPCR
Fold change over wild-type
10000
1000
100
10
1
0.1
wt BM only
wt GVHD
IFN-γR-/- BM only
IFN-γR-/- GVHD


CD4 qPCR
Fold change over wild-type
2.5
2.0
1.5
1.0
0.5
0.0
wt BM only
wt GVHD
IFN-γR-/- BM only
IFN-γR-/- GVHD

D


Wt donors
IFN-γ-/- donors
Percent survival
Time
wt
IDO-/-
100
75
50
25
0
0
25
50
75
100

E

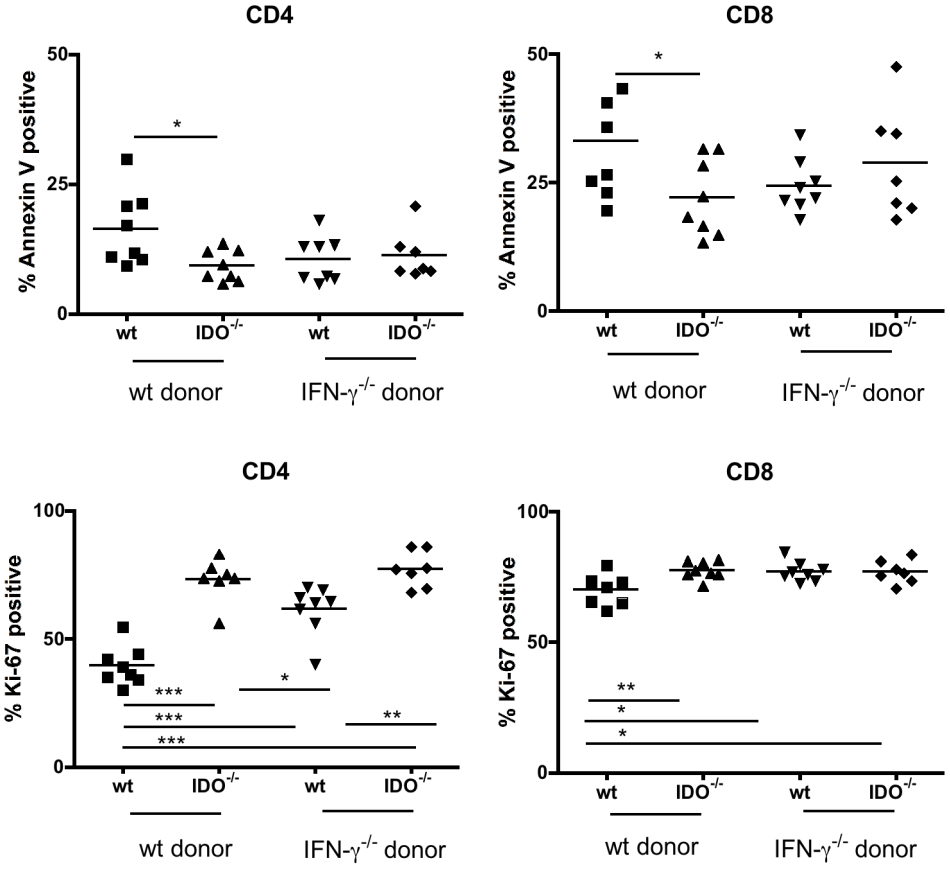
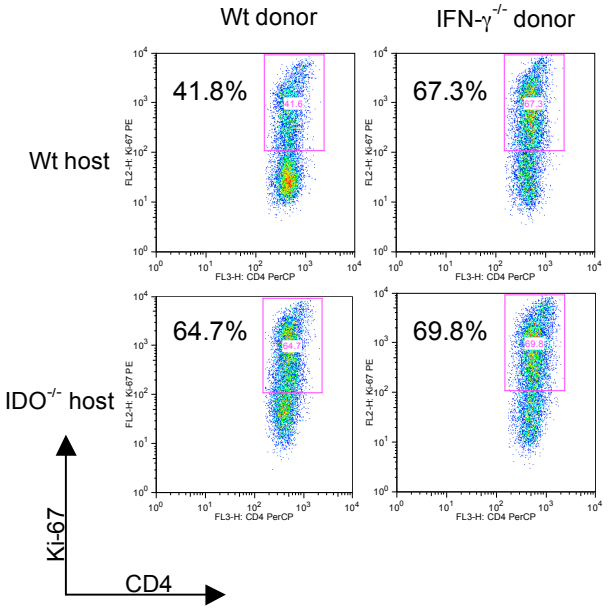

