The role of host dendritic cells during the effector phase of intestinal graft-versus-host disease

Die Rolle der dendritischen Zellen in der akuten intestinalen Graft-versus-Host Reaktion

Doctoral thesis for a doctoral degree at the Graduate School of Life Sciences,
Julius-Maximilians-Universität Würzburg,
Experimentelle Stem Cell Transplantation

Submitted by

Ana-Laura Jordán Garrote

from

Barcelona, Spain

Würzburg, 2014

CC BY

Submitted on: ……………………………………………………………..……..
Office stamp

Members of the PhD Thesis Committee:

Chairperson: Prof. Dr. Thomas Hünig

Primary Supervisor: Dr. Andreas Beilhack

Supervisor (Second): Prof. Dr. Manfred B. Lutz

Supervisor (Third): Prof. Dr. Natalio Garbi

Date of Public Defence: …………………………………………….…………

Date of Receipt of Certificates: ……………………………………………….

For my family

Table of Contents

List of Figures 7

List of Tables 8

List of Abbreviations 9

Abstract 12

Zusammenfassung 14

1 Introduction 16

1.1 Hematopoietic stem cell transplantation and graft-versus-host disease (GVHD) 16

1.1.1 GVHD physiology 16

1.1.2 GVHD pathology 17

1.1.3 Intestinal GVHD 18

1.1.4 Antigen presenting cells in GVHD 19

1.2 Dendritic cells 23

1.2.1 Intestinal dendritic cells 25

1.2.2 Dendritic cells in immunity and tolerance 27

1.2.3 Tolerogenic cytokines and co-inhibitory molecules in the control of T cell immunity by DCs 28

2 Specific aims 31

3 Material and Methods 32

3.1 Material 32

3.1.1 Chemical reagents 32

3.1.2 Buffers and solutions 32

3.1.3 Antibodies and secondary reagents 33

3.1.4 Commercially available kits 34

3.1.5 Consumables 34

3.1.6 Mice 35

3.2 Methods 36

3.2.1 HCT 36

3.2.2 Generation of bone marrow chimeras 37

3.2.3 Pathologic scoring of intestinal GVHD 37

3.2.4 Immunofluorescence microscopy 37

3.2.5 Isolation of immune cells from the intestinal tract 38

3.2.6 Flow cytometry and cell sorting....38
3.2.7 Cytokine Bead Array (CBA) analysis....39
3.2.8 *Ex vivo* BLI....39
3.2.9 Multicolor Light Sheet Microscopy (LSFM) and sample preparation....39
3.2.10 T cell stimulation and intracellular staining....40
3.2.11 Cell size and morphology measurement....40
3.2.12 Statistics....41
4 Results....42
4.1 Activated host intestinal DCs interact with donor T cells during aGVHD....42
4.2 Host DCs protect from lethal aGVHD during the effector phase....44
4.3 Host intestinal DCs protect from intestinal pathology by limiting donor T cell infiltration and inflammatory cytokine production....47
4.4 Host intestinal DCs are the major source of intetsial PD-L1 expression after allo-HCT....51
4.5 Host DCs limit alloreactive cell responses via PD-L1....53
4.6 Protective intestinal DCs are of monocytic origin....56
4.7 Host intestinal DCs arise from tissue resident CX_3CR1 non-classical monoytes....62
5 Discussion....67
5.1 Non-classical monocyte-derived DCs....67
5.2 DCs in GVHD: immunogenic or tolerogenic?....69
5.3 PD-L1 in aGVHD....71
5.3.1 PD-L1 expression on nc-moDCs proves critical for T-cell alloreactivity suppression....71
5.4. Molecular signatures of dendritic cells and macrophages....72
6 Graphical summary....74
References....76
Acknowledgments....84
Curriculum vitae....86
Affidavit (Eidesstattliche Erklärung)....90

List of Figures

Figure 1. Antigen presentation 20

Figure 2. Acute GVHD pathophysiology 21

Figure 3. Dendritic cell maturation 24

Figure 4. Activated host intestinal DCs interact with donor T cells during aGVHD 42

Figure 5. Activated host intestinal DCs interact with donor T cell 43

Figure 6. Selective depletion of DCs via diphtheria toxin administration 44

Figure 7. Intestinal DCs protect from lethal aGVHD during the effector phase 46

Figure 8. Diphtheria toxin administration in WT mice after GVHD induction did not present any cytotoxic effects 47

Figure 9. Host DCs protect from acute intestinal GVHD pathology by limiting donor T cell infiltration and inflammatory cytokine production 48

Figure 10. Increased inflammatory cytokine production in the absence of host DC 49

Figure 11. Administration of DTx on a later time point after allo-BMT resulted in increased mortality 50

Figure 12. Depletion of neutrophils after DTx administration did not improve weight loss and survival 51

Figure 13. Host DCs are the major source of intestinal PD-L1 expression after allo-HCT 52

Figure 14. Host monocyte-derived DCs limit alloreactive T cell responses via PD-L1 53

Figure 15. PD-L1 expression particularly on host intestinal DCs pervades alloreactivity suppression 55

Figure 16. Shift from migratory $CD103^+$ into $CD11b^+$ intestinal DCs during acute intestinal GVHD 56

Figure 17. $CD103^+$ steady-state DCs migrated out of the LP in a CCR7 independent manner shortly after allo-HCT 57

Figure 18. $CD11b^+CD11c^-$ cells in the intestinal mucosa shortly after irradiation and become $CD11b^+CD11c^+$ after 70 h of allo-HCT 58

Figure 19. Change of size and morphology during the first 70h after allo-HCT 59

Figure 20. Detection of radio resistant monocytes in bone marrow and spleen 20 h after allo-HCT 59

Figure 21. $CD11b^+$ cells detected close to the blood vessels with the help of LSFM ….60

Figure 22. Intestinal DCs are from monocytic origin 61

Figure 23. Host intestinal DCs during aGVHD are monocyte-derived 62

Figure 24. Monocyte recruitment appears CCR2-independent 63

Figure 25. Intestinal DCs differentiate *in situ* in a CX_3CR1 dependent manner 65

Figure 26. Intestinal DCs precursors do not proliferate *in situ* 66

Figure 27. The function of intestinal DCs during the effector phase of aGVHD remained elusive 74

Figure 28. Tissue-resident non-classical monocyte-derived DCs suppress aGVHD via PD-L1 75

List of Tables

Table 1. Murine primary antibodies used for FACS staining and fluorescence microscopy 33

Table 2. Secondary antibodies for FACS staining and fluorescence microscopy 34

Table 3. Mice 35

List of Abbreviations

aGVHD	Acute Graft-versus-Host Disease
Ab	Antibody
Ag	Antigen
AICD	Activation-induced cell death
Allo-HCT	Allogeneic hematopoietic cell transplantation
APC	Antigen presenting cell
APC	Allophycocyanine
BLI	Bioluminescence Imaging
BM	Bone marrow
BSA	Bovine serum albumin
BW	Body weight
CCR	Chemokine receptor
CD	Cluster of differentiation
cLN	Cervical lymph node
CTL	Cytotoxic T lymphocyte
Cy	Cytochrome
DAPI	4′,6-Diamidin-2-phenylindol
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacidic acid
FACS	Fluorescence activated cell sorting
FC	Flow cytometry
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one

FoxP3	Forkhead box P3
g	Gram
GI	Gastrointetsinal
GIT	Gastrointestinal tract
GVHD	Graft-versus-Host Disease
GVL	Graft-versus-Leukemia
GVTE	Graft-versus-Tumor Effect
Gy	Gray
HCT	Hematopoietic cell transplantation
HLA	Human Leukocyte Antigen
HSC	Hematopoietic stem cells
HPF	High Power Field
IDO	Indoleamine 2,3-dioxygenase
IFM	Immunofluorescence microscopy
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IL	Interleukin
iLN	Inguinal lymph node
kg	Kilogram
LN	Lymph node
LP	Lamina Propria
LPS	Lipopolysaccharide
MDSC	Myeloid-derived suppressor cell
mg	Milligram
min	Minute
MHC	Major histocompatibility complex
ml	Milliliter
mLN	Mesenteric lymph node

mM	Millimolar
moDC	Monocyte-derived DC
µg	Microgram
nc-moDC	Non-classical monocyte-derived DC
ng	Nanogram
NRS	Normal rat serum
OVA	Ovalbumin
PB	Pacific blue
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD1	Programmed death 1
PD-L1	Programmed death ligand 1
PE	Phycoerythrin
PerCP	Peridinin chlorophyll
PFA	Paraformaldehyde
PI	Propidium iodide
pLN	Peripheral lymph node
PMA	Phorbol 12-myristate 13-acetate
PP	Peyer's patch
RA	Retinoic acid
RT	Room temperature
SD	Standard deviation
SLO	Secondary lymphoid organ
TGF-ß	Transforming growth factor-ß
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
WT	Wild type

Abstract

Monocytes can be functionally divided in two subsets, both capable to differentiate into dendritic cells (DCs): $CX_3CR1^{lo}CCR2^+$ classical monocytes, actively recruited to the sites of inflammation and direct precursors of inflammatory DCs; and $CX_3CR1^{hi}CCR2^-$ non-classical monocytes, characterized by CX_3CR1-dependent recruitment to non-inflamed tissues. Yet, the function of non-classical monocyte-derived DCs (nc-mo-DCs), and the factors, which trigger their recruitment and DC differentiation, have not been clearly defined to date. Here we show that *in situ* differentiated nc-moDCs mediate immunosuppression in the context of intestinal graft-versus-host disease (GVHD).

Employing multi-color confocal microscopy we observed a dramatic loss of steady state host-type $CD103^+$ DC subset immediately after transplantation, followed by an enrichment of immune-regulatory $CD11b^+$ nc-moDCs. Parabiosis experiments revealed that tissue-resident non-classical CX_3CR1^+ monocytes differentiated *in situ* into intestinal $CD11b^+$ nc-moDCs after allogeneic hematopoietic cell transplantation (allo-HCT). Differentiation of this intestinal DC subset depended on CSF-1 but not on Flt3L, thus defining the precursors as monocytes and not pre-DCs. Importantly, CX_3CR1 but not CCR2 was required for this DC subset differentiation, hence defining the precursors as non-classical monocytes. In addition, we identify PD-L1 expression by CX_3CR1^+ nc-moDCs as the major mechanism they employ to suppress alloreactive T cells during acute intestinal GVHD. All together, we demonstrate that host nc-moDCs surprisingly mediate immunosuppression in the context of murine intestinal GVHD – as opposed to classical "inflammatory" monocyte-derived dendritic cells (mo-DCs) – via coinhibitory signaling. This thorough study unravels for the first time a biological function of a - so far only *in vitro* and phenotypically described - DC subset. Our identification of this beneficial immunoregulatory DC subset points towards alternate future strategies in underpinning molecular pathways to foster their function. We describe an unexpected

mechanism of nc-moDCs in allo-HCT and intestinal GVHD, which might also be important for autoimmune disorders or infections of the gastrointestinal tract.

Zusammenfassung

Monozyten (MO) lassen sich funktionell in zwei Subpopulationen unterteilen, die sich in dendritische Zellen (DC) differenzieren können: 1) Klassische Monozyten ($CX_3CR1^{lo}CCR2^+$), die direkte Vorläuferzellen von inflammatorischen DCs (mo-DCs) sind und aktiv an die Stelle der Entzündung rekrutiert werden und 2) nicht-klassische Monozyten ($CX_3CR1^{hi}CCR2^-$). Bisher ist weder die Funktion der, aus nicht-klassischen Monozyten abstammenden, DC-Subpopulation (nc-mo-DCs) bekannt, noch ist geklärt, welche Faktoren die Ausdifferenzierung und die Rekrutierung der nc-mo-DCs in periphere Gewebe ermöglichen. In dieser Dissertationsarbeit zeige ich, dass nc-mo-DCs *in situ* differenzieren und in der akuten intestinalen Graft-versus-Host Erkrankung (GVHD) immunsuppressiv wirken. Konfokale Mikroskopie verdeutlichte den Rückgang der, im gesunden Gleichgewicht im intestinalen Gewebe dominierenden, $CD103^+$ DCs direkt nach allogener hämatopoetischer Zelltransplantation (HCT) zugunsten immunregulatorischer $CD11b^+$ nc-moDCs. Weiterhin zeigten Parabiose Experimente, dass nicht-klassische CX_3CR1^+ Monozyten im intestinalen Gewebe ansässig sind und sich nach allogener HCT *in situ* zu $CD11b^+$ nc-moDCs ausdifferenzieren. Die Differenzierung findet in Abhängigkeit von CSF-1 und unabhängig von Flt3L statt, ein Beweis dafür, dass dieser Zelltyp aus Monozyten und nicht aus DC-Vorläuferzellen gebildet wird. Die Differenzierung dieser intestinalen DC Subpopulation hängt von CX_3CR1, nicht jedoch von CCR2 ab, ein Beweis dafür, dass dieser Zelltyp aus nicht-klassischen Monozyten gebildet wird. Mechanistisch zeigen wir, dass nc-moDCs alloreaktive T-Zellen durch die Expression von PD-L1 supprimieren.

Zusammenfassend beschreiben wir erstmalig nc-moDCs als neue DC Subpopluation, die im Mausmodell die intestinale GVHD unterdrückt und sich damit wesentlich von den bisher beschriebenen, klassischen proinflammatorischen mo-DCs unterscheidet. Die von den nc-moDCs vermittelte immunregulatorische Wirkung könnte weiterhin eine wichtige Rolle bei

Autoimmunerkrankungen und Infektionen im Magen-Darm-Trakt spielen und sollte daher in zukünftigen Therapieansätzen berücksichtigt werden.

1 Introduction

1.1 Hematopoietic stem cell transplantation and graft-versus-host disease (GVHD)

1.1.1 GVHD physiology

Hematopoietic cell transplantation (HCT) is a potential curative therapy for malignant and non-malignant hematological diseases. Based on the source of the donated stem cells, the graft is categorized as autologous (from the same patient) or as allogeneic (from a foreign donor). The most frequent indications for autologous HCT in Europe are multiple myeloma, non-Hodgkin lymphoma and Hodgkin lymphoma. Smaller numbers of autologous transplants are performed to treat solid tumors, and about 150 autologous transplants are performed each year for autoimmune disorders. The risks of autologous HCT include organ damage due to high dose chemotherapy (especially liver and lungs injury), risk of viral, bacterial and fungal infections, bleeding due to the marrow aplasia, and lack of graft-versus-tumor (GVT) effect (Copelan, 2006; Passweg et al., 2012). Allogeneic HCT (allo-HCT) is used to replace the hematopoietic system in patients with congenital or acquired marrow failure, and more commonly to exploit the GVT effect. Risks are different for allo-HCT. Infections, engraftment failure and leukemia relapse are causes of morbidity and mortality. However, the major complication after allogeneic HCT is GVHD, an immune syndrome initiated by donor T cells that recognize recipient tissues as foreign and primarily attack the gastrointestinal (GI) tract, liver and skin as target tissues (Ferrara and Reddy, 2006; Shlomchik, 2007). The frequency and severity of GVHD depends on the match degree of the human leukocyte antigen (HLA) between donor and recipient. The HLA system is the human analogue of the major histocompatibility complex

(MHC) genes that are found in most vertebrates. This group of genes encode cell-surface antigen-presenting proteins. The proteins encoded by HLAs determine the biological identity of an individual's cells and tissues. The immune system uses the HLAs to differentiate between self and non-self antigens (alloantigens) and represent the major force in T cell activation. In the context of GVHD alloantigens can be recognized in two diferent forms depending on whether the presenting MHC molecules are matched or mismatched between donor and recipient. Differences in MHC molecules themselves are refered as major antigen mismatch leading to a severe, hyperacute form of GVHD. If MHC molecules between donor and recipient are matched, but the presented peptide is recognized by T cells are foreign, this is referred to a minor histocompatibility antigen (miHAg) mismatch, which leads to a less severe form of GVHD

1.1.2 GVHD pathology

The overall incidence of GVHD remains between 30% and 60% and is associated with an approximately 50% mortality rate (Barton-Burke et al., 2008). GVHD can be either acute (aGVHD) or chronic (cGVHD). In the past the type of GVHD was defined by the time of disease onset. Acute GVHD was defined to occur in the first 100 days after allo-HCT, whereas chronic GVHD after day 100 post-transplant. However, as transplant practice has changed, so too the timing of GVHD occurrence and clinical manifestations, which are now a better definition than timing alone. cGVHD is a chronic inflammatory condition and its manifestation resembles those of autoimmune diseases. It represents the major cause of long-term morbidity and mortality. aGVHD major targets are the GI tract, the liver and skin, and is graded according to the degree of skin involvement, the amount of diarrhea and the bilirubin levels. In allo-HCT myeloablative doses of chemotherapy and total body irradiation (TBI) were thought necessary to eradicate malignancy, to provide immunosuppression to the recipient, and to create space for the donor hematopoietic cells. Nowadays the intensity of the conditioning regimens is reduced, trying to keep treatment intensity high enough to avoid graft rejection but preserving the graft versus tumor effect, the so called mini-transplants

(Djulbegovic et al., 2003). Mini-transplants are HCTs that use non-myeloablative conditioning regimens, being well tolerated by most patients who are ineligible for conventional high-dose conditioning prior to transplantation. Mini-transplants, also called mixed chimeric transplants, do not rely on high-dose cytotoxic therapy, but rather rely on graft-versus-tumor effects for killing the patient's cancer cells. The low-dose conditioning regimen is administered to weaken the patient's immune system so that it accepts the donor's stem cells. Ideally, the transplanted cells engraft and a new immune system develops alongside the patient's remaining immune system. For a time, the patient has two immune systems (mixed chimerism) before the patient's system eventually disappears.

1.1.3 Intestinal GVHD

Although GVHD may affect any organ, intestinal GVHD is particularly important because of its frequency, severity and impact on the general condition of the patient. Manifestations are variable, but the most common presenting symptoms are nausea, vomiting, anorexia, and secretory diarrhea. In severe cases even gastrointestinal (GI) bleeding may be seen (Washington and Jagasia, 2009). Histologic grading for GI GVHD is most common as follows: grade 1 GVHD, isolated apoptotic epithelial cells, without crypt loss; grade 2, loss of isolated crypts, without loss of contiguous crypts; grade 3, loss of 2 or more contiguous crypts; and grade 4, extensive crypt loss with mucosal denudation (Kaplan et al., 2004; Lerner et al., 1974)

Acute intestinal GVHD remains a major source of morbidity after allo-HCT (Takatsuka et al., 2003). Around 60% of patients undergoing GVHD present gastrointestinal involvement (Jacobsohn et al., 2012). Evidence in experimental allo-HCT suggests that damage to the GI tract during aGVHD plays a major pathophysiologic role in the amplification of systemic disease and in the propagation of inflammation, characteristic of aGVHD (Aoyama et al., 2013; Chen et al., 2013; Hill and Ferrara, 2000). Damage of the intestinal tract during GVHD is due to both, TBI and allogeneic donor T cells. Detection of damage associated molecular patterns (DAMPs), like ATP; and pathogen associated molecular patterns (PAMPs) regulates

GVHD by the activation of the inflammasome, leading to an increase of intestinal IL-1ß, a key inflammatory cytokine (Hill and Ferrara, 2000; Jankovic et al., 2013; Wilhelm et al., 2010). Furthermore, tissue damage leads to the translocation of endotoxins and lipopolysaccharide (LPS), which promotes further inflammation and additional gastrointestinal damage (Takatsuka et al., 2003). LPS, a normal constituent of the bowel flora, is a potent immuno-stimulator and inducer of inflammatory cytokine production, such as tumor necrosis factor (TNF-α), interleukin-1 (IL-1) or IL-12, which are important mediators of clinical and experimental GVHD. High levels of inflammatory cytokines may perpetuate GVHD since TNF- α has been shown to be directly toxic to the GI tract during GVHD (Cooke et al., 1998; Piguet et al., 1987). It has been demonstrated, that the increase of TBI dose, directly correlates to the severity of GVHD (Nash et al., 1992). Thus, the pathophysiologic mechanisms involved in this increase in GVHD seem to be focused on the GI tract damage and the following amplification of the inflammatory effectors of GVHD rather than on the effects on donor T cells.

1.1.4 Antigen presenting cells in GVHD

Antigen presenting cells (APCs) are immune cells with the capability to present antigens via major histocompatibility complexes (MHCs). There are different cell populations that may participate in the process of antigen presentation with diverse efficiencies. Professional APCs are hematopoietic cells specialized in processing and presenting antigens that can efficiently promote an adaptive immunes response. They include B cells, macrophages and dendritic cells (DCs). Professional APCs are located on sites of potential exposure to foreign antigens. Antigens can be loaded via an endogenous or exogenous pathways onto MHC class I or class II, respectively. In the endogenous pathway cytosolic proteins are processed and peptides are loaded onto MHC class I molecules. In the exogenous pathway, soluble antigens are taken up by phagocytosis, processed and loaded onto MHC class II. DCs have in addition the special capacity to present exogenous antigens loaded onto MHC class I.

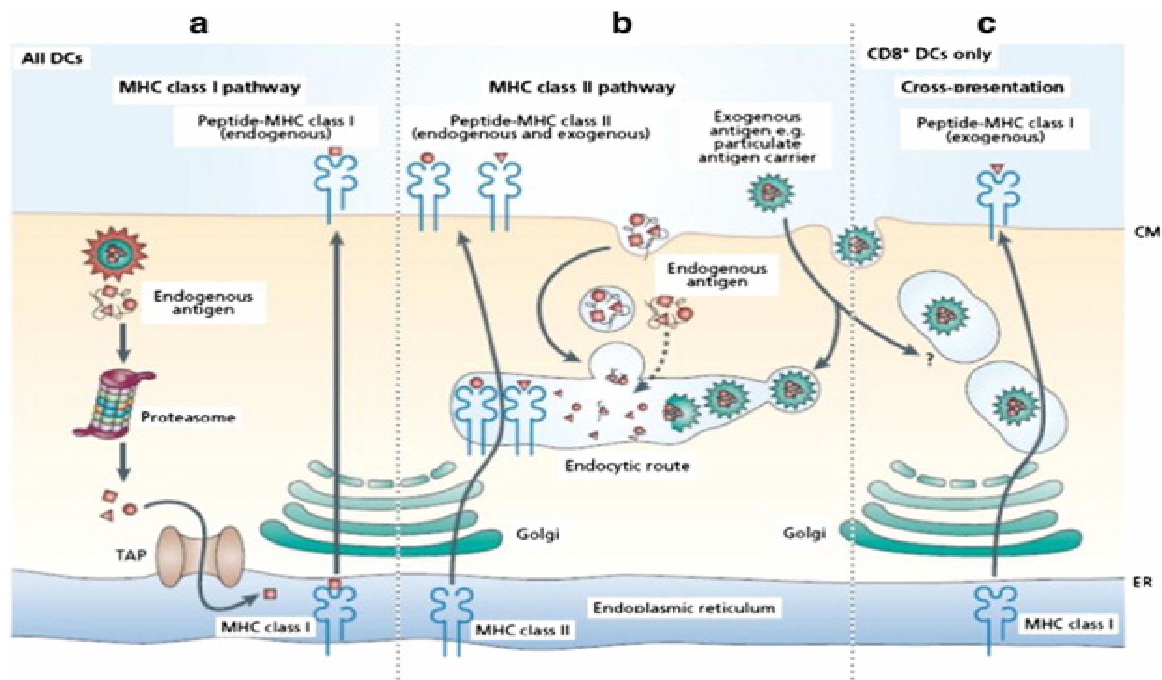

Figure 1. **Antigen presentation**. (A) Endogenous pathway. Cytosolic proteins are degraded by proteasomes in the APCs, and the resulting peptides are loaded onto MHC class I molecules. The peptide-MHC class I complex is transported to the cell surface where peptides can be recognized by $CD8^{+}$T cells. (B) Exogenous pathway. Antigens are endocytosed by APCs and processed by the class II pathway. (C) APCs can also activate $CD8^{+}$ T cells by cross-presenting exogenously acquired antigens on MHC class I molecules. Sketch taken from Joshi et al., 2012

This phenomenon is called cross-presentation and is essential for the development of immunity to infectious agents (in which APCs are not infected) and tumors (**Figure 1**). Myeloablative conditioning and allo-HCT result in the dramatic reduction of the number of hematopoietic APCs. However, few radioresistant APCs survive the conditioning and persist during a considerable time after allo-HCT (Auffermann-Gretzinger et al., 2006; Auffermann-Gretzinger et al., 2002; Durakovic et al., 2006; Zhang et al., 2002).Thus, early after allo-HCT host APCs represent the dominant antigen presenting cells available to prime donor T cells.

Currently, the pathogenesis of aGVHD is understood to occur in three sequential phases (Ferrara et al., 1999) (**Figure 2**).

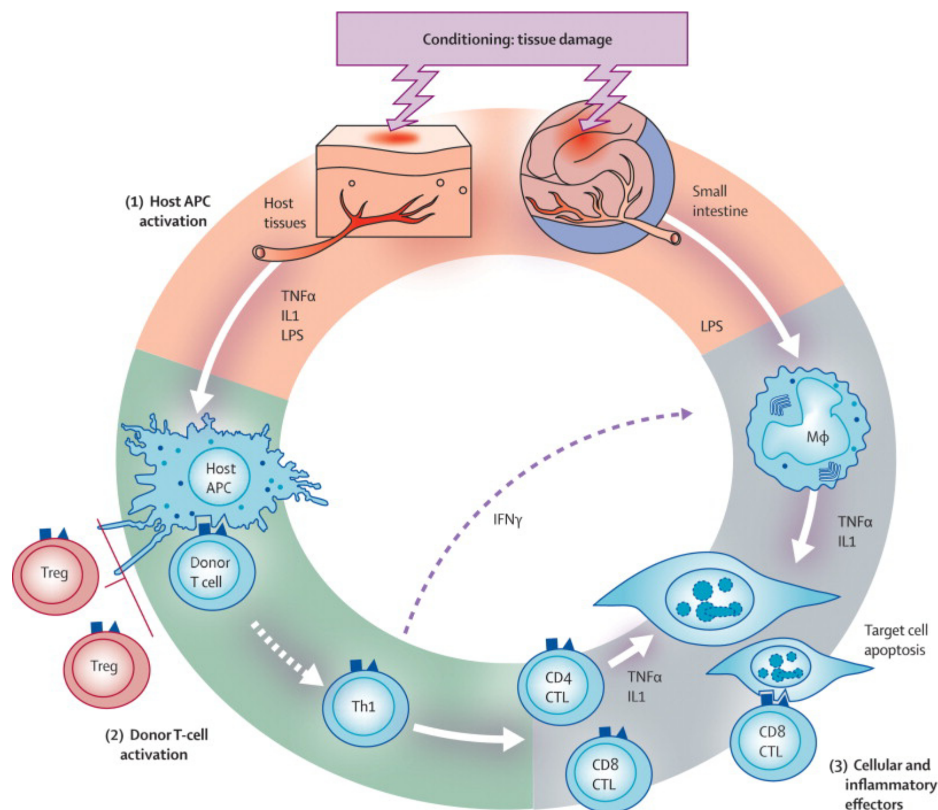

Figure 2. Acute GVHD pathophysiology. GVHD pathology in three sequential phases. 1, recipient conditioning; 2, donor T cell activation by host APCs and cytokine production, 3, inflammation and migration to the periphery and tissue specific destruction during the effector phase. Sketch taken from Ferrara et al., 1999.

