Aus der Abteilung für Klinische Pharmakologie

Leiter: Prof. Dr. med. S. Endres

Medizinische Klinik Innenstadt

Klinikum der Universität

Ludwig-Maximilians-Universität München

Direktor: Prof. Dr. med. M. Reincke

Tissue-specific migration: The effect of innate immune activation on lymphocyte homing to the gastrointestinal tract

Dissertation

zum Erwerb des Doktorgrades der Medizin

an der Medizinischen Fakultät der

Ludwig-Maximilians-Universität zu München

vorgelegt von

Simon Heidegger

aus Weingarten

2010

Mit Genehmigung der Medizinischen Fakultät
der Universität München

1. Berichterstatter:	Prof. Dr. med. Stefan Endres
2. Berichterstatter:	Prof. Dr. med. Georg Enders
Mitberichterstatter:	Prof. Dr. med. Reinhard Lorenz
	Prof. Dr. med. Burkhard Göke
Mitbetreuung durch die promovierte Mitarbeiterin:	PD Dr. med. Dr. rer. nat. Carole Bourquin (Arbeitsgruppenleiterin)
Dekan:	Prof. Dr. med. Dr. h.c. Reiser, FACR, FRCR
Tag der mündlichen Prüfung:	02.12.2010

Dedicated to my parents Anita and Erwin
and to my sister Patrizia

INDEX

1 INTRODUCTION

1.1 The human immune system

Our body's integrity is constantly threatened by the invasion of microorganisms and the development of neoplastic cells. But we have evolved systems of immune defense to eliminate infective pathogens and tumor cells. As in all jawed vertebrates, there are two types of defense in humans: the innate and the adaptive (also known as acquired) immunity. Multiple interactions between these two branches of the immune system are required for cooperative elimination of foreign pathogens and transformed host cells.

1.1.1 The two arms of the immune system

The innate immune system is a phylogenetically very old and evolutionarily conserved system. It provides a first line of defense against invading pathogens and is mainly mediated by phagocytes such as dendritic cells and macrophages. These cells can effectively sense microbial infection, rapidly contain it locally and subsequently induce a systemic inflammatory response. In contrast, adaptive immunity is involved in elimination of pathogens in the late phase of infection but is highly specific and long-lasting by generating an immunological memory. This specificity of the adaptive immune system is assured by antigen-specific receptors expressed on the surface of T and B lymphocytes. By individual somatic recombination of the genes encoding these receptors, a nearly infinite repertoire of receptors with random but narrow specificities is generated in a process called gene rearrangement. Each T and B cell expresses antigen receptors of only a single specificity. In response to a certain pathogen, only particular populations of lymphocytes are selected to clonally expand, which is the basis for immunological memory.

Unlike the adaptive immune system, the innate immune system is not a single entity. It is a collection of distinct subsystems that carry out different functions in host defense. Mucosal epithelia and the skin, for example, form physical barriers and also produce antimicrobial peptides and mucins preventing pathogen attachment and entry. Another module, the acute phase proteins and complement produced by hepatocytes are a cascade of serum proteins leading to opsonization and lysis of pathogens as well as chemotactic attraction of leukocytes. Phagocytes take up and process antigens to later present these to cells of adaptive immunity. However, the innate immune response is not completely nonspecific as originally assumed, but rather is able to discriminate

between self and a variety of pathogens. The innate immune system recognizes microorganisms via a limited number of germ line-encoded pattern-recognition receptors (PRR), in contrast to the huge repertoire of randomly rearranged receptors utilized by the acquired system.

1.1.2 Pattern-recognition receptor families

Pattern-recognition receptors recognize microbial components known as pathogen-associated molecular patterns (PAMP) and were first hypothesized twenty years ago by Charles Janeway [Janeway, 1989]. PRR are germ line encoded, non-clonal and expressed constitutively on all cells of a given type. Each receptor has a broad specificity and the potential to bind to a variety of molecules that share a common motif. These pathogen-associated molecular patterns are generally unique to microorganisms and thereby allow discrimination between self (host) and non-self molecules. They are invariant among a class of microorganisms and have essential roles in microbial physiology, hence limiting the ability to evade immune recognition through adaptation and alteration of these molecules. In addition to PAMP, innate receptors have the potential to also recognize endogenous molecules that are released by host cells as a result of infection, damage or necrosis. The recognition of these molecules is tightly linked to the pathogenesis of chronic inflammatory and autoimmune diseases.

1.1.2.1 Membrane-bound receptors

The best characterized class of PRR is the family of Toll-like receptors (TLR). In 1996, it was demonstrated that *Drosophila* carrying mutations in a receptor called *'toll'* were highly susceptible to fungi infection as they showed defective induction of anti-fungal peptides [Lemaitre et al., 1996]. Later, a human homologue of *toll* – now known as Toll-like receptor 4 – was discovered and shown to induce innate immunity by production of inflammatory cytokines and expression of co-stimulatory molecules [Medzhitov et al., 1997]. These and other studies led to the identification of the family of membrane-bound TLR. So far, 13 members of the TLR family have been described [Akira et al., 2006]. TLR1 to 9 are conserved between humans and mice although TLR8 is non-functional in mice as well as TLR10 due to a retrovirus insertion. TLR11 to 13 were lost in the human genome. Regarding their cellular location, the members of the TLR family can be divided into two subpopulations: On the one hand, TLR1, 2, 4, 5, 6 and

11 are expressed on the cell surface and thereby recognize components of microbial membranes or fungal cell walls such as lipids, lipoproteins or proteins. On the other hand, TLR3, 7, 8 and 9 are localized in the membrane of intracellular vesicles such as endo-/lysosomes and the endoplasmatic reticulum. This subpopulation of receptors predominately recognizes microbial nucleic acids such as bacterial DNA containing unmethylated CpG motifs (CpG-DNA) thus differing from mammalian DNA. Table 1.1 gives an overview of TLR and their most important ligands.

TLR	PAMP/activator	Species	Reference
TLR1	Heterodimers with TLR2		
TLR2	Triacyl lipopeptides	Bacteria	[Takeuchi et al., 1999]
	Diacyl lipopeptides	Mycoplasma	[Takeuchi et al., 2001]
	Zymosan	Fungus	[Ozinsky et al., 2000]
TLR3	dsRNA	Virus	[Alexopoulou et al., 2001]
TLR4	Lipopolysaccharid (LPS)	Bacteria	[Poltorak et al., 1998]
	Envelope proteins	Virus	[Kurt-Jones et al., 2000]
TLR5	Flagellin	Bacteria	[Hayashi et al., 2001]
TLR6	Heterodimers with TLR2		
TLR7	ssRNA	RNA virus	[Hemmi et al., 2002]
hTLR8	ssRNA	RNA virus	[Heil et al., 2004]
TLR9	CpG DNA	Bacteria	[Hemmi et al., 2000]
	DNA	DNA virus	[Lund et al., 2003]
	Malaria hemozoin	Parasites	[Coban et al., 2005]
hTLR10	Not determined		
mTLR11	Not determined	Uropathogenic bacteria	[Zhang et al., 2004]
	Profilin-like molecule	Parasites	[Yarovinsky et al., 2005]
mTLR12	Not determined		
mTLR13	Not determined		

Table 1.1: Overview of Toll-like receptors and their main ligands

TLR are predominantly expressed in tissues involved in immune function, such as spleen and peripheral blood leukocytes, as well as those exposed to the external environment such as epithelia and fibroblasts in the gastro intestinal (GI) tract, skin, urinary tract and the lung. Their expression profiles vary amongst tissues and cell types. Focusing on immune cells, myeloid cells constitutively express TLR1 and 6; macrophages preferentially express TLR2, 3, 4 and 8. B cells express TLR7, 9 and 10 while $CD4^{pos}$ T cells and natural killer cells were reported to express TLR2; some publications describe the expression of TLR2, 3, 5, and 9 on T cells [Ishii et al., 2006;

Kabelitz, 2007]. Notably, TLR expression on dendritic cells (DC) shows considerable differences between mice and men: human myeloid DC express all TLRs except TLR9 while plasmacytoid DC only express TLR1, 6, 7, and 9. Murine DC subsets have a broader distribution of TLR expression, with mouse plasmacytoid DC expressing all TLR but TLR3 and 4. In contrast to human DC, TLR7 and 9 are expressed by most subsets of murine DC [Mazzoni and Segal, 2004].

Dectin-1 is another recently discovered membrane-bound PRR which is TLR-independent but synergistic as they share partially common signaling pathways. Like other members of the diverse family of C-type lectins – many of which have yet unknown functions – Dectin-1 is a transmembrane receptor present on dendritic cells and macrophages [Robinson et al., 2006]. Dectin-1 specifically binds to β -glucans which are glucose polymers found in the cell walls of fungi [Brown et al., 2003]. It has been shown that recognition of β-glucans is TLR-2 independent [Ikeda et al., 2008] and subsequent signaling is unique to Dectin-1 [Gross et al., 2006]. However, recent data suggests that Dectin-1 and TLR2/TLR6 signaling combine to synergistically enhance the responses triggered by each receptor in anti-fungal immunity [Gantner et al., 2003; Dennehy and Brown, 2007].

1.1.2.2 Cytosolic receptors

The TLR family and other transmembrane receptors detect PAMP either on the cell surface or the lumen of intracellular vesicles such as endosomes. Now, recent studies revealed the existence of a cytosolic branch of innate immunity to detect intracellular PAMP. These cytosolic detection PRR include retinoic acid-inducible gene-I (RIG-I)-like receptors and nucleotide-binding oligomerization domain (NOD)-like receptors.

RIG-I-like receptors - also known as RIG-I-like helicases - constitute a family of cytoplasmatic RNA helicases that are critical for host antiviral responses. The RIG-I-like receptor family has three known members present in both immune and non-immune cells: RIG-I, melanoma differentiation associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). RIG-I and MDA-5 sense double-stranded RNA (dsRNA), either from genomic RNA of dsRNA viruses or as a replication intermediate from single-stranded RNA (ssRNA)-viruses, both leading to potent production of type I interferons (IFN) in infected cells [Kawai and Akira, 2009]. Foreign RNA is also recognized by TLR3, 7 and 8, which are located in cell endosomes. Recognition of viral RNA by these receptor families is cell-type dependent. In myeloid

DC, macrophages and fibroblasts, RIG-I-like receptors are the major sensors for viral infection, while in plasmacytoid DC, TLR play a more important role [Kato et al., 2005].

Despite a structural similarity between RIG-I and MDA5, they detect specific viral species, presumably through recognition of distinct structures of viral RNA [Kato et al., 2006]. Notably, RIG-I was demonstrated to bind to ssRNA bearing a 5'-triphosphate moiety (3p-RNA) [Hornung et al., 2006; Pichlmair et al., 2006]. Self RNA is either capped or contains base modifications suggesting a discrimination mechanism between self and non-self RNA. Recently, it was shown that RIG-I binds preferentially to short dsRNA while MDA-5 recognizes preferentially long dsRNA [Kato et al., 2008].

LGP2 was initially implicated to serve as a negative regulator of virus-induced immune responses [Yoneyama and Fujita, 2008], but later studies suggested a two-sided role in regulation of RIG-I and MDA5 depending on the type of RNA virus [Venkataraman et al., 2007].