CD4
CD8
% Annexin V positive
% Ki-67 positive
wt
IDO-/-
wt donor
IFN-γ-/- donor
*
**
***

Figure 2


Wt donor
IFN-γ-/- donor
Wt host
IDO-/- host
41.8%
67.3%
64.7%
69.8%
Ki-67
CD4

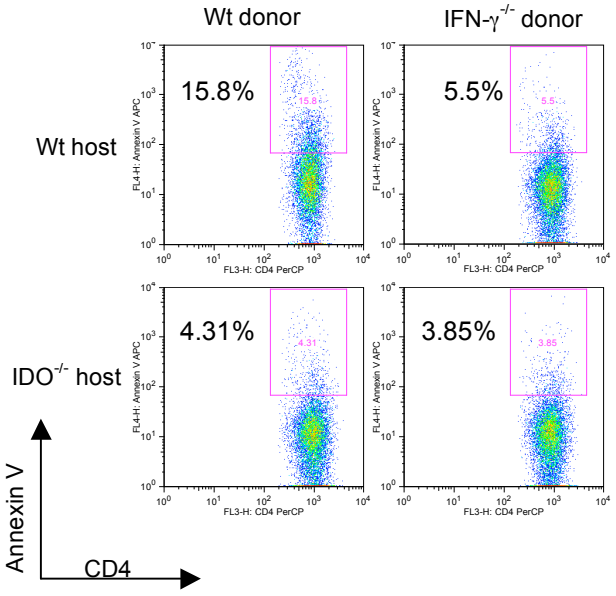
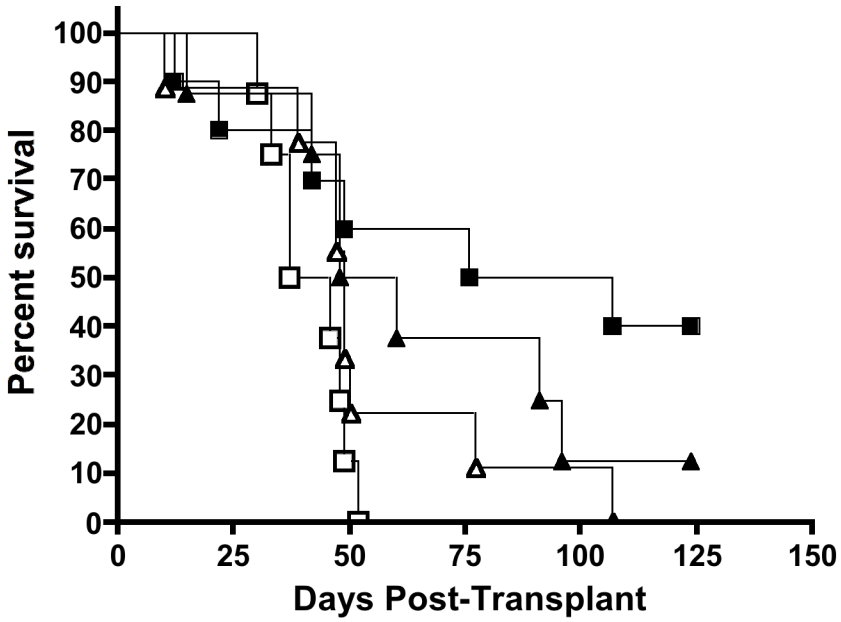

Wt donor
IFN-γ-/- donor
Wt host
IDO-/- host
15.8%
5.5%
4.31%
3.85%
Annexin V
CD4

Figure 3

A


Percent survival
Days Post-Transplant
0
10
20
30
40
50
60
70
80
90
100
0
25
50
75
100
125
150