The first phase involves recipient tissues damage due to conditioning regimen, which leads to cytokine induced activation of radioresistant APCs. Second, Donor T cells within the secondary lymphoid organs (SLOs) recognize alloantigens presented by host APCs. This leads to the activation and proliferation of allogeneic T cells and their migration to the periphery and, tissue specific destruction during the effector phase (Anderson et al., 2005; Beilhack et al., 2005). Several studies within the last decade have highlighted the importance of host APCs contribution to the initiation of aGVHD (Duffner et al., 2004; Shlomchik et al., 1999; Teshima et al., 2002).

The current dogma of antigen presentation and the requirement of host APCs for GVHD induction has been based on seminal studies by Shlomchik and colleagues (1999). The authors demonstrated that host APCs were required to induce aGVHD in a CD8$^+$ dependent model in which cross-presentation from recipient-derived antigens by donor APCs alone

failed to induce aGVHD (Shlomchik et al., 1999). *Teshima et al.*(2002) showed that antigen presentation by hematopoietic cells was sufficient for GVHD onset. Yet, expression of antigens on non-hematopoietic target tissues was not required. Duffner and colleagues (2004) consolidated the notion that host DCs are required to induce for GVHD by comparing aGVHD induction in the presence of host or donor DC and B cells. Based on these studies it was concluded that host hematopoietic APCs served essential functions to induce GVHD. Additionally, based on the fact that DCs are the most efficient APC population, it was extrapolated that DCs serve as the critical APC subset for GVHD onset.

However, only through the recent development of mouse models that allow to conditionally deplete defined APC subsets it has become possible to address the relative contribution of these APC populations and specific subsets. Recent data questioned whether professional hematopoietic host APCs (Hashimoto et al., 2011; Koyama et al., 2011; Toubai et al., 2011) particularly DCs, are essential to induce GVHD. Instead, these studies suggested that nonhematopoietic recipient APCs within target organs suffice to induce aGVHD (Koyama et al., 2011; Toubai et al., 2011), and rather pointed towards an immunomodulatory role of certain host APCs subsets during GHVD initiation (Hashimoto et al., 2011; Rowe et al., 2006). *Hashimoto e. al.* showed GVHD amelioration after host macrophages expansion, prior to GVHD induction. In the same line, Rowe and colleagues proposed host B cells as important to attenuate GVHD because of their IL-10 production following TBI. In this direction, recent data shows in both MHC class II- and class I-dependent models that, depletion of host DCs failed to demonstrate their requirement for GVHD onset (Koyama et al., 2011; Li et al., 2012). Indeed their depletion led to an exarcerbation of T cell proliferation (Koyama et al., 2011). This findings rather attribute a predominantly regulatory function to host DCs rather than plainly activating alloreactive T cells. Thus, the function of host APCs to prime and modulate alloreactive T cells in secondary lymphoid organs remains important to understand the initiating events of aGVHD.

However, it remains unresolved how tissue-resident APCs, particularly DCs as the most potent APCs, may impact alloreactive effector T-cell function in peripheral tissues.

1.2 Dendritic cells

DCs are unique and specialized APCs, with the principal function to present captured antigens, and induce an immune response in resting naïve T cells. They are found in lymphoid and non-lymphoid tissues. DCs are present almost in all organs, but they mainly line the surface of the airway, skin and the intestine, where they constantly sample antigens. In steady-state and infection, DCs migrate from non-lymphoid to lymphoid tissues, after antigen uptake, where they direct the T cells to mount an appropriate immune response. The ability to migrate from peripheral sites to lymphoid organs to prime an immune response is hallmark of DCs. They can exist as immature or mature DCs. Only mature DCs are able to induce an immunogenic response. Phenotypically mature DCs can be defined by their upregulation of MHC class II molecules and the costimulatory molecules CD40, CD80, CD86 and CD83. Immature DCs receive maturation signals from microbial patterns, danger signals or inflammatory cytokines, which are recognized mainly by toll-like receptors (TLRs), a class of pattern recognition receptors engaged by microbial products, and tumor necrosis factor (TNF)–receptors. Importantly, maturation leads to an increase in immunogenicity and decrease in antigen acquisition and processing (Reis e Sousa, 2006) (**Figure 3**).

Steady-state DCs are immature cells, which are very efficient at processing exogenous antigens for MHC class II presentation, but unable to prime immune responses. There is evidence that steady-state DCs can tolerize peripheral T cells by inducing deletion, anergy or regulation, depending on the model and the environmental factors (Steinman et al., 2003; Steinman and Nussenzweig, 2002). Tolerogenic DCs, are then, immature DCs which efficiently present processed antigens via MHC class II (signal 1), but fail to deliver signal 2, known as the costimulatory signals to induce immunity; and therefore, resulting in tolerance.

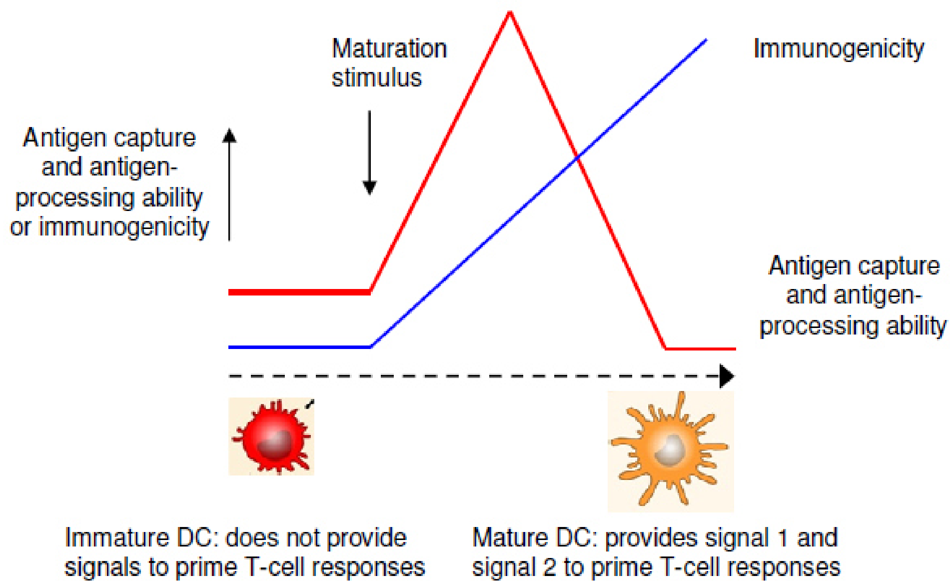

Figure 3. Dendritic cell maturation. Immature DCs are very efficient at capturing and processing exogenous antigens. Maturation stimuli increase their ability to prime an immune response, whereas the antigen acquisition capability is downregulated. Figure adapted from Reis e Sousa (Reis e Sousa, 2006).

DCs are a heterogeneous population that can be classified in distinct subsets, based on their phenotype, localization (including lymphoid and non-lymphoid tissues), and functions. Three types of DCs have been described in mice and humans (Helft et al., 2010; Merad et al., 2013).

- Classical DCs (cDCs), or tissue resident steady-state DCs, are very heterogeneous. Arise from BM pre-classical DC (pre-cDC), able to differentiate in cDCs but not in pDCs, and their maintenance and development depends on tyrosine kinase receptor fms-like tyrosine kinase 3 (Flt3). cDCs can be found in lymphoid and non-lymphoid tissues, although the major cDCs networks are found in the lung, skin and intestines. They have an enhanced ability to sense tissue injuries, capture environmental and cell-associated antigens and process and present them. Through this process cDCs can induce immunity to any foreign antigen and enforce tolerance to self-antigens. Different subsets from cDCs have been described and certain functions have been ascribed depending on their location, phenotype and microenvironmental conditions.
- Monocyte-derived DCs arise from monocytes that develop in the BM. Monocytes are a heterogeneous population, which can be divided in two functional subsets, both

subsets capable to differentiate into DCs *in vivo* (Geissmann et al., 2003; Jakubzick et al., 2008) :

- $CX_3CR1^{lo}CCR2^+$ subset, actively recruited to inflamed tissues, known as classical monocytes, and considered to be the direct precursors of inflammatory DCs (Auffray et al., 2009). Recognition of bacterial antigens though toll-like-receptors (TLRs) or T cell activation signals are requirements for monocyte differentiation into DCs.
- $CX_3CR1^{hi}CCR2^-$ subset, or non-classical monocytes, is characterized by CX_3CR1-dependent recruitment to non-inflamed tissues. Their differentiation into DCs *in vivo* has been described in both inflamed and non-inflamed tissues (Balazs et al., 2002; Geissmann et al., 2003; Yoneyama et al., 2001). Yet, the function of non-classical monocytes and non-classical monocyte-Derived DCs (nc-mo-DCs), and the factors, which trigger their recruitment and DC differentiation, has not been clearly defined to date.

- Plasmacytoid DCs (pDCs) arise from common DC progenitors (CDPs), which can give rise to classical DCs (cDCs) or pDCs. They represent a small DC subset that accumulates mainly in the blood and lymphoid organs in steady-state. They express low levels of MHC and costimulatory molecules, and low levels as well of the CD11c integrin. Upon recognition of foreign nucleic acids, they produce massive amounts of type I IFN and acquire the capacity to present foreign antigens and very efficiently prime T cells (Reizis et al., 2011; Villadangos and Schnorrer, 2007).

1.2.1 Intestinal dendritic cells

Intestinal DCs during steady-state conditions, can be functionally classified in two major classes according to their ability to exert tolerogenic or immunogenic functions. These two DC subsets are distinguished by the expression of CD103 (αE integrin), the receptor for the epithelial cell adhesion molecule E-cadherin. In functional terms, DCs could be classified as

CD103 tolerogenic or CD11b immunogeneic DCs (Miller et al., 2012; Rescigno and Di Sabatino, 2009).

Intestinal $CD103^+$ DCs are steady-state tissue-resident DCs, which are described as tolerogenic (Mann et al., 2013; Rescigno and Di Sabatino, 2009; Schulz et al., 2009). They originate from a BM pre-DC precursor, which give rise to $CD103^+$ DCs in the presence of retinoic acid (RA) and TGF-ß released by epithelial cells. $CD103^+$ DCs efficiently drive the differentiation of gut homing regulatory T cells (T_{regs}) by producing retinoic acid (RA) and transforming growth factor-ß (TGF-ß), and migrate very efficiently to the mesenteric lymph nodes (mLNs), were they can present the captured antigens to naïve T cells (Rescigno, 2011). Nonetheless, $CD103^+$ DCs are efficient cross-presenters and hence the most potent at stimulating $CD8^+$ T cells (Miller et al., 2012). They can also change to an inflammatory phenotype during intestinal inflammation and induce interferon-γ^+ (IFN-γ) cells (Miller et al., 2012; Rescigno and Di Sabatino, 2009; Scott et al., 2011).

Immunogenic CD11b expressing DCs in steady-state are characterized by the expression of CX_3CR1. Under steady-state $Ly6C^{hi}$ monocytes differentiate into $CD11b^+CX_3CR1^+$ DCs under the influence of ATP-producing bacteria. CX_3CR1 DCs extend their protrusions in to the lumen, participate actively in antigen capture and naïve T cell activation, and induce vigorous Th17 T cell differentiation in a TLR independent manner, due to their high TLRs expression, which allow them to sense microbe-associated molecular patterns. Yet, $CD11b^+$ intestinal DCs have been recently described as a heterogenous population. The fact that they arise from circulating monocytes on a Flt3-independent manner and their poor migratory ability, defines them closer to macrophages than to DCs (Mazzini et al., 2014; Miller et al., 2012).

During intestinal inflammation newly formed monocyte-derived $CD11b^{hi}$ DCs (moDCs) are rapidly recruited. They arise from $CX_3CR1^{lo}CCR2^+$ monocytes (classical monocytes) and may out-number other tissue-resident DCs to dominate the interaction with incoming T cells (Domínguez and Ardavín, 2010). Classical monocytes develop in the BM and their mobilization from the circulation to the tissues is accompanied by their differentiation into

DCs or macrophages (Geissmann et al., 2010). However, Swirski and colleagues showed that they can also be mobilized from the spleen (Swirski et al., 2009). moDCs promote inflammation and participate in the induction of both adaptive and innate immune responses (Domínguez and Ardavín, 2010; Segura and Amigorena, 2013). Yet, another type of moDCs has been described. They arise from $CX_3CR1^{hi}CCR2^{-}$ non-classical monocytes, which develop in the BM and enter tissues in a CX_3CR1 dependent manner. Monitoring of blood vessels and tissues, early response and tissues repair functions have been ascribed to non-classical monocytes (Shi and Pamer, 2011). This monocyte subset, known as non-classical anti-inflammatory monocytes, presents the ability to differentiate into DCs both *in vitro*, and *in vivo* (Geissmann et al., 2003; Leon et al., 2005). However, their function *in vivo* has not been well defined to date.

Although particular functions have been confined to certain subsets, DCs retain a remarkable plasticity. They can promote either immunostimulatory or tolerogenic effects, depending on their state of maturity, their cytokine or tissue environment (Lutz and Schuler, 2002; Steinman and Nussenzweig, 2002).

1.2.2 Dendritic cells in immunity and tolerance

DCs are potent immune cells that directly trigger and control T cell responses. They are important stimulators and can activate both CD4 T helper cells and cytotoxic CD8 T cells and provide them with co-stimulatory molecules and signals to begin to grow and function, influencing the type and quality of the immune response. Once activated, T cells can interact with other APCs to produce an additional immune response. Therefore, DCs need to mature in response to external stimuli related to infection, vaccination, or other scenarios like transplantation. Maturation is induced by various agents, but the two major receptor families are TLRs and TNF–receptors, especially CD40 (Liu et al., 2002; Sparwasser et al., 2000).

Maturation stimuli for DCs differ in their ability to induce tolerogenic or immunogenic DCs and their capacity to produce certain cytokines. Activated immunogenic DCs produce large amounts of inflammatory cytokines (IL-12, TNF-α, IL-6, IL-1ß, and type I IFN) (Rutella et al., 2006), which are crucial to induce T cell immunity. Thus, the maturation status and cytokine milieu by DCs is critical for the determination of the appropriate immune response. Most studies have focused on the DCs' role in activating T cells to resist foreign antigens, especially infections, but DCs exert an important function in the polarization between Th1, Th2, Th17, regulatory T cell (T_{reg}) development and even to improve T cell memory.

In the absence of infection or inflammation, DCs remain in an immature state, but they are not quiescent. They efficently acquire antigens from the enviroment (signal 1) but are poorly immunogenic, due to the low levels of MHC molecules and the lack of co-stimulation (signal 2), and the abcense of proinflammatory cytokines (signal 3). It has been suggested, that these might be the main factors that lead to DC tolerogenicity. However, while immaturity appears to be a good indicator of DC tolerogenicity, phenotypically mature DCs not always induce immunity but, depending upon prior exposure to certain differentiation signals, may retain their tolerogenic function (Maldonado and von Andrian, 2010). The cytokine environment, such as IL-10, TGF-ß or retinoic acid (RA), is crucial to shape tolerogenic DCs and consequently the induction of tolerance. Interestingly, higher numbers of DCs lead to a T_{reg} number increase, whereas elimination of T_{regs} increases the number of DCs. Thus, DCs and T_{regs} regulate each other.

1.2.3 Tolerogenic cytokines and co-inhibitory molecules in the control of T cell immunity by DCs

Recent studies highlighted the importance of DC-T cell interactions in peripheral tissues as an additional control required to shift the balance between tolerance and immunity (Dolfi et al., 2011; Hufford et al., 2011; McGill et al., 2008; McLachlan et al., 2009). Effector T cell activity is regulated by a balance of positive and negative costimulatory signals (Rothstein and Sayegh, 2003; Steinman et al., 2000). The effect of antigen (Ag) dose, DC lineage,

maturational status and cytokines milieu determines whether an immunogenic or tolerogenic response will develop.

DC function in peripheral tolerance implies the promotion of T_{reg} conversion by the release of IL-10 and TGF-ß and inhibition of effector T cell responses via secreted or membrane-bound immunosuppressive cytokines. The inability to produce IL-12 together with enhanced release of IL-10 is an unique functional feature of cytokine-modulation by tolerogenic DCs. In addition, immature DCs residing in almost all organs can induce T cell anergy due to their low levels of MHC, absence of costimulation and lack of inflammatory cytokines.

Co-signaling molecules are cell surface molecules that can fine-tune T-cell-receptor (TCR) signals. They can be co-stimulatory or co-inhibitory, which promote or suppress T cell activation, respectively. Co-signaling molecules are among the first responding elements of the immune system to antigens. The specific T cell response is defined by both co-stimulators and/or co-inhibitors, which are often provided simultaneously by DCs. The B7 family of co-signaling molecules is the best studied, and it includes both negative regulators or stimulators.

One of the better-characterized co-inhibitory signals is the B7-1/B7-2/CD28/CTLA-4 pathway. This pathway consists of two B7 family members B7-1 (CD80) and B7-2 (CD86), which bind to CD28 and CTLA-4. The engagement of B7-1 or B7-2, on APCs, to CD28 on naïve T cells provides a potent co-stimolatory signal. However, engagement of CTLA-4 on activated T cells deliver negative signals that inhibit TCR- and CD28-mediated signal transduction. Due to its dominant inhibitory role, CTLA-4 has been involved in several therapeutic approaches in animal models of autoimmune diseases, transplantation rejection and allergies (Linsley and Nadler, 2009).

Moreover, DCs acquire indoleamine 2,3 dioxygenase (IDO) activity after B7-1/B7-2 ligation by CTLA4 on T_{regs}. IDO is a potent immunosuppressive mediator, which involves production of tryptophan metabolites by DCs (Rutella et al., 2006), responsible for killing of activated T cells (Terness et al., 2002). Accordingly, Jasperson and colleagues (2009) showed the

importance of IDO expression by APCs in order to mitigate T cell alloreactivity in a GVHD model.

The B7-H1/PD-1 pathway has been intensively studied over the last decade, and it is described as a critical inhibitory signalling pathway that regulates T cell responses and maintains peripheral tolerance. Programmed death 1 (PD1) is a co-inhibitor in the regulation of T and B cell response (Riella et al., 2012). Whereas PD1 is inducibly expressed by T and B cells, PD-L1 is constitutively expressed on DCs, although it can be expressed on other hematopoietic and non-hematopoietic cells (including epithelial and endothelial cells) and inflammation and IFN-γ drives its upregulation (Freeman et al., 2000; Liang et al., 2003; Yamazaki et al., 2002). There is evidence of its immunosuppressor features *in vivo*. Antagonist PD1-specific antibodies (Abs) were shown to lead to acceleration of experimental autoimmune encephalomyelitis (EAE), autoimmune diabetes and GVHD (Ansari et al., 2003; Blazar et al., 2003; Salama et al., 2003).

Another member ob the B7 family, B7-H4, has been recently identified as co-inhibitor, inducibly expressed on T cells, B cells, monocytes and especially on DCs. B7-H4 binds particularly to activated T cells, and not naïve T cells, and ihibits their proliferation and cytokine production. Administration of B7-H4-Ig successfully reduced the cytolytic activity of alloreactive CD8 T cells in a GVHD model (Sica et al., 2003), whereas its endogenous blocking exacerbated T cell responses in EAE *in vivo* (Prasad et al., 2003).

2 Specific aims

Acute intestinal GVHD remains a major source of morbidity and mortality after allo-HCT. Therefore, it is important to identify key cellular subsets and mechanisms that modulate immunity in GVHD for developing novel therapeutic strategies to mitigate GVHD pathogenesis.

The aim of this work is to study the function of peripheral host APCs, particularly of DCs in the intestinal tract, during the GVHD effector phase and their influence on GVHD pathophysiology.

The specific aims of my thesis project are:

1. To study the composition of DC subsets in the intestinal tract during acute intestinal GVHD.
2. To define the origin, recruitment and subset involved in intestinal aGVHD.
3. To elucidate their contribution to intestinal GVHD during the effector phase.
4. To determine possible cellular mechanism relevant for future therapies.

3 Material and Methods

3.1 Material

3.1.1 Chemical reagents

Aceton	Sigma (Deisenhofen, Germany)
Baytril	Bayer (Leverkusen, Germany)
D-Luciferin	Biosynth (Staad, Switzerland)
Entellan	Merck (Darmstadt, Germany)
Ethanol	Sigma (Deisenhofen, Germany)
Fetal Calf Serum (FCS)	Invitrogen (Darmstadt, Germany)
Ketamine	Pfizer (Berlin, Germany)
Normal Rat Serum (NRS)	Invitrogen (Darmstadt, Germany)
O.C.T.	Sakura (Staufen, Germany)
Paraformaldehyde	Roth (Karlsruhe, Germany)
Trypan blue	Sigma (Deisenhofen, Germany)
Xylazine 2%	CP-Pharma (Burgdorf, Germany)

3.1.2 Buffers and solutions

- Lysis buffer (10x): NH_4Cl (89.9 g), $KHCO_3$ (10 g), EDTA (0.37 g) in 1000 ml distilled water, sterile filtered
- PBS (10x): NaCl (80 g), Na_2HPO_4-$2H_2O$ (14,2 g), KCL (2 g), KH_2PO_4 (2 g) in 1000 ml distilled water, pH: 6.8
- Dynal buffer: BSA (0.5 g), EDTA 0.5 M (0.2 ml) in 500 ml (1x) PBS
- PFA 4 %: 4 g PFA in 100 ml (1x) PBS, dissolved at 65 °C, pH: 7.4.

- Anesthetics: 8 ml Ketamine (25 mg/ml, Ketanest, Pfizer Pharma, Berlin, Germany), 2 ml Xylazin (2%) (Rompun, CP-Pharma, Burgdorf, Germany), 15 ml (1x) PBS

- cRPMI-1640: RPMI-1640 medium supplemented with 10% FCS, Penicillin (100 U/ml), Streptomycin (100 µg/ml), L-glutamine (2 mM) and β-mercaptoethanol (50 µM) (all Invitrogen, Darmstadt, Germany)

3.1.3 Antibodies and secondary reagents

Table1: Murine primary antibodies used for FACS staining and fluorescence microscopy.

Antibody	Clone	Conjugation	Isotype	Purchased from
CD11c	N418	Alexa647 or Alexa488	armenian hamster	Biolegend
CD103	2E7	Alexa488	armenian hamster	Biolegend
CD11b	M1/70	PE-Cy7 or biotin	rat	Biolegend
CD169	3D6.112	Purified	rat	Biolegend
CD274	10F.9G2	Purified, APC	rat	Biolegend
CD31	MEC13.3	biotin	rat	Biolegend
CD31	390	biotin	rat	Biolegend
CD3ε	145-2C11	purified	armenian hamster	BD Pharming
CD4	RM4-5	Alexa488, APC, APC-CY7 or PE	rat	Biolegend
CD4	GK1.5	Alexa647	rat	Biolegend
CD44	IM7	Pacific Blue	rat	Biolegend
CD45.1	A20	Alexa647, APC-Cy7, PE	mouse	Biolegend
CD45.2	104	Alexa647, APC-Cy7, FITC	mouse	Biolegend
CD8α	53-6.7	Alexa488, APC, APC-CY7, biotin or PE-CY7	rat	Biolegend
CD8α	53-6.7	FITC or PE	rat	eBioscience
CD80	16.10A1	Purified or Alexa488	armenian hamster	Biolegend
CD90.1	OX-7	Alexa488, Alexa647 or FITC	mouse	Biolegend
CD90.1	HIS51	APC or biotin	mouse	eBioscience
CD90.2	30-H12	biotin, FITC or PE	rat	Biolegend
FoxP3	FJK-16s	Purified	rat	eBisocience
I-A^b	AF6-120.1	PE	mouse	Biolegend
I-A^q	KH116	Alexa488	mouse	Bolegend
IL2	JES6-5H4	Alexa647	rat	eBioscience

Antibody	Clone	Conjugation	Isotype	Purchased from
IL17	eBio17B7	APC or PE	rat	Biolegend
IFN-γ	XMG1.2	Brilliant Violet 412	rat	Biolegend
Ly6C	HK1.4	FITC	rat	Biolegend
Ly6G	1A8	Alexa647 or Purified	rat	Biolegend or B-X-Cell
TNFα	MP6-XT22	PE	rat	Biolegend

Secondary antibodies: Donkey anti goat Cy3 purchased from Dianova (Hamburg, Germany), goat anti mouse IgG AlexaFluor® 568 purchased from Invitrogen (Karlsruhe, Germany).

Secondary reagents: Streptavidin-conjugated AlexaFluor® 488, AlexaFluor® 532, AlexaFluor® 546, AlexaFluor® 568 or AlexaFluor® 647 all purchased from Invitrogen (Karlsruhe, Germany).

Table 2: Secondary antibodies and reagents for FACS staining and fluorescence microscopy.

Reagent	Binds to	Conjugate	Company
Streptavidin	Biotin	Alexa750	Invitrogen
Streptavidin	Biotin	eFluor450	Invitrogen
Streptavidin	Biotin	Alexa456	Invitrogen
Strptavidin	Biotin	Alexa647	Invitrogen
AffiniPure donkey anti-rat IgG	Purified rat anti-mouse Ab	Cy3	Jackson Immunoresearch
AffiniPure donkey anti-rat IgG	Purified rat anti-mouse Ab	Cy3	Jackson Immunoresearch

3.1.4 Commercially available kits

Avidin-Biotin blocking Kit	Vector Laboratories (Burlingame, CA)
Vektashield mounting medium	Vector Laboratories (Burlingame, CA)
Dynabeads® Magnetic Beads	Invitrogen (Karlsruhe, Germany)
Cytometric Bead Array	BD Bioscience (Heidelberg, Germany)

3.1.5 Consumables

6 well flat bottom culture plates	Greiner Bio-One (Frickenhausen, Germany) or Sarstedt (Newton, USA)

96 well flat bottom culture plates	Sarstedt (Newton, USA)
96 well U bottom culture plates	Sarstedt (Newton, USA)
96 well V bottom culture plates	Sarstedt (Newton, USA)
5, 15 and 25 ml single use pipettes	Greiner Bio-One (Frickenhausen, Germany)
10 µl tips	Sarstedt (Newton, USA)
200 µl tips	Sarstedt (Newton, USA)
1000 µl tips	Sarstedt (Newton, USA)
15 ml and 50 ml centrifuge tube	Greiner Bio-One (Germany)
Cell strainer 70 µm	BD Biosciences (CA, USA)
Cryomolds	Sakura (Staufen, Germany)
SuperFrost Microscope Slides	R. Langenbrinck (Emmendingen, Germany)
U-100 Insulin Syringes	BD Bioscience (Heidelberg, Germany)
5, 10 and 15 ml Syringes	BD Bioscience (Heidelberg, Germany)

3.1.6 Mice

BALB/C, C57BL/6 (B6) and FVB/N mice between 8 and 12 weeks old were purchased from Charles River Laboratories (Sulzfeld, Germany). Following (transgenic) mice included in the study were bred in our own mouse colony at the Center for Experimental Molecular Medicine (ZEMM) Würzburg, or purchased from Jackson Laboratory and Taconic:

Table 3: Mice

Scientific name	Transgene	Coat color	Short name
C57Bl/6.L2G85.CD90.1	Luciferase	black	B6.L2G85.CD90.1
C57Bl/6.L2G85.CD45.1	Luciferase	black	B6.L2G85.CD45.1
C57Bl/6.CD11c.DOG	DTR-OVA-GFP	black, white	B6.CD11c.DOG
FVB/N-L2G85	Luciferase	white	FVB.L2g85
C57Bl/6.B7H1$^{-/-}$	B7H1$^{-/-}$	black	PD-L1^{ko}
C57BL/6.CCR7$^{-/-}$	CCR7	black	B6.CCR7$^{-/-}$
B6.129S4-Ccr2^{tm1Ifc}/J	CCR2	black	B6.CCR2$^{-/-}$
B6.129P-Cx3cr1^{tm1Litt}/J	CX_3CR1	black	B6.CX^{3}CR1$^{-/-}$
B6;C3Fe a/a-Csf1^{op}/J	Csf1	black	B6.m-csf$^{-/-}$
C57BL/6-flt3L^{tm1Imx}	Flt3 L	black	B6.Flt3 L$^{-/-}$

Transgenic mice B6.L2G85.CD90.1 expressing firefly luciferase (luciferase^{+}) were generated by backcrossing the luciferase^{+} FVB/N-L2G85 founder line (Cao et al., 2004; Beilhack et al., 2005) with the C57Bl/6 mice for more than 12 generations.