Another large family of cytosolic PRR, the NOD-like receptors, comprises at least 23 human and 34 murine members; for most of which the physiological function remains unclear [Ting et al., 2008]. Best characterized are NOD1 and NOD2 as well as NALP3. NOD1 and NOD2 recognize distinct structural motifs of bacterial cell walls derived from peptidoglycans [Chamaillard et al., 2003; Girardin et al., 2003; Girardin et al., 2003]. NALP3 is one of several large caspase-1-activating cytosolic protein complexes called inflammasomes [Martinon et al., 2002]. They can recognize not only a wide variety of PAMP but different endogenous signs of cell stress and injury, conveniently called danger-associated molecular patterns. Activation of the inflammasome leads to potent release of cytokines of the interleukin-1 family [Franchi et al., 2009].

Recent studies suggested a yet undefined cytosolic DNA sensor promoting anti-viral and inflammatory responses independent of TLR9 and RIG-I-like receptors [Ishii and Akira, 2006; Stetson and Medzhitov, 2006]. DNA-dependent activator of IRF (DAI) has been proposed to be this sensor [Takaoka et al., 2007]. However, DAI-deficient mice showed an unaffected immune response against double stranded-DNA, suggesting a redundant or non-essential role of DAI [Ishii et al., 2008]. Very recently, several groups have identified AIM2 as a new receptor for cytoplasmatic DNA, which forms an inflammasome with the ligand and activates caspase-1 [Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009].

1.1.3 Signaling of pattern-recognition receptors

Signaling pathways via TLR, RIG-I-like helicases, NOD1 and 2 as well as Dectin-1 culminate in the activation of nuclear factor (NF)-κB and/or mitogen-activated protein kinases (MAPK) and activated protein 1 (AP-1), respectively, all transcription factors which regulate the expression of various immune and inflammatory genes. Furthermore, some TLR and the RIG-I-like receptors also activate members of the interferon regulatory factor (IRF) family of transcription factors inducing expression of type I IFN separately from inflammatory genes [Kawai and Akira, 2006]. Figure 1.1 gives a basic overview of PRR and their main target transcription factors; details are given in the text.

TLRs belong to a superfamily called the Toll/IL-1 receptor (TIR) family. The TLR signaling cascade is initiated by the ectodomain binding to a ligand and subsequent dimerization of the receptor. Receptor dimers facilitate the recruitment of four different TIR domain-containing cytosolic adapter molecules, the myeloid differentiation primary response gene 88 (MyD88), TIR-containing adapter inducing IFN-β (TRIF), TIR domain-containing adapter molecule (TIRAP) and TRIF-related adapter molecule (TRAM) [Kawai and Akira, 2007]. These adapter molecules are specifically recruited to their respective receptor in order to elicitate appropriate immune responses depending on the type of PAMP recognized. MyD88 is utilized by all TLR except TLR3 and ultimately activates NF-κB and MAPK to control inflammatory responses by triggering the production of a variety of pro-inflammatory cytokines such as IL-1, IL-6, IL-10, IL-12 and tumor necrosis factor (TNF)-α. TIRAP is exclusively recruited to TLR2 and 4 to act as a link to MyD88 [Horng et al., 2002]. In contrast, TRIF is only used by TLR3 and 4, initiating an alternative pathway eventually leading to NF-κB and MAPK activation as well as IRF3-mediated induction of type I IFNs. TRAM uniquely serves to link TRIF to TLR4, but not TLR3 [Yamamoto et al., 2003].

Thus, TLR4 recruits two adapter molecule complexes: TIRAP/MyD88 which leads to production of pro-inflammatory cytokines and TRAM/TRIF inducing the same cytokines as well as type IFNs. Notably, this is a biphasic activation pathway. TLR4 first engages TIRAP/MyD88 leading to NF-κB and MAPK activation and is subsequently internalized and relocated to the endosome where it promotes a second-phase NF-κB and MAPK activation as well as IRF3-mediated type IFN induction [Kagan and Medzhitov, 2006; Kagan et al., 2008; Tanimura et al., 2008].

Beside TLR3, TLR7, 8 and 9 recognize nucleic acids and are therefore capable of effectively inducing type I IFN. Signaling mechanisms leading to the induction of type I IFN differ depending on the TLR activated. They involve the interferon regulatory factors, a growing family of transcription factors known to play a vital role in antiviral defense, cell growth and immune regulation. Four IRF function as direct transducers of TLR signaling. TLR3 and TLR4 activate IRF3 and IRF7 via the adapter TRIF [Doyle et al., 2002], while TLR7, 8 and 9 activate IRF5 and IRF7 via MyD88 [Schoenemeyer et al., 2005]. In myeloid DC, TLR9 additionally activates IRF1 [Schmitz et al., 2007]. IRF8 cooperates with the other transcription factors to facilitate target gene expression.

RIG-I-like helicases signal via the adaptor IFN-β promoter stimulator 1 (IPS-1) which is bound to the mitochondrial membrane eventually resulting in activation of NF-κB, MAPK, IRF3 and 7. IPS-1 is also known as mitochondrial anti-viral signaling (MAVS) CARD adapter inducing IFN-β (Cardif) or virus-induced signaling adapter (VISA) [Kawai and Akira, 2009]. The yet undefined cytosolic dsDNA sensor apart from recently discovered AIM2 inflammasome, shares at least partly common pathways with these receptor molecules culminating in activation of transcription factors NF-κB and IRF3 [Yanai et al., 2009].

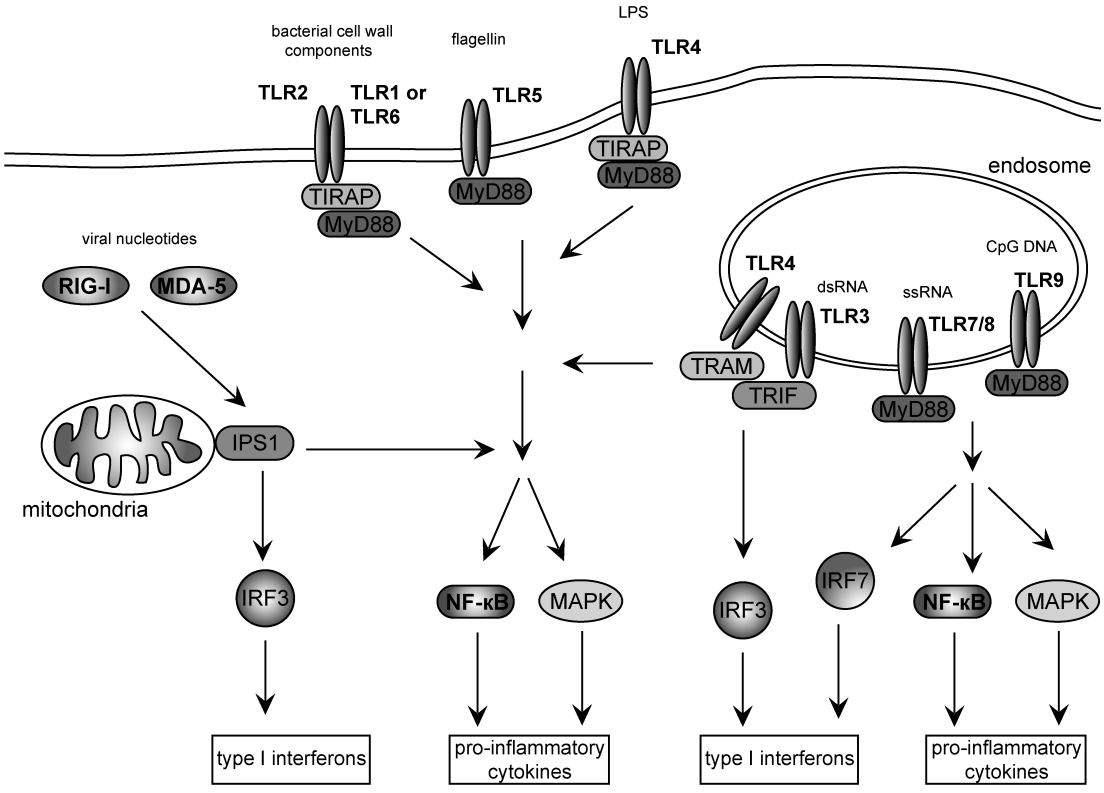

Figure 1.1: Overview of pattern-recognition receptors and their main target transcription factors relevant to this work. This schematic overview is reduced to receptors, TIR domain-containing adapter molecules and main target transcription factors that vary between the different PRR.

1.2 Dendritic cells: mediators between innate and adaptive immunity

1.2.1 Dendritic cell function

Antigen-presenting cells (APC) that process antigenic material and present it on their surface to other cells of the immune system act as mediators between the innate and adaptive immune system. There are three main types of APC that can activate T cells: dendritic cells, macrophages, and B cells. The most important APC is the highly specialized dendritic cell, named for their branch-like projections [Steinman and Cohn, 1973]. Dendritic cells are present in virtually all peripheral body tissues, forming a network and constantly sampling their environment by taking up extracellular components through phago- or endocytosis. The ingested microbial pathogens, dead or dying cells, immune complexes and other antigens are processed and presented by major histocompatibility complex (MHC) molecules at the cell surface [Rossi and Young, 2005]. Once they have made contact with a danger signal, they mature and migrate to regional lymph nodes. Here, a critical interaction with T cells from the adaptive branch of the immune system takes place. Depending on their level of activation, dendritic cells will induce tolerance towards the presented antigen or will initiate a specific T cell immune response. Induction of such an adaptive immune response is achieved in a two-step process: T cells need to bind their cognate antigen by T cell receptor-MHC complex interaction (signal 1) and must then be additionally activated by DC co-stimulatory molecules (signal 2). Recent studies have shown that the co-stimulatory potential of DC plays a critical role in inducing an effective T cell response. Immature DC only present an antigen without appropriate co-stimulation, thus inducing tolerance to this antigen or anergy, respectively [Dhodapkar et al., 2001]. Only fully matured DC which also supply an adequate second signal, will induce a potent T cell-mediated immune response [Shortman and Heath, 2001].

These findings move DC to the center of adaptive immunity; as it is the DC who determine the fate of T cells deciding between anergy and activation by their co-stimulatory function. The mechanism of this regulation remained poorly understood until Charles Janeway's hypothesis of pattern-recognition receptors that link innate and adaptive immunity proved true. Further studies showed that effective maturation of DC and subsequent triggering of a potent immune response is mainly achieved by stimulation with microbial cell components [Medzhitov and Janeway, 1997]. However, endogenous danger signals can also alert dendritic cells. Only necrotic but not apoptotic cells lead to DC maturation although both are processed and presented to T

cells by DC [Gallucci et al., 1999]. As described previously (chapter 1.2.1), potential danger signals are identified by pattern-recognition receptors recognizing highly conserved PAMP. Considering their main function as the body's scavenger cells it is not surprising that DC express a broad spectrum of PRR [Kadowaki et al., 2001; Krug et al., 2001].