B

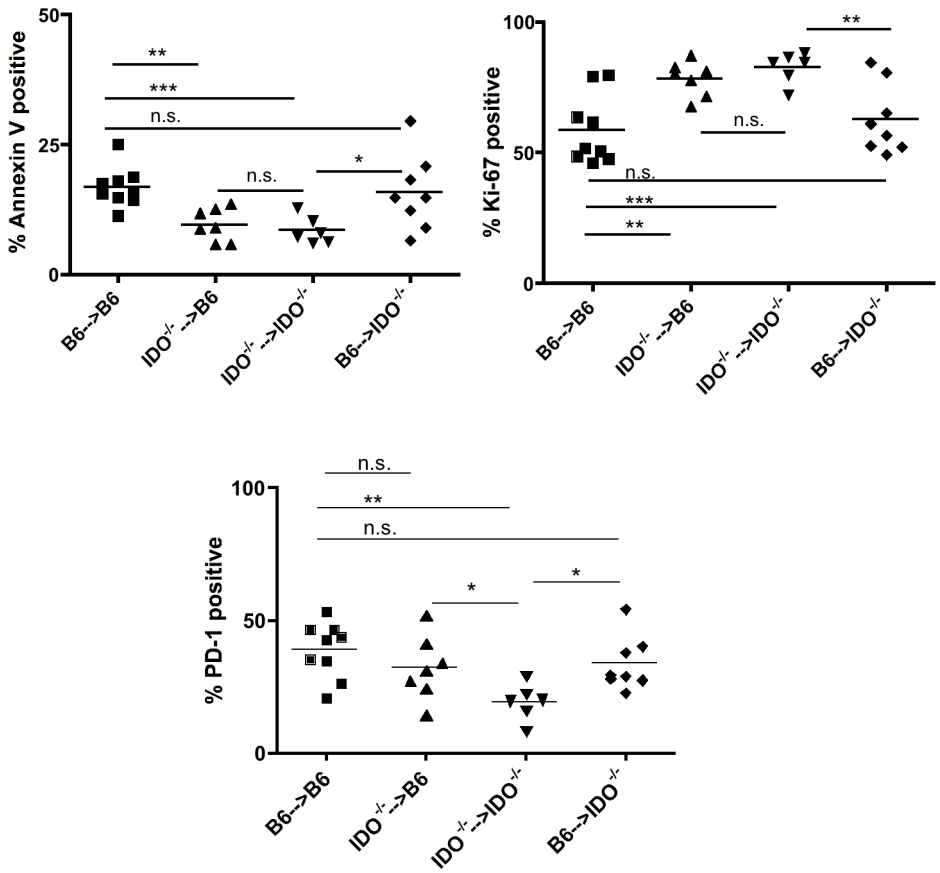

% Annexin V positive
% Ki-67 positive
% PD-1 positive
B6-->B6
IDO-/- -->B6
IDO-/- -->IDO-/-
B6-->IDO-/-
n.s.
*
**
***