C57BL/6.CD11c.DOG (B6.CD11c.DOG, H-2^{b}, CD90.2, CD45.2) transgenic mice, were kindly provided from Günter Hämmerling (German Cancer Research Center, Heidelberg, Germany). C57Bl/6.B7H1$^{-/-}$ (PD-L1^{ko}, H-2^{b}, CD90.2, CD45.2) and C57BL/6.CCR7$^{-/-}$ (B6.CCR7$^{-/-}$, H-2^{b}, CD90.2, CD45.2) were kindly provided by Manfred B. Lutz (Institute for Virology and Immunobiology, Würzburg University). B6.CCR2$^{-/-}$, B6.CX^{3}CR1$^{-/-}$, B6.m-csf$^{-/-}$ (H-2^{b}, CD90.2, CD45.2) were purchased from The Jackson Laboratory, and B6.Flt3 L$^{-/-}$ from Taconic. Mice were maintained in specific pathogen-free conditions at the Center for Experimental Molecular Medicines (ZEMM), Würzburg. All animal experiments were approved by local authorities (Regierung von Unterfranken) and complied with German animal protection law.

3.2 Methods

3.2.1 HCT

Sex matched 8 to 12 week old recipient mice received myeloablative total body irradiation (TBI). Balb/c received 8Gy, whereas C57Bl/6, B6.CD11c.DOG, B6.CCR7$^{-/-}$, B6.CCR2$^{-/-}$, B6.CX^{3}CR1$^{-/-}$, B6.m-csf$^{-/-}$, B6.Flt3 L$^{-/-}$, and PDL-1$^{-/-}$ received 9Gy using a Faxitron CP-160 X-ray irradiation system (Faxitron X-Ray, Lincolnshire, IL, USA). Two to three hours after irradiation mice were injected with 5x10^{6} FVB/N BM cells for hematopoietic reconstitution. In order to induce GVHD Balb/c recipients received 1.2x10^{6}, B6.CD11c.DOG and PDL-1^{ko} recipients received 6x10^{5} FVB.L2G85 splenic T cells intravenously. T cells were purified from the spleen using a Dynal T cell negative isolation Kit (Invitrogen, Darmstadt, Germany) according to the manufacturer’s instructions. Cell purity was assessed by flow cytometry analysis (>90%). For DC depletion recipient mice were injected intraperitoneally (i.p.) with diphtheria toxin (DTx, Sigma-Aldrich, München) for DC depletion at doses of 20 ng/g body

weight (bw) on days 2.5, 3.5 and 5 after allo-HCT. For neutrophil depletion allogeneic transplanted recipients received 500 µg Ly6G (1A8) (Bio-X-Cell, New Hampshire, USA) depleting Ab i.p. on day 3.5 and 5.5 after allo-HCT. Survival and weight change were daily monitored.

3.2.2 Generation of bone marrow chimeras

B6, B6.DOG.CD11c and PD-L1^{ko} recipient mice were irradiated with 9 Gy and received BM from either B6 (B6 → B6), B6.DOG.CD11c (B6.DOG.CD11c → B6, B6.DOG.CD11c → PD-L1^{ko}) or PD-L1^{ko} (PD-L1^{ko} → B6) for hematopoietic reconstitution. 1x10^{7} bone marrow cells in 200 µl PBS were injected i.v. into recipient mice. Three months after bone marrow reconstitution chimerism in PBMCs, and small bowel was assessed and mice were used for further transplantation experiments.

3.2.3 Pathologic scoring of intestinal GVHD

Small intestine and large bowel were fixed in 4% neutral buffered formalin and subsequently embedded in paraffin. 2µm sections were slide mounted, stained with hematoxylin and eosin (H&E) and scored by a pathologist (Anja Mottok) blinded to experimental groups. Biopsies were assessed for the presence and amount of apoptotic bodies in the crypts and for inflammation as described previously (Kaplan et al., 2004). Based on these criteria a score ranging from 0 (none) to 4 (severe) GvHD was established.

3.2.4 Immunofluorescence microscopy

For immunofluorescent analysis, organs were embedded in O.C.T. compound, frozen on dry ice and cut into fresh frozen sections of 5 µm thickness were mounted on microscope slides (R. Langenbrinck) and stored at -20°C. Sections were thawed for 15 min at room temperature. Samples were fixed with Acetone (7 min) and air dried (5 min). Unspecific binding sites were blocked with PBS (PAN, Aidenbach, Germany) containing 2% FCS (15

min) and Avidin/Biotin (30 min) (Vector Laboratories, Burlingame, CA, USA). Incubation with primary antibodies was performed for 1 h at room temperature. Washing steps in PBS (3x 5 min) were performed after the first incubation. For secondary antibodies and/or streptavidin a second incubation was performed for 30 min at room temperature. Sections were washed in PBS after the second incubation (3x 5 min). 4,6-diamidino-2-phenylindole (DAPI) was used for nuclear staining and Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) was used to prevent photo bleaching. Fluorescence microscopic images were obtained with a Zeiss Imager. Z1m fluorescent microscope (Carl Zeiss, Göttingen, Germany) equipped with a AxioCam MRm camera (Carl Zeiss). Standard magnification were 200x or 400x using a 20x Plan-Apochromat with 0.8 numerical aperture [NA] or a 40x Plan-Neofluar with 1,3 [NA] /Oil objective lens respectively.

3.2.5 Isolation of immune cells from the intestinal tract

On day+6 after allo-HCT intestinal tract from transplanted mice was collected, flushed with PBS, cut longitudinally and minced with the help of a scalpel. Tissue was digested for 30 min at 37°C with PBS containing calcium, magnesium, 2 mg/ml collagenase D and 0.1 mg/ml DNase I (Roche, Mannhein, Germany). Tissue pieces were mashed through a cell strainer and washed with PBS containing 0.5 % BSA. Cell suspension was centrifuged and the resulting pellet was resuspended in PBS and used for flow cytometry or cell sorting.

3.2.6 Flow cytometry and cell sorting

Fluorescence-activated cell sorting (FACS) analyses were performed using a BD FACS-Canto II (Becton Dickinson, BD, Heidelberg, Germany). Unspecific binding sites were blocked with normal rat serum (1:20) and surface markers were stained at 4°C for 30 min. To exclude dead cells from the analysis cells were stained with propidium iodide or fixable violet dead cell stain kit (Invitrogen, Darmstadt, Germany). Data was analyzed with FlowJo Software (Tree Star, Ashland, OR, USA). Gates were set using the fluorescence minus one-gating strategy (Tung et al., 2007).

Alloreactive T cells (CD90.1$^+$) from small intestine of allo-HCT recipients were purified with a FACSAria III cell sorter (Becton Dickinson, BD, Heidelberg, Germany). For T cell purification tissues were digested (Isolation of immune cells from the intestinal tract**, Materials and Methods)** and subsequently stained as described above.

3.2.7 Cytokine Bead Array (CBA) analysis

On day+6 after allo-HCT, serum and tissues were collected for cytokine analysis. Serum was obtain by centrifugation of 200 µl of blood during 15 min centrifugation at 4 °C. Small bowel (2 cm) was homogenized (3x 50 min each) at 5000 rpm (Precellys 24, Peqlab, Erlangen, Germany) and supernatant was kept for cytokine analysis. Cytokine concentrations were determined using a BD Cytometric Bead Array Kit (BD Biosciences Pharmingen, Heidelberg) according to the manufacturer's protocol. Data were analyzed with FCAP Array v2.0 Software.

3.2.8 *Ex vivo* BLI

On day+6 after allo-HCT mice were injected 150 µg/g BW D-Luciferin (Biosynth AG, Staad, Switzerland) and 10 min later euthanized. Organs were harvested and imaging was performed using an IVIS Spectrum CCD imaging system (Caliper Xenogen, Alameda, CA). Data was analyzed with Living Image 4.0 software (Caliper Xenogen).

3.2.9 Multicolor Light Sheet Microscopy (LSFM) and sample preparation

Organs were removed from transcardially perfused mice (20 ml ice-cold PBS followed by 40 ml of 4 % paraformaldehyde (pH 7.4) and prepared as previously described (Brede et al., 2012). Samples were blocked with 2 % FCS/PBS in 0.1 % Triton-X for 18-24 h, incubated with the respective antibodies for 24 h at 4 °C. Tissue specimens rendered optically transparent after dehydration in a graded ethanol series (30 % - 100 % for 2 h each) followed by incubation for 2 h in 100 % n-hexane and subsequently stored in clearing solution

consisting of 1 part benzyl alcohol in 2 parts benzyl benzoate (both Sigma). For 3D imaging we used a homebuilt LSFM similar to a previously described setup (Brede et al. 2012). Of note, the light sheet illumination was created by scanning the laser beam with an optical scanner (Electro-Optical Products Crp.). We acquired multicolor stacks, by imaging each plane sequentially by each wavelength and emission filter combination with an increment from 1 to 5 microns. Exposure times for image acquisition were 200 or 500 ms per frame. Resulting multicolor stacks were processed and analyzed with ImageJ (US National Institutes of Health).

3.2.10 T cell stimulation and intracellular staining

FACS-sorted donor T cells ($CD90.1^{+}CD45.1^{+}$) from the small intestine were stimulated for 5 h with of phorbol 12-myristate 13-acetate (PMA) (20 ng/ml), ionomycin (1 µM) (both Sigma-Aldrich, München,Germany) and Monensin (Golgi Stop, BD Biosciences Pharmingen, Heidelberg, Germany) to avoid cytokines release. Surface markers were stained and cells were fixed with Fixation/Permeabilization buffer (eBioscience, Frankfurt, Germany). For intercellular staining antibodies were resuspended in 1x Permeabilization buffer (eBioscience, Frankfurt, Germany) and cells were stained for 30 min at 4 °C. Cell suspension was centrifuged and the resulting pellet was resuspended in PBS and used for flow cytometry.

3.2.11 Cell size and morphology measurement

We analyzed the morphology of cells with the software Volocity (Improvision, UK). Therefore, we calculated area, skeletal length and circularity of cells (Volocity Measurements: Find objects using intensity > Clip objects to ROI > Exclude objects touching edge of image) within a ROI roughly encircling a cell. The skeletal length of an object is measured as continuous line stretching from one end of the cell to the other. The circularity indicates the shape of a cell and is given as a numeric value between 0 and 1, being 1 a perfect circle.

3.2.12 Statistics

Data is shown as mean ± standard deviation (SD). Graphs were prepared using GraphPad Prism 5 software (La Jolla, CA, USA). Different groups were compared by two-tailed unpaired student's t-tests using GraphPad InStat 3 software. The level of significance was set at $P < 0.05$.

4 Results

4.1 Activated host intestinal DCs interact with donor T cells during aGVHD

First, we aimed to investigate the presence of intestinal DCs during aGVHD. Therefore, we used a major mismatch transplantation model, whose fast kinetics and severity allow analysis of the first GVHD symptoms and immune cell involvement within the first 6 days. We transferred splenic T cells together with bone marrow (BM) cells from FVB/N donors into BALB/c recipients after myeloablative total-body irradiation (TBI).

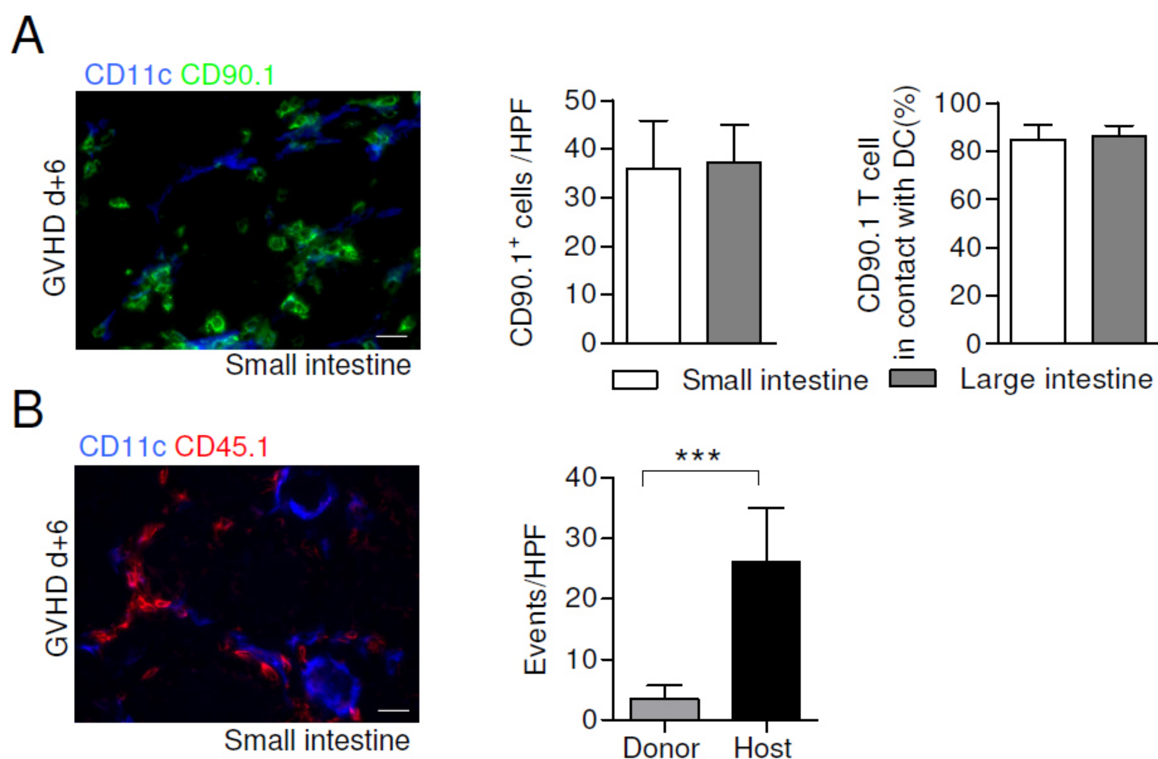

Figure 4. Activated host intestinal DCs interact with donor T cells during aGVHD.
Balb/c recipients were lethally irradiated and transplanted with BM cells and splenocytes from FVB/N donors. The congenic marker CD45.1 was utilized to differentiate donor (CD45.1) origin from host origin (CD45.2) cells. Organs were harvested at day 6 after transplantation for further analysis. (A) Immunofluorescence microscopy (IFM) of intestinal mucosa from Balb/c at day 6 after allo-BMT. Donor T cells displayed in green (CD90.1 Alexa488) and DCs in red (CD11c Alexa647). DCs present contact with donor T cells. Graphs show the quantification of (right) donor T cells detected in the small and large bowel on d+6 after allo-HCT and (left) percentage of donor T cells presenting contact with DC. (B) IFM of intestinal mucosa DCs (CD11c) displayed in blue and donor hematopoietic cells (CD45.1) displayed in red. Graph shows the absolute numbers/high power field (HPF) of donor versus host DCs. Mean ± SD, *** $p < 0.0001$.

Previous studies showed that transferred T cells proliferation and activation take place during the first 3 days after allo-HCT (Beilhack et al., 2005). On day 4 alloreactive T cells leave the secondary lymphoid organs and subsequently infiltrate the GVHD target organs. Consequently, organs were harvested on day +6 after allo-HCT, and embedded in OCT compound for further analysis. Immunofluorescence microscopy (IFM) revealed numerous DCs (CD11c) in the small and large bowel. Of note, high frequency of donor T cells (CD90.1) were in contact with DCs (**Figure 4A**). Congenic markers allowed distinguishing cells from host (CD45.2) and donor (CD45.1) origin. Remarkably, intestinal DCs remained of host origin (CD11c^{+}CD45.2^{+}) 6 days after allo-HCT despite myeloablative TBI (**Figure 4B**). To elucidate the activation and maturation phenotype of host intestinal DCs, we next analyzed the expression of co-stimulatory molecules on DCs with both IFM and flow cytometry. Host intestinal DCs interacting with donor T cells expressed moderate levels of co-stimulatory molecules, including CD86 and CD80 (**Figure 5A**), compared to mature splenic DCs (**Figure 5A**). These findings suggested a hitherto underestimated function of radio-resistant host intestinal DCs in regulating alloreactive T cells during aGVHD.

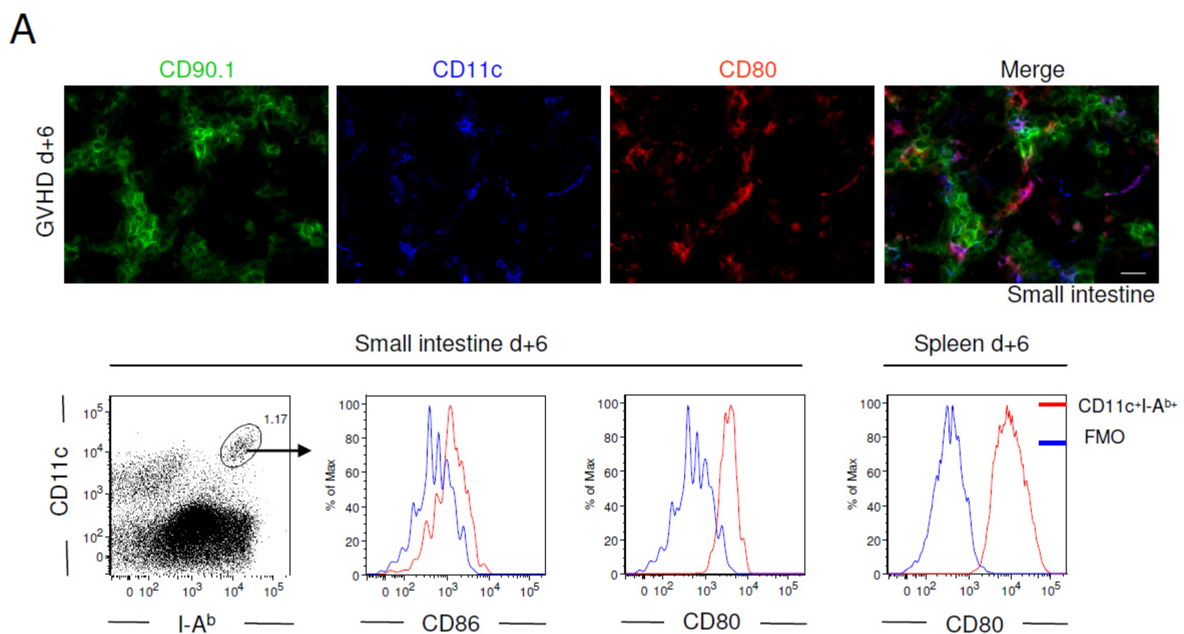

Figure 5. Activated host intestinal DCs interact with donor T cells.
(A) IFM and flow cytometry analysis show the expression of costimulatory molecules on host DCs in the intestinal mucosa on day 6 after allo-HCT. As a positive control histogram showing the costimulatory levels of splenic DC on d+6 after allo-HCT. Pictures depict single color channels and merged color for stained donor T cells (CD90.1 Alexa488), DCs (CD11c Alexa647) and the co-stimulatory molecule CD80 (Alexa546). Data are representative of two independent experiments with no fewer than three mice per group. Bar indicates 20 µm.

4.2 Host DCs protect from lethal aGVHD during the effector phase

After the identificaton of host intestinal DCs interacting with donor T cells, we aimed to elucidate the influence of DCs on T cell alloreactivity in peripheral tissues during the aGVHD effector phase. The prevailing opinion has been that DCs in peripheral tissues are important for stimulating, recruiting, or even “licensing” alloreactive T cells to exert tissue damage after allo-HCT (Bennett and Chakraverty, 2012).

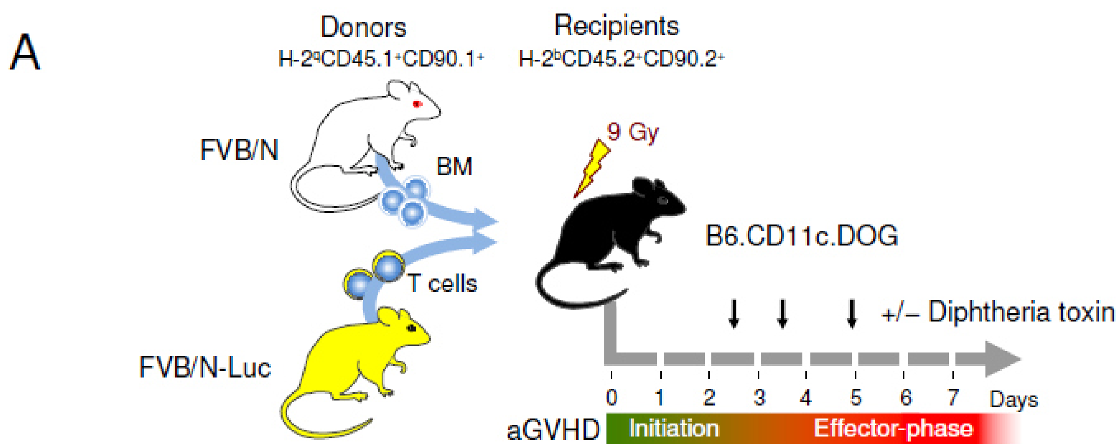

Figure 6. Selective depletion of DCs via diphtheria toxin administration.
(A) Experimental set-up. B6.CD11c.DOG mice were lethally irradiated (9Gy) and transplanted with BM cells ($5x10^6$ cells) and enriched luciferase$^+$ T cells ($6x10^5$ cells) from FVB/N donors. Recipients received 20ng/g bw diphtheria toxin (DTx) or PBS intraperitoneally (i.p.) on days 2.5, 3.5 and 5 after allo-BMT. On day 6 organs were harvested for further analysis. Intestinal tract was analyzed to confirm the depletion of host DCs via IFM and flow cytometry.

To elucidate whether DCs would propagate inflammation in the context of intestinal aGVHD, we employed a transgenic mouse model to selectively deplete host DCs at defined time-points during the course of aGVHD. In this model, the CD11c promoter drives expression of the diphtheria toxin (DTx) receptor (DTR) (B6.CD11c.DOG) (Hochweller et al., 2009), rendering CD11c$^+$ cells susceptible to DTx mediated depletion. First, we examined whether efficient DTx-induced DC depletion could be achieved in the intestinal tract of B6.CD11c.DOG transgenic mice. B6.CD11c.DOG mice were lethally irradiated and transplanted with BM from FVB/N and enriched T cells from FVB.L2G85 luciferase

transgenic (luc$^+$) donors. We depleted DCs by administering DTx at 2.5 days after allo-HTC, when alloreactive T cells were already primed and just before effector T cells infiltrated the GI tract to initiate aGVHD (Bauerlein et al., 2013; Beilhack et al., 2005) (**Figure 6A**). Intestinal host DC depletion was highly efficient (>90%) as shown with both flow cytometry and IFM (**Figure 7A and 7B**), and importantly, DTx treatment did not affect cells with intermediate CD11c expression levels (**Figure 7A**). Thus, indicating that B6.CD11c.DOG mice are a suitable model to investigate host intestinal DC function in acute intestinal GVHD.

Next, we assessed survival and GVHD severity in the presence and absence of host DC. Unexpectedly, aGVHD mortality was exacerbated by host DC depletion after alloreactive T-cell priming and during the effector phase of the immune response. All experimental animals died by day 8, while all control recipients survived (**Figure 7C**). Recipients lacking host DCs suffered from severe intestinal aGVHD (**Figure 7D**). Histopathology of the small and large bowel indicated epithelial crypt loss, high numbers of apoptotic bodies in the crypts, and inflammation (**Figure 7E**). We excluded any toxic effects mediated by DTx administration itself that would affect aGVHD progression, because B6 wild-type (WT) recipients that were treated every second day during 10 days with DTx or with phosphate-buffered saline (PBS) did not differ in behavior, clinical performance, or weight loss (**Figure 8A**). These experiments revealed a hitherto underestimated protective function of intestinal host DCs in limiting the on-going alloreactive T-cell response during the effector phase of intestinal aGVHD.