1.2.2 Dendritic cell subtypes

Since their initial description by Steinman and Cohn, it has become evident that DC are a heterogeneous population and comprise many distinct DC subtypes. Although all DC share the capability of antigen uptake, processing and presentation to naïve T and B cells, the DC subtypes differ considerably in terms of location, migratory pathways, detailed immunological function and dependence on infections or inflammatory stimuli for their generation. DC subtypes in the steady state, that is in the absence of infection, include type I interferon-producing plasmacytoid DC (pDC), migratory DC located in peripheral tissues such as Langerhans cells, and lymphoid tissue-resident myeloid DC (mDC), also called conventional DC [Shortman and Liu, 2002]. In mice, mDC can be further subdivided according to their expression of CD8α on the cell surface [Vremec et al., 2000]. These two subsets differ in their immune functions, with only the $CD8^{pos}$ mDC being able to cross-present exogenous antigens on MHC I complexes [den Haan et al., 2000]. DC among other APC usually present antigens acquired from the extracellular environment on class II MHC complexes binding to T-cell receptors on $CD4^{pos}$ T helper cells, whereas class I MHC molecules bear peptides synthesized in the cytosol and activate $CD8^{pos}$ cytotoxic T cells. However, $CD8^{pos}$ mDC are capable of cross-presenting exogenous antigens on class I MHC complexes, thus allowing the induction of a cytotoxic T-cell response to pathogens that do not infect the DC themselves [Groothuis and Neefjes, 2005; Rossi and Young, 2005].

Lymphoid tissue-resident DC collect and present antigens in a single lymphoid organ without migrating. In contrast, migratory DC are the classical DC that act as sentinels in peripheral tissues and migrate to the lymph nodes through the lymphatics, bearing antigens from the periphery. Plasmacytoid DC represent a rare but important cell type and are characterized by fast production of high amounts of type I interferons in response to viral infections. Immature pDC are present in the blood, bone marrow and secondary lymphoid organs and can be recruited to the skin, gut, lung and cerebrospinal fluid under stimulatory conditions. Moreover, pDC are known to infiltrate many tumors.

1.3 Effector cells of adaptive immunity

1.3.1 T and B cell function

Conventional T (most αβ T cells) and B cells (also known as B2 cells) are the major cellular components of the adaptive immune response characterized by their huge arsenal of antigen-specific receptors generated by gene rearrangement. In contrast, there are so-called innate-like lymphocytes, that are B1 cells, marginal-zone B cells, natural-killer T cells and subsets of γδ T cells whose assembly process is not entirely random [Medzhitov, 2007]. Their receptor diversity is biased towards a characteristic set of specificities for each subset of innate-like lymphocytes [Bendelac et al., 2001]. Accordingly, effector function of these lymphocytes and the sites where they reside are often predetermined.

T cells are involved in cell-mediated immunity whereas B cells are primarily responsible for humoral immunity, which is for the production of antibodies. Once properly activated by APC (see chapter 1.2.1), these cells mount specific immune responses that are tailored to maximally eliminate specific pathogens or pathogen-infected or transformed cells. B cells respond to pathogens by producing large quantities of antibodies which then mark and/or neutralize foreign pathogens like bacteria and viruses. There are two groups of conventional T cells: T-helper (T_H) cells, which are characterized by the co-receptor CD4 on the cell surface, and cytotoxic T cells, which express CD8. T_H cells are the middlemen of adaptive immunity and can differentiate into several types of effector cells, characterized by the production of distinct sets of cytokines [Glimcher and Murphy, 2000; Reinhardt et al., 2006]. T_H1 cells produce IFN-γ and thereby boost the cellular immune system by maximizing the killing efficacy of macrophages and the proliferation of cytotoxic T cells. T_H2 secrete IL-4, 5 and 13 and enhance humoral immune responses by induction of B cell proliferation, antibody class switching and increased antibody production. Recently described T_H17 cells produce IL-17 and are involved in protection against extracellular bacteria and fungi [Harrington et al., 2005; LeibundGut-Landmann et al., 2007]. Regulatory T (T_{reg}) cells are a specialized subpopulation of T_H cells that act to suppress immune responses and thereby maintain immune homeostasis and tolerance to self-antigens. They are commonly characterized by their expression of CD4, CD25 and the transcription factor forkhead box p3 (foxp3) [Vignali et al., 2008]. Cytotoxic T cells kill infected or otherwise damaged and dysfunctional cells and thereby play an important role in immune response against intracellular pathogens and tumor cells.

1.3.2 Lymphocytic migration patterns

Since lymphocytes respond to pathogens only upon direct contact with antigens derived from these pathogens, they are forced to migrate to sites where antigen is found. The repertoire of naïve T cells – that are T cells that have never encountered their cognate antigen – consists of 25 to 100 million distinct clones [Arstila et al., 1999]. However, the number of identical cells whose T-cell receptors recognize the same individual antigen is very limited; several thousand at most [von Andrian and Mackay, 2000]. Clearly, a complex guidance system must be at work to accomplish this task.

To facilitate the encounter between APC and the rare antigen-specific lymphocytes, the immune system is compartmentalized so that activation of naïve lymphocytes is concentrated to secondary lymphoid organs, for example spleen and peripheral lymph nodes (PLN) which stand in contrast to internal lymph nodes found in thoracic and abdominal body cavities. The secondary lymphoid organs of the gastro-intestinal tract form a somehow separate group and are made up of mesenteric lymph nodes (MLN) and Peyer's patches (PP). The PP are large lymphoepithelial structures localized within the small intestinal wall in humans while murine PP are distributed in the small and large bowl.

Naïve lymphocytes constantly patrol secondary lymphoid organs in search for their cognate antigen presented by APC. They enter these sites via the blood stream and leave in the lymph fluid. Upon activation by antigen contact, they relocate to peripheral sites and exert their effector activities. To achieve these tasks, lymphocytes must travel between lymphoid and non-lymphoid organs via the blood stream and finally exit the circulation to enter antigen-containing tissues. Therefore, in a process called the multi-step adhesion cascade, lymphocytes are captured ("tethering") and interact loosely with the endothelial cells ("rolling"), then undergo "activation" and establish firm arrest ("sticking") on the epithelial surface to finally transmigrate into a tissue (diapedesis) [Springer, 1994]. The molecules involved in the different steps vary not only depending on the lymphocyte population but first and foremost on target-tissue and inflammatory context [von Andrian and Mackay, 2000]. Generally, initial tethering and rolling is mediated by selectins or $\alpha 4$ integrins. Chemokines presented on the vascular endothelium interacting with corresponding chemokine receptors (CCR) activate further integrins on lymphocytes which then mediate rolling, sticking and diapedesis.

1.3.2.1 Homing of naïve T and B cells

By their set of surface migratory molecules, naïve lymphocytes are mainly excluded from nonlymphoid peripheral tissues, but effectively migrate to secondary lymphoid organs where they can meet their cognate antigen and undergo activation. Basically, lymphocytes use L-Selectin (also named CD62L) to tether and roll on specialized microvessels found in PLN, MLN and PP known as high endothelial venules (HEV) [Bargatze et al., 1995]. L-Selectin binds to a molecule called peripheral lymph node addressin (PNAd) which is found on HEV. In addition, the chemokines CCL19 and CCL21 are expressed on HEV, interacting with CCR7 on lymphocytes and thereby triggering activation of the integrin lymphocyte function-associated antigen 1 (LFA-1) and lymphocyte arrest in HEV [Warnock et al., 1998]. In contrast, HEV in PP do not at all and in MLN only partially express PNAd in the lumen. Instead, they express high levels of mucosal addressin cell adhesion molecule-1 (MAdCAM-1). MadCAM-1 can interact with both L-Selectin and the $\alpha_4\beta_7$ integrin [Berlin et al., 1993]. $\alpha_4\beta_7$ which is also called leukocyte Peyer's patches adhesion molecule 1 (LPAM-1) is expressed only at low levels on naïve lymphocytes but plays a critical role in their migration to MLN and PP [Wagner et al., 1996]. Table 1.2 shows the most important molecules involved in lymphocyte migration.

Target tissue	**PLN**	**MLN / PP**	**Skin**	**Intestinal lamina propria**
Cell subset	Naïve	Naïve	Effector	Effector
Tethering/ Rolling	L-Selectin *PNAd*	L-Selectin *PNAd* $\alpha_4\beta_7$ *MAdCAM-1*	P-/E-Lig *P-/E-Selectin*	$\alpha_4\beta_1$ / $\alpha_4\beta_7$ *MAdCAM-1*
Integrin activation	CCR7 *CCL19/21*	CCR7 *CCL19/21*	CCR4 *CCL17* CCR10 *CCL27*	CCR9 *CCL25* Others in colon?
Firm adhesion	LFA-1 *ICAM-1*	LFA-1 *ICAM-1* $\alpha_4\beta_7$ *MAdCAM-1*	LFA-1 *ICAM-1* $\alpha_4\beta_1$ *VCAM-1*	$\alpha_4\beta_7$ *MAdCAM-1*

Table 1.2: Overview of steady-state lymphocyte homing receptors and endothelial vascular addressins. Corresponding pairs of migratory molecules are shown with endothelial structures depicted in *italic.* The table describes the situation in the non-inflamed steady state.

1.3.2.2 Homing of antigen-experienced T and B cells

Upon activation in secondary lymphoid organs, lymphocytes change their set of surface migratory molecules and then migrate more efficiently to nonlymphoid tissue and sites of inflammation. In addition, antigen-experienced T and B cells often display tissue specificity to improve their chances of reencountering their cognate antigen.

T and B cells that migrate to the small intestine express the integrin $\alpha_4\beta_7$ and the chemokine receptor CCR9 [Zabel et al., 1999]. These traffic molecules, as well as their corresponding ligands are essential for efficient lymphocyte homing to the small bowel [Hamann et al., 1994]. The main $\alpha_4\beta_7$ ligand, MAdCAM-1 and the CCR9 ligand CCL25 are expressed on intestinal lamina propria (LP) venules and epithelial cells, respectively. Unlike CCR9, $\alpha_4\beta_7$ is also important for lymphocyte migration into the colon. Other yet unknown chemoattractant pathways have been suggested for T and B cell migration to colon mucosa [Kunkel and Butcher, 2002].

In contrast, skin-tropic lymphocytes express E- and P-selectin ligands (E-Lig, P-Lig) as well as CCR4 and/or CCR10. Non-inflamed skin venules constitutively express their corresponding partners E- and P-selectin as well as CCL17 and CCL27. These traffic molecules are critical for efficient lymphocyte homing into the skin.
The best understood tissue-selective homing pathways are in the intestine and skin, but there may be other selective migration streams, such as to the lungs, joints and central nervous system [Salmi et al., 1992]. Indeed, the large number of leukocyte-adhesion receptors and endothelial counterparts as well as chemokines and corresponding receptors allow hundreds of possible combinations, comparable to a postal code system. In this context, endothelial adhesion molecules with a predominant role in tissue-specific cell migration are often called “vascular addressins” while their counterparts on T and B cells are called “homing receptors” [Butcher and Picker, 1996].

1.3.2.3 Tissue-specific homing: imprinting mechanisms

Previous studies have suggested that lymphocytes acquire homing receptors randomly or independently of their site of activation [Davenport et al., 2000]. However, a considerable amount of evidence indicates that the tissue where T and B cells encounter their cognate antigen influences the traffic pattern they acquire. Several studies showed that T cells activated in MLN express higher levels of $\alpha_4\beta_7$ and CCR9 while skin-specific receptors E- and P-Lig are preferentially induced in skin-draining

PLN [Campbell and Butcher, 2002]. Thus, local environments within lymph nodes differentially direct T- and B-cell expression of adhesion and chemoattractant receptors.