Figure 4

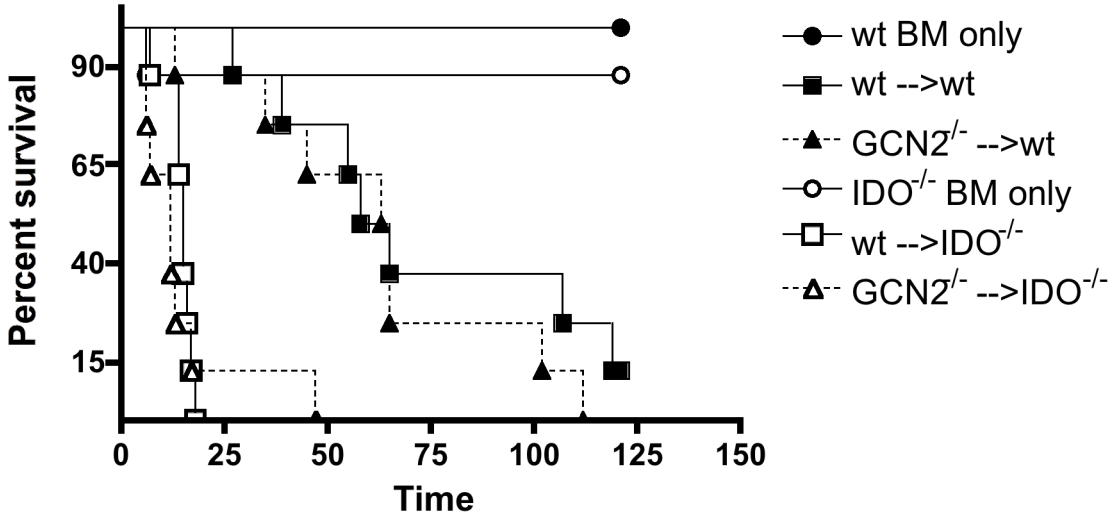
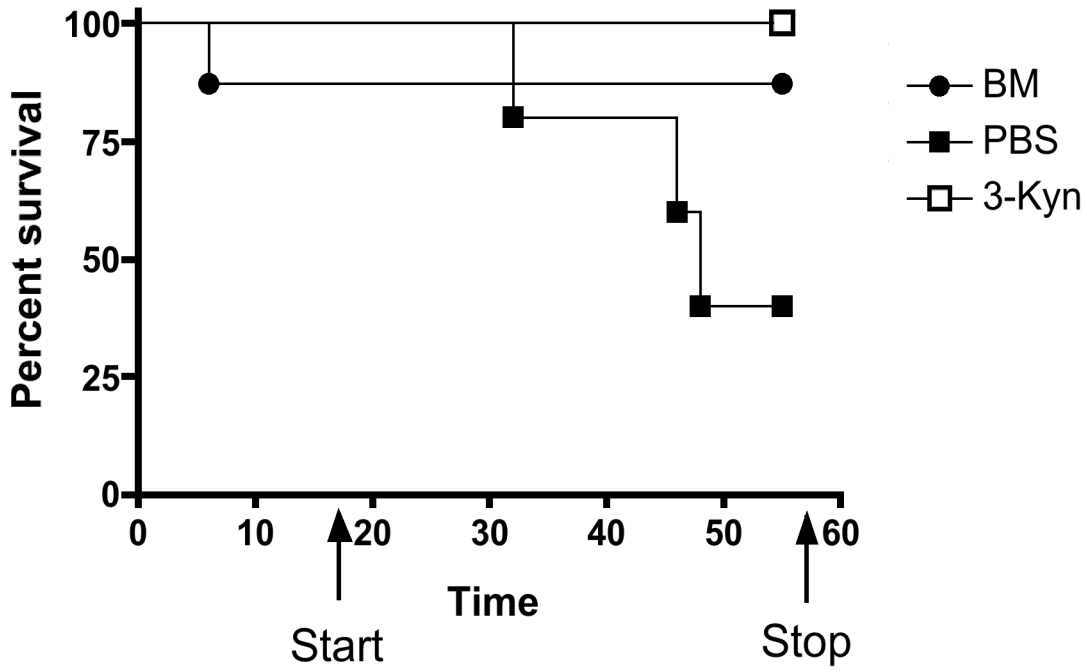

Percent survival
90
65
40
15
0
25
50
75
100
125
150
Time
wt BM only
wt -->wt
GCN2-/- -->wt
IDO-/- BM only
wt -->IDO-/-
GCN2-/- -->IDO-/-