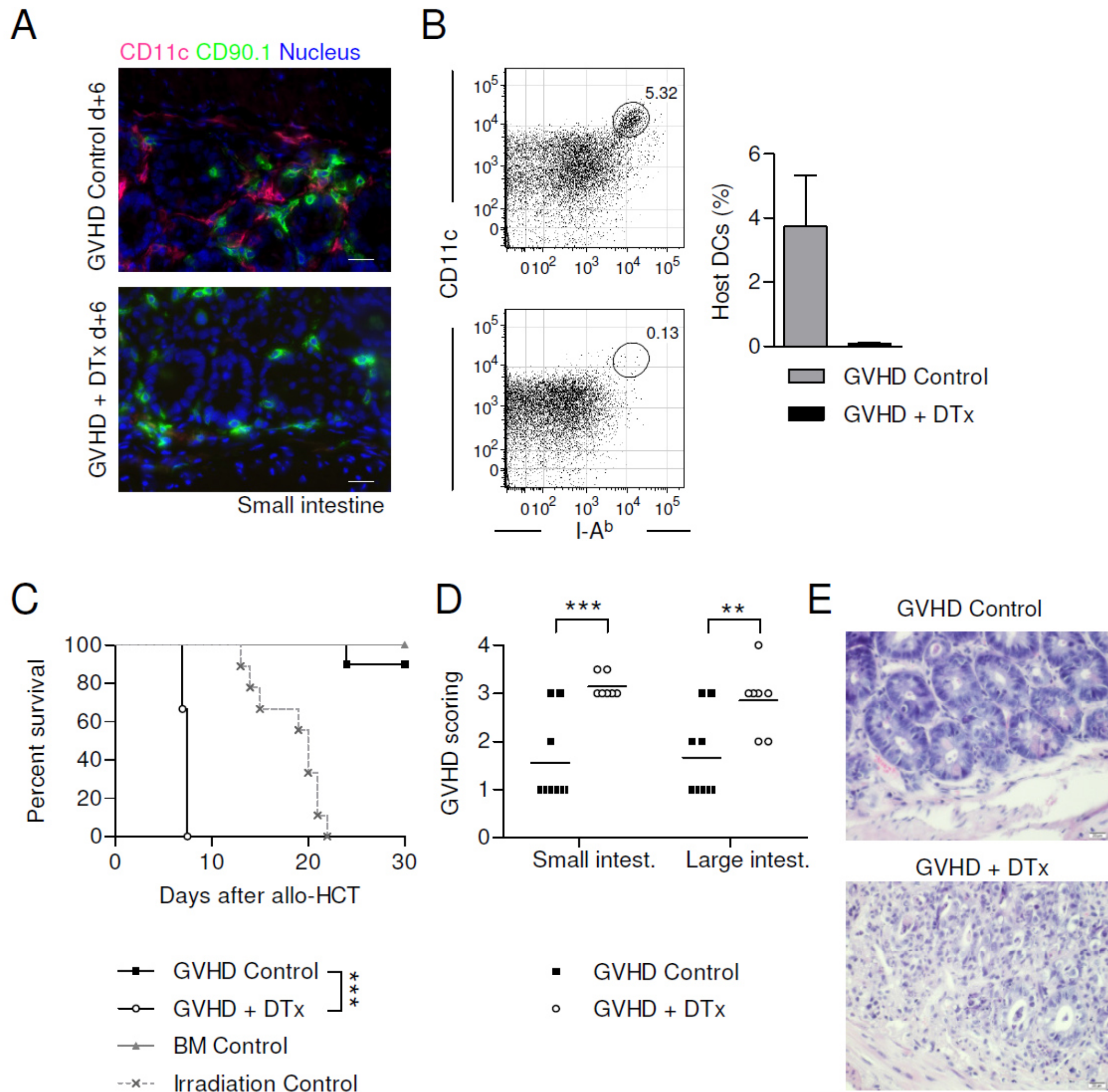

Figure 7. Intestinal DCs protect from lethal aGVHD during the effector phase.
(A) IFM from the intestinal mucosa from B6.CD11c.DOG recipients injected with PBS (GVHD Control) or DTx (GVHD + DTx) treated recipients. DCs (CD11c Alexa647) are displayed in red, donor T cells (CD90.1 Alexa 488) in green and nuclei (DAPI) in blue. (B) Flow cytometry was used as a second method to validate our data. Dot plots show cell suspension from digested small bowel using Collagenase D and DNAse I. After digestion cell suspension was stained for CD45.2, CD11c and MHC-II. Graph shows quantification of flow cytometry data. Shown frequency of live host DCs (n=6/group). (C) Survival of B6.CD11c.DOG + DTx (GVHD + DTx) and C57BL/6 (B6) (GVHD Control) transplanted recipients. GVHD Control n=10, GVHD + DTx n=15, *** $p < 0.0001$. (D) Histopahtology in small and large intestine at day 6 after allo-HCT. Biopsies were assessed for the presence and amount of apoptotic bodies in the crypts and for inflammation. GVHD Control n=9, GVHD + DTx n=7, ** $p < 0.0091$, *** $p < 0.0006$. (E) H&E staining of small intestine sections harvested 6 days after transplant from mice treated with DTx (GVHD + DTx) or PBS (GVHD Control). Bar indicates 20 µm.

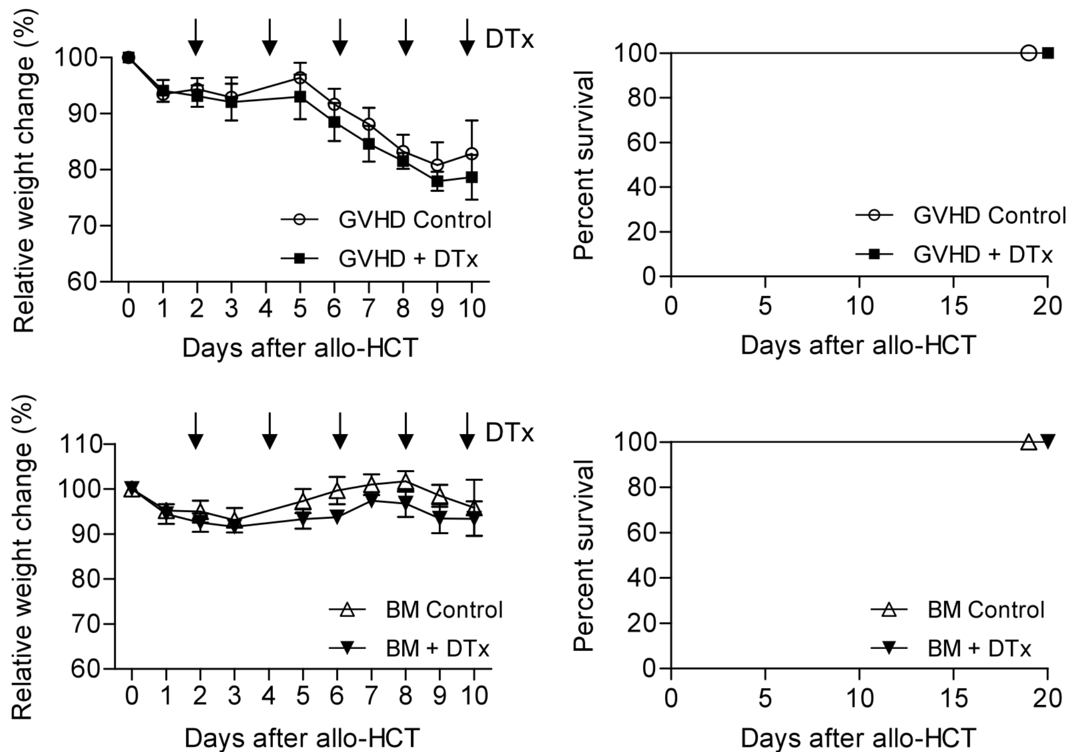

Figure 8. Diphtheria toxin administration in WT mice after GVHD induction did not present any cytotoxic effects.
(A) B6 WT mice were irradiated and received either $5x10^6$ bone marrow (BM) cells together with $6x10^5$ T cells (GVHD groups), or just BM cells (BM groups). Both groups were daily treated with diphteria toxin (20 ng/gbw) or PBS i.p. (A) Relative weight loss and survival after daily administration of either DTx or PBS after allo-BMT. GVHD Control (PBS) n=5, GVHD + DTx n=5, BM Control (PBS) n=5, BM + DTx n=5.

4.3 Host intestinal DCs protect from intestinal pathology by limiting donor T cell infiltration and inflammatory cytokine production

After uncovering that host DCs protect from GI tract GVHD, we further investigated the effects of host DC depletion in the intestinal tract after allo-HCT. Bioluminesce imaging (BLI) was applied to detect donor luciferase$^+$ T cells within priming sites, GVHD target and non-target organs. Significantly more donor T cells infiltrated the intestinal tract and mesenteric lymph nodes (mLNs) in the absence of host DCs at day 6 after allo-HCT (**Figure 9A and 9B**). BLI signal intensities (photons/seconds/cm^2) were comparable in all other analyzed

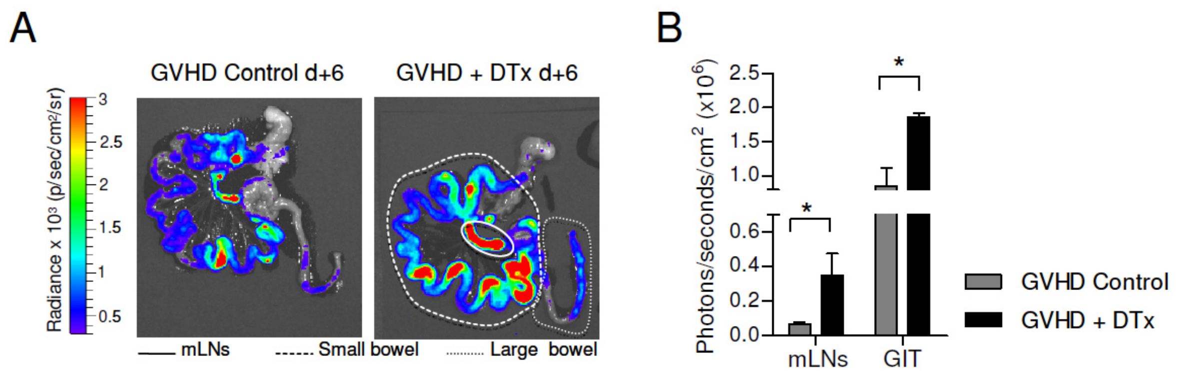

Figure 9. Host DCs protect from acute intestinal GVHD pathology by limiting donor T cell infiltration and inflammatory cytokine production.
(A) *Ex vivo* BLI of a representative mouse of each group at day 6 after allo-HCT. (B) Quantification of donor T cells infiltration and expansion in the intestinal tract and mLNs. (C) Quantification of donor T cells infiltration and expansion in the lungs, cLNs, thymus, heart, iLNs, kidneys, liver and spleen. One out of two independent experiments is shown. GVHD Control n=4, GVHD + DTx n=4, Mean ± SD * $p < 0.05$.

organs (spleen, cervical and inguinal lymph nodes, liver and GVHD non-target organs like kidneys and heart) (**Figure 9C**). Furthermore, we measured the amount of inflammatory cytokines detectable in the presence and absence of intestinal DCs. The pro-inflammatory cytokines MCP-1 and TNF were significantly elevated in serum and as well in the small bowel in allo-HCT recipients lacking host DCs (**Figure 10A and 10B**), and IFN-γ levels were slightly increased. In contrast, these cytokines were not markedly produced in syngeneic DC-depleted HCT recipients (C57BL/6 → B6.CD11c.DOG + DTx). Thus, ruling out a cytokine storm due to DC killing. We next, analyzed the cytokines produced by the infiltrating donor T cells. Alloreactive T cells isolated, via FACS-sorting, from the small intestine on day 6 after allo-HCT from DC depleted recipients produced higher amounts of IFNγ, TNF, IL-2 and IL-17 than control recipients (**Figure 10C**). To preclude that DC depletion 2,5 days after TBI could exacerbate GVHD due to the inflammatory environment at the moment of depletion, and to assure that the timing of DC depletion would not interfere with T cell priming during the initiation phase, we depleted host DCs at a later time point after allo-HCT (d+8), when inflammation caused by TBI is reduced and alloreactive T cells are predominantly in the GI tract (Beilhack et al., 2005). However, also host DC depletion on day 8 after GVHD induction significantly increased mortality and morbidity compared to control animals (**Figure 11A**).

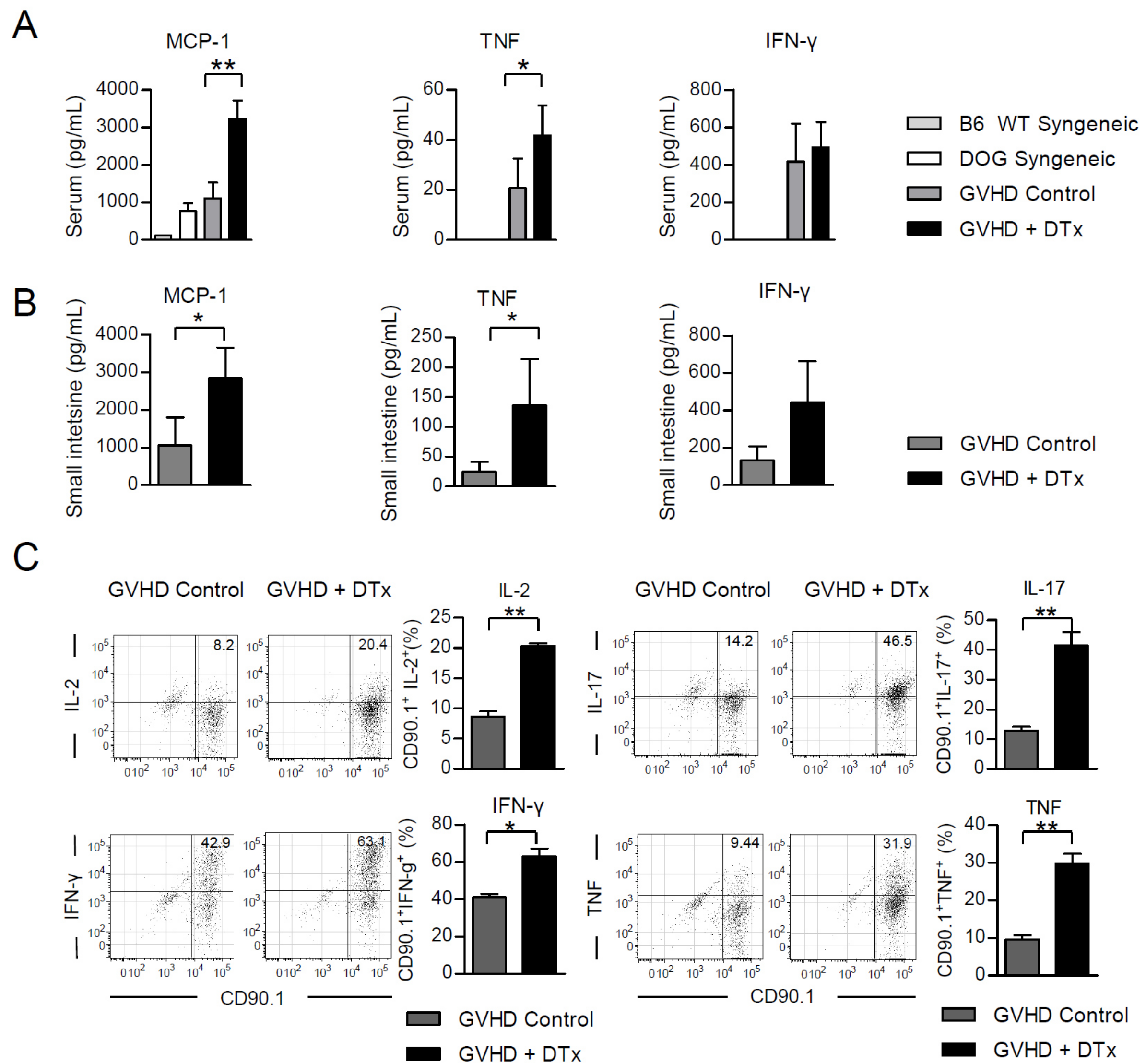

Figure 10. Increased inflammatory cytokine production in the absence of host DCs.
(A) Cytokine Bead Array (CBA) analysis. Serum IFN-γ, MCP-1 and TNF at day 6 from B6 WT syngeneic, B6.CD11c.DOG syngeneic, B6.CD11cDOG allogeneic and B6.CD11c.DOG + DTx allogeneic transplanted mice. Data from one representative experiment (n=5/group). (B) CBA analysis. IFN-γ, MCP-1 and TNF at day 6 from the small intestine from GVHD control and GVHD + DTx mice. Data are representative of one out of three independent experiments with no fewer than three mice per group. (C) Representative dot plots and graphs from cytokine secretion profile of facs-sorted donor T cells from the small intestine on day 6 after allo-BMT. Facs-sorted T cells were stimulated and intracellularly stained for IL-2, IFN-γ, TNF and IL-17. Representative data for two independent experiments with no fewer than three mice per group. Mean ± SD, * $p < 0.05$, ** $p < 0.0095$.

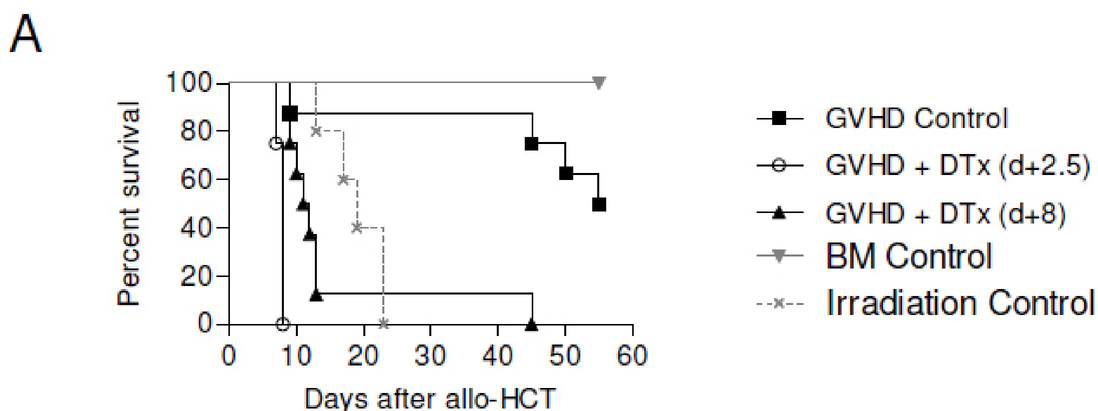

Figure 11. Administration of DTx on a later time point after allo-BMT resulted in increased mortality.
To rule out enhancement of inflammation after lethal irradiation and therefore interference of T cell priming by host DCs depletion, B6.CD11c.DOG were treated with either DTx or PBS 8 days after allo-BMT. (A) Survival of lethally irradiated B6.CD11c.DOG recipients, transplanted with BM and T cells from FVB/N donor to induce GVHD. Transplanted recipients were injected i.p. with 20 ng/g bw DTx or PBS either on d2,5, d3,5 and d5, or on d8, d9 and d+11 after allo-BMT. GVHD + DTx (d+2,5) n=5, GVHD + DTx (d+8) n=8, GVHD Control (PBS) n=8.

Tittel et al. reported induced neutrophilia caused by DC depletion under steady-state conditions. To exclude aGVHD aggravation due to induced neutrophilia in the intestinal tract upon host DC depletion we additionally eliminated neutrophils in allo-HCT recipients. After aGVHD induction recipient mice were treated with DTx (day 2,5 after allo-HCT) and 24 hours later were injected either with neutrophil-depleting Ab (Ly6G mAb) or PBS. Depletion of neutrophils was efficient (**Figure 12A)**, but did not improve aGVHD lethality and pathology after DC depletion (**Figure 12B and 12C**). Histopathological analysis on day 6 showed no signs of neutrophilia in the intestinal tract of DC depleted recipients as compared to undepleted GVHD controls. These results demonstrate that host intestinal DC depletion during the effector phase exacerbates aGVHD morbidity and mortality, increases donor T cell infiltration into the intestinal tract and enhances inflammatory cytokines release.

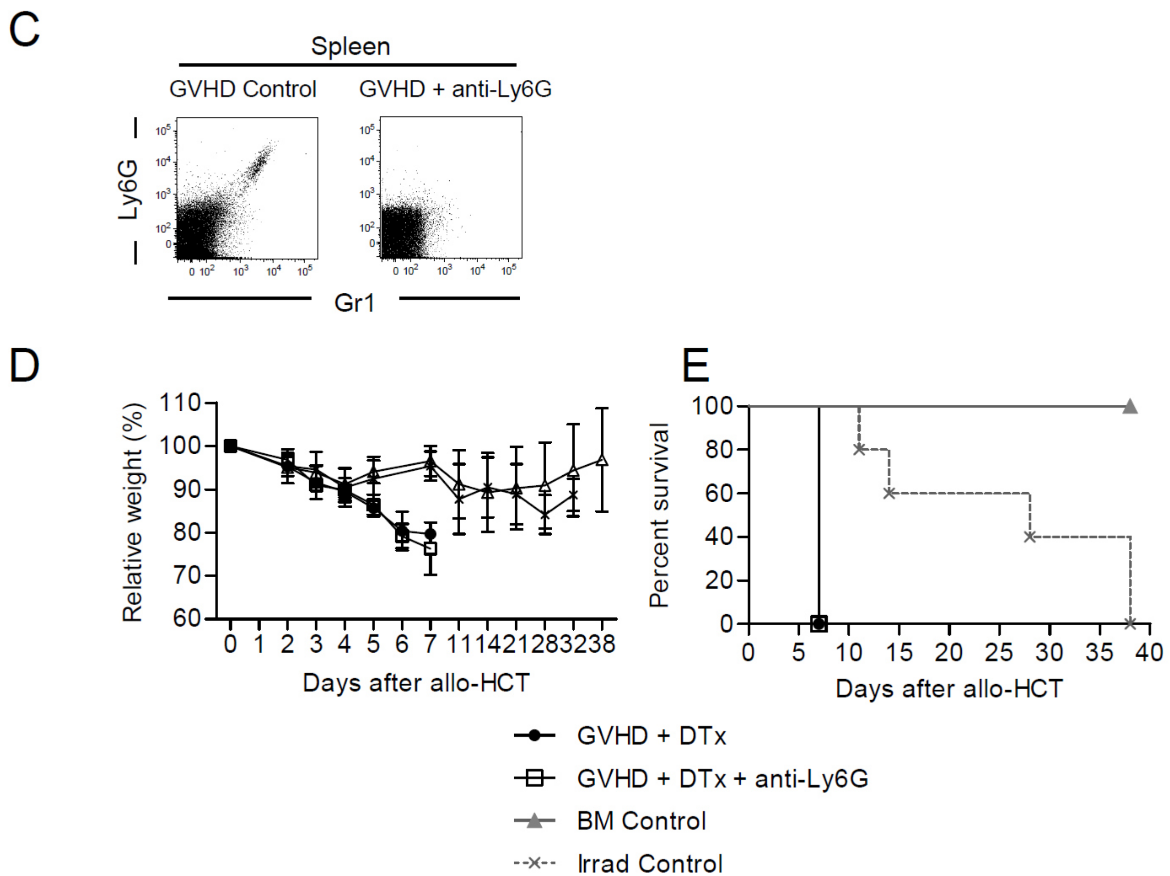

Figure 12. Depletion of neutrophils after DTx administration did not improve weight loss and survival.
(A) To investigate to which extent neutrophils could play a role in GVHD exacerbation after host DC depletion, we eliminated neutrophils with a depleting Ab (Ly6G, clone 1A8). All B6.CD11c.DOG transplanted mice were treated with DTx for DC depletion and Ly6G depleting Ab or PBS. (B) Relative weight loss and survival (C) of allo-BMT recipients after DC depletion in the presence or absence of neutrophils. GVHD + DTx n=7, GVHD + DTx + anti-Ly6G n=7, BM control n=5, Irrad. control n=5.

4.4 Host intestinal DCs are the major source of intetsial PD-L1 expression after allo-HCT

After revealing the deleterious effect of DC depletion, we intended to determine the mechanism how host DCs suppressed alloreactive T cells. Previous studies showed that programmed cell death 1 ligand (PD-L1) plays a major role in suppressing T cell responses, and blocking its interaction with PD-1 increases GVHD lethality after allo-HCT (Blazar et al., 2003; Riella et al., 2012). We then investigated PD-L1 expression on host intestinal DCs, as a potentially important mechanism for limiting alloreactive T-cell responses (Fife and Bluestone, 2008; Probst et al., 2005). Flow cytometry of the small intestine revealed on day 6

after allo-HCT that PD-L1 was broadly expressed on host-hematopoietic and non-hematopoietic cells (CD45^{-}), with stronger PD-L1 contribution by the hematopoietic compartment **(Figure 13A).** Yet, IFM showed that PD-L1 was significantly reduced in the LP of recipients lacking host DCs (**Figure 13B**). Compared to host DCs, CD11c$^{low/-}$ host hematopoietic cells expressed PD-L1 only marginally **(Figure 13C).** Overall, host DCs served as the major source of PD-L1 in the intestinal tract during aGVHD, suggesting that loss of this major PD-L1 source accelerated aGVHD after DC depletion (**Figure 7**).

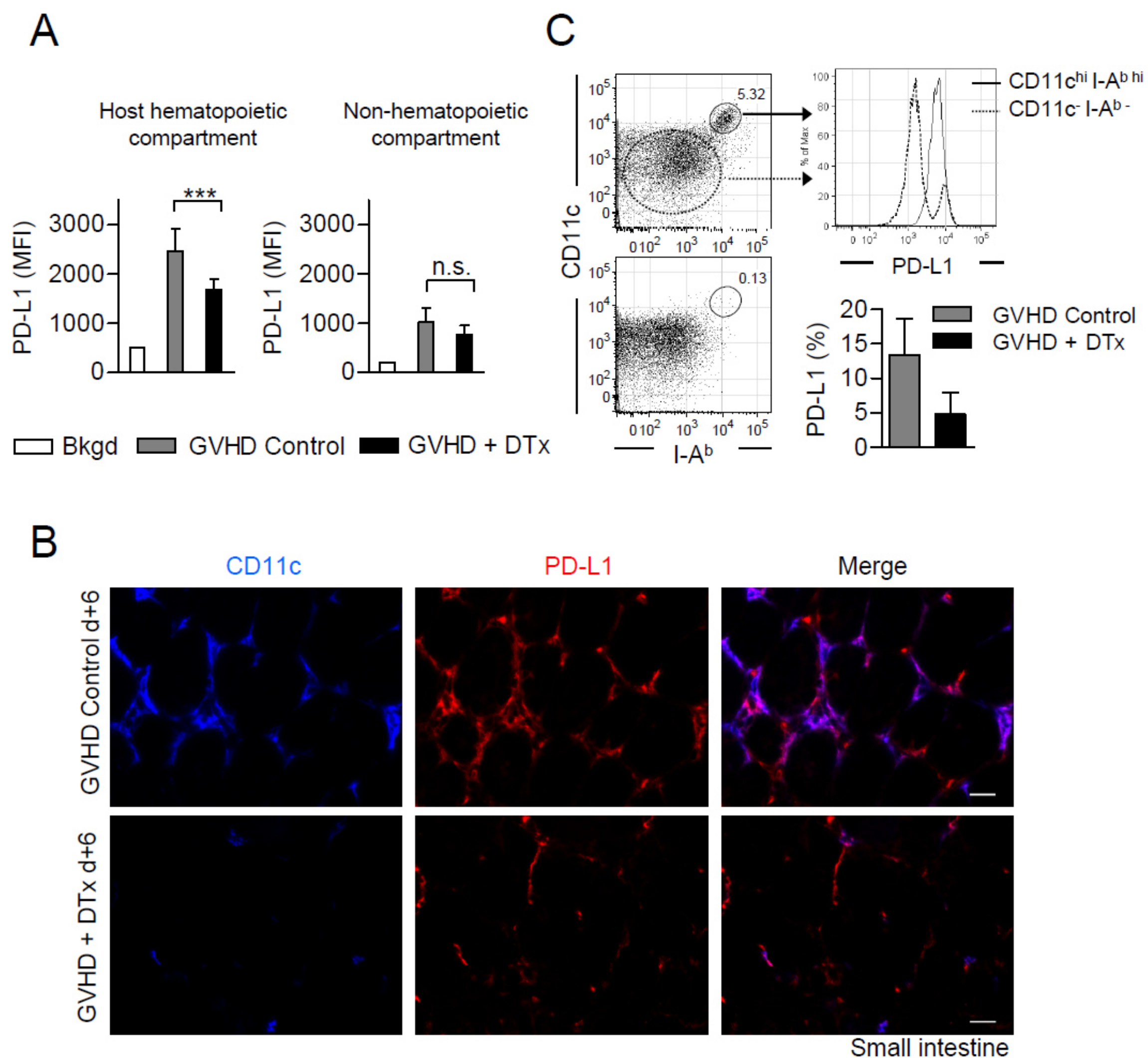

Figure 13. Host DCs are the major source of intestinal PD-L1 expression after allo-HCT.
(A) Contribution of hematopoietic and non-hematopoietic compartment to PD-L1 expression in the small intestine. Graphs show the mean fluorescence intensity (IFM) of PD-L1 expressed in the small intestine on day 6 after allo-HCT in the presence (GVHD Control) or absence (GVHD + DTx) of host DCs. GVHD Control n=7, GVHD + DTx n=7 and background (unstained transplanted recipient) n=1. (B) IFM from the small bowel of allogeneic B6.CD11c.DOG transplanted recipients at day 6 with or without DTx treatment. DCs (CD11c Alexa647) displayed in blue and PDL-1 (PD-L1 Cy3) in red. Double positive cells (CD11c^{+}PD-L1^{+}) displayed in purple. (C) Representative dot plots and histograms of PD-L1 expression on host hematopoietic cells and host DCs in the small intestine in the presence or absence of host DCs. Shown is a representative experiment of three with 3 mice per group. Mean ± SD, * $p < 0.05$, ** $p < 0.0095$, *** p <0,001. Bar indicates 20µm.