Subsequent reports have shown that DC underlie the differential regulation of homing receptors in mesenteric versus peripheral lymph nodes and that DC are sufficient to imprint tissue-specific homing on T and B cells. DC from PP [Mora et al., 2003] or MLN [Johansson-Lindbom et al., 2003; Mora et al., 2005] induce $\alpha_4\beta_7$ and CCR9 on activated T cells. In contrast, high levels of skin-specific E- and P-Lig were induced by DC from PLN [Dudda et al., 2004]. Regarding their tissue-specific homing commitment, T and B cells can also be reprogrammed when they are re-stimulated under different homing conditions [Mora et al., 2005].

Recently, it has been demonstrated that the selective capacity of intestinal DC to generate gut-specific lymphocytes involves retinoic acid (RA). RA is an oxidative metabolite of dietary vitamin A (retinol) and is essential for numerous physiological processes including embryonic growth and development, reproduction, maintenance of epithelial surfaces and immunity. Its major physiologically active metabolite, all-*trans* RA, is sufficient to induce $\alpha_4\beta_7$ and CCR9 on activated T cells even in the absence of DC [Iwata et al., 2004] which then effectively home to the GI tract. Blocking RA-receptors significantly reduces the induction of $\alpha_4\beta_7$ and CCR9 by intestinal DC. Compatibly, intestinal DC are not homogeneous in their capacity to imprint gut-specificity as recent studies showed that only $CD103^{pos}$ MLN-derived DC imprint gut homing on T cells [Johansson-Lindbom et al., 2005]. That is consistent with their higher expression of retinol aldehyd dehydrogenase (RALDH) messenger RNA (mRNA), a key enzyme in the synthesis of retinoic acid [Coombes et al., 2007].

Upon stimulation by intestinal DC, B cells are not only imprinted with a gut-homing phenotype, upregulating $\alpha_4\beta_7$ and CCR9, but also with a specific set of antibodies. Intestinal DC induce IgA class-switching on activated B cells in a partially RA dependent manor [Mora et al., 2006]. IgA is the principle immunoglobulin isotype in secretions, the most important being those in the endothelium lining the GI and respiratory tract. In contrast to T cells, gut-specific B cells have been implicated with CCR10 and its ligand CCL28 which is expressed by most mucosal epithelial cells. However, it is still heavily debated whether IgA-secreting B cells need both CCR9 and CCR10 for optimal migration to the small bowel or whether these chemokines play a redundant role in B cell gut-homing [Hieshima et al., 2004; Feng et al., 2006].

Recent studies have shown that vitamin D3, which is mainly produced in sun-exposed skin, induces T cell tropism for the skin [Sigmundsdottir et al., 2007]. With vitamin A which enters the body exclusively through the diet being linked to gut migration, evolution has cleverly co-opted tissue-specific, external environmental stimuli to imprint homing. DC play a dual role in this process, processing both local antigen and the environmental signals themselves for presentation to lymphocytes [Sigmundsdottir and Butcher, 2008]. Adapted to the GI tract, antigens sampled in the lamina propria are taken up by $CD103^{pos}$ DC and are transported via draining lymphatic vessels to MLN or PP. Here, by simultaneous presentation of antigen and co-stimulatory molecules as well as secretion of RA, DC imprint gut-homing specificity on T and B cells. However, it is poorly understood how DC themselves are educated to acquire tissue-specific imprinting potential such as their RA-secreting capacity.

1.4 Pattern-recognition receptor ligands in immunotherapy

The increasing knowledge of innate immune cells with their pattern-recognition receptors and their central role in triggering inflammatory signals has moved PRR in the focus of immunotherapy. Their ligands make up attractive immunomodulators in clinical use for different reasons: In contrast to distinct cytokines like IFN-α or IL-1, PRR ligands lead to a co-ordinated immune response which resembles the natural situation. Secondly, selective activation of professional APC and subsequent induction of antibody production and cellular immunity make PRR ligands to an eligible adjuvant for both prophylactic and therapeutic vaccinations. Furthermore, induction of a T_H1-weighted immune response with potent production of type I IFN defenses against intracellular pathogens and prevents allergic reactions which are based upon T_H2-directed immune conditions. Finally, PRR ligand-induced T_H1-accentuated stimulation of adaptive immunity supports immunological defense of host tumor cells.

Therefore, PRR ligands are the subject of intensive research in immunotherapy and many have found their way to clinical trials or have been established in routine therapies. Because their function and ligands have been characterized in most detail, Toll-like receptors have played a pioneer role in different approaches in the human system. Many TLR ligands are now tested as vaccine adjuvants to boost long-lasting adaptive immune responses. Synthetic TLR4 ligands are part of the commercial hepatitis B virus vaccine Fendrix [Baldridge et al., 2004] and CpG-ODN are used in combination with the vaccines Engerix-B1 (for use against hepatitis B virus) and Fluarix1 (for use against influenza) in late phase II trials [Cooper et al., 2004; Cooper et

al., 2004]. Because of their potent induction of type I IFN and thereby profound antiviral effect, TLR7 and 8 agonists have especially been used to target infectious diseases. Synthetic TLR7 agonists showed promising effects in chronic hepatitis C infections [Horsmans et al., 2005] while imiquimod, another synthetic TLR7 ligand now approved for topical application in the treatment of papilloma-virus-induced genital wards, had initially contributed to the increased interest in the TLR field [Beutner et al., 1998].

Furthermore, TLR ligands are an integral part of anti-cancer immunotherapy research and here our work group is especially interested in the function of TLR7 and 9 ligands in murine tumor models [Bourquin et al., 2007; Bourquin et al., 2008; Wurzenberger et al., 2009]. TLR ligands have been used as anti-cancer monotherapy, in combination with anti-tumor antibodies or as adjuvants in cancer vaccines. In monotherapy, unspecific activation of the immune system can fight tumor growth which leaded to the approved use of imiquimod in superficial basal cell carcinoma [Geisse et al., 2004]. Inducing type I IFN and IL-12, TLR agonists can indirectly increase antibody-dependent cellular cytotoxicity by macrophages and natural killer (NK) cells thereby boosting the effect of anti-tumor antibodies. Two clinical trials have shown encouraging results combinating CpG-ODN with rituximab (an anti-CD20 monoclonal antibody) in the treatment of patients with non-Hodgkin's lymphoma [Friedberg et al., 2005]. Probably the most promising approach in tumor immunology is to immunize cancer patients with autologous, patient-derived DC loaded with tumor antigens *ex vivo*. TLR agonists promote maturation of DC that can then present cancer-specific antigens and produce type I IFN and inflammatory cytokines, which in turn promote direct cellular effector functions. Both mechanisms might participate in breaking the tolerance towards cancer cells that often secrete immunosuppressant cytokines, and eventually lead, in certain cases, to activation of tumor-specific adaptive immunity [Romagne, 2007]. However, recent studies suggest that tumor cells also bear TLR and that their signaling may promote tumor growth and immune evasion [Huang et al., 2005; Kelly et al., 2006].

1.5 Objectives

The ability of T and B cells to traffic to distinct tissue compartments is vital for the maintenance of immune homeostasis and the generation of an effective immune response. It is mediated by adhesion molecules, also called homing receptors, on lymphocytes that have cognate ligands at peripheral or mucosal sites such as the gut. Intestinal lymphocytes express the gut-homing receptors $\alpha_4\beta_7$ integrin and chemokine

receptor CCR9 that mediate lymphocyte migration to the gastrointestinal tract, thus promoting regional immunity. The imprinting of $\alpha_4\beta_7$ and CCR9 on T and B cells is effected by intestinal dendritic cells during T cell activation. The expression of the correct pattern of adhesion molecules is crucial for mounting efficient gut-tropic immune responses but also the induction of chronic inflammatory autoimmune diseases such as Crohn's disease and ulcerative colitis.

From a therapeutic perspective, controlling the expression pattern of adhesion molecules that direct lymphocyte homing to the gastrointestinal tract represents an attractive tool for the manipulation of localized immune and inflammatory responses. Understanding the regulation of homing receptors such as $\alpha_4\beta_7$, is thus a vital prerequisite for their therapeutical utilization. Little is known however, about the expression patterns of these gut-homing receptors during a systemic inflammatory state. Using Toll-like receptor ligands to induce innate immune activation such as is seen during infection, this work was designed to answer the following questions:

(1) Does stimulation of Toll-like and other innate immune receptors change the gut-homing phenotype of T and B cells?
(2) If so, which are the cellular and molecular mechanisms involved?
(3) What is the resulting effect of Toll-like receptor stimulation on lymphocyte migration patterns *in vivo*?

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Technical equipment

Balance (LP 6209)	Sartorius, Göttingen, Germany
Cell culture CO2 incubator (BD 6220)	Heraeus, Hanau, Germany
Cell culture laminar flow	Heraeus, Hanau, Germany
Centrifuge 5417 R	Eppendorf, Hamburg, Germany
Centrifuge 5424	Eppendorf, Hamburg, Germany
DynaMag 15/50 magnet	Invitrogen Dynal, Carlsbad, USA
FACSCalibur, FACSCanto II	Becton Dickinson, San Jose, USA
LightCycler 2.0 System	Roche, Mannheim, Germany
Microscope Axiovert 25	Zeiss, Jena, Germany
MiniMACS, QuadroMACS	Miltenyi, Bergisch Gladbach, Germany
Mithras LB940 multilabel plate reader	Berthold, Bad Wildbad, Germany
Multifuge 3L-R	Heraeus, Hanau, Germany
Nanodrop ND-1000	NanoDrop, Wilmington, USA
Omnifuge 2 ORS	Heraeus, Hanau, Germany
pH meter	WTW, Weilheim, Germany
Power Supply 200/2.0	Biorad, Munich, Germany
Refrigerators (4°C, -20°C, -80°C)	Thermo Scientific, Waltham, USA
Shaker	NeoLab, Heidelberg, Germany
Thermocycler T3	Biometra, Göttingen, Germany
Thermomixer	Eppendorf, Hamburg, Germany
Vortex VF2	Janke & Kunkel, Staufen, Germany

2.1.2 Chemicals, reagents and buffers

Agarose LE	Biozym, Hess. Oldendorf, Germany
Aqua ad injectabilia	Braun AG, Melsungen, Germany
Bovine serum albumine (BSA)	Sigma Aldrich, Steinheim, Germany
Collagense D	Roche, Mannheim, Germany
Chloroform	Sigma Aldrich, Steinheim, Germany
DNase II	Roche, Mannheim, Germany
Dimethyl sulfoxide (DMSO)	Sigma Aldrich, Steinheim, Germany