Figure 5

A


Percent survival
100
75
50
25
0
0
10
20
30
40
50
60
Time
Start
Stop
BM
PBS
3-Kyn

B

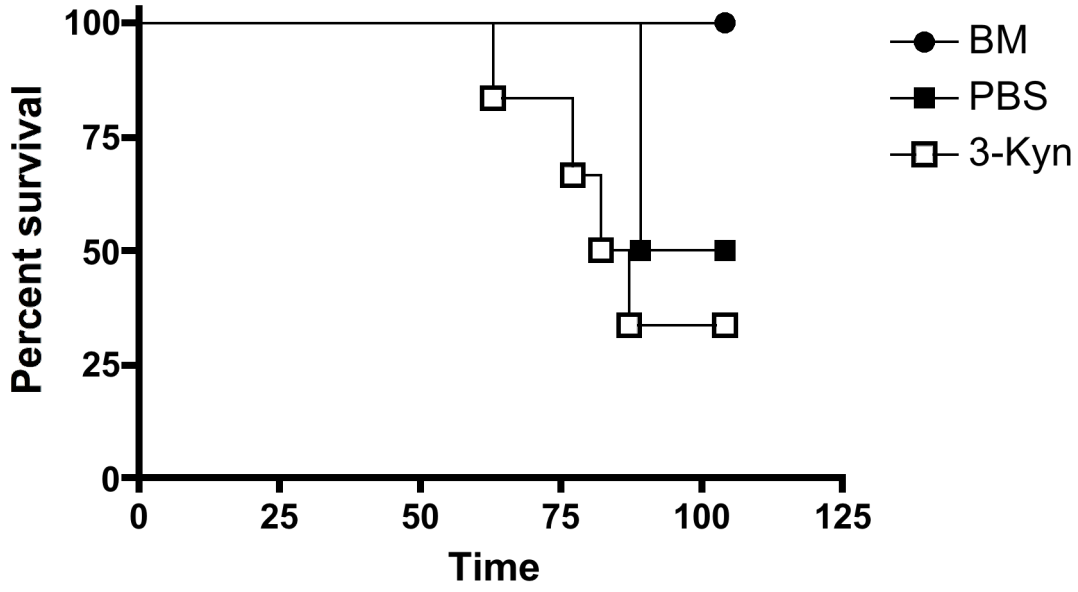

Percent survival
100
75
50
25
0
0
25
50
75
100
125
Time
BM
PBS
3-Kyn

Figure 6

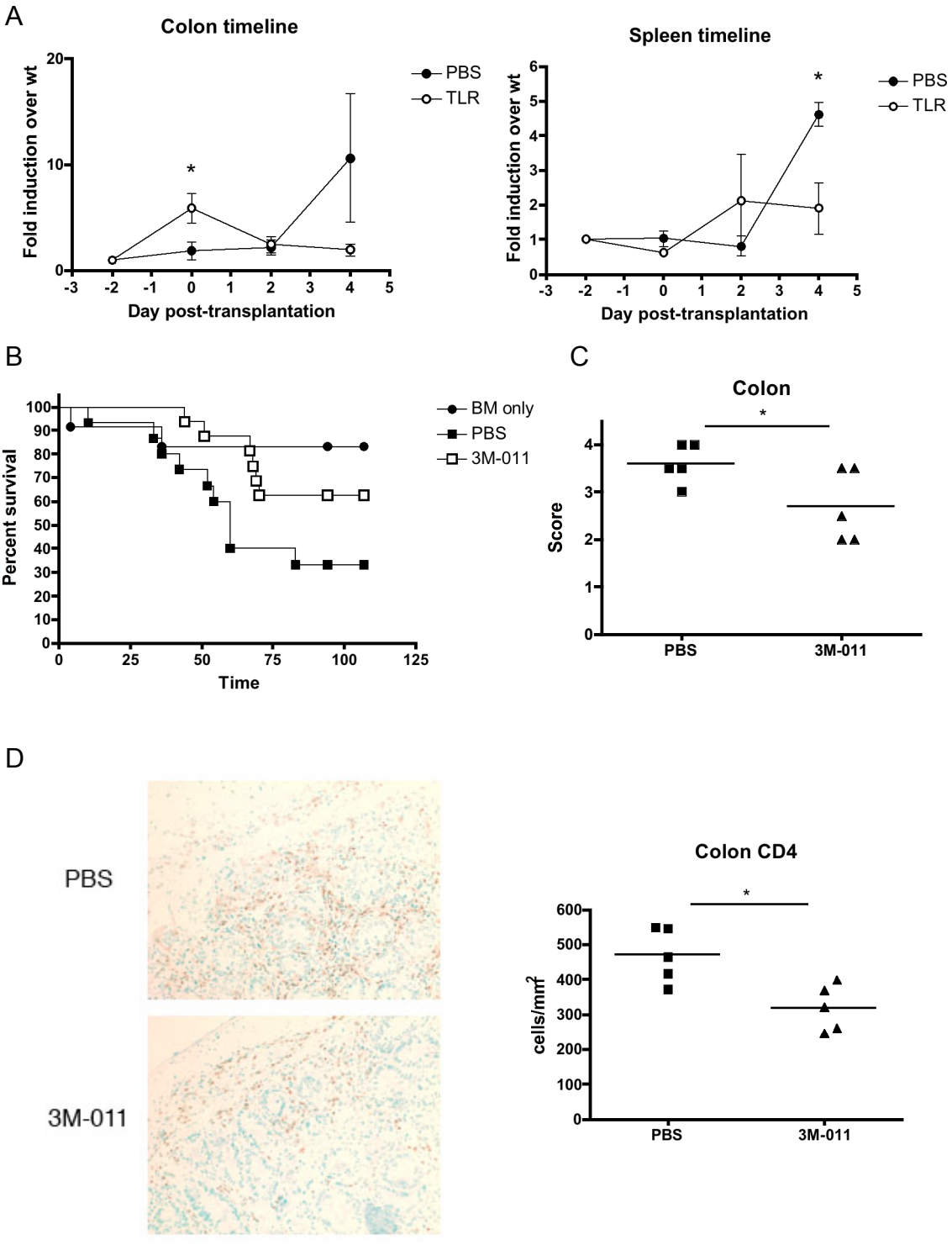
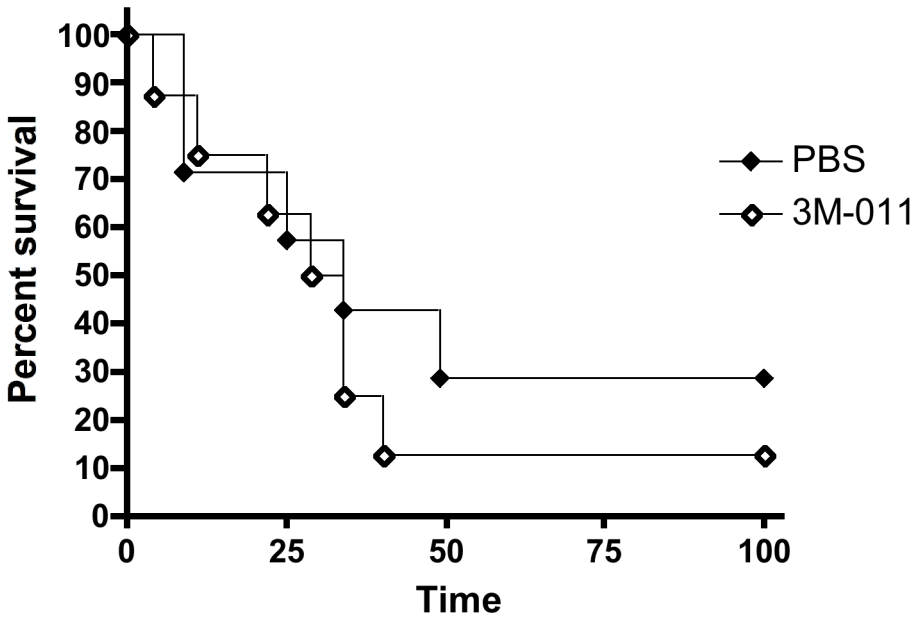