4.5 Host DCs limit alloreactive cell responses via PD-L1

Next, we analyzed the relevance of PD-L1 expression in aGVHD by comparing the post-allo-HCT survival of PD-L1^{ko} (B7-H1$^{-/-}$) (Dong et al., 2004) and DC-depleted B6.CD11c.DOG recipients (**Figure 14A**). Both groups showed similar survival rates, which were significantly decreased compared to that of control mice (**Figure 14B**). This indicated that the absence of PD-L1 expression on cells surviving radiation for at least 6 days—such as intestinal DCs (**Figure 13B**)—had the same detrimental effect on aGVDH progression as a lack of DCs.

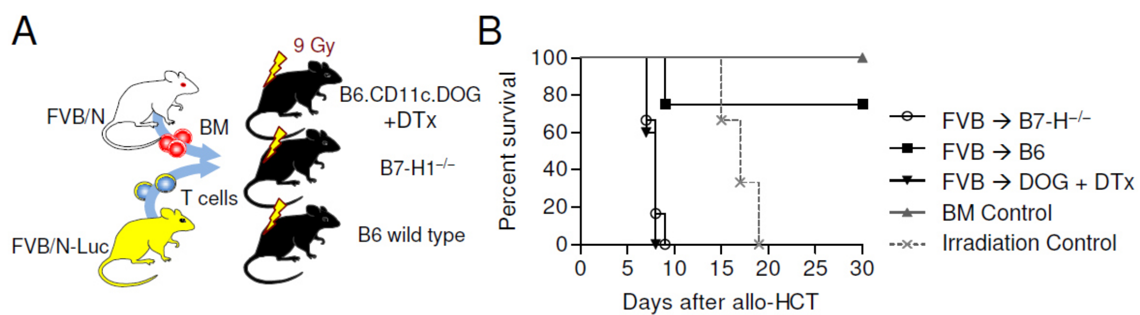

Figure 14. Host monocyte-derived DCs limit alloreactive T cell responses via PD-L1.
(A) Allo-HCT of B7-H1$^{-/-}$ (PD-L1^{ko}), B6.CD11c.DOG, and B6 recipients after TBI with BM and T cells from FVB/N donor mice. B6.CD11c.DOG received 20 ng/g bw DTx. (B) Survival after allo-HCT with FVB→ B7-H1$^{-/-}$ (n = 6), FVB→B6.CD11c.DOG + DTx (n = 5), and FVB→B6 (n = 5).

We further investigated whether PD-L1 on host DCs controlled T-cell alloreactivity in acute intestinal GVHD. We generated different BM chimeras to separately investigate the function of PD-L1 on host DCs, host immune cells, and tissue stroma (**Figure 15A)**. B7-H1$^{-/-}$ and B6 WT mice were reconstituted with B6.CD11c.DOG or B7-H1$^{-/-}$ BM cells (B6.CD11c.DOG → B7-H1$^{-/-}$, B6.CD11c.DOG → B6, and B7-H1$^{-/-}$ → B6). These chimeras enabled examination of the contribution of PD-L1 to aGVHD when expressed exclusively in the hematopoietic compartment in the presence or absence of DCs (B6.CD11c.DOG → B7-H1$^{-/-}$ +/– DTx), in both stroma and hematopoietic cells in the presence or absence of host DCs (B6.CD11c.DOG → B6 +/– DTx), or exclusively on stroma cells (PD-L1 B7-H1$^{-/-}$ → B6) (**Figure 15A**). Three months after reconstitution, BM chimeric mice were then irradiated, and received allo-HCT combined with allogeneic T-cell transfer to induce aGVHD (**Figure 15A**). On day 6 after allo-HCT, we analyzed PD-L1 expression in the intestinal tract (**Figure 15B**).

Only host DCs of FVB → (B6.CD11c.DOG → B7-H1$^{-/-}$) chimeras expressed PD-L1, whereas the rest of the tissue was PD-L1 negative. In FVB → (B6.CD11c.DOG → B7-H1$^{-/-}$) + DTx mice, almost no PD-L1 expression was detectable after DC depletion. Both DCs and stromal cells in FVB → (B6.CD11c.DOG → B6) chimeras expressed PD-L1 when DCs were not depleted, as observed in primary allo-HCT recipients (**Figure 15B**). After DC depletion of FVB → (B6.CD11c.DOG → B6) + DTx chimeras, PD-L1 expression only remained on stromal cells. FVB → (B7-H1$^{-/-}$→ B6) recipients presented normal PD-L1 expression on stromal cells, whereas host DCs were PD-L1 negative (**Figure 15B**). Importantly, PD-L1 expression on host DCs was decisive for improved survival. PD-L1 expression on stroma or other hematopoietic cells did not provide any survival advantage (**Figure 15C**). We concluded that PD-L1 expression particularly on host intestinal DCs served as an important mechanism to limit T-cell alloreactivity during intestinal aGVHD.

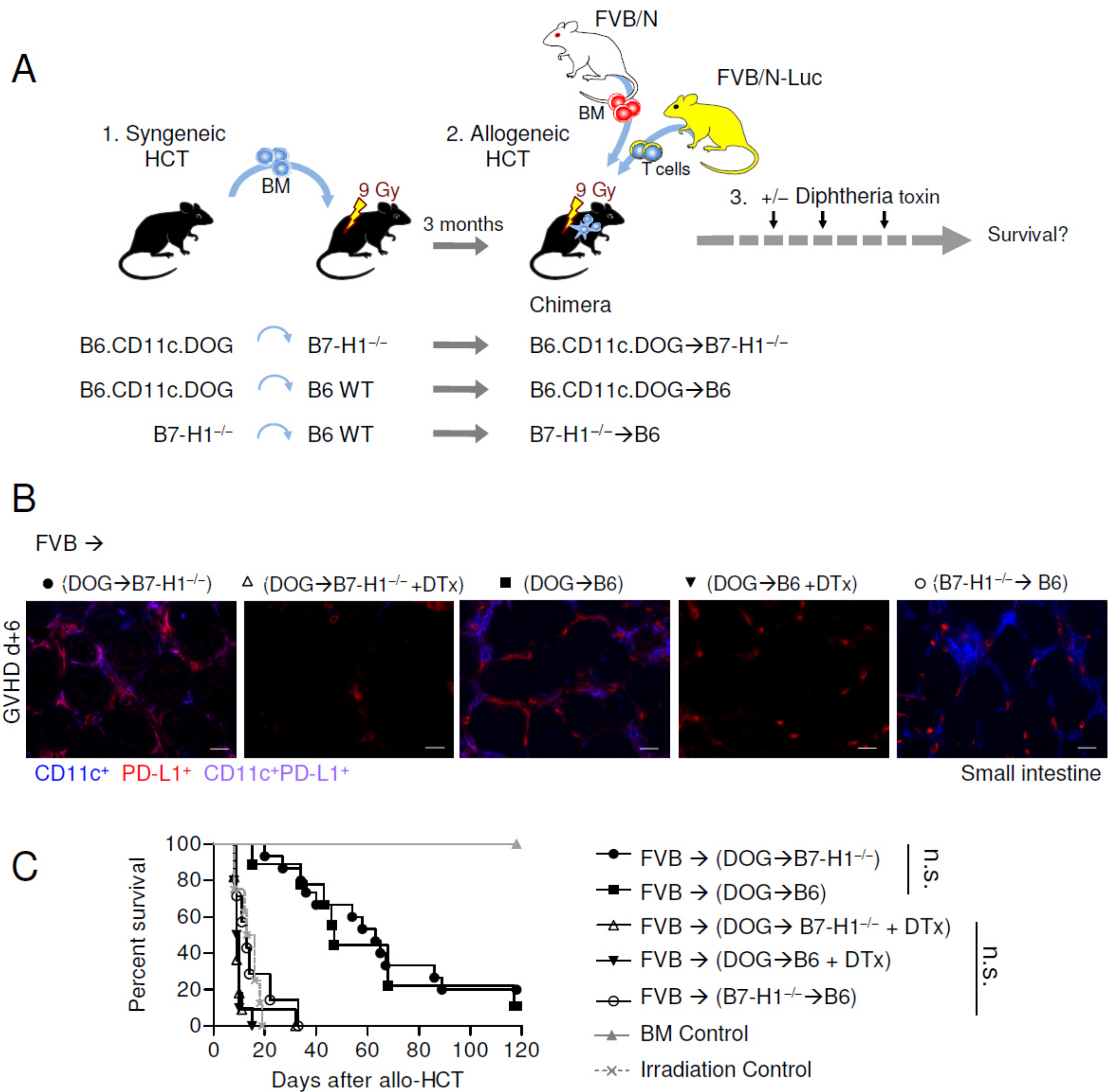

Figure 15. PD-L1 expression particularly on host intestinal DCs pervades alloreactivity suppression. (A) Generation of BM chimeras for analysis of PD-L1 on DCs during aGVHD, and the contribution when expressed in other compartments: B6.CD11c.DOG→ B7-H1$^{-/-}$, B6.CD11c.DOG→B6, B7-H1$^{-/-}$ → B6 and B6→ B6. Three months after cell transfer, chimeric mice were used as allo-HCT recipients and transplanted with BM and T cells from allogeneic FVB/N donors. Recipients were either treated with DTx [*(FVB→(B6.CD11c.DOG→ B7-H1$^{-/-}$ + DTx)* and *FVB→(B6.CD11c.DOG→ B6 + DTx)*], or PBS [*(FVB→(B6.CD11c.DOG→ B7-H1$^{-/-}$)*, and *(FVB → (B7-H1$^{-/-}$→ B6)* and *(FVB →(B6.CD11c.DOG→ B6)*]. (B) IFM of the intestinal tract to confirm chimerism, localization of PD-L1 expression, and host DC depletion at day +6 after allo-HCT. Blue, DCs (CD11c Alexa647); Red, PDL-1 (Cy3); Purple, Double-positive (CD11c^{+}PD-L1^{+}). (C) Survival after allo-HCT. n = 7–15 per group. p = 0.26 (n.s.) B6.CD11c.DOG→ B7-H1$^{-/-}$ + DTx versus B6.CD11c.DOG→B6 + DTx and B7-H1$^{-/-}$ → B6. p = 0.78 (n.s.) B6.DOG.CD11c → B7-H1$^{-/-}$ + PBS versus B6.CD11c.DOG→ B6 + PBS. Scale bar represents 20 µm.

4.6 Protective intestinal DCs are of monocytic origin

We next aimed to identify the intestinal DC subset responsible to suppress alloreactive T cells during intestinal GVHD. Intestinal DCs during steady-state conditions can be functionally classified in two major classes according their ability to act as tolerogenic or immunogenic. These two DC subsets are distinguished by the expression of CD103, the receptor for the epithelial cell adhesion molecule E-cadherin. During intestinal inflammation newly formed monocyte-derived CD11b^{hi} DCs (moDCs) are rapidly recruited and may out-number other tissues-resident DCs to dominate the interaction with incoming T cells (Domínguez and Ardavín, 2010).

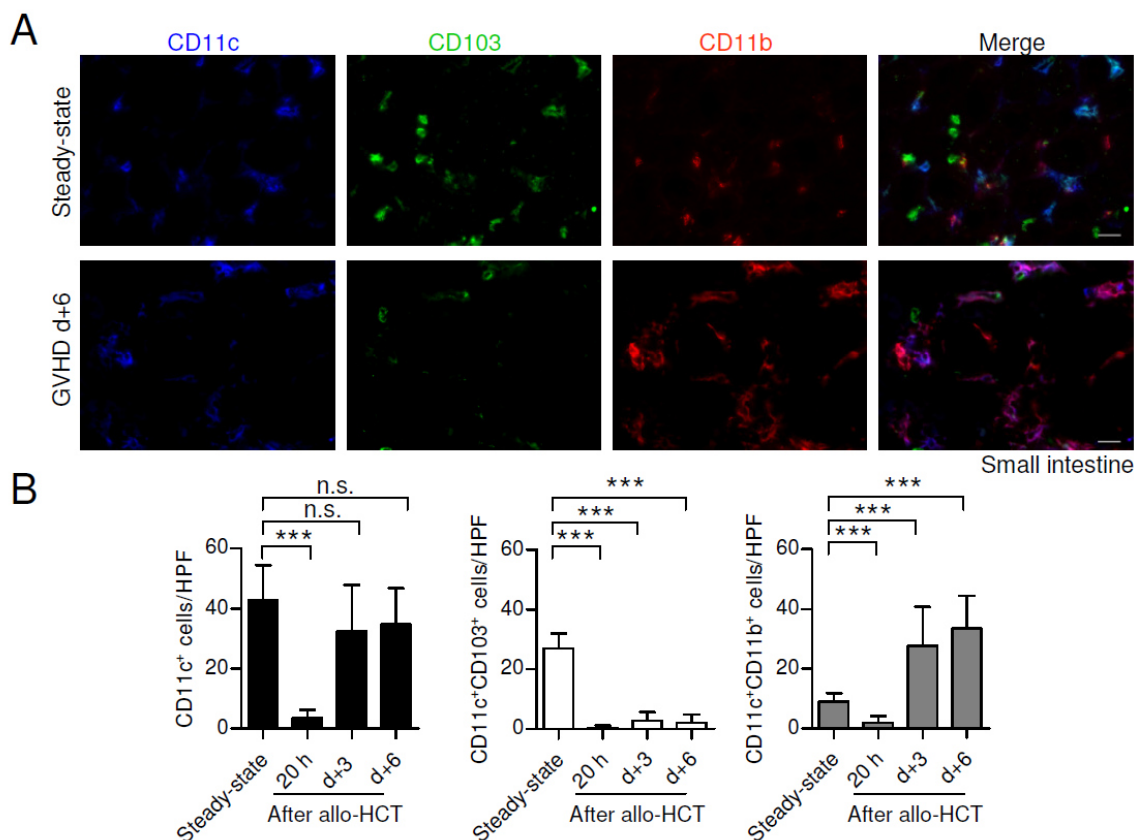

Figure 16. Shift from migratory CD103^{+} into CD11b^{+} intestinal DCs during acute intestinal GVHD.
IFM to discern DC subsets in the intestinal mucosa in steady-state and during aGVHD. (A) IFM of intestinal mucosa from a representative Balb/c mouse in steady-state and on day 6 after allo-HCT (GVHD d+6). CD11c^{+} DCs shown in blue (Alexa647), CD103 in green (Alexa488) and CD11b in red (Alexa546). Double positive CD11c^{+}CD103^{+} appear turquoise and CD11c^{+}CD11b^{+} in purple. (B) Absolute numbers/HPF of CD11c expressing cells and the CD103 and CD11b subsets in steady-state, day 3 (GVHD d+3) and day 6 (GVHD d+6) after allo-HCT. One out of two independent experiments is shown, with $\geq$ three mice per group. Mean ± SD, *** $p < 0.0002$. Bar indicates 20 µm.

Therefore, we aimed to identify the DC subsets involved in intestinal GVHD. IFM revealed that over the course of aGVHD development, the DC compartment shifted dramatically from the migratory CD11c^{+}CD103^{+} DCs, predominantly found in steady-state conditions, to CD11c^{+}CD11b^{hi} DCs (**Figure 16A and 16B**) that dominated during the aGVHD effector phase. CCR7 controls peripheral DCs migration to the draining LNs (Sallusto and Lanzavecchia, 2000). We employed used B6.CCR7$^{-/-}$ recipients to elucidate whether the prevention of CD103 DCs emigration from the LP to the SLOs might influence the dramatic shift of DC populations after allo-HCT. IFM on d+3 after allo-HCT showed again the disappearance of CD103^{+} DCs from the LP in both CCR7$^{-/-}$ and B6 WT recipients (**Figure 17A**). Thus, DCs disappearance of CD103^{+} DCs from the LP was not impaired by the lack of CCR7. This suggested that other redundant mechanisms are involved in DC emigration. Whether intestinal DCs could efficiently reach the T cell ares, still needs to be clarified. To examine the kinetics of CD11b^{hi} DC enrichment in the intestinal mucosa, we quantified intestinal DC numbers and phenotypes during acute intestinal GVHD pathogenesis with the help of IFM.

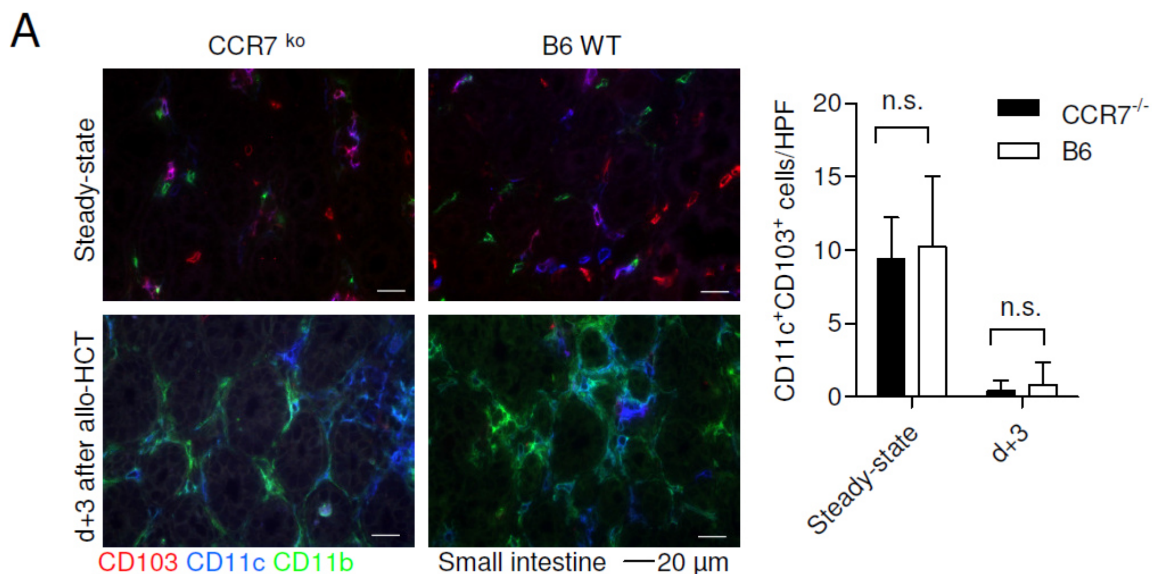

Figure 17. CD103 steady-state DCs migrated out of the LP in a CCR7 independent manner shortly after allo-HCT.
(A) Left. IFM of the small intestine during steady-state and d+3 after allo-HCT from both B6.CCR7$^{-/-}$ and B6 WT mice. Right. Quantification shows comparable amounts of CD103 DCs in B6.CCR7$^{-/-}$ and B6 WT animals prior and after transplantation. CCR7$^{-/-}$ steady-state n=2, d+3 n=4. B6 WT steady-state n=2, d+3 n=4. Mean ± SD, n.s > 0.05. Bar indicates 20 µm.

At 20 h after allo-HCT, CD103^{+}CD11c^{+} cells disappeared (**Figure 16A**), and CD11b^{hi}CD11c^{-} cells were detected in the LP (**Figure 18A**). Between 40 and 70 hours after allo-HCT, the size, morphology, and phenotype diverged from small circular CD11c^{-} cells into bigger cells with expanding protrusions, increasing cell volume, cytoskeletal lengthening (**Figure 19A and C**), and loss of circularity (**Figure 19B and 19D**), which exhibited retained CD11b and upregulated CD11c expressions (**Figure 18A**). These findings suggested a monocytic origin of protective host intestinal DCs. Therefore, we wanted to corroborate our hypothesis and elucidate the origin of CD11b^{hi} DCs.

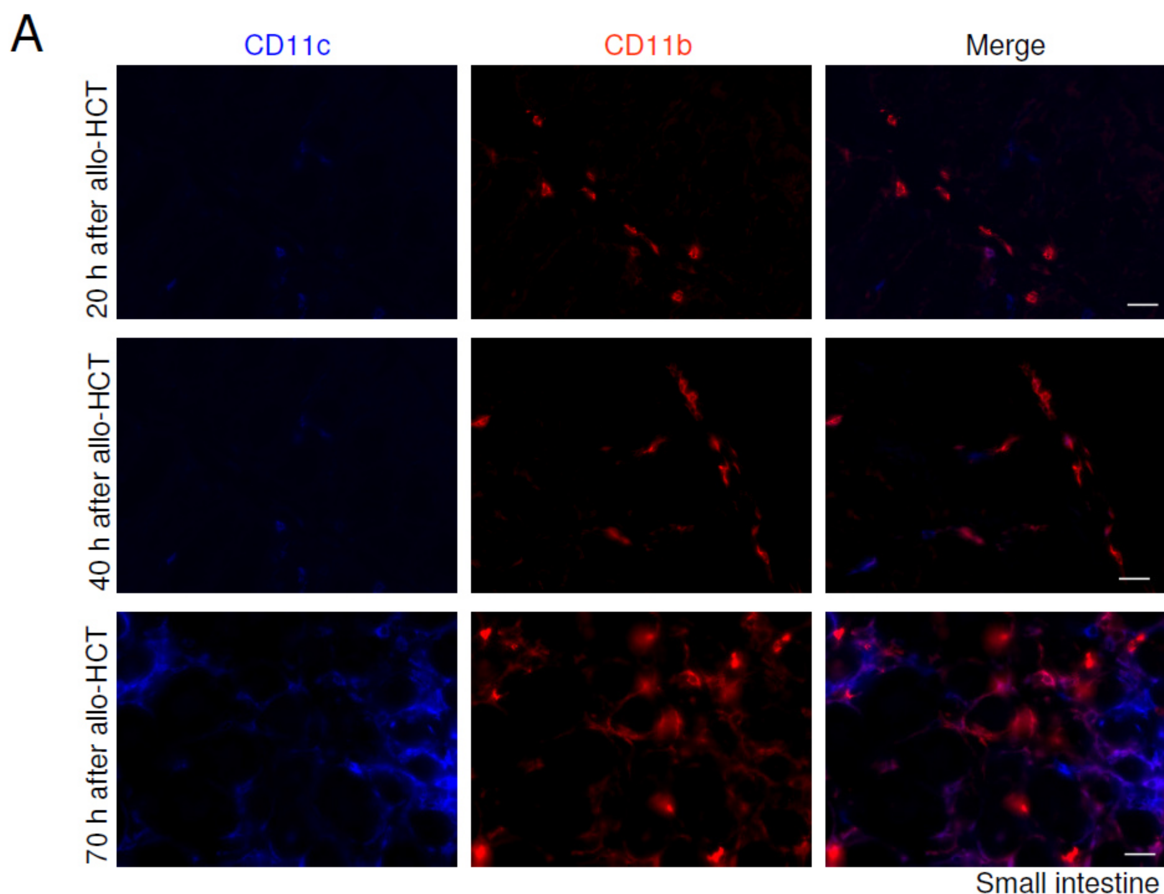

Figure 18. CD11b^{+}CD11c^{-} cells in the intestinal mucosa shortly after irradiation and become CD11b^{+}CD11c^{+} after 70 h of allo-HCT.
To study when does the shift from CD11c^{+}CD103^{+} to CD11c^{+}CD11b^{+} subsets take place, the intestinal mucosa of transplanted recipients was analyzed 20, 40 and 70h after allo-HCT and stained for CD11c (Alexa 647), CD103 (Alexa 488) and CD11b (Alexa 546). (A) IFM of single color channel and merged images of small intestine at 20, 40 and 70 h after allo-HCT, CD11c displayed in blue, CD11b in red and double positive CD11c^{+}CD11b^{+} in purple. No CD103^{+} cells were found. aGVHD samples at 20 h n=4, at 40 h n=3 and at 70 h n=3. Bar indicates 20µm.

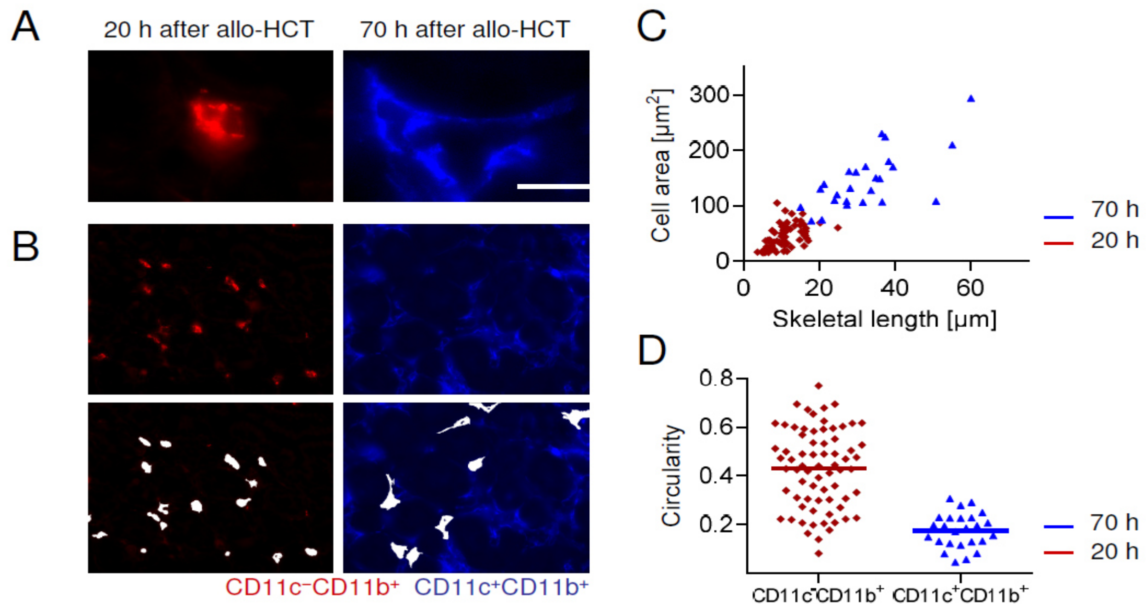

Figure 19. Change of size and morphology during the first 70h after allo-HCT.
(A-D) Analysis of morphology and phenotype from the first cells infiltrating the intestinal mucosa after allo-HCT. (A and B) IFM of the small intestine 20 and 70h after allo-HCT. CD11b (Alexa 546) are displayed in red and CD11c (Alexa 647) in blue. (A) IFM of single cells at 20 and 70 h. (B) IFM and analysis with Volocity software to determine the morphology of single cells within the tissue. (C) Correlation between area and skeletal length from the single cells detected after 20 hours displayed in red, and 70 hours, displayed is blue. (D) Circularity of the cells detected 20 and 70 hours after HCT. The circularity indicates the shape of a cell and is given as a numeric value between 0 and 1, being 1 a perfect circle. Every measurement represents a single cell.