Dulbecco's PBS (1x)	PAA, Pasching, Germany
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich, Steinheim, Germany
FACSFlow, FACSSafe	Becton Dickinson, San Jose, USA
Heparin-Natrium 25000 I.E./5 ml	Braun AG, Melsungen, Germany
Isoflurane (Forene®)	Abbott, Zug, Switzerland
Isopropanol (70 Vol%)	Apotheke Innenstadt, LMU Munich
Paraformaldehyde (PFA)	Sigma Aldrich, Steinheim, Germany
Sodium azide (NaN3)	Sigma Aldrich, Steinheim, Germany
Sodium chloride (NaCl 0.9%)	Apotheke Innenstadt, LMU Munich
Trypan blue Sigma	Aldrich, Steinheim, Germany
Trypsin (10x)	PAA, Pasching, Austria

MACS buffer	Cell Fixation buffer
2 mM EDTA	2% PFA
10% FCS	in PBS
in PBS	

ELISA coating buffer	ELISA assay diluent
0.2 M sodium phosphate	10% FCS
in water	in PBS
pH 6.5	pH 7.0

ELISA wash buffer
0.05% Tween 20
in PBS

2.1.3 Cell culture materials, reagents and media

β-Mercaptoethanol	Sigma-Aldrich, Steinheim, Germany
DOTAP (N-[1-(2,3-Dioleoyloxy)propyl]-3N-trimethylammonium-methylsulfate	Roche, Mannheim, Germany
Dulbecco's modified Eagle´s medium (DMEM), high glucose	PAA, Pasching, Austria
Fetal calf serum (FCS)	GibcoBRL, Karlsruhe, Germany
Hank's balanced salt solution (HBSS)	PAA, Pasching, Austria
Iscove's modified Dulbecco's medium (IMDM)	PAA, Paschin, Austria

L-glutamine 200mM	PAA, Pasching, Austria
MEM-NEAA (non-essential amino acids)	GibcoBRL, Karlsruhe, Germany
Phosphate buffered saline (PBS)	PAA, Pasching, Austria
Penicillin / Streptomycin (100x)	PAA, Pasching, Austria
Roswell Park Memorial Institute (RPMI) 1640 medium	PAA, Pasching, Austria
Sodium pyruvate	PAA, Pasching, Austria

RPMI complete medium	IMDM complete medium
10% FCS	10% FCS
2 mM L-glutamine	2 mM L-glutamine
100 IU/ml penicillin	100 IU/ml penicillin
100 µg/ml streptomycin	100 µg/ml streptomycin
1 mM sodium pyruvate	1 mM sodium pyruvate
1% non-essential amino acids (MEM-NEAA)	1% MEM-NEAA
3.75 x 10-4 % β-mercaptoethanol	in IMDM
in RPMI 1640	

DMEM complete medium	Cryo medium
10% FCS	50% DMEM complete medium
2 mM L-glutamine	40% FCS
100 IU/ml penicillin	10% DMSO
100 µg/ml streptomycin	

Cytokines and growth factors

anti-CD3ε (clone 500A2, syrian hamster IgG2,κ)	BD Biosciences, San Diego, USA
Flt3-Ligand, human recombinant	tebu-bio,Offenbach, Germany
granulocyte-macrophage colony-stimulating factor (GM-CSF), mouse recombinant	tebu-bio, Offenbach, Germany
Interleukin 1-β, mouse recombinant	Biolegend, San Diegeo, USA
IFN-α, human, recombinant	InvivoGen, San Diego, USA
Interleukin-2, mouse recombinant	R&D Systems, Wiesbaden, Germany
Interleukin-4, mouse recombinant	R&D Systems, Wiesbaden, Germany
Interleukin-6, mouse recombinant	R&D Systems, Wiesbaden, Germany
Interleukin-10, mouse recombinant	R&D Systems, Wiesbaden, Germany
Interleukin-12p40, mouse recombinant	BioLegend, San Diego, USA
Interleukin 17, mouse recombinant	Biolegend, San Diegeo, USA

Blocking antibodies:

Description	Isotype	Clone	Distributor
anti-IL-2	Rat IgG_{2a}	JES6-1A12	R&D Systems
anti-IL-6	Rat IgG_1	MP5-20F3	R&D Systems
anti-IL-10	Rat IgG_1	JES052A5	R&D Systems
anti-IL12p40	Rat IgG_{2a}, κ	C17.8	BioLegend
anti-IFN-α	Rat IgG_1	RMMA-1	PBL Biomedical Lab
anti-TGF-β	Rat IgG_{2a}	A75-2	BD Biosciences
anti-TNF-α	Rat, IgG_1, κ	MP6-XT22	BioLegend

Disposable plastic materials for cell culture experiments were purchased from Becton Dickinson (Heidelberg, Germany), Bibby Sterilin (Stone, Staffordshire, Great Britain), Corning (Corning, USA), Eppendorf (Hamburg, Germany), Falcon (Heidelberg, Germany), Greiner (Frickenhausen, Germany), Henke-Sass Wolf (Tuttlingen, Germany), Nunc (Rochester, USA) or Sarstedt (Nümbrecht, Germany).

2.1.4 Oligonucleotides, TLR ligands and other stimuli

CL097	InvivoGen, San Diego, USA
CpG 1826 (CpG)	Coley, Langenfeld, Germany
Curdlan	Roche, Mannheim, Germany
Flagellin	InvivoGen, San Diego, USA
Lipopolysaccharide (LPS) (*Salmonella enterica ssp. enterica*)	Sigma, St. Louis, USA
PAM3CysSerLys4 (PAM3CSK)	tebu-bio, Offenbach, Germany
polyI:C	InvivoGen, San Diego, USA
5'-triphosphate 2.2ds RNA	Eurogentec, Köln, Germany
9.2s double right RNA	CureVac, Tübingen, Germany

Listing of all oligonucleotides used in this work:

Description	Nucleotide sequence (5' --> 3')
CpG 1826	TCCATGACGTTCCTGACGTT
9.2s double right RNA	UGUCCUUCAAUGUCCAA
Poly A 20 RNA	AAA AAA AAA AAA AAA AAA AA
5'-triphosphate 2.2ds RNA	GCAUGCGACCUCUGUUUGA

2.1.5 Kits

Cell Trace CFSE Cell Proliferation Kit	Molecular Probes, Eugene, USA
Cell Tracker Violet BMQC dye	Molecular Probes, Eugene, USA

RNA isolation, reverse transcription, qRT-PCR

High pure RNA isolation kit	Roche, Mannheim, Germany
Transcriptor first strand cDNA synthesis kit	Roche, Mannheim, Germany
LightCycler TaqMan Master kit	Roche, Mannheim, Germany
Universal ProbeLibrary	Roche, Mannheim, Germany

Magnetic-activated cell sorting

CD3 Dynabeads, murine	Invitrogen Dynal, Carlsbad, USA
CD8a Micro Beads, murine	Miltenyi, Bergisch Gladbach, Germany
CD8 T Cell Isolation Kit, murine	Miltenyi, Bergisch Gladbach, Germany
CD11c Micro Beads, murine	Miltenyi, Bergisch Gladbach, Germany
CD19 Micro Beads, murine	Miltenyi, Bergisch Gladbach, Germany
CD45R/B220 Micro Beads, murine	Miltenyi, Bergisch Gladbach, Germany

Cytokine ELISA sets

IL-2 murine	BD Bioscences, San Diego, USA
IL-6 murine	BD Bioscences, San Diego, USA
IL-10 murine	BD Bioscences, San Diego, USA
IL-12p40 murine	BD Bioscences, San Diego, USA

Cytokine ELISA antibodies

Detection of murine IFN-α:

Capture Ab: Anti-IFN-α (RMMA-1)	PBL, New Brunswick, USA
Detection Ab: Anti-IFN-α (polyclonal, rabbit anti mouse)	PBL, New Brunswick, USA
HRP-conjugated F(ab´)2 fragments (donkey anti rabbit)	Biomeda, Foster City, USA

2.1.6 FACS antibodies

Description	Isotype	Clone	Distributor
anti-CD3	Rat IgG_{2b}	17A2	BD/ Pharmingen
anti-CD4	Rat (DA) IgG_{2a}, κ	RMA4-5	BD/ Pharmingen
anti-CD8a	Rat (LOU/Ws1/M) IgG_{2a}, κ	53-6.7	BD/ Pharmingen
anti-CD11b	Rat (DA) IgG_{2b}, κ	M1/70	BD/ Pharmingen
anti-CD11c	Armenian Hamster IgG_{1a}, λ2	HL3	BD/ Pharmingen
anti-CD19	Rat, IgG_{2a}, κ	1D3	BD/ Pharmingen
anti-CD25	Rat (Lewis) IgM, κ	7D4	BD/ Pharmingen
anti-CD45R/B220	Rat IgG_{2a}, κ	RA3-6B2	BD/ Pharmingen
anti-CD69	Armenian Hamster IgG_1, λ3	H1.2F3	BD/ Pharmingen
anti-CD80 (B7-1)	Armenian Hamster IgG_2, κ	16-10A1	BD/ Pharmingen
anti-CD86 (B7-2)	Rat (Louvain) IgG_{2a}, κ	GL1	BD/ Pharmingen
anti-foxp3	Rat IgG_{2b}, κ	TH6	BD/ Pharmingen
anti-LPAM-1 ($\alpha_4\beta_7$)	Rat IgG_{2a}, κ	DATK32	BD/ Pharmingen
anti-MHC II	Rat IgG_{2b}	NIMR-4	Southern Biotech

2.1.7 Software

Adobe Illustrator CS3	Adobe System, San Jose, USA
CellQuest	BD Biosciences, San Diego, USA
Endnote X2	Thompson Reuter, Carlsbad, USA
FlowJo	Tree Star, Ashland, USA
Microsoft Office	Mircosoft, Redmond, USA
SPSS 16	SPSS, Chicago, USA

2.2 Animal experimentation

2.2.1 Animals

Female C57BL/6 mice were purchased from Harlan-Winkelmann (Borchen, Germany). IL-6-deficient, IL-12p40-deficient and IFN-γ-deficient mice were purchased from Jackson Laboratories (Bar Harbor, MA). IL-10-deficient mice, IFN-α receptor (IFNaR)-deficient mice, IRF3/7-deficient mice and p50-deficient mice were kindly provided by Prof. J. Heesemann

(Max von Pettenkofer Institute for Hygiene and Medical Microbiology, Ludwig-Maximilian University Munich, Germany), Prof. H.J. Anders (Medizinische Poliklinik Innenstadt, Ludwig-Maximilian University Munich, Germany), PD Dr. A. Krug (Department of Internal Medicine II, Klinikum Rechts der Isar, Technical University Munich, Germany) and PD Dr. S. Frantz (Medizinische Klinik und Poliklinik I, University Würzburg, Germany). Mice were 8-12 weeks of age at the onset of experiments. Animal studies were approved by the local regulatory agency (Regierung von Oberbayern, Munich, Germany).

2.2.2 Organ and single cell preparation

2.2.2.1 Isolation of splenocytes

Mice were anesthetized with isoflurane and sacrificed by cervical dislocation. Spleens were resected and pressed through a 40 µm cell strainer to disintegrate tissue structure. Single cell solutions were centrifuged (400 G, 7 minutes) and resuspended in erythrocyte lysis buffer. Red blood cell debris was removed by a second centrifugation step. Splenocytes were kept in IMDM complete medium for subsequent experiments.