A
Colon timeline
Fold induction over wt
Day post-transplantation
PBS
TLR
Spleen timeline
Fold induction over wt
Day post-transplantation
PBS
TLR
B
Percent survival
Time
BM only
PBS
3M-011
C
Colon
Score
PBS
3M-011
D
PBS
3M-011
Colon CD4
cells/mm2
PBS
3M-011

Figure 7


Percent survival
100
90
80
70
60
50
40
30
20
10
0
0
25
50
75
100
Time
PBS
3M-011

Chapter IV: Concluding Statement

GVHD is the most serious complication of HSCT. Better GVHD treatment and prevention will lead to increased survival and better quality of life for patients with conditions currently treated with transplantation and will also allow HSCT to be applied to other hard-to-treat conditions that cause significant morbidity and mortality. A more complete understanding of GVHD biology and its regulation will identify potential therapeutic targets and strategies. Results detailed here identify IDO as a critical regulator of acute GVHD. Its effects are largely confined to the colon, where donor T cell-derived IFN-γ strongly upregulates IDO. IDO-deficient recipients suffer accelerated lethality and markedly worse pathology in the colon, accompanied by increased numbers of infiltrating T cells. Other GVHD target organs are unaffected by IDO, as are serum cytokines, early T cell proliferation and activation, and T cell effector function. In the absence of IDO, whether due to IDO-deficient recipients or donor T cells lacking IFN-γ, T cells in the colon undergo more proliferation and less apoptosis. The effects of IDO are independent of GCN2 kinase, suggesting kynurenine production, and not tryptophan depletion, is the mechanism of suppression. These results support the model depicted in Figure 1. Together, this evidence suggests IDO exerts its control over T cell numbers, decreasing the intensity but not the nature of the alloreactive T cell response. As GVHD seems to be critically dependent on the accumulation of sufficient numbers of T cells and tissue damage, the ability

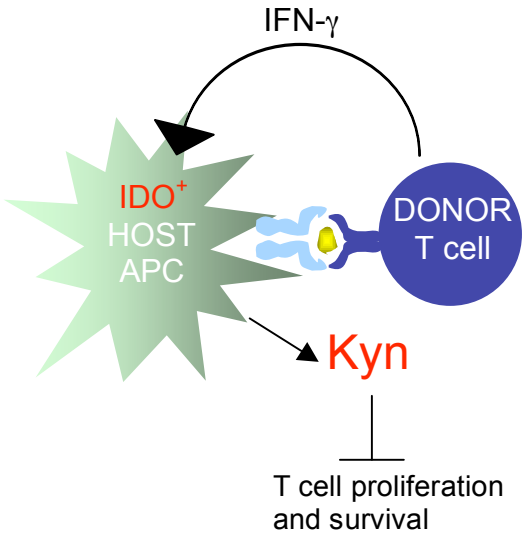


Figure 1. Model of T cell suppression by IDO expressing colon APCs. Donor T cells produce IFN-γ, which upregulates IDO in host APCs and results in kynurenine production, suppressing T cell survival and proliferation.