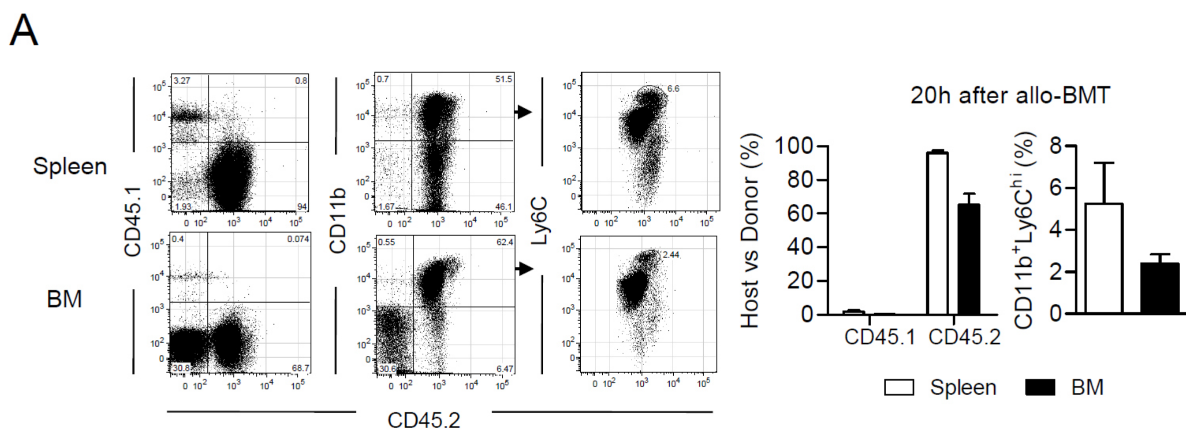

Figure 20. Detection of radio resistant monocytes in bone marrow and spleen 20 h after allo-HCT.
In order to identify the possible source of host monocytes shortly after transplant, mice were sacrificed 20 h after allo-HCT, and BM and spleen were analyzed. (A) Remaining host hematopoietic cells (CD45.2+) and host monocytes (CD45.2+CD11b+Ly6Ghi) 20 h after allo-HCT detected in BM and spleen. Flow cytometry of live cells from BM and spleen of transplanted recipients. Graphs show quantification of remaining host hematopoietic cells and monocytes. n=5. Mean ± SD.

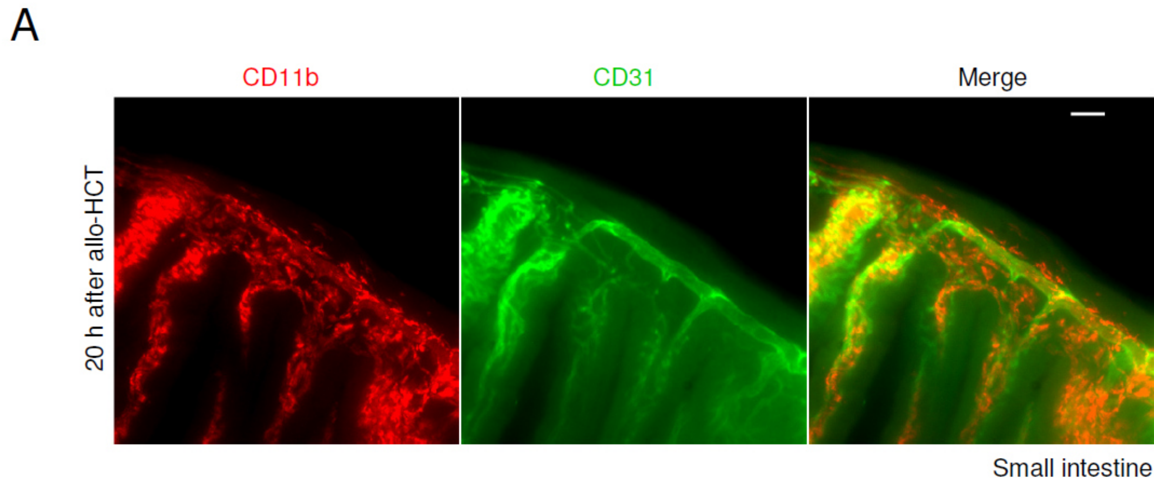

Figure 21. CD11b^{+} cells detected close to the blood vessels with the help of LSFM.
(A) CD11b^{hi}CD11c^{-} cells infiltrating the intestinal mucosa located close to or within CD31^{+} blood vessels. Light sheet fluorescence microscopy (LSFM) was used to perform a Z projection of small intestine at 20 h after allo-HCT. Green, Blood vessels (CD31 Cy3); Red, CD11b (Alexa546) cells. Scale bar represents 20 µm.

The BM and spleen have been described as reservoirs for monocytes (Geissmann et al., 2010; Swirski et al., 2009). To identify the source of intestinal CD11b^{hi} DCs, we analyzed BM and spleens of the transplanted recipients. At 20 h after allo-HCT, 70–90% of hematopoietic cells were still of host origin (**Figure 20A**) and host monocytes (Ly6C^{hi}CD11b^{hi}CD45.2^{+}) persisted in both the spleen, and BM (**Figure 20A**). Analysis of the small bowel shortly after allo-HCT with light sheet fluorescence microscopy (LSFM) (Brede et al., 2012) revealed the first CD11b^{hi} cells located near blood vessels (CD31^{+}) in the intestinal mucosa (**Figure 21A**). To confirm the monocytic origin of intestinal DCs during aGVHD we used tyrosine kinase receptor fms-like tyrosine kinase 3 ligand (Flt3L) and colony stimulating factor-1 (Csf-1) deficient mice as recipients. Flt3L is crucial for cDC development and maintenance, whereas Cfs-1 is a growth factor involved in the proliferation, differentiation, and survival of monocytes Flt3L deficit leads to cDCs deficiency but not moDCs. Csf-1 default affects monocytes and macrophage numbers, but not cDCs. As expected B6.Flt3 L$^{-/-}$ mice lacked DCs during steady-state conditions (**Figure 22A**), yet three days after allo-HCT flow cytometry showed similar numbers of intestinal DCs as wild type controls (**Figure 22B**). In contrast Csf-1$^{-/-}$ mice presented normal DC numbers in steady-state (**Figure 22A**), but were deficient in intestinal

DCs after allo-HCT (**Figure 22A and 22B**). Hence, these results clearly demostrate the monocytic origin of intestinal DCs during aGVHD.

To corroborate our results we transferred $50x10^6$ BM enriched $CD11b^+Ly6G^-$ monocytes from irradiated B6 donor mice (CD45.2) into congenic B6 recipients (CD45.1) at the time of allo-HCT before aGVHD development (**Figure 23A and 23B**). Since the first $CD11b^+CD11c^-$ infiltrating cells required 70 hours to change their morphology and phenotype to $CD11b^{hi}CD11c^+$ cells (**Figure 18A**), we analyzed the small bowel for donor-derived $CD45.2^+$ DCs at this time-point. Flow cytometry (**Figure 23C)** and IFM (**Figure 23D**) of the small intestine revealed the generation of $CD45.2^+$ DCs during aGVHD. Nevertheless transferred monocytes migrated with a very low efficiency (0.1%) to the intestinal tract (**Figure 23C).** Although we clearly illuminated the monocytic origin of DCs, monocyte transfer experiments did not appear conclusive. The intestinal recruitment of monocytes was so surprisingly inefficient that it raised the concern whether vascular monocytes mobilization from BM derived $CD11b^+Ly6G^-$ monocytes was the major source of newly arising $CD11b^+C11c^+$ DCs in intestinal aGVHD

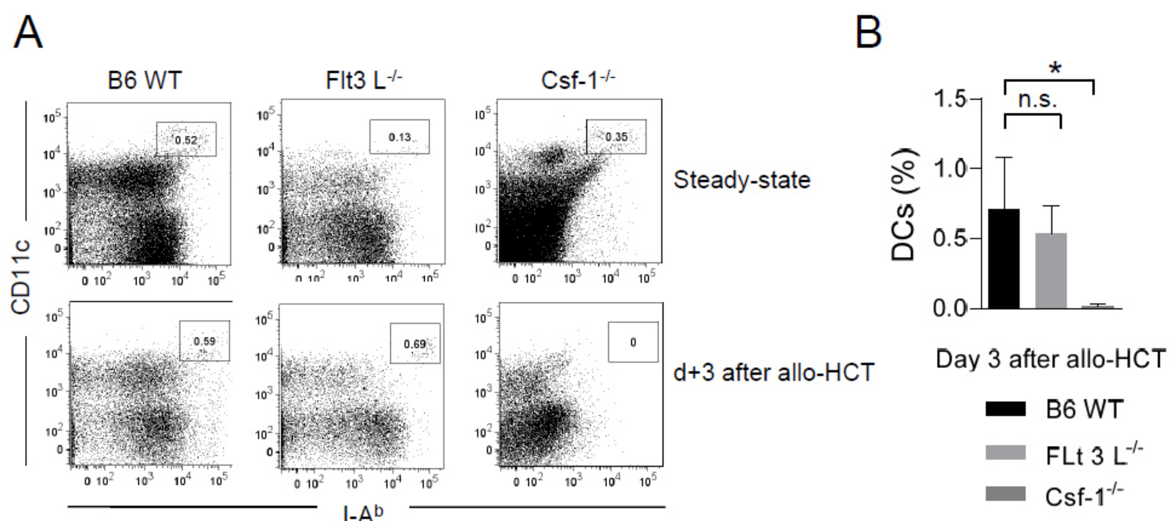

Figure 22. Intestinal DCs are from monocytic origin.
(A) Intestinal DC compartment from B6 WT, Flt3 $L^{-/-}$ and Csf-$1^{-/-}$ mice analyzed by flow cytometry in steady-state conditions and d+3 after allo-HCT. (B) Quantification of intestinal DCs of B6 WT, Flt3 $L^{-/-}$ and Csf-$1^{-/-}$ recipients on d+3 after allo-transplant. Steady-state B6 WT n=1, Flt3 $L^{-/-}$ n=1 and Csf-$1^{-/-}$ n=1. d+3 after allo-HCT B6 WT n=7, Flt3 $L^{-/-}$ n=4 and Csf-$1^{-/-}$ n=4. Mean$\pm$ SD, *p>0.05.

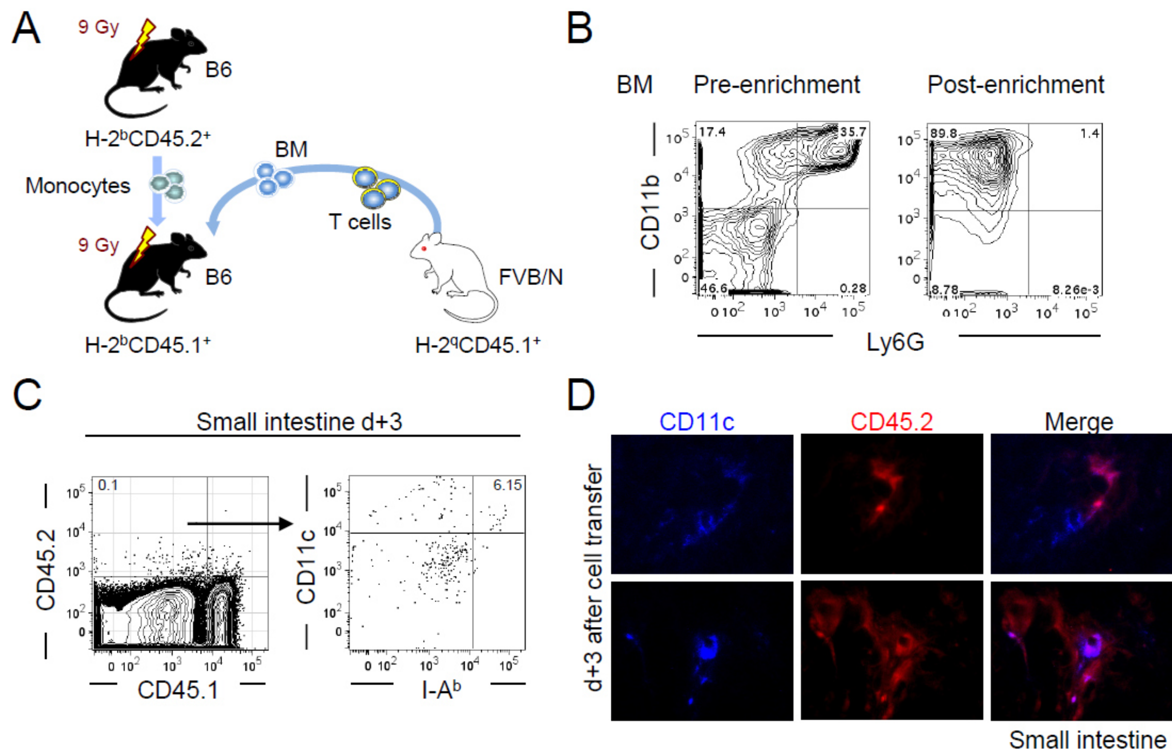

Figure 23. Host intestinal DCs during aGVHD are monocyte-derived.
(A) Confirmation of monocytic origin of intestinal DCs during aGVHD. Immediately after TBI, 5 × 10^7 enriched monocytes from B6 WT donor mice (CD45.2) were transferred i.v. into B6 congeneic recipients (CD45.1) that were simultaneously transplanted with allogeneic BM and T cells from FVB/N (CD45.1). (B) Monocyte purity after enrichment. (C) Flow cytometry of the small intestines 3 days after cell transfer to confirm the presence of $CD45.2^+$ cells (left) and $CD45.2^+CD11c^+MHCII^+$ DCs (right). (D) IFM to confirm the presence of $CD45.2^+$ DCs (purple) in the small bowel.

4.7 Host intestinal DCs arise from tissue resident CX_3CR1 non-classical monoytes

To test whether circulating monocytes served as the predominant source of intestinal DCs, we used $CCR2^{-/-}$ mice as recipients for an allo-HCT, considering that classical inflammatory monocytes require CCR2 to migrate to sites of inflammation (Geissmann et al., 2003; Grainger et al., 2013; Kuziel et al., 1997). Surprisingly, flow cytometry analysis and IFM from the intestinal tract three days after allo-HCT revealed the presence of intestinal DCs despite CCR2 deficiency (**Figure 24A and 24B**).

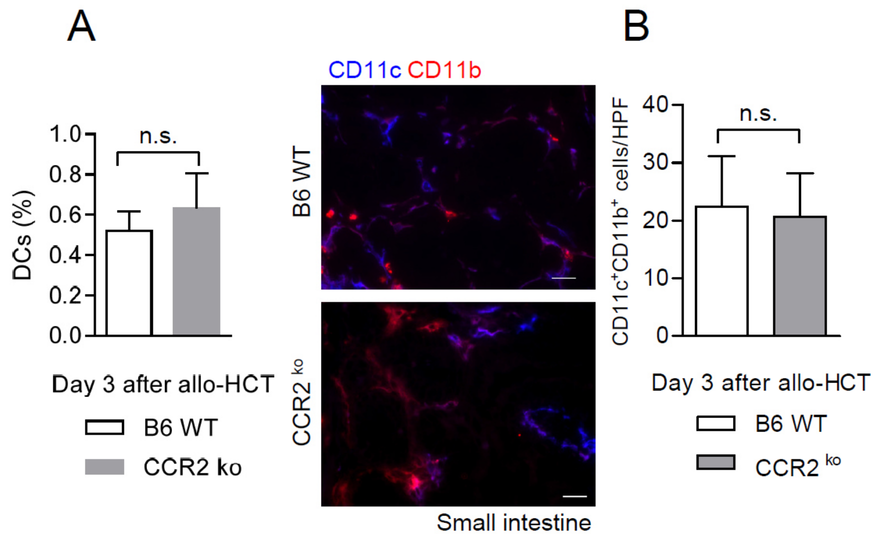

Figure 24. Monocyte recruitment appears CCR2-independent.
B6.CCR2$^{-/-}$ deficient mice and B6 WT received BM and T cells from FVB donor mice after TBI. Three days after transplant the small intestines were analyze for DC presence. (A) Flow cytometry and (B) IFM of the small intestine showed presence of host DCs in comparable numbers. Mean $\pm$ SD, $p>0.05$. B6 WT n=4, B6.CCR2$^{-/-}$ n=3. Scale bar indicates 20µm.

Next, to illuminate whether DCs precursors arise from circulating monocytes in a CCR2 independent manner or from tissue resident monocytes, we generated parabiotic mice. Parabiosis involves the surgical joining of two mice that develop a common, anastomosed blood circulation. Blood chimerism is achieved 5-8 days after joining (Wright et al., 2001). Parabiotic partners consisted of a B6.CCR2$^{-/-}$CD45.1 mouse sharing the blood circulation with a wild type B6.CD45.2 partner (CD45.1 CCR2$^{-/-}$: CD45.2 WT). Congenic markers allow the identification of the origin of the intestinal DCs in case of exchange and additionally allow us to further illuminate the role of CCR2 for monocyte recruitment to the sites of inflammation. Full peripheral blood chimerism was confirmed 2 weeks after surgical joining (**Figure 25B**). Subsequently parabiotic mice were lethally irradiated and allogeneically transplanted with BM and enriched T cells from FVB/N mice (**Figure 25A**). Strikingly, flow cytometry of the small intestines on day 3 after allo-HCT showed intestinal DCs in both parabiotic mice. However, these intestinal DCs did not arise from the parabiotic partner (**Figure 25C**) precluding vascular recruitment of precursor cells as the origin of intestinal CD11b^{+}CD11c^{+} DCs. Thus, parabiosis experiments revealed that intestinal CD11b^{+}CD11c^{+}DCs predominantly evolved

from tissue resident monocytes after HCT and not from circulating classical inflammatory monocytes. Two principal monocyte subsets with different migratory capacities have been defined to date. A $CX_3CR1^{lo}CCR2^{+}$ classical subset that is actively recruited to inflamed tissues, and a non-classical $CX_3CR1^{hi}CCR2^{-}$ subset, characterized by CX_3CR1-dependent recruitment to non-inflamed tissues. Both subsets posses the potential to differentiate into DCs in vivo (Geissmann et al., 2003). To answer whether intestinal DCs after allo-HCT arise from resident non-classical monocytes, we transplanted B6 WT, B6.$CCR2^{-/-}$ and B6.$CX_3CR1^{-/-}$ mice (**Figure 25D**). Three days after allo-HCT flow cytometry revealed significantly reduced numbers of $CD11b^{+}CD103^{-}CD11c^{+}$ DCs in B6.$CX_3CR1^{-/-}$ recipients compared to B6 WT or B6.$CCR2^{-/-}$ recipients (**Figure 25E**). Additionally, Ki67 analysis on day 1 and two after allo-HCT revealed that CD11b precursors did not proliferate (**Figure 26A**), excluding self-renewing of resident cells, and supporting differentiation of resident precursors into DCs.

These experiments established a hitherto unknown function of tissues resident non-classical monocytes, which differentiate into intestinal DCs after allo-HCT, and suppress alloreactive T cells during acute intestinal GVHD.

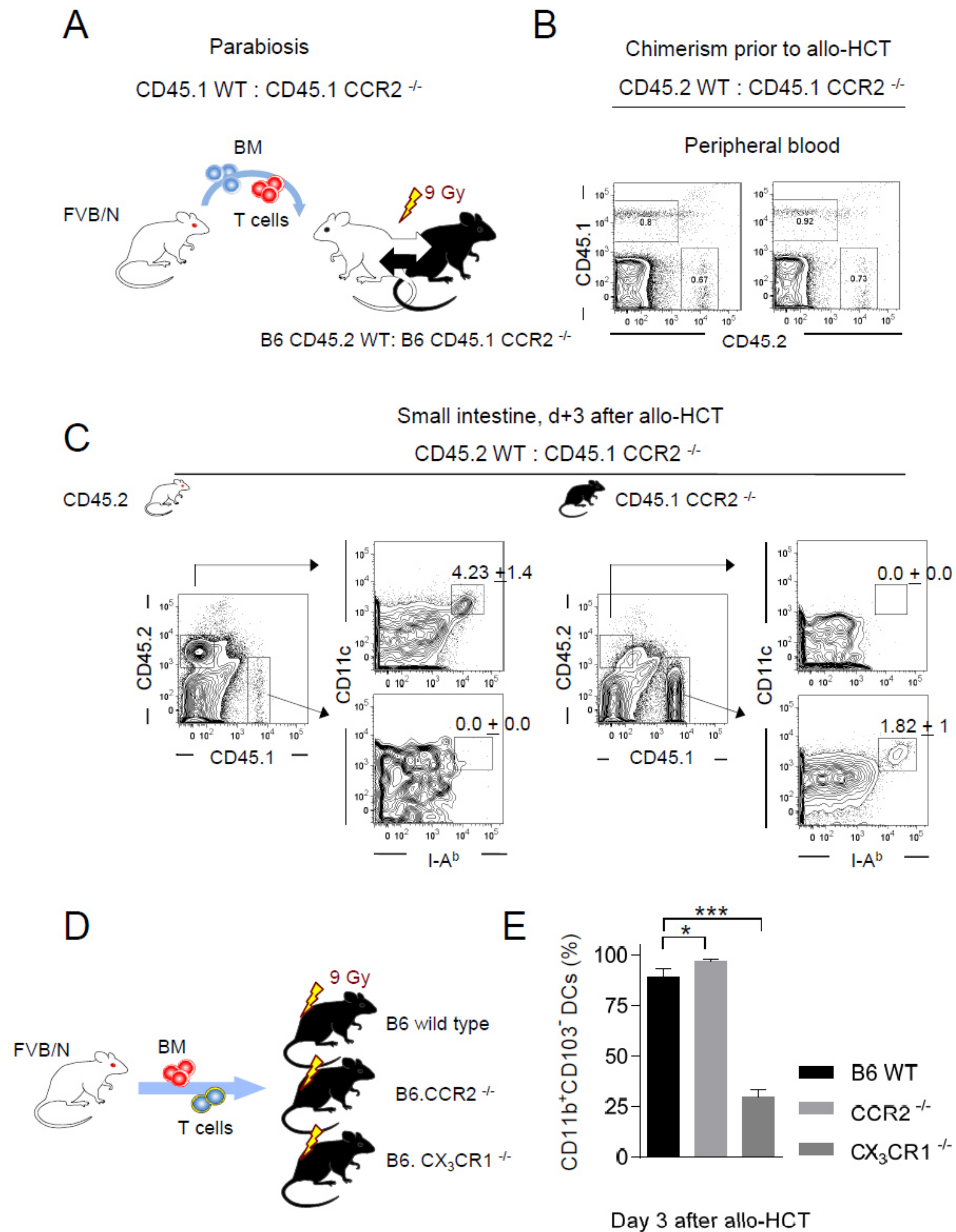

Figure 25. Intestinal DCs differentiate *in situ* in a CX_3CR1 dependent manner.
(A) Two weeks after surgery parabiotic mice (CD45.2 WT : CD45.1 $CCR2^{-/-}$) were transplanted with BM and T cells from FVB/N donor mice after myeloablative conditioning. (B) Blood chimerism from parabiotic mice was analyzed shortly prior allo-HCT. (C) Three days after allo-HCT the presence and origin of intestinal DCs was assessed by flow cytometry. Mean ± SD, n=3 parabiotic pairs. (D) Lethal irradiated B6. $CX3CR1^{-/-}$, B6.$CCR2^{-/-}$ and B6 WT recipients were transplanted with BM and T cells from FVB/N donor mice.(E) Quantification of $CD11b^{+}CD103^{-}$ DCs ($CD11c^{hi}$I-$A^{b\ hi}$) from small intestines 3 days after allo-HCT. B6. $CX3CR1^{-/-}$n=4, B6.$CCR2^{-/-}$ n=4 and B6 WT n=4. Mean ± SD, , * $p < 0.05$, ** , *** $p < 0{,}001$.

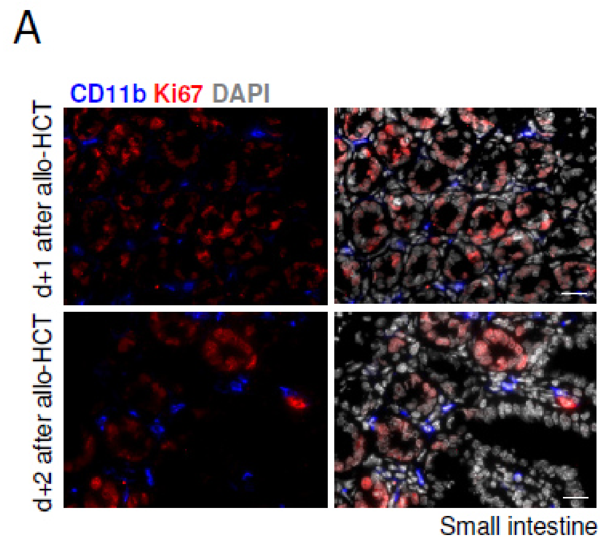

Figure 26. Intestinal DCs precursors do not proliferate *in situ*.
(A) IFM of the small intestinal on day 1 and 2 after allo-HCT. Blue, CD11b (Alexa 647); Red, Ki67 (Cy3), White, Nuclei (DAPI). Scale bar represents 20 µm.

5 Discussion

We report for the first time a biological function of non-classical (CX_3CR1^+) monocyte derived DCs (nc.moDCs) in regulating acute intestinal GVHD. We demonstrate that this DC subset pervades an immune protective purpose limiting T-cell alloreactivity and cytokine production. Furthermore, we established that nc-moDCs arise from tissues resident monocytic precursors upon allo-HCT, and identify PD-L1 expression by CX_3CR1^+ nc-moDCs as the major mechanism they employ to suppress alloreactive T cells during acute intestinal GVHD.

5.1 Non-classical monocyte-derived DCs

Non-classical monocytes ($CX_3CR1^{hi}CCR2^-$) are characterized by a CX_3CR1-dependent recruitment to non-inflamed tissues. Monitoring of blood vessels and tissues, early response and tissues repair functions have been ascribed to non-classical monocytes (Shi and Pamer, 2011). Intravital microscopy studies demonstrate that they adhere to and migrate along the luminal surface of endothelial cells that line small vessels in a process referred as patrolling (Auffray et al., 2007). Yet, the cues for directing their differentiation into DCs and particularly their function *in vivo* remained elusive. We illuminate a function of nc-moDCs in regulating inflammatory response, as opposed to classical “inflammatory” ($CCR2^+$) moDCs, which reportedly play an important role in defense mechanisms by promoting inflammation (Segura and Amigorena, 2013). Here we found that host nc-moDCs constituted the predominant DC subset in the intestinal tract during the aGVHD effector phase. However, these cells did not enhance intestinal aGVHD. Surprisingly, these nc-moDCs promoted tolerogenic effects in the intestinal tract, limiting alloreactive T-cell infiltration.