2.2.2.2 Preparation of lymph node and Peyer's patch cells

Mice were anesthetized with isoflurane and sacrificed by cervical dislocation. Cervical, brachial, axillary, inguinal and popliteal lymph nodes pooled as peripheral lymph nodes as well as mesenteric lymph nodes and Peyer's patches were resected. Tissues were enzymatically digested with collagenase D (1 mg/ml) and DNase I (0.05 mg/ml) in IMDM complete medium at 37°C and moderate stirring for 25 to 35 minutes. The digested tissues were passed through a 40 µm-pore cell strainer. Single-cell suspensions were washed with PBS and were then kept in IMDM complete medium for later experiments.

2.2.2.3 Isolation of lung, liver, small bowel and colonic cells

Mice were anesthetized with isoflurane and sacrificed by cervical dislocation. Small bowel, colon, lung and liver were resected. For the intestine, remaining feces, complete mesenteries and Peyer's patches were carefully removed. In a first step, organs were disintegrated manually. Then, tissues were enzymatically digested and passed through a cell strainer as described above (see chapter 2.2.2.2). Digestion and separation with the cell strainer were

repeated once. Red blood cells were then lysed with erythrocyte lysis buffer. Single-cell solutions were kept in IMDM complete medium for subsequent experiments.

2.2.2.4 Preparation of bone marrow cells

Mice were anesthetized with isoflurane, sacrificed by cervical dislocation and femur and tibia were bilaterally dissected. Remaining muscle tissue was removed and bones were externally cleaned with isopropanol. Bone marrow was extracted by flushing RPMI complete medium through the bones and passing the cell suspension through a 40 µm cell strainer. After resuspension of centrifuged cells, erythrocytes were lysed by PharmLyse ammonium chloride buffer. Cells were then washed with PBS and kept in RPMI complete medium for later experiments.

2.2.3 Immunostimulation of mice

To determine the role of Toll-like and other pattern-recognition receptors in tissue-specific regulation of the homing receptor $\alpha_4\beta_7$, mice were treated with different immunostimulants. On the one hand, 100 µg CpG-oligodesoxyribonucleotides (ODN) were solved in 120 µl PBS and injected subcutaneously. On the other hand, 20 µg oligoribonucleotide (ORN) 9.2dr with a phosphothiorated (PTO) ribose-backbone to prevent enzymatic degredation, were incubated with 100 µg DOTAP - a liposomal transfection reagent - and 40 µl PBS for 20 min and were then injected intravenously into the retro-bulbar venous plexus. In some experiments, CpG-ODN were injected intraperitoneally or intravenously. These mice were treated with 100 µg CpG solved in 100 µl PBS or 20 µg CpG complexed with 100 µg DOTAP and 40 µl PBS, respectively. 48 hours after the injection, mice were sacrificed and spleen, PLN, MLN and PP were preparated as described above (see chapter 2.2.2). For analysis of $\alpha_4\beta_7$ surface expression, cells were stained with fluorochrome-coupled antibodies and analyzed by flow cytometry.

2.2.4 Tumor experiments

The experimental use of DC as vectors for anti-tumor and infectious disease immunotherapy is limited by their trace levels and accessibility in normal tissue and terminal state of differentiation. For this reason we used a murine B16 melanoma cell line secreting the hematopoietic growth factor Flt3-Ligand (B16-FL, see chapter 2.3.2). Mice were subcutaneously injected with 5×10^6 B16-FL tumor cells. Secondary lymphoid organs were harvested after eleven to 14 days. At this time spleens were 3- to 5-fold enlarged over that of untreated mice because of extramedullary hematopoesis with all lineages of hematopoietic

cells fully represented [Shurin et al., 1997]. Tumor-secreted Flt3-Ligand provoked a time-dependent and reversible accumulation of DC in spleen, bone marrow, lymph nodes and liver. These cells exhibited veiled and dendritic processes and were as efficient as rare, mature DC isolated from the spleens of untreated mice at presenting antigen to T cells, or in priming an antigen-specific T cell response *in vivo* [Maraskovsky et al., 1996]. Thus, Flt3-Ligand secreted by the B16-FL melanoma cell line proved to be a valuable tool to expand the number of functionally mature DC *in vivo* for utilization in subsequent experiments.

Single-cell solutions were preparated from secondary lymphoid organs as described above and dendritic cells were sorted by magnetic cell sorting. Purified DC from spleen, PLN, MLN and PP were kept in IMDM complete medium for subsequent experiments.

2.2.5 Lymphocyte *in vivo* migration assay

In order to assess *in vivo* migration patterns of lymphocytes after CpG stimulation, fluorescence-marked splenocytes were adoptively transferred into recipient mice. Therefore, splenocytes were cultured in IMDM complete medium with or without added CpG-ODN (4 µg/ml). After 48 hours, cells were incubated for 15 minutes with 1 mM or 0.1 mM CFSE at 4°C. Thereafter, cells were centrifuged over complete RPMI medium and extensively washed. 2 - 4 x 10^7 cells from both preparations (stimulated $CFSE^{high}$ and unstimulated $CFSE^{low}$ splenocytes) were mixed and injected intravenously into each recipient mouse. An aliquot was saved to assess the input ratio. Four to six hours after injection, recipient tissues were harvested. For the intestine, complete mesenteries and PP were carefully removed before further preparation. Single cell suspensions were generated by mechanical disintegration, following enzymatic digestion and filtration through cell strainers as described earlier. Each tissue cell preparation was analyzed for adoptively transferred lymphocyte subpopulations with flow cytometry and $CFSE^{high}$ / $CFSE^{low}$ ratios were measured. The homing index (HI) was calculated as the ratio of $[CFSE^{high}]_{tissue}$ / $[CFSE^{low}]_{tissue}$ to $[CFSE^{high}]_{input}$ / $[CFSE^{low}]_{input}$.

2.2.6 T cell adoptive transfer assay

To assess antigen-specific T cell responses *in vivo,* OT-I transgenic T cells were adoptively transferred into naïve recipient mice. In OT-I transgenic mice, all $CD8^{pos}$ T cells have the same T cell receptor that recognizes the model antigen ovalbumin. $CD8^{pos}$ T cells were

purified by magnetic sorting (see chapter 2.3.4) from splenocyte single cell suspensions of wild-type and OT-I transgenic mice. OT-I $CD8^{pos}$ T cells were fluorescently labeled with CFSE while WT $CD8^{pos}$ T cells were marked with the fluorochrome Cell Tracker Violet (see chapter 2.4.1.4). The labeled $CD8^{pos}$ T cells were mixed at equal numbers and 5 - 10 x 10^6 cells were injected intravenously into naïve WT recipient mice. One day after the adoptive T cell transfer, mice were immunized intraperitoneally with 100 µg LPS and 500 µg endotoxin-free ovalbumin. Two to three days after immunization, mice were killed and secondary lymphoid organs were obtained. The surface expression of $\alpha_4\beta_7$ on antigen-specifically activated OT-I $CD8^{pos}$ T cells and unspecific WT $CD8^{pos}$ T cells was analyzed by flow cytometry.

2.3 Cell culture

2.3.1 General culture conditions and cell viability testing

Culture of all cell lines was carried out in incubators at 37°C, 5% CO_2/air mixture and 95% atmospheric humidity using disposable tissue culture flasks. All cell and reagent manipulations were performed using sterile technique under *a* laminar flow hood. Cell numbers and viability were tested by trypan blue exclusion. With decreasing membrane integrity of dying cells the dye is absorbed where as trypan is not able to penetrate living cells. Thus, dead cells show unique blue intracellular staining under the light microscope. After appropriate dilution cell numbers were determined by counting in a Neubauer hemocytometer.

2.3.2 B16-FL tumor cell line

The B16 melanoma cell line expressing Flt3-Ligand (B16-FL) was provided by Prof. T. Mempel (Bosten, USA). These B16 melanoma cells of C57BL/6 background were prepared by transfection of murine Flt3-Ligand cDNA using the retroviral vector MFG [Shi et al., 1999]. The cell line was cultured in DMEM complete medium and repeatedly checked for signs of cell death and microbial contamination by light microscopy. Medium was substituted every two to three days according to cell growth. Additionally, tumor cells were regularly transferred to new culture flasks. Therefore, adherent growing cells were detached with 1% trypsin-EDTA solution, washed with PBS and then re-disseminated. A possible mycoplasmatic contamination was excluded by frequently repeated tests.

2.3.3 Generation of bone marrow-derived dendritic cells

Immature murine DC were raised from bone marrow as described previously (Brasel et al., 2000; Brawand et al., 2002), with some modifications. Preparation of bone marrow cells was described above (see chapter 2.2.2.4). Following washing with PBS, cells were diluted to 1 x 10^6 cells/ml in RPMI complete medium. To generate myeloid DC (GM-DC) GM-CSF (20 ng/ml) and interleukin 4 (20 ng/ml) were added to the culture assay. After four days, fresh medium supplemented with GM-CSF and IL-4 (40% of original volume) was added to the culture. Differentiated dendritic cells were harvested on day 7 to 8. Therefore, culture medium was gathered and flasks were rinsed with cold PBS to detach loosely adherent cells. DC ($CD11c^{pos}$ cells) generally represented > 75% of the preparation.

A second protocol was used to generate a mixture of myeloid and plasmacytoid dendritic cells. Here, bone marrow precursors were cultured at 2 - 3 x 10^6 cell/ml in complete RPMI medium supplemented with recombinant Flt3-Ligand (20 ng/ml). Culture and harvesting of differentiated cells (FL-DC) were identical to the GM-DC protocol. Dendritic cells ($CD11c^{pos}$) generally represented > 80% of the preparation's cells with a typical proportion of 40% being pDC ($B220^{pos}$) and 60% being mDC ($B220^{neg}$, $CD11b^{pos}$). For subsequent sorting of DC subpopulations, see chapter 2.3.4.

2.3.4 Cell purification with magnetic-activated cell sorting

Magnetic-activated cell sorting (MACS) is a sorting technique used to isolate viable and functionally active cells with minimal interference with subsequent experiments. Cells are labeled with magnetic beads, superparamagnetic particles of approximately 50 nanometers in diameter. These are composed of a biodegradable matrix; thus it is not necessary to remove them from purified cells after the separation process. The magnetic beats are coated with antibodies specific for the desired surface antigen. Hence, the beads attach to cells expressing this particular antigen. Labeled cells are then transferred into a plastic column which is placed in a high-gradient magnetic field induced by a strong permanent magnet. The field is strong enough to retain cells attached to the superparamagnetic beads while unlabeled cells pass through and can be collected. After removal from the magnetic field, retained cells are eluted from the column. Using this method, cells can be sorted positively or negatively. Positive selection means that the desired target cells are magnetically labeled and isolated as the magnetically retained cell fraction. Negative selection or so-called untouched isolation is performed by depletion of undesired cells. Non-target cells are

magnetically labeled and eliminated from the cell mixture. The non-magnetic, untouched cell fraction contains the target cells.

In this work, materials and reagents were used from Miltenyi Biotec if not stated otherwise. $CD8^{pos}$ T cells were negatively sorted using a $CD8^{pos}$ T cell isolation kit. B cells were purified by positive sorting with CD19 beads. Plasmacytoid DC were sorted from FL-DC by positive selection using B220 microbeads, while mDC differentiated by Flt3L were obtained by depleting $B220^{pos}$ plasmacytoid DC from FL-DC. Splenic DC were separated from total splenocytes using CD11c beads.