of IDO to titer these levels makes it a promising target for GVHD modulation. As shown in Figure 2A, IFN-γ-producing donor T cells induce IDO after the disease process has begun, counteracting the inflammation and slowing the accumulation of T cells. In the absence of IDO, T cells continue to survive and proliferate unchecked, reaching the threshold that causes lethality more quickly than when IDO is present. Induction of IDO via TLR agonist before the arrival of T cells in the colon restrains T cell accumulation to an even greater extent (Fig. 2B). Kynurenine administration keeps T cell growth in check below the lethality threshold, but once treatment is stopped, the T cells rebound (Fig. 2C). Future experiments will determine if these IDO-based treatments can be combined for greater effect.

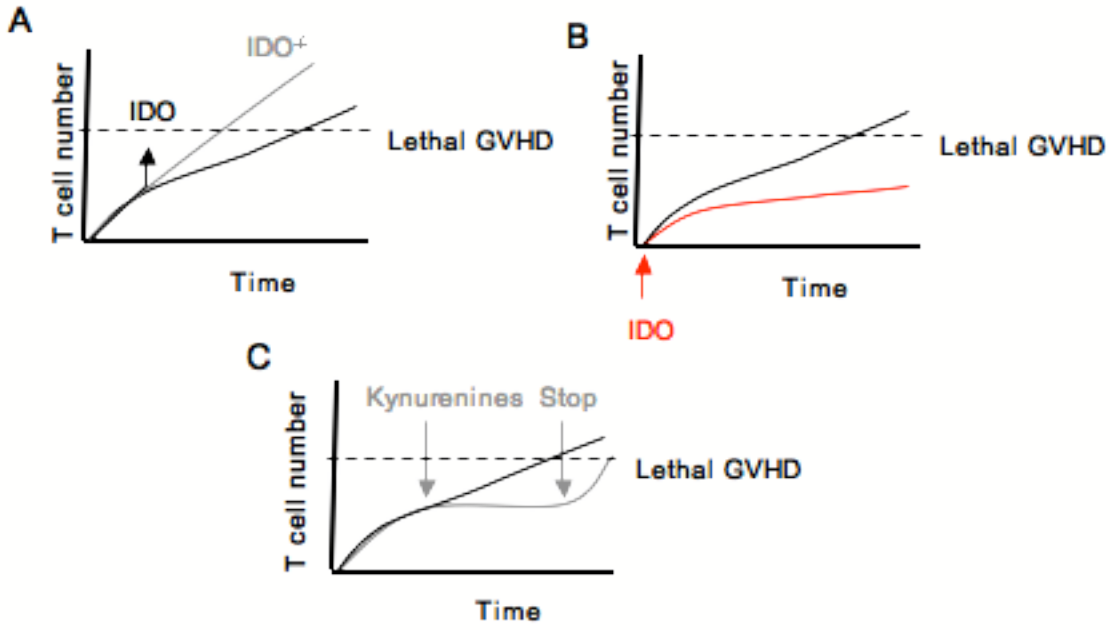


Figure 2. Effects of IDO and IDO-based treatments on T cell number. A. IDO induction by donor T cells deflects the growth of T cell numbers, delaying lethality. B. Pre-transplant IDO induction acts earlier to dampen T cell expansion and prevent the accumulation of lethal numbers of T cells. C. Exogenous kynurenine administration further depresses T cell accumulation, which rebounds after kynurenine withdrawal.