The prevailing understanding of moDCs recruitment is that under inflammatory conditions, classical monocytes migrate to the inflamed sites—in a CCR2 dependent manner—and differentiate into moDCs to promote inflammation as a defense mechanism (León et al., 2005). Importantly, we show that in the context of intestinal GVHD, classical inflammatory monocytes were not the main precursors of DCs in the inflamed gut, as shown by CCR2 deficient recipients, in which the development of intestinal DCs was not impaired. Flt3L deficient recipients showed that intestinal DCs were present after allo-HCT. In contrast in Csf-1 recipients there were no host-type intestinal DCs visible. Flt3 is known to control the differentiation and maintenance of cDCs. Consequently, Flt3 deficient mice presented decreased numbers of cDCs, whereas moDCs development was not affected. On the other hand, Csf-1 deficiency impaired monocytes and macrophages development. Under inflammatory conditions the formation of moDCs was defective. Furthermore, CX_3CR1—the key chemokine receptor that allows non-classical monocytes to enter peripheral tissues—appeared critical for $CD11b^+$ DCs differentiation under GVHD inflammatory conditions. Additionally, parabiosis experiments clearly established that intestinal DCs arose from tissue resident monocytic precursors. Zigmond and colleagues reported the presence of CX^3CR1^{int} cells in the inflamed colon, which developed into $CD11c^+MHC^{hi}$ cells in a DSS-induced colitis model (Zigmond et al., 2012a). Yet, contrary to our model, the appearance of these cells was impaired in CCR2 defective recipients, implying their classical monocyte origin and contributing to improve the understanding of the complexity of inflammatory monocytes. Many tissues contain diverse populations of local self-renewing and peripherally derived cells, especially macrophages. However the intestinal tract is the only organ which contains resident macrophages all of them blood born classical monocyte-derived (Davies et al., 2013). Reportedly, on the recovery from inflammation tissue resident macrophages exhibit enhanced proliferation (Davies et al., 2013). Our own analysis of proliferating cells with Ki67 staining on day 1 and 2 after allo-HCT, excluded the possibility of self-renewing proliferating

macrophages, but instead supported a mechanism of local CCR2-independent CX_3CR1-dependent monocytic precursor differentiation.

Taken all together, our data supports the monocytic origin of intestinal DCs during aGVHD, but, importantly, excludes classical monocytes as the immediate precursors. Furthermore, CX_3CR1—which allows non-classical monocytes entering peripheral tissues—appeared critical for $CD11b^+$ DCs differentiation under GVHD inflammatory conditions, as shown by $CX_3CR1^{-/-}$ allo-HCT recipients. Additionally, parabiosis experiments clearly established that intestinal DCs arise from tissues resident monocytic precursors, and again corroborated the CCR2-idependency for DC emergence. To our knowledge, we report for the first time an alternative model in the context of murine GVHD, in which tissue resident non-classical monocytes (CX_3CR1^+) differentiate *in situ* into intestinal DCs, revealing their key role in regulating mucosal inflammation. Establishing the origin of these nc-moDCs is essential to develop future therapeutic strategies to foster their function to prevent or even treat patients from aGVHD.

5.2 DCs in GVHD: immunogenic or tolerogenic?

In the last decade it had been assumed that recipient DCs, as the most potent APCs, were crucial for GVHD onset, based on seminal work from Shlomchik and colleagues (1999). However, this hypothesis has been recently reconsidered through cause-and-defect studies in immune competent recipients (Koyama et al., 2011; Toubai et al., 2011). Koyama et al. (2011) recently demonstrated aGVHD aggravation when DCs were depleted prior to allo-HCT, before alloreactive T-cell priming. This suggested, firstly, that host DCs were not required to induce severe GVHD, and, secondly, that predominantly steady-state DC subsets exert a protective effect during the initiation phase of aGVHD. Thus, the relative contribution of defined host DC subsets to the aGVHD pathophisiology during the priming phase still needs to be clarified. In contrast, in our study we eliminated host DCs after T-cell priming took place in lymphoid organs, during a time-period when alloreactive effector T cells had already started to infiltrate the intestinal tract (Bäuerlein et al., 2013; Beilhack et al., 2005).

This implies that we targeted different DC subsets, as steady-state intestinal DCs had been already replaced by nc-moDCs. Our study results indicate that nc-moDCs protect from aGVHD during the effector phase of the disease. Therefore we conclude that specialized host DC subpopulations confer aGVHD protection at specific stages and locations of disease progression.

To date, the prevailing opinion has been that intestinal DCs are important for stimulating, recruiting, or even "licensing" alloreactive T cells to exert tissue damage after allo-HCT (Bennett and Chakraverty, 2012). This hypothesis has been mainly based on the efficiency of DCs to initiate antigen-specific adaptive immune responses, and on infection model studies, where the recruitment of inflammatory DCs to the site of infection together with DC co-stimulation appeared crucial for antigen-specific effector T cells function (McGill et al., 2008). Accordingly, moDCs have been shown to be responsible for T cell recruitment and IFN- γ production in several murine viral infection models (Iijima et al., 2011). Yet, our detailed study clearly shows that depletion of host nc-moDCs during the effector phase especially contributed to intestinal aGVHD aggravation, leading to enhanced donor T cell infiltration and inflammatory cytokine production. These results reveal a key role of peripheral DCs in regulating mucosal inflammation and consequently contribute to our own understanding of the complexity of DC function. Of note, histopathological analysis on day 6 after allo-HCT did not reveal signs of aggravated liver and skin aGVHD if host DCs were depleted. BLI analysis demonstrated increased donor T-cell infiltration in the intestinal tract and mesenteric lymph nodes in DC-depleted recipients, yet no differences were detected in other priming sites (spleen and peripheral lymph nodes) nor in GVHD target organs, such as the liver. Although we cannot rule out divergent functions of host APCs in different organs, the lack of pathology in liver and skin during aGVHD after DC depletion may be ascribed to the early time-points of our analysis (day 6 after allo-HCT), when most alloreactive T cells infiltrate the GI tract (Beilhack et al., 2008; Beilhack et al., 2005). Importantly, whether the presence of non-classical monocytes is particularly significant in the intestinal tract, and

whether the environment after allo-HCT is appropriate in other organs for DC differentiation and exertion of immunoregulatory mechanisms, still needs to be addressed.

5.3 PD-L1 in aGVHD

PD-L1 fulfills key functions in regulating T-cell responses to maintain peripheral tolerance. Programmed death 1 (PD1) is a co-inhibitor in the regulation of T and B cell responses (Riella et al., 2012). This pathway is emerging as a major regulator converting effector T cells into exhausted T-cells during chronic infection and inflammation (Hofmeyer et al., 2011).

PD-L1 is constitutively expressed on DCs, although it can be expressed on other hematopoietic and non-hematopoietic cells (including epithelial and endothelial cells). Inflammation and IFN-γ drives its upregulation (Freeman et al., 2000; Liang et al., 2003; Yamazaki et al., 2002). There is evidence of its immunosuppressor features *in vivo*. Blocking the interaction between PD-1 and PD-L1, resulted in aGVHD exacerbation by an IFN-γ-dependent mechanism (Blazar et al., 2003). In the same line, it has been reported that the PD-1/PD-L1 pathway is involved in suppressing T-cell alloreactivity in GVHD-non-target organs, like the heart (Schilbach et al., 2007) or kidney (Al-Chaqmaqchi et al., 2013) and suggest that this mechanism might indicate is role in protecting organs against GVHD.

5.3.1 PD-L1 expression on nc-moDCs proves critical for T-cell alloreactivity suppression

As previously discussed, Blazar et al. reported GVHD exacerbation after blocking PD-1/PD-L1 engagement. However, PD-L1 expression is not limited to DCs or to hematopoietic cells. Therefore, the complete blockade of this inhibitory pathway could not determine which PD-L1-expressing cells exert immune modulation, and which organs are mainly affected after its blockade. Here we identified host nc-moDCs as the major source of PD-L1 in the intestinal mucosa during aGVHD. Furthermore, PD-L1 expression by host DCs—but not by stromal or other host hematopoietic cells—proved essential for improved survival and reduced aGVHD

mortality. These findings substantiate the importance of PD-L1 expression particularly on host nc-moDCs for limiting T-cell responses during intestinal aGVHD by dampening alloreactive T-cell-cytokine production. Hence, we propose PD-L1 on nc-moDCs as a promising therapeutic target to modulate immunity in GVHD.

5.4. Molecular signatures of dendritic cells and macrophages

Recently, several studies have focused to distinguish DC subsets from macrophages (Hume et al., 2013). Under homeostatic conditions, the divergence between macrophages and DCs has been better defined based on their original precursors, their requirements to develop and survive, and the transcriptional characterization of their lineage (Liu et al., 2009). DC or macrophage differentiation is determined at the stage of “macrophage and DC precursor” (MDP). DC commitment occurs during the transition from MDP to “common DC precursor” (CDP) (Miller et al., 2012a). cDCs differentiate in a Flt3L-dependent and Csf-1R ligand-independent manner, whereas macrophages arise independently of CDP and require Csf-1R ligand but not Flt3L for their development. Moreover, DCs are much more capable to efficiently emigrate from peripheral tissues to draining LNs when compared to macrophages.

Monocytes are morphologically and phenotypically heterogeneous. They possess considerable developmental plasticity, are able to differentiate into both, macrophages and their tissue specific representatives, as well as into DCs under homeostatic and inflammatory conditions (Zigmond et al., 2012). Consequently, monocytes and their macrophage and DC progeny exert diverse functions, which makes it complex to distinguish between these two cell types. It has been proposed, that monocytes could constitute a considerable systemic reservoir of myeloid precursors. How monocyte differentiation occurs under physiological conditions *in vivo* remains largely unknown. Most of the studies on the transcriptional regulation of macrophage differentiation have been done *in vitro* (Auffray et al., 2009; Friedman, 2007; Geissmann et al., 2010), and the exact role of these factors in driving macrophage subsets differentiation *in vivo* remains to be examined. Several markers have been ascribed to monocyte-derived macrophages and DCs. Yet, none of them is unique for

one cell type. We define non-classical monocyte-derived cells as DCs grounded on their morphology and a phenotypical and comparative analysis. Therefore, we describe nc-moDCs based on their DC-like shape with expanding protrusions, levels of MHC class II, CD11c, and the costimulatory molecules CD80 and CD86, which appeared the highest compared to other CD11c, MHC II expressing immune cells in the intestinal tract during GVHD. Yet, whether the cell phenotype epitomizes the identity of these cells could spark scientific controversy. Further analysis at the transcriptional and functional level should help to establish the identity of this immunoregulatory DC subpopulation. Thus, more work is required to truly understand the role and fate of functional monocyte-derived immune subsets *in vivo*.

In conclusion, in this thesis project I established a GVHD model that efficiently allowed DC depletion at any time point after transplantation to study DC function, specifically during the effector phase of aGVHD. DC elimination after alloreactive T-cell priming and at the time of GVHD target organ infiltration aggravated GVHD pathology and mortality. PD-L1 on intestinal DCs proved essential to dampen T cell cytokine production *in vivo*. Additionally, we could elucidate the monocytic origin of this DC subpopulation, and importantly, we found that tissue-resident non-classical CX_3CR1^+ monocytes differentiate *in situ* into intestinal $CD11b^+$ nc-moDCs after allo-HCT.

Our thorough study unraveled for the first time a biological function of nc-moDC *in vivo*, and demonstrates a PD-L1-dependent protective role of host nc-moDCs during intestinal aGVHD. The identification of the beneficial effects of this immune-regulatory nc-moDCs in the context of intestinal GVHD defines them as attractive targets for manipulating allo-responses, and points to future strategies to target molecular pathways that will specifically foster their function.

6 Graphical summary

Tissue-resident non-classical monocyte-derived dendritic cells

suppress GVHD via PD-L1

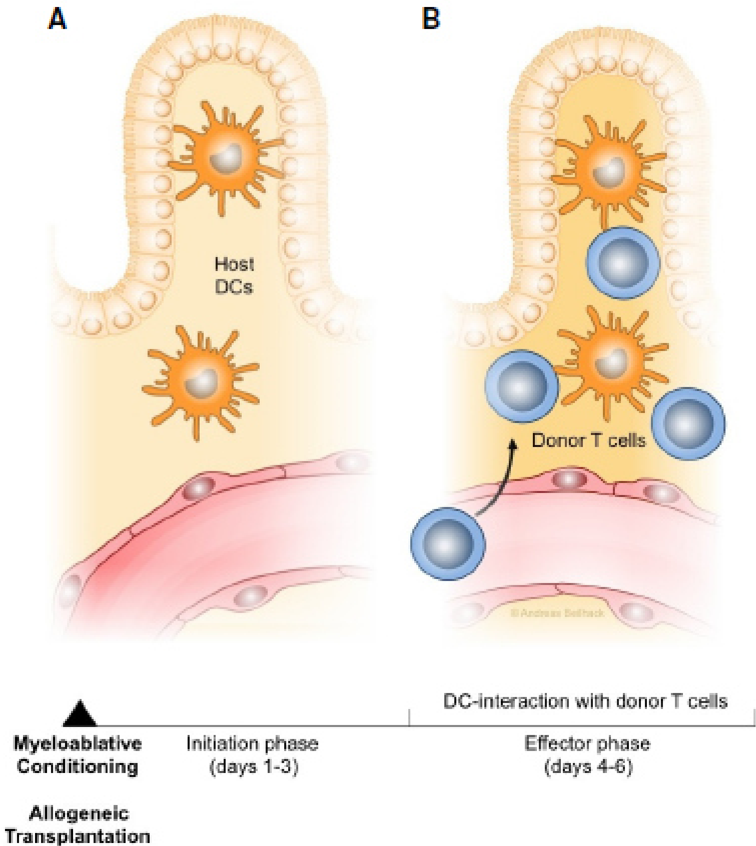

Figure 27. The function of intestinal dendritic cells (DCs) during the effector phase of acute graft-versus-host disease (GVHD) remained elusive.
(A) Alloreactive donor T cells are primed outside the intestinal tract within secondary lymphoid organs during the initiation phase of acute GVHD and (B) infiltrate the intestinal tract during the effector phase to cause tissue destruction. Peripheral DCs were postulated to recruit, enhance or even license alloreactive donor T cells to cause tissue damage. Sketch by Andreas Beilhack.

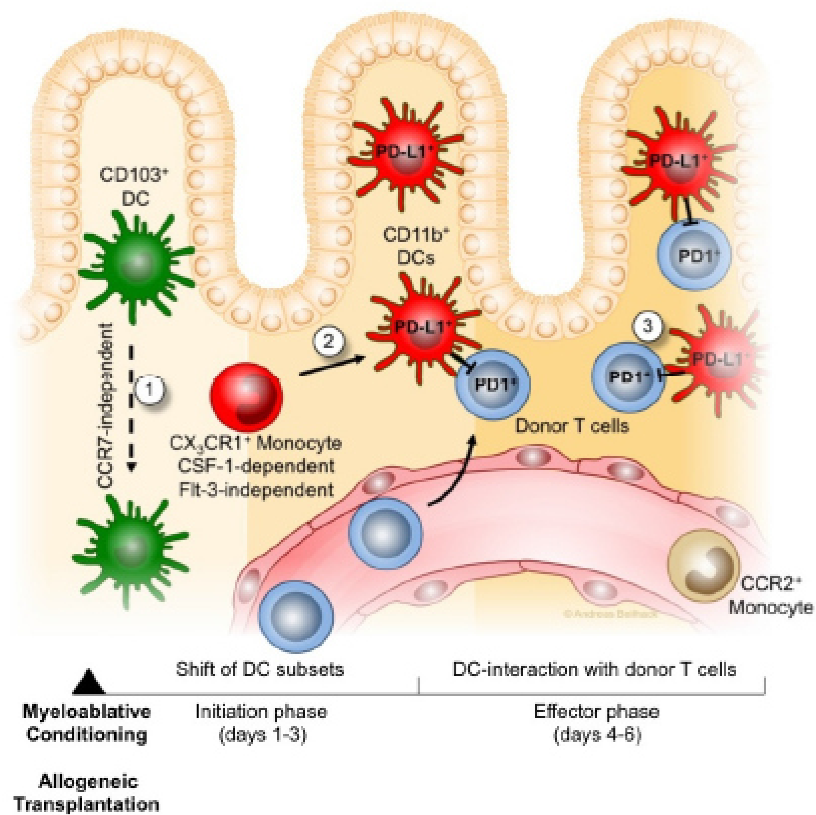

Figure 28. Tissues-resident non-classical monocyte-derived dendritic cells (nc-moDCs) suppress GVHD via PD-L1.

A rigorous analysis of DC subsets after allogeneic hematopoietic cell transplantation (allo-HCT) unraveled several surprising findings:

1.) Shortly after allo-HCT $CD103^+$ intestinal DCs (green) migrate out of the lamina propria in a CCR7-independent manner. 2.) Tissue-resident intestinal non-classical monocytes (CX_3CR1) differentiate *in situ* into nc-moDCs (red). Nc-moDC-differentiation depends on CSF-1 and CX_3CR1 but not on Flt3l and CCR2. 3.) PD-L1 expression on nc-moDCs proved critical to suppress alloreactive T cells during acute intestinal GVHD. Sketch by Andreas Beilhack.

References

Al-Chaqmaqchi, H., B. Sadeghi, M. Abedi-Valugerdi, S. Al-Hashmi, M. Fares, R. Kuiper, J. Lundahl, M. Hassan, and A. Moshfegh. 2013. The role of programmed cell death ligand-1 (PD-L1/CD274) in the development of graft versus host disease. *PLoS One*. 8:e60367.

Anderson, B.E., J.M. McNiff, D. Jain, B.R. Blazar, W.D. Shlomchik, and M.J. Shlomchik. 2005. Distinct roles for donor- and host-derived antigen-presenting cells and costimulatory molecules in murine chronic graft-versus-host disease: requirements depend on target organ. *Blood*. 105:2227-2234.

Ansari, M.J., A.D. Salama, T. Chitnis, R.N. Smith, H. Yagita, H. Akiba, T. Yamazaki, M. Azuma, H. Iwai, S.J. Khoury, H. Auchincloss, Jr., and M.H. Sayegh. 2003. The programmed death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice. *J Exp Med*. 198:63-69.

Aoyama, K., A. Saha, J. Tolar, M.J. Riddle, R.G. Veenstra, P.A. Taylor, R. Blomhoff, A. Panoskaltsis-Mortari, C.A. Klebanoff, G. Socie, D.H. Munn, W.J. Murphy, J.S. Serody, L. Fulton, T. Teshmia, R.A. Chandraratna, E. Dmitrovsky, Y. Guo, R.J. Noelle, and B.R. Blazar. 2013. Inhibiting retinoic acid signaling ameliorates graft-versus-host disease by modifying T-cell differentiation and intestinal migration. *Blood*.

Auffermann-Gretzinger, S., L. Eger, M. Bornhauser, K. Schakel, U. Oelschlaegel, M. Schaich, T. Illmer, C. Thiede, and G. Ehninger. 2006. Fast appearance of donor dendritic cells in human skin: dynamics of skin and blood dendritic cells after allogeneic hematopoietic cell transplantation. *Transplantation*. 81:866-873.

Auffermann-Gretzinger, S., I.S. Lossos, T.A. Vayntrub, W. Leong, F.C. Grumet, K.G. Blume, K.E. Stockerl-Goldstein, R. Levy, and J.A. Shizuru. 2002. Rapid establishment of dendritic cell chimerism in allogeneic hematopoietic cell transplant recipients. *Blood*. 99:1442-1448.

Auffray, C., D. Fogg, M. Garfa, G. Elain, O. Join-Lambert, S. Kayal, S. Sarnacki, A. Cumano, G. Lauvau, and F. Geissmann. 2007. Monitoring of Blood Vessels and Tissues by a Population of Monocytes with Patrolling Behavior. *Science*. 317:666-670.

Auffray, C., M.H. Sieweke, and F. Geissmann. 2009. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. *Annu Rev Immunol*. 27:669-692.

Balazs, M., F. Martin, T. Zhou, and J. Kearney. 2002. Blood dendritic cells interact with splenic marginal zone B cells to initiate T-independent immune responses. *Immunity*. 17:341-352.

Barton-Burke, M., D.M. Dwinell, L. Kafkas, C. Lavalley, H. Sands, C. Proctor, and E. Johnson. 2008. Graft-versus-host disease: a complex long-term side effect of hematopoietic stem cell transplant. *Oncology (Williston Park, N.Y.)*. 22:31-45.

Bäuerlein, C.A., S.S. Riedel, J. Baker, C. Brede, A.L. Garrote, M. Chopra, M. Ritz, G.F. Beilhack, S. Schulz, R. Zeiser, P.G. Schlegel, H. Einsele, R.S. Negrin, and A. Beilhack. 2013. A diagnostic window for the treatment of acute graft-versus-host disease prior to visible clinical symptoms in a murine model. *BMC medicine*. 11:134.

Beilhack, A., S. Schulz, J. Baker, G.F. Beilhack, R. Nishimura, E.M. Baker, G. Landan, E.I. Herman, E.C. Butcher, C.H. Contag, and R.S. Negrin. 2008. Prevention of acute graft-versus-host disease by blocking T-cell entry to secondary lymphoid organs. *Blood*. 111:2919-2928.

Beilhack, A., S. Schulz, J. Baker, G.F. Beilhack, C.B. Wieland, E.I. Herman, E.M. Baker, Y.-A. Cao, C.H. Contag, and R.S. Negrin. 2005. In vivo analyses of early events in acute graft-versus-host disease reveal sequential infiltration of T-cell subsets. *Blood*. 106:1113-1122.

Bennett, C.L., and R. Chakraverty. 2012. Dendritic cells in tissues: in situ stimulation of immunity and immunopathology. *Trends in Immunology*. 33:8-13.

Blazar, B.R., B.M. Carreno, A. Panoskaltsis-Mortari, L. Carter, Y. Iwai, H. Yagita, H. Nishimura, and P.A. Taylor. 2003. Blockade of Programmed Death-1 Engagement Accelerates Graft-Versus-Host Disease Lethality by an IFN-Î³-Dependent Mechanism. *The Journal of Immunology*. 171:1272-1277.

Brede, C., M. Friedrich, A.L. Jordan-Garrote, S.S. Riedel, C.A. Bauerlein, K.G. Heinze, T. Bopp, S. Schulz, A. Mottok, C. Kiesel, K. Mattenheimer, M. Ritz, V. von Krosigk, A. Rosenwald, H. Einsele, R.S. Negrin, G.S. Harms, and A. Beilhack. 2012. Mapping immune processes in intact tissues at cellular resolution. *J Clin Invest*. 122:4439-4446.

Chen, Y.B., S. McDonough, H. Chen, J. Kennedy, C. Illiano, E.C. Attar, K.K. Ballen, B.R. Dey, S.L. McAfee, M. Jagasia, R. Soiffer, T.R. Spitzer, and J. Ritz. 2013. Expression of [alpha]4[beta]7 integrin on memory CD8+ T cells at the presentation of acute intestinal GVHD. *Bone Marrow Transplant*. 48:598-603.

Cooke, K.R., G.R. Hill, J.M. Crawford, D. Bungard, Y.S. Brinson, J. Delmonte, Jr., and J.L. Ferrara. 1998. Tumor necrosis factor- alpha production to lipopolysaccharide stimulation by donor cells predicts the severity of experimental acute graft-versus-host disease. *J Clin Invest*. 102:1882-1891.

Copelan, E.A. 2006. Hematopoietic stem-cell transplantation. *The New England journal of medicine*. 354:1813-1826.

Davies, L.C., S.J. Jenkins, J.E. Allen, and P.R. Taylor. 2013. Tissue-resident macrophages. *Nat Immunol*. 14:986-995.

Djulbegovic, B., J. Seidenfeld, C. Bonnell, and A. Kumar. 2003. Nonmyeloablative allogeneic stem-cell transplantation for hematologic malignancies: a systematic review. *Cancer control : journal of the Moffitt Cancer Center*. 10:17-41.

Dolfi, D.V., P.A. Duttagupta, A.C. Boesteanu, Y.M. Mueller, C.H. Oliai, A.B. Borowski, and P.D. Katsikis. 2011. Dendritic Cells and CD28 Costimulation Are Required To Sustain Virus-Specific CD8+ T Cell Responses during the Effector Phase In Vivo. *The Journal of Immunology*. 186:4599-4608.

Domínguez, P.M., and C. Ardavín. 2010. Differentiation and function of mouse monocyte-derived dendritic cells in steady state and inflammation. *Immunological Reviews*. 234:90-104.

Dong, H., G. Zhu, K. Tamada, D.B. Flies, J.M.A. van Deursen, and L. Chen. 2004. B7-H1 Determines Accumulation and Deletion of Intrahepatic CD8+ T Lymphocytes. *Immunity*. 20:327-336.

Duffner, U.A., Y. Maeda, K.R. Cooke, P. Reddy, R. Ordemann, C. Liu, J.L.M. Ferrara, and T. Teshima. 2004. Host Dendritic Cells Alone Are Sufficient to Initiate Acute Graft-versus-Host Disease. *The Journal of Immunology*. 172:7393-7398.

Durakovic, N., K.B. Bezak, M. Skarica, V. Radojcic, E.J. Fuchs, G.F. Murphy, and L. Luznik. 2006. Host-derived Langerhans cells persist after MHC-matched allografting

independent of donor T cells and critically influence the alloresponses mediated by donor lymphocyte infusions. *Journal of immunology (Baltimore, Md. : 1950)*. 177:4414-4425.

Ferrara, J.L., R. Levy, and N.J. Chao. 1999. Pathophysiologic mechanisms of acute graft-vs.-host disease. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation*. 5:347-356.

Ferrara, J.L.M., and P. Reddy. 2006. Pathophysiology of Graft-Versus-Host Disease. *Seminars in Hematology*. 43:3-10.

Fife, B.T., and J.A. Bluestone. 2008. Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways. *Immunological Reviews*. 224:166-182.

Freeman, G.J., A.J. Long, Y. Iwai, K. Bourque, T. Chernova, H. Nishimura, L.J. Fitz, N. Malenkovich, T. Okazaki, M.C. Byrne, H.F. Horton, L. Fouser, L. Carter, V. Ling, M.R. Bowman, B.M. Carreno, M. Collins, C.R. Wood, and T. Honjo. 2000. Engagement of the Pd-1 Immunoinhibitory Receptor by a Novel B7 Family Member Leads to Negative Regulation of Lymphocyte Activation. *The Journal of Experimental Medicine*. 192:1027-1034.

Friedman, A.D. 2007. Transcriptional control of granulocyte and monocyte development. *Oncogene*. 26:6816-6828.

Geissmann, F., S. Jung, and D.R. Littman. 2003. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity*. 19:71-82.

Geissmann, F., M.G. Manz, S. Jung, M.H. Sieweke, M. Merad, and K. Ley. 2010. Development of Monocytes, Macrophages, and Dendritic Cells. *Science*. 327:656-661.

Grainger, J.R., E.A. Wohlfert, I.J. Fuss, N. Bouladoux, M.H. Askenase, F. Legrand, L.Y. Koo, J.M. Brenchley, I.D.C. Fraser, and Y. Belkaid. 2013. Inflammatory monocytes regulate pathologic responses to commensals during acute gastrointestinal infection. *Nat Med*. 19:713-721.

Hashimoto, D., A. Chow, M. Greter, Y. Saenger, W.-H. Kwan, M. Leboeuf, F. Ginhoux, J.C. Ochando, Y. Kunisaki, N. van Rooijen, C. Liu, T. Teshima, P.S. Heeger, E.R. Stanley, P.S. Frenette, and M. Merad. 2011. Pretransplant CSF-1 therapy expands recipient macrophages and ameliorates GVHD after allogeneic hematopoietic cell transplantation. *The Journal of Experimental Medicine*. 208:1069-1082.

Helft, J., F. Ginhoux, M. Bogunovic, and M. Merad. 2010. Origin and functional heterogeneity of non-lymphoid tissue dendritic cells in mice. *Immunological Reviews*. 234:55-75.

Hill, G.R., and J.L.M. Ferrara. 2000. The primacy of the gastrointestinal tract as a target organ of acute graft-versus-host disease: rationale for the use of cytokine shields in allogeneic bone marrow transplantation. *Blood*. 95:2754-2759.