According to the manufacturer's protocol, cells were washed with MACS buffer then incubated with the microbeads at 4°C for 15 min and washed again twice. LS columns were rinsed with 3 ml MACS buffer, loaded with labeled cells diluted in 2 ml of buffer and then placed in a MACS separator magnet. Columns were washed three times with 3 ml MACS buffer. Effluent was gathered as negative fraction. After removal from the magnetic field, positive cells were eluted by flushing the column with 2 ml MACS buffer using the provided plunger. For further enrichment of desired cells, previous steps were repeated with already purified target cells. According to remaining cell numbers, LS or MS columns were used. MS columns were rinsed, loaded, twice washed and eluted with 1ml MACS buffer each. Splenic DC were depleted using LD columns. After rinsing with 2.5 ml MACS buffer, LD columns were loaded with CD11c-labeled splenocytes diluted in 4 ml buffer. Columns were washed once with 2 ml buffer and negative flowthrough was re-applied on another LD column. Previous steps were repeated and total effluent was gathered as DC-depleted splenocytes.

Purity of magnetically sorted T and B lymphocytes was > 95 %. After DC-depletion, residual dendritic cells made up < 0.045 % of total splenic cells. Figure 2.1 shows defined cell populations after lymphocyte purification and DC depletion, respectively. T cells are gated on the lineage marker CD3 and CD8 to define cytotoxic T cells (Figure 2.1 A). B cells are shown as CD19 / B220 double-positive cells. Splenic DC are gated on their lineage marker CD11c and B220 to distinguish plasmacytoid and myeloid dendritic cells (Figure 2.1 B).

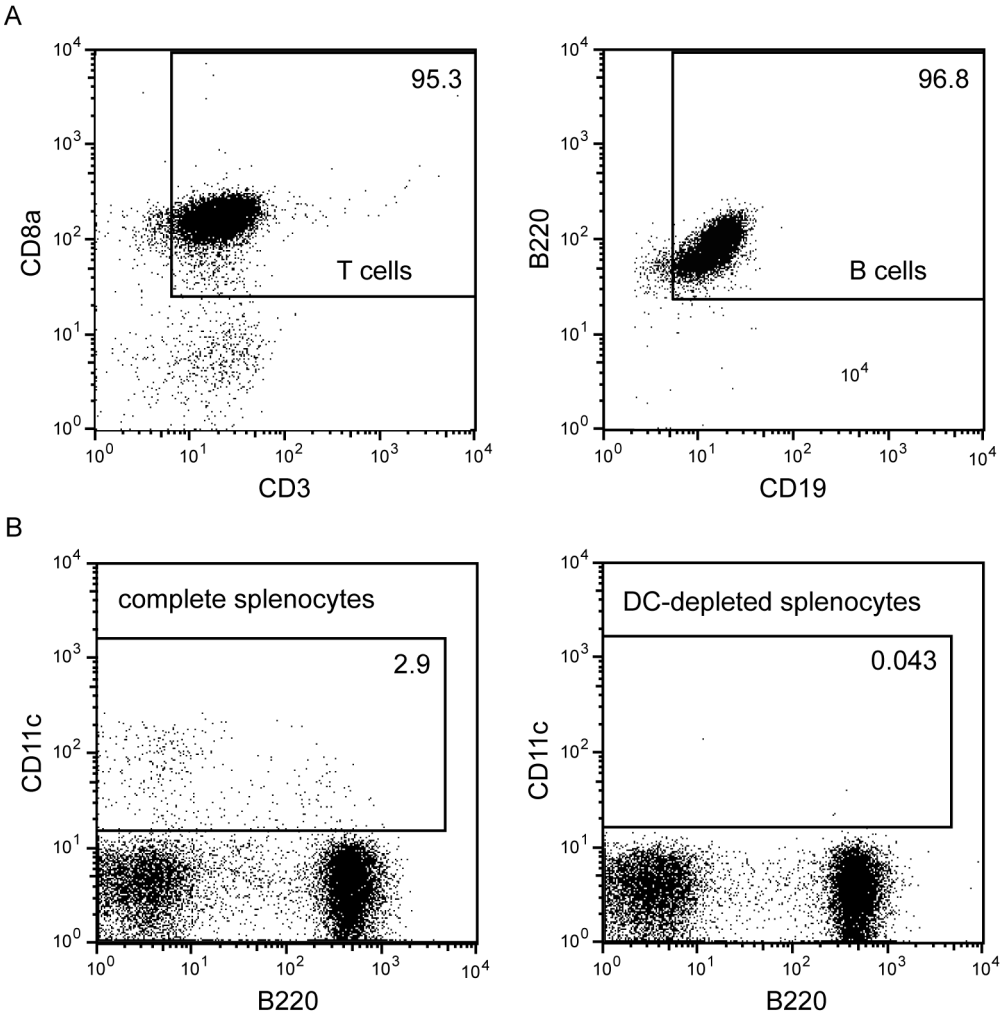

Figure 2.1: Purity of defined splenic cell populations after magnetic-activated cell sorting. Representative flow cytometry data is shown as dot plots. T cells are gated on the lineage marker CD3 and CD8 to define cytotoxic T cells. B cells are shown as CD19 / B220 double-positive cells. Splenic DC are gated on their lineage marker CD11c and B220 to distinguish plasmacytoid ($B220^{pos}$) and myeloid ($B220^{neg}$) dendritic cells. Numbers show percentage of the indicated subset relative to all viable cells.

2.4 Immunological methods

2.4.1 Flow cytometry

Flow cytometry - also called fluorescent-activated cell sorting (FACS) - is a technique used for counting, examining and eventually sorting cells and any other given microscopic particles. Beforehand prepared single-cell suspensions are necessary for flow cytometry analysis. Various antibodies conjugated with fluorochromes can be attached to the cellular antigen of interest. The cell suspension is aspirated through a capillary into a flow cell where, surrounded by a narrow sheath fluid stream, they pass one-by-one through a focused laser beam. The light is either absorbed or scattered when hitting a cell. Such absorbed light of the appropriate wavelength will be re-emitted as fluorescence if the cell contains a naturally

fluorescent substance or if the cell is labeled with one or more fluorochrome-conjugated antibodies at surface or intracellular structures. Light scatter is dependent on the internal structure of the cell and its size and shape. Light scattered at a low angle depends on relative cell size and is recorded as so-called forward scatter (FSC). Whereas light scattered orthogonal to the fluid stream depends on cell granularity as well as surface configuration and is logged as side scatter (SSC). Fluorescence and light scatter signals are detected and amplified by multiple photodiodes. Optical filters help to block unwanted light and permit light of the desired wavelength to reach the photodetector.

2.4.1.1 Multicolor flow cytometry

In this work, flow cytometry analysis was performed with a FACSCalibur or a FACSCanto II, respectively. In both devices, cells are illuminated by an argon-ion laser at a wavelength of 488 nm. The FACSCalibur three-color capability system facilitates detection of the following fluorochromes: fluorescein isocyanate (FITC), phycoerythrin (PE) and peridinin chlorophyll protein (PerCP). The FACSCanto II allows for the additional detection of the Cy7-coupled tandem-dye PE-Cy7. A second red diode laser (633 nm) was used to excite allophycocyanin (APC) or the tandem-dye APC-Cy7. With the FACSCanto II, a third violet diode laser (405 nm) allows for the usage of the fluorescent dyes Pacific Blue and -Orange amongst others.

The emission spectra of the available fluorochromes for flow cytometry overlap and despite appropriate optical filters, this ‘spillover’ of light to photodetectors allocated to other dyes can compromise data acquisition. To minimize the potential of spectral overlap, a special combination of fluorochromes was selected for 8-color analysis with the FACSCanto II. The following table gives fluorescent dyes and the commonly coupled antibodies in these assays.

Fluorochrome	Excitation laser [nm]	Peak excitation [nm]	Peak emission [nm]	Common target
Pacific Blue	405	401	452	anti-CD80
Pacific Orange	405	405	551	anti-CD4
FITC	488	494	519	anti-CD3
PE	488	496, 564	578	anti-$\alpha_4\beta_7$
PerCP-Cy5.5	488	482	695	anti-CD19
PE-Cy7	488	496, 564	785	anti-CD69
APC	633	650	660	anti-CD11
APC-Cy7	633	650	785	anti-CD8a

2.4.1.2 Analysis of cell surface antigens

For analysis of surface antigens, cells were diluted at 1 x 10^6 to 2 x 10^6 in PBS supplemented with 10% FCS. A mixture of up to four flourochrome-conjugated monoclonal antibodies directed against antigens of interest were added at a concentration of 0.5 to 0.7 µl/ml. Cells and staining antibodies were incubated for 25 minutes at 4°C. Cells were then washed twice with PBS and finally re-suspended in PBS supplemented with FCS for subsequent analysis. For intermediate storage, cells were suspended in fixation buffer and kept at 4°C. During the staining process, exposure of cells to light was kept to a minimum.

Flow cytometry data of a given surface antigen is expressed as percentage of cells staining positive for the flourochrome-coupled antibody directed against the antigen of interest or as the mean fluorescence intensity (MFI). The MFI is calculated directly by the flow cytometer and is a non-dimensional index for the amount of cell-bound fluorescent dye. Therefore, the MFI reflects expression levels of the investigated antigen. In the research field of lymphocyte migration, the MFI is commonly used to illustrate expression levels of homing receptors like $\alpha_4\beta_7$.

2.4.1.3 Analysis of intracellular antigens

For some experiments, intracellular transcription factor foxp3 was analyzed to characterize regulatory T cell subsets using a Treg staining Kit (BD Biosciences). In a first step, surface antigens including CD4 were stained as described above (chapter 2.4.1.1). Externally bound antibodies were fixated and the cell membranes were permeabilisized to later allow the anti-foxp3 antibody to penetrate the cell. Therefore, cells were incubated in the kit's fixation/permeabilisation buffer for 30 minutes at 4°C. After being washed with buffer, cells were incubated with 0.5 µl anti-foxp3 antibody at 4°C for another 30 minutes. Finally, cells were washed again twice and suspended in PBS supplemented with 10% FCS for subsequent FACS analysis.

2.4.1.4 CFSE staining

Very often used to trace cell movement and growth, carboxyfluorescein diacetate succinimidyl ester (CFSE) in its pre-active form is a colorless molecule that passively diffuses into cells. Here, its two acetate groups are cleaved by cytosolic esterases to yield highly fluorescent carboxyfluorescein succinimidyl ester. The succinimidyl ester groups then react with intracellular amines, forming stable fluorescent conjugates. These conjugates are

retained by the cells throughout maturation and are inherited to daughter cells during mitotic cell division. Thus, labeled cells are marked and cell divisions can be traced by decreasing fluorescence intensity. A similar fluorescent cell tracer is the Cell Tracker Violet and the simultaneous use of both markers is possible as their excitation spectra differ strongly (peak excitation: CFSE 492 nm, Cell Tracker Violet 415 nm). They are therefore excited and detected by two different laser systems of the flow cytometer.