Expression of IDO by APCs appears to be responsible for the bulk of IDO-mediated suppression, as $IDO^{-/-}$ APCs led to increased lethality accompanied by greater T cell proliferation and survival, regardless of IDO expression by the tissue. Decreased PD-1 expression was associated with IDO expression in both tissues and APCs, although this did not affect survival. While T cell-APC interactions are typically thought to occur in lymphoid organs, the effects of IDO^{+} APC in GVHD are mostly restricted to the colon, although $IDO^{-/-}$ spleens did show increased pathology at late time points. Two possibilities explain this finding. APCs in the mLN, where gut-reactive T cells are primed, could be induced to express IDO during GVHD. Their proximity to the intestine and possible trafficking between the mLN and the tissue could lead to detection of increased IDO in the tissue, especially when induced by TLR ligation, which did not appear to affect epithelial cell IDO expression (not shown). Second, tissue-resident APCs, or those recruited there by inflammation, could express IDO and suppress T cells in the tissue. This could occur as activated T cells arrive following priming in spleen or LN and engage local APCs. The role of tissue APCs in GVHD, outside of Langerhans cells in the skin, has not been fully defined. However, depletion of APCs from the liver decreased the recruitment of activated alloreactive $CD8^{+}$ T cells and ameliorated liver GVHD[254], supporting a crucial role for tissue APCs in mediating local tissue damage.

While crucial for GVHD generation, host APCs are rapidly eliminated following irradiation. Therefore, the critical interactions between T cells and APCs must occur very early after transplantation. In fact, the kinetics of host DC disappearance and donor T cell activation indicate that the priming of T cells occurs within the first 24 hours following transplantation[255]. The finding that host APCs are the critical IDO-expressing cells indicates IDO-based suppression must also occur at an early time point, even

though the effects of IDO on T cells, tissues, and survival do not become evident until at least two weeks after transplantation, at which time host APCs will have been eliminated. This requires that the initial contact between T cell and APC begins a process that slowly builds until the full effect can be observed. This is consistent with the notion of GVHD as an accrual of damage and immune activation; small changes at early timepoints affect the cycle and lead to significant differences in outcome, long after those first events take place. It is also consistent with the ability of transient IDO induction by TLR7/8 ligation at the time of transplant to affect late events in the colon.

pDCs are the intended target of the TLR7/8 agonist and would be expected to upregulate IDO in response to autocrine IFN-α. The expression of TLR7 by pDCs and their propensity for IDO expression, especially in response to TLR ligation, support this scenario, but this was not formally proven. Future experiments will examine the identity of the TLR7/8 agonist's target and the role of IFN-α in IDO induction. This data will contribute to the development of other IDO-inducing strategies for GVHD prevention, which may include CTLA-4 and other TLR agonists.

The effect of IDO on GVL or antimicrobial immune responses was not examined. There are reasons to predict manipulation of IDO for therapy, specifically superinduction of IDO, will not adversely influence these immune functions. First is the tissue-specific expression of IDO. IDO has a propensity to be expressed at mucosal sites such as the lung and the gut, where contact with large numbers of harmless foreign antigens requires a relatively suppressive environment. Accordingly, in the GVHD system used here, IDO mediates its effects specifically in the colon, and TLR7/8 ligation induces colon, but not spleen, IDO. Since GVL is mediated in the hematopoietic compartment, it

may be relatively separated from events in IDO-expressing tissues. In addition, IDO induction by the TLR7/8 agonist is transient and likely will not have long-term effects on immunity to infection. Again, the crucial role of the gut in initiating and amplifying the GVHD reaction supports the prospect that modest and transient IDO upregulation there can cause significant differences in disease outcome without affecting other immune processes. Second, IDO acts to restrict the number of T cells in the colon. If, as has been described earlier, GVHD requires more activated T cells than GVL, it is possible that IDO can decrease T cell numbers to below the GVHD threshold but still sufficient to mediate GVL. These notions require further investigation.

Additional investigation will also be required to identify the relationships between IDO and other mechanisms of GVHD suppression. It will be especially important to determine the association between IDO and Tregs, as both are promising treatments for GVHD and evidence suggests the two are closely linked. It is possible IDO induction or kynurenine treatment will increase the efficacy of Tregs in vivo. It is also possible that the IDO pathway can be manipulated to increase the yield and potency of ex vivo cultured Tregs used to treat GVHD.

This thesis presents a body of work detailing the previously unidentified role for IDO in acute GVHD, outlining its induction, timing, location and mechanism. Further, it develops two clinically relevant strategies for treating and preventing GVHD: pre-transplant IDO induction and kynurenine administration. These data have increased our understanding of a crucial GVHD regulatory pathway and provide strategies for developing new therapies that hold promise for decreasing the morbidity and mortality of this devastating disease.

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