Hochweller, K., T. Miloud, J.r. Striegler, S. Naik, G.n.J. HÃ¤mmerling, and N. Garbi. 2009. Homeostasis of dendritic cells in lymphoid organs is controlled by regulation of their precursors via a feedback loop. *Blood*. 114:4411-4421.

Hofmeyer, K.A., H. Jeon, and X. Zang. 2011. The PD-1/PD-L1 (B7-H1) pathway in chronic infection-induced cytotoxic T lymphocyte exhaustion. *Journal of biomedicine & biotechnology*. 2011:451694.

Hufford, M.M., T.S. Kim, J. Sun, and T.J. Braciale. 2011. Antiviral CD8+ T cell effector activities in situ are regulated by target cell type. *The Journal of Experimental Medicine*. 208:167-180.

Hume, D.A., N. Mabbott, S. Raza, and T.C. Freeman. 2013. Can DCs be distinguished from macrophages by molecular signatures? *Nat Immunol*. 14:187-189.

Iijima, N., L.M. Mattei, and A. Iwasaki. 2011. Recruited inflammatory monocytes stimulate antiviral Th1 immunity in infected tissue. *Proceedings of the National Academy of Sciences of the United States of America*. 108:284-289.

Jacobsohn, D.A., B.F. Kurland, J. Pidala, Y. Inamoto, X. Chai, J.M. Palmer, S. Arai, M. Arora, M. Jagasia, C. Cutler, D. Weisdorf, P.J. Martin, S.Z. Pavletic, G. Vogelsang, S.J. Lee, and M.E. Flowers. 2012. Correlation between NIH composite skin score, patient-reported skin score, and outcome: results from the Chronic GVHD Consortium. *Blood*. 120:2545-2552; quiz 2774.

Jakubzick, C., F. Tacke, F. Ginhoux, A.J. Wagers, N. van Rooijen, M. Mack, M. Merad, and G.J. Randolph. 2008. Blood monocyte subsets differentially give rise to CD103+ and CD103- pulmonary dendritic cell populations. *Journal of immunology (Baltimore, Md. : 1950)*. 180:3019-3027.

Jankovic, D., J. Ganesan, M. Bscheider, N. Stickel, F.C. Weber, G. Guarda, M. Follo, D. Pfeifer, A. Tardivel, K. Ludigs, A. Bouazzaoui, K. Kerl, J.C. Fischer, T. Haas, A. Schmitt-Graff, A. Manoharan, L. Muller, J. Finke, S.F. Martin, O. Gorka, C. Peschel, J. Ruland, M. Idzko, J. Duyster, E. Holler, L.E. French, H. Poeck, E. Contassot, and R. Zeiser. 2013. The Nlrp3 inflammasome regulates acute graft-versus-host disease. *J Exp Med*. 210:1899-1910.

Joshi, M.D., W.J. Unger, G. Storm, Y. van Kooyk, and E. Mastrobattista. 2012. Targeting tumor antigens to dendritic cells using particulate carriers. *Journal of controlled release : official journal of the Controlled Release Society*. 161:25-37.

Kaplan, D.H., B.E. Anderson, J.M. McNiff, D. Jain, M.J. Shlomchik, and W.D. Shlomchik. 2004. Target Antigens Determine Graft-versus-Host Disease Phenotype. *The Journal of Immunology*. 173:5467-5475.

Koyama, M., R.D. Kuns, S.D. Olver, N.C. Raffelt, Y.A. Wilson, A.L.J. Don, K.E. Lineburg, M. Cheong, R.J. Robb, K.A. Markey, A. Varelias, B. Malissen, G.J. Hammerling, A.D. Clouston, C.R. Engwerda, P. Bhat, K.P.A. MacDonald, and G.R. Hill. 2011. Recipient nonhematopoietic antigen-presenting cells are sufficient to induce lethal acute graft-versus-host disease. *Nat Med*. 18:135-142.

Kuziel, W.A., S.J. Morgan, T.C. Dawson, S. Griffin, O. Smithies, K. Ley, and N. Maeda. 1997. Severe reduction in leukocyte adhesion and monocyte extravasation in mice deficient in CC chemokine receptor 2. *Proceedings of the National Academy of Sciences of the United States of America*. 94:12053-12058.

Leon, B., M. Lopez-Bravo, and C. Ardavin. 2005. Monocyte-derived dendritic cells. *Semin Immunol*. 17:313-318.

León, B., M. López-Bravo, and C. Ardavín. 2005. Monocyte-derived dendritic cells. *Seminars in Immunology*. 17:313-318.

Lerner, K.G., G.F. Kao, R. Storb, C.D. Buckner, R.A. Clift, and E.D. Thomas. 1974. Histopathology of graft-vs.-host reaction (GvHR) in human recipients of marrow from HL-A-matched sibling donors. *Transplantation proceedings*. 6:367-371.

Li, H., A.J. Demetris, J. McNiff, C. Matte-Martone, H.S. Tan, D.M. Rothstein, F.G. Lakkis, and W.D. Shlomchik. 2012. Profound Depletion of Host Conventional Dendritic Cells, Plasmacytoid Dendritic Cells, and B Cells Does Not Prevent Graft-versus-Host Disease Induction. *The Journal of Immunology*. 188:3804-3811.

Liang, S.C., Y.E. Latchman, J.E. Buhlmann, M.F. Tomczak, B.H. Horwitz, G.J. Freeman, and A.H. Sharpe. 2003. Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses. *European Journal of Immunology*. 33:2706-2716.

Linsley, P.S., and S.G. Nadler. 2009. The clinical utility of inhibiting CD28-mediated costimulation. *Immunol Rev*. 229:307-321.

Liu, K., T. Iyoda, M. Saternus, Y. Kimura, K. Inaba, and R.M. Steinman. 2002. Immune tolerance after delivery of dying cells to dendritic cells in situ. *J Exp Med*. 196:1091-1097.

Liu, K., G.D. Victora, T.A. Schwickert, P. Guermonprez, M.M. Meredith, K. Yao, F.F. Chu, G.J. Randolph, A.Y. Rudensky, and M. Nussenzweig. 2009. In vivo analysis of dendritic cell development and homeostasis. *Science*. 324:392-397.

Lutz, M.B., and G. Schuler. 2002. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? *Trends in Immunology*. 23:445-449.

Maldonado, R.A., and U.H. von Andrian. 2010. How tolerogenic dendritic cells induce regulatory T cells. *Advances in immunology*. 108:111-165.

Mann, E.R., J.D. Landy, D. Bernardo, S.T.C. Peake, A.L. Hart, H.O. Al-Hassi, and S.C. Knight. 2013. Intestinal dendritic cells: Their role in intestinal inflammation, manipulation by the gut microbiota and differences between mice and men. *Immunology Letters*. 150:30-40.

Mazzini, E., L. Massimiliano, G. Penna, and M. Rescigno. 2014. Oral Tolerance Can Be Established via Gap Junction Transfer of Fed Antigens from CX3CR1(+) Macrophages to CD103(+) Dendritic Cells. *Immunity*. 40:248-261.

McGill, J., N. Van Rooijen, and K.L. Legge. 2008. Protective influenza-specific CD8 T cell responses require interactions with dendritic cells in the lungs. *The Journal of Experimental Medicine*. 205:1635-1646.

McLachlan, J.B., D.M. Catron, J.J. Moon, and M.K. Jenkins. 2009. Dendritic Cell Antigen Presentation Drives Simultaneous Cytokine Production by Effector and Regulatory T Cells in Inflamed Skin. *Immunity*. 30:277-288.

Merad, M., P. Sathe, J. Helft, J. Miller, and A. Mortha. 2013. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annu Rev Immunol*. 31:563-604.

Miller, J.C., B.D. Brown, T. Shay, E.L. Gautier, V. Jojic, A. Cohain, G. Pandey, M. Leboeuf, K.G. Elpek, J. Helft, D. Hashimoto, A. Chow, J. Price, M. Greter, M. Bogunovic, A. Bellemare-Pelletier, P.S. Frenette, G.J. Randolph, S.J. Turley, and M. Merad. 2012b. Deciphering the transcriptional network of the dendritic cell lineage. *Nat Immunol*. 13:888-899.

Nash, R.A., M.S. Pepe, R. Storb, G. Longton, M. Pettinger, C. Anasetti, F.R. Appelbaum, R.A. Bowden, H.J. Deeg, K. Doney, and et al. 1992. Acute graft-versus-host disease: analysis of risk factors after allogeneic marrow transplantation and prophylaxis with cyclosporine and methotrexate. *Blood*. 80:1838-1845.

Passweg, J.R., J. Halter, C. Bucher, S. Gerull, D. Heim, A. Rovo, A. Buser, M. Stern, and A. Tichelli. 2012. Hematopoietic stem cell transplantation: a review and recommendations for follow-up care for the general practitioner. *Swiss medical weekly*. 142:w13696.

Piguet, P.F., G.E. Grau, B. Allet, and P. Vassalli. 1987. Tumor necrosis factor/cachectin is an effector of skin and gut lesions of the acute phase of graft-vs.-host disease. *J Exp Med*. 166:1280-1289.

Prasad, D.V., S. Richards, X.M. Mai, and C. Dong. 2003. B7S1, a novel B7 family member that negatively regulates T cell activation. *Immunity*. 18:863-873.

Probst, H.C., K. McCoy, T. Okazaki, T. Honjo, and M. van den Broek. 2005. Resting dendritic cells induce peripheral CD8+ T cell tolerance through PD-1 and CTLA-4. *Nat Immunol*. 6:280-286.

Reis e Sousa, C. 2006. Dendritic cells in a mature age. *Nat Rev Immunol*. 6:476-483.

Reizis, B., A. Bunin, H.S. Ghosh, K.L. Lewis, and V. Sisirak. 2011. Plasmacytoid dendritic cells: recent progress and open questions. *Annu Rev Immunol*. 29:163-183.

Rescigno, M., and A. Di Sabatino. 2009. Dendritic cells in intestinal homeostasis and disease. *J Clin Invest*. 119:2441-2450.

Riella, L.V., A.M. Paterson, A.H. Sharpe, and A. Chandraker. 2012. Role of the PD-1 Pathway in the Immune Response. *American Journal of Transplantation*. 12:2575-2587.

Rothstein, D.M., and M.H. Sayegh. 2003. T-cell costimulatory pathways in allograft rejection and tolerance. *Immunological Reviews*. 196:85-108.

Rowe, V., T. Banovic, K.P. MacDonald, R. Kuns, A.L. Don, E.S. Morris, A.C. Burman, H.M. Bofinger, A.D. Clouston, and G.R. Hill. 2006. Host B cells produce IL-10 following TBI and attenuate acute GVHD after allogeneic bone marrow transplantation. *Blood*. 108:2485-2492.

Rutella, S., S. Danese, and G. Leone. 2006. Tolerogenic dendritic cells: cytokine modulation comes of age. *Blood*. 108:1435-1440.

Salama, A.D., T. Chitnis, J. Imitola, M.J. Ansari, H. Akiba, F. Tushima, M. Azuma, H. Yagita, M.H. Sayegh, and S.J. Khoury. 2003. Critical role of the programmed death-1 (PD-1) pathway in regulation of experimental autoimmune encephalomyelitis. *J Exp Med*. 198:71-78.

Schilbach, K., J. Schick, M. Wehrmann, G. Wollny, P. Simon, P.G. Schlegel, and M. Eyrich. 2007. PD-1-PD-L1 pathway is involved in suppressing alloreactivity of heart infiltrating t cells during murine gvhd across minor histocompatibility antigen barriers. *Transplantation*. 84:214-222.

Schulz, O., E. Jaensson, E.K. Persson, X. Liu, T. Worbs, W.W. Agace, and O. Pabst. 2009. Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. *The Journal of Experimental Medicine*. 206:3101-3114.

Scott, C.L., A.M. Aumeunier, and A.M. Mowat. 2011. Intestinal CD103+ dendritic cells: master regulators of tolerance? *Trends in Immunology*. 32:412-419.

Segura, E., and S. Amigorena. 2013. Inflammatory dendritic cells in mice and humans. *Trends in Immunology*.

Shi, C., and E.G. Pamer. 2011. Monocyte recruitment during infection and inflammation. *Nat Rev Immunol*. 11:762-774.

Shlomchik, W.D. 2007. Graft-versus-host disease. *Nat Rev Immunol*. 7:340-352.

Shlomchik, W.D., M.S. Couzens, C.B. Tang, J. McNiff, M.E. Robert, J. Liu, M.J. Shlomchik, and S.G. Emerson. 1999. Prevention of Graft Versus Host Disease by Inactivation of Host Antigen-Presenting Cells. *Science*. 285:412-415.

Sica, G.L., I.H. Choi, G. Zhu, K. Tamada, S.D. Wang, H. Tamura, A.I. Chapoval, D.B. Flies, J. Bajorath, and L. Chen. 2003. B7-H4, a molecule of the B7 family, negatively regulates T cell immunity. *Immunity*. 18:849-861.

Sparwasser, T., R.M. Vabulas, B. Villmow, G.B. Lipford, and H. Wagner. 2000. Bacterial CpG-DNA activates dendritic cells in vivo: T helper cell-independent cytotoxic T cell responses to soluble proteins. *Eur J Immunol*. 30:3591-3597.

Steinman, R.M., D. Hawiger, and M.C. Nussenzweig. 2003. Tolerogenic dendritic cells. *Annu Rev Immunol*. 21:685-711.

Steinman, R.M., and M.C. Nussenzweig. 2002. Avoiding horror autotoxicus: The importance of dendritic cells in peripheral T cell tolerance. *Proceedings of the National Academy of Sciences*. 99:351-358.

Steinman, R.M., S. Turley, I. Mellman, and K. Inaba. 2000. The Induction of Tolerance by Dendritic Cells That Have Captured Apoptotic Cells. *The Journal of Experimental Medicine*. 191:411-416.

Swirski, F.K., M. Nahrendorf, M. Etzrodt, M. Wildgruber, V. Cortez-Retamozo, P. Panizzi, J.-L. Figueiredo, R.H. Kohler, A. Chudnovskiy, P. Waterman, E. Aikawa, T.R. Mempel, P. Libby, R. Weissleder, and M.J. Pittet. 2009. Identification of Splenic Reservoir Monocytes and Their Deployment to Inflammatory Sites. *Science*. 325:612-616.

Takatsuka, H., T. Iwasaki, T. Okamoto, and E. Kakishita. 2003. Intestinal graft-versus-host disease: mechanisms and management. *Drugs*. 63:1-15.

Terness, P., T.M. Bauer, L. Rose, C. Dufter, A. Watzlik, H. Simon, and G. Opelz. 2002. Inhibition of allogeneic T cell proliferation by indoleamine 2,3-dioxygenase-expressing dendritic cells: mediation of suppression by tryptophan metabolites. *J Exp Med*. 196:447-457.

Teshima, T., R. Ordemann, P. Reddy, S. Gagin, C. Liu, K.R. Cooke, and J.L.M. Ferrara. 2002. Acute graft-versus-host disease does not require alloantigen expression on host epithelium. *Nat Med*. 8:575-581.

Toubai, T., I. Tawara, Y. Sun, C. Liu, E. Nieves, R. Evers, T. Friedman, R. Korngold, and P. Reddy. 2011. Induction of acute GVHD by sex-mismatched H-Y antigens in the absence of functional radiosensitive host hematopoieticâ€“derived antigen-presenting cells. *Blood*. 119:3844-3853.

Tung, J.W., K. Heydari, R. Tirouvanziam, B. Sahaf, D.R. Parks, L.A. Herzenberg, and L.A. Herzenberg. 2007. Modern Flow Cytometry: A Practical Approach. *Clinics in Laboratory Medicine*. 27:453-468.

Villadangos, J.A., and P. Schnorrer. 2007. Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo. *Nat Rev Immunol*. 7:543-555.

Washington, K., and M. Jagasia. 2009. Pathology of graft-versus-host disease in the gastrointestinal tract. *Human Pathology*. 40:909-917.

Wilhelm, K., J. Ganesan, T. Muller, C. Durr, M. Grimm, A. Beilhack, C.D. Krempl, S. Sorichter, U.V. Gerlach, E. Juttner, A. Zerweck, F. Gartner, P. Pellegatti, F. Di Virgilio, D. Ferrari, N. Kambham, P. Fisch, J. Finke, M. Idzko, and R. Zeiser. 2010. Graft-versus-host disease is enhanced by extracellular ATP activating P2X7R. *Nat Med*. 16:1434-1438.

Wright, D.E., A.J. Wagers, A.P. Gulati, F.L. Johnson, and I.L. Weissman. 2001. Physiological Migration of Hematopoietic Stem and Progenitor Cells. *Science*. 294:1933-1936.

Yamazaki, T., H. Akiba, H. Iwai, H. Matsuda, M. Aoki, Y. Tanno, T. Shin, H. Tsuchiya, D.M. Pardoll, K. Okumura, M. Azuma, and H. Yagita. 2002. Expression of Programmed Death 1 Ligands by Murine T Cells and APC. *The Journal of Immunology*. 169:5538-5545.

Yoneyama, H., K. Matsuno, Y. Zhang, M. Murai, M. Itakura, S. Ishikawa, G. Hasegawa, M. Naito, H. Asakura, and K. Matsushima. 2001. Regulation by Chemokines of Circulating Dendritic Cell Precursors, and the Formation of Portal Tract–Associated Lymphoid Tissue, in a Granulomatous Liver Disease. *The Journal of Experimental Medicine*. 193:35-50.

Zhang, Y., J.P. Louboutin, J. Zhu, A.J. Rivera, and S.G. Emerson. 2002. Preterminal host dendritic cells in irradiated mice prime CD8+ T cell-mediated acute graft-versus-host disease. *J Clin Invest*. 109:1335-1344.

Zigmond, E., C. Varol, J. Farache, E. Elmaliah, A.T. Satpathy, G. Friedlander, M. Mack, N. Shpigel, I.G. Boneca, K.M. Murphy, G. Shakhar, Z. Halpern, and S. Jung. 2012a.

Ly6C hi monocytes in the inflamed colon give rise to proinflammatory effector cells and migratory antigen-presenting cells. *Immunity*. 37:1076-1090.

Zigmond, E., C. Varol, J. Farache, E. Elmaliah, AnsumanÂ T. Satpathy, G. Friedlander, M. Mack, N. Shpigel, IvoÂ G. Boneca, KennethÂ M. Murphy, G. Shakhar, Z. Halpern, and S. Jung. 2012b. Ly6Chi Monocytes in the Inflamed Colon Give Rise to Proinflammatory Effector Cells and Migratory Antigen-Presenting Cells. *Immunity*. 37:1076-1090.

Acknowledgments

I would like to thank several special persons who made possible this PhD thesis over the last four years:

First of all I want to thank Dr. Andreas Beilhack for giving me the opportunity to perform my PhD thesis under his supervision and introducing me into the exciting world of experimental hematopoietic stem cell transplantation. I am very grateful for his support, his encouragement and freedom to develop this project. Furthermore, he gave me the opportunity to broaden my horizons by allowing me to attend several international conferences and to conduct scientific work in several renowned institutions.

I am deeply thankful to all the members of the Beilhack laboratory for providing a very nice working atmosphere, for their patience, for helpful and constructive discussions, for excellent technical assistance, and especially for their friendship.

Thanks to the graduate school of life sciences (GSLS, Würzburg University) for awarding me with a prestigious stipend from the German Excellence Initiative during my PhD project. I am very grateful to them for creating a wonderful working environment, for their personal support and encouragement throughout the last four years.

Thanks to the members of my Supervisory Committee Prof. Dr. Manfred B. Lutz, Prof. Dr. Natalio Garbi for their involvement in the project, their important contributions, their enormous support and inspiring discussions. Their experience and assistance made this project possible.

I am grateful to Prof. Dr. Thomas Hünig for constructive discussions and for the great opportunity to be part of the Graduate College Immunomodulation (GK520). It was a fantastic opportunity to exchange knowledge among graduate students and project leaders.

My parents, my brother and friends deserve special mention for their endless support and for believing in me. Finally I want to express my gratitude to Axel for his love, endless patience, support, and for always believing in me.

Curriculum vitae

Ana-Laura Jordán Garrote
University Hospital Würzburg
Medical Clinic II
Experimental Stem Cell Transplantation
Zinklesweg 10, ZEMM
97078 Würzburg
Phone: +49 (0)931 201 27637
email: Jordan_A@ukw.de

PERSONAL DATA

Place and date of birth	Barcelona, Spain, October 30th, 1984
Nationality	Spanish
Marital Status	Single

EDUCATION

2010 – Present	University of Würzburg, Germany PhD student (Experimental stem cell transplantation) under the supervision of Dr. Andreas Beilhack.
2008-2009	Master thesis in the laboratory of Dr. Andreas Beilhack at the Department of Medicine II at the Universitätsklinik Würzburg and at the laboratory of Dr. Stephan Schulz at the Institute of Pathology at the Technische Universität München.
2002-2009	Masters degree in Biology at the University of Barcelona, Spain (June 30, 2009), including one year at the Universidad de Salamanca with a Seneca Scholarship.
2000 – 2002	High School: IES Joaquin Rubio i Ors, Sant Boi de llobregat, Barcelona. Spain University entrance exam: June 2002

FELLOWSHIPS AND AWARDS

Sep – Feb 2009	STIBET-fellowship from the Deutscher Akademischer Austausch Dienst, DAAD
Mar 2010 - Aug 2013	Fellowship from the Excellence Initiative at the Graduate School of Life Sciences, University of Würzburg
Mar 2013	Else Kröner-Poster-Award for Translational Immunology, Else Kröner Symposium “”Translational Immunology-From Target to Therapy”, Würzburg, Germany

SCIENTIFIC WORK ABROAD

Jul – Oct 2007	Purpose: 3-month internship in the department of Cell Biology under the supervision of Dr. Audrey Gerard Host group: John Collard Lab Institution: Netherlands Cancer Institute-Antoni Van Leeuwenhoek
April, July, and August 2010	Purpose: several experiments in diverse experimental sessions were performed during this period under the supervision of Prof. Garbi. Host group: Natalio Garbi Lab Institution: DKFZ, Heidelberg, Germany.
11-22.12.2013	Purpose: Collaborative experiments under the supervision of Prof. Wagers. Host group: Amy Wagers Lab Institution: Stem Cell Institute, Harvard University, Boston, USA

LANGUAGE SKILLS

Spanish	Native Language
Catalan	Native Language
English	Fluent in writing and speaking
German	Good in writing and fluent in speaking

PUBLICATIONS

Chopra M, Lang I, Salzmann S, Pachel C, Kraus S, Bäuerlein C, Brede C, **Jordán Garrote AL**, Mattenheimer K, Ritz M, Schwinn S, Graf C, Schäfer V, Frantz S, Einsele H, Wajant H, Beilhack A. (2013). Tumor necrosis factor induces tumor promoting and anti-tumoral effects on pancreatic cancer via TNFR1. PLoS ONE 8(9): e75737.

Bäuerlein CA, Riedel SS, Baker J, Brede, C, **Jordán Garrote AL**, Ritz M, Schulz S, Grether M, Chopra M, Beilhack GF, Zeiser R, Schlegel PG, Einsele H, Negrin RS, Beilhack A. (2013). A diagnostic window for the treatment of acute GVHD prior to visible clinical symptoms in a murine model. BMC Medicine 11(1): 134.

Chopra M, Riedel SS, Biehl M, Donat S, von Krosigk V, Bäuerlein CA, Brede C, **Jordan-Garrote AL**, Schäfer V, Ritz M, Mattenheimer K, Degla A, Mottok A, Einsele H, Wajant H, Beilhack A. (2013). Tumor necrosis factor receptor 2-dependent homeostasis of regulatory T cells as player in TNF-induced experimental metastasis. Carcinogenesis 34(6): 1296-1303.

Brede C, Friedrich M, von Krosigk V, **Jordán-Garrote AL**, Riedel SS, Bäuerlein CA, Heinze K, Bopp T, Schulz S, Mottok A, Rosenwald A, Einsele H, Negrin RS, Harms GS, Beilhack A. (2012) Mapping immune processes in intact tissues at cellular resolution. The Journal of Clinical Investigation, 122 (12): 4439-4446.

Riedel SS, Mottok A, Brede C, Bäuerlein CA, **Jordán Garrote AL**, Ritz M, Mattenheimer K, Rosenwald A, Einsele, H, Bogen B, Beilhack A.

(2012). Non-invasive imaging provides spatiotemporal information on disease progression and response to therapy in a murine model of multiple myeloma. PLoS ONE 7: e52398.

SCIENTIFIC CONFERENCES

10 - 13 June 2010	15th Congress of the European Hematology Association (EHA), Barcelona, Spain Talk: CD11b^{+} CD11c^{+} dendritic cells interact with alloreactive T cells in the intestinal mucosa in acute GVHD
01 - 05 October 2010	Deutsche, österreichische und schweizer Gesellschaft für Hämatologie und Onkologie (DGHO), Jahrestagung 2010, Berlin, Germany Talk: Transition from CD103+ to CD11b+ dendritic cells correlates with acute intestinal graft-versus-host disease
1 - 5 February 2012	American Association of Bone and Marrow Transplantation (ASBMT), San Diego, California, USA Poster: Depletion of host dendritic cells during the effector phase of GVHD enhances acute GVHD and mortality.
7 - 11 October 2012	12th International Symposium on Dendritic Cells, Daegu, Korea Poster: Host dendritic cell depletion during the effector phase exacerbates acute GVHD

RESEARCH RETREATS AND WORKSHOPS

3 - 4 Mar 2009	Immunofluorescence Microscopy – IFM Workshop I Würzburg (München-Würzburg)
Nov 2009	Basic Course in Flow Cytometry (Becton and Dickinson, Heidelberg, Germany)
Apr 2009 - Jul 2013	Graduate College : Inmunomodulation, Würzburg University
10 - 12 Aug 2009	Immunofluorescence Microscopy – IFM Workshop II Würzburg (München-Würzburg)
13 - 14 Nov 2009	1st EBMT (Experimental Blood and Marrow Transplantation) Retreat in Schauinsland (Freiburg-Erlangen-Würzburg)
15 - 16 Jul 2010	2nd EBMT Retreat Schwanberg (Freiburg-Erlangen-Würzburg)

16 - 17 Jun 2011	3rd EBMT Retreat Feuerstein (Freiburg-Erlangen-Würzburg)
May 12 2012	GVHD - Symposium on murine GVHD including training of pathological GVHD scoring, Mainz (PD Dr. med. R. Meyer)
12 - 13 Jul 2012	4th EBMT Retreat Schauinsland (Freiburg-Erlangen-Würzburg)
25 - 26 Oct 2012	Course in FACS-Sorting (Aria II) by Becton and Dickinson
Oct 15 2013	Workshop: ALZET-Pumps, Charles River, Würzburg University
26 - 28 Mar 2014	12th International Symposium GvH/GvL, Regensburg, Germany

Würzburg, April 2014

Ana-Laura Jordán Garrote

Affidavit (Eidesstattliche Erklärung)

I hereby declare that my thesis entitled 'The role of host dendritic cells during acute intestinal graft-versus-host disease' is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I verify that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Hiermit erkläre ich an Eides statt, die Dissertation „Die Rolle der dendritischen Zellen in der akuten intestinalen Graft-versus-Host Reaktion" eigenständig, d.h. insbesondere selbstständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Würzburg……………………………………………………………………………………

Date Signature