Generally, cells were suspended in PBS at 2 x 10^7 cells/ml. For imprinting and adoptive transfer experiments, CFSE or the Cell Tracker Violet were added at a concentration of 5 μM and cells were incubated for 15 min at 37°C. For the *in vivo* homing assay, cells were either stained with 10 μM or 1 μM CFSE and incubated for 3 minutes at 4°C. Then, cells were washed extensively and re-suspended in IMDM complete medium for subsequent experiments.

2.4.2 Enzyme-linked immunosorbent assay

The Enzyme-linked immunosorbent assay (ELISA) is a method used to detect a particular antigen of interest in a given sample by antigen-specific, enzyme-linked antibodies. Binding of the antibody in the presence of antigen leads to the subsequent conversion of a colorless substrate to a colored reagent by the coupled enzyme.

In this work, cytokines from murine cell culture supernatants were investigated by so-called sandwich ELISA. Herefore, ELISA detection plates were coated with antibodies binding the particular cytokine. Fixated cytokines were exposed by a second, biotinylated antibody that in turn was bound by streptavidin coupled to peroxydase. This enzyme catalyzes the oxidation of tetramethylbenzidine with hydrogen peroxide to a blue fluorochrome. The investigation of all cytokines except IFN-α was performed with commercially available detection kits according to the manufacturers' protocol. The samples were generally diluted 1:2; for the detection of IL-12p40 and IFN-γ, samples were diluted 1:5 or 1:10.

Meassurement of IFN-α was accomplished by a set of antibodies: detection plates were coated with 50 µl/well capture antibody in coating buffer (1 µg/ml) and incubated overnight at 4°C. Unspecific binding on the plates was blocked by 150 µl/well assay diluent, applied for 3 hours at room temperature. Coated plates were cleaned several times with washing buffer, than samples and standard protein dilutions were transferred on the plates and again incubated overnight at 4°C. The highest level standard was set at 105 IU/ml, samples were applied undiluted. The detection antibody (625 ng/ml) in assay diluent (50 µl/well) was added

for 3 h at RT. Following extensive washing, horseradish peroxidase-conjugated F(ab´)2 fragments (15 µg/ml) were used to identify plate-bound detection antibody. After the fragments' incubation for 3 h at room temperature, tetramethylbenzidine with hydrogen peroxide served as substrate for the horseradish peroxidase. Therefore, substrate solution (50 µl) was added to each well after washing the plates extensively. The enzymatic reaction was stopped by adding sulfuric acid (25 µl/well). All ELISA assays were read-out at a wavelength of 450 nm with correctional subtraction at 590 nm.

2.5 Molecular biology methods

Cells in all organisms effectively regulate gene expression and turnover of gene transcripts (mRNA). The number of copies of a gene's transcripts in a cell or tissue is determined by the rates of its expression and degradation. In order to reliably detect and quantify gene expression from small amounts of mRNA, amplification of the gene transcript is necessary. The polymerase chain reaction is a common method for amplifying DNA; for mRNA-based PCR the RNA sample is first reversely transcribed to DNA. In this work, PCR was used to quantitatively determine mRNA expression of the enzyme retinalaldhyde dehydrogenase 2 (RALDH2) by quantitative real-time PCR.

2.5.1 Isolation of cytoplasmatic RNA

To examine gene expression of RALDH2 in dendritic cells before and after CpG stimulation, total RNA was extracted using the High pure RNA isolation kit (Roche). Therefore, DC were magnetically purified from PLN, MLN and PP. Dendritic cells as well as additionally prepared hepatocytes were stimulated with CpG-ODN and cultured for 18 hours. After being washed in cold PBS twice, 1 x 10^6 cells were re-suspended in 200 µl PBS at each. By adding 400 µl of lysis-/binding buffer and shaking for 15 seconds, cells were lysed and RNases were inactivated simultaneously. The samples were then transferred into a filter tube and centrifuged at 8000 g for 15 seconds. Flowthrough liquid was discarded and 100 µl DNase mix was added to the nucleic acids retained in the filter. Disintegrated DNA and the enzyme mix were removed from the filter in three consecutive washing steps. First, 500 µl washing buffer I and II were added and probes were centrifuged at 8000 g for 15 seconds each. Then, another 200 µl washing buffer II were added and probes were centrifuged at 13,000 g for 2 minutes to remove any residual washing buffer. Retained RNA was re-suspended in 50 µl elution buffer and eluted from the filter tube by centrifugation at 8000 g for 1 minute. Extracted complete RNA was kept at -80°C for further analysis.

2.5.2 Reverse transcription

To be later amplified by polymerase chain reaction (PCR), complete RNA had to be reversely transcribed into a so-called copy-DNA single strand (cDNA) using the Transcriptor first strand cDNA synthesis kit (Roche). According to the manufacturer's protocol, the reaction assay was composed of the following: 10mM Tris Buffer, 50 mM KCl, 5 mM $MgCl_2$, 1mM desoxynucleotide mix, 2.5 µM anchored-oligo$(dT)_{15}$ primer, 20 U Protector RNase inhibitor and 10 U Transcriptor reverse transcriptase. 1 µg of previously isolated RNA was mixed with 11.8 µl of the reaction assay and sterile water was added to a final volume of 20 µl. Probes were incubated at 25°C for 10 minutes and then heated to 42°C for another 60 minutes. To exclude interference of the reverse transcriptase with subsequent PCR steps, probes were finally heated to 95°C for 5 minutes to denaturize the enzyme. Sterile water was added to the cDNA to a final volume of 500 µl and probes were kept at -80°C for subsequent amplification and analysis.

2.5.3 Polymerase chain reaction

2.5.3.1 Functional principle

The PCR technique allows amplification of a specific DNA sequence in a complex mix of nucleic acids e.g. genomic DNA or previously synthesized cDNA. The specific DNA matrix which is to be amplified, is called template. The reaction is facilitated by a thermoresistent DNA polymerase and a sequence-specific pair of oligonucleotide primers, which are homologue to the template's ends. The amplification proceeds with the following three repetitive steps: thermal separation of both strands in the matrix-DNA (denaturation), specific hybridization of the primers with the homologue target sequence in the DNA-templates (annealing) and final DNA synthesis beginning at the primers (elongation). In subsequent amplification rounds, newly synthesized DNA fragments will act as matrices themselves. Thus, multiple repetitive cycles allow exponential accumulation of arbitrary DNA sequences located between the both primers origination from only trace amount of template-DNA.

However, quantification of initial DNA amounts by conventional PCR is only possible as long as the PCR reaction is in the linear interval of amplification efficacy. Here, in every cycle the amount of product is doubled. Later, the DNA polymerase gradually loses its activity and consumption of primers and nucleotides slow the reaction to finally reach a plateau when no more product accumulates due to exhaustion of reagents and enzyme. This plateau phase is reached at varying time points and thus is very hard to be verified. Therefore, an aliquot would have to be saved after every PCR cycle and be quantified by gel electrophoresis

without altering the conditions in the amplification assay. This verification is obsolete if amounts of synthesized DNA can be measured in the reaction assay during the PCR; which is possible by quantitative real-time PCR (see chapter 2.5.3.2).

2.5.3.2 Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) is a technique based on the polymerase chain reaction, which is used to amplify and simultaneously quantify one or several targeted DNA molecules. The procedure follows the general principle of PCR. As its key feature, amplified DNA is quantified as it accumulates in the reaction in *real time* after each amplification cycle. Therefore, a fluorescent dye is added to the PCR probes. The fluorochrome is able to bind only to double-stranded DNA. Following every amplification cycle, probes kept in glass capillaries were excited by a laser and thus emitted a fluorescent signal proportionally to the amount of synthesized DNA. The signal is measured by an appropriate detector and electronically stored by a computer. Increasing fluorescence intensity is adjusted to the number of amplification cycles. Using adequate reference probes with a known number of templates, the original number of copies in the samples of interest can be extrapolated. To ultimately conclude quantitative mRNA expression of a certain gene, the measured number of copies is normalized to the number of transcripts in a so-called reference gene. Typically, an ubiquitaryly expressed gene whose expression is not influenced by the experimental assay is chosen as the reference gene and additionally measured in every sample. In this work, hypoxanthine-guanine phosphoribosyltransferase (HPRT), an enzyme in purine metabolism, is used as the reference gene.

In this work, all qRT-PCR assays were performed using a LightCycler TaqMan Master kit with fluorescent hydrolysis probes from the Universal ProbeLibrary Set. Primers were designed according to the Universal ProbeLibrary Assay Design Center. The experimental assay for each sample was as follows:

Component	**Volume**	**Final concentration**
LightCycler TaqMan Master mix	2.0 µl	1x
Hydrolysis probe	0.1 µl	0.1 µM
Primer *sense*	0.2 µl	0.5 µM
Primer *antisense*	0.2 µl	0.5 µM
Water, PCR-grade	4.5 µl	
cDNA	3.0 µl	

Immediately after preparation in a cooling block at 4 °C, all samples were amplificated and analyzed on a LightCycler 2.0 system using LightCycler Software 4.1. Vital PCR parameters were programmed as follows:

Cycles	Component	Target temperature	Hold time
1	Pre-Incubation	90 °C	10 min
45	Denaturation	95 °C	10 s
	Annealing	60 °C	30 s
	Elongation	72 °C	1 s
1	Cooling	40 °C	30 s

2.6 Statistical analysis

In this work, for multiple experiments all data is given as arithmetic mean values. Variance of mean values is indicated as standard error of the mean (SEM). Statistical significance of different experimental findings was calculated with the independent two-tailed Student's t-test. Significance was implicated at p levels < 0.05, $p < 0.01$ and $p < 0.001$ and was then indicated with an asterisk (*, ** and ***). If not stated otherwise, an asterisk illustrates significant difference in comparison to the experiment's unstimulated control group. In some figures, statistical significance among several experimental groups is calculated and indicated with asterisk-marked brackets. All statistical calculations were performed using SPSS software. Mathematical analysis and graphical design were done with Microsoft Office and Adobe Illustrator.

3 RESULTS

3.1 Effects of TLR ligands in different immunological compartments *in vivo*

3.1.1 Activation of lymphocytes in secondary lymphoid organs after systemic TLR stimulation

In our lab, previous experiments have shown varying response rates to immunotherapy with TLR9 ligands in murine cancer models from different immunological compartments. In particular tumors of the gastro-intestinal tract proved resistant against CpG-ODN treatment. Therefore, in a first experiment, we investigated the immunostimulatory capacity of CpG-ODN - our most intensively studied TLR ligand - in different secondary lymphoid organs. Here we focused on the opposing compartments of peripheral immunity (represented by PLN) and intestinal immunity (MLN and PP). We considered the spleen to be a 'neutral' organ in terms of tissue-specific lymphocyte homing as the spleen lacks high endothelial venules and no adhesion pathway appears to be essential for homing to that organ [von Andrian and Mackay, 2000].

Mice were injected with 100 µg CpG-ODN subcutaneously; a dosage and application type resembling typical anti-tumor treatment regimens. At the onset of this work TLR7 ligands – although not as well understood as their TLR9 counterparts - emerged as another promising approach in immunotherapy because of the restricted expression pattern of TLR9 on human DC. Therefore, another group of mice was treated with 20 µg RNA intravenously according to the standard experimental therapeutic protocol at the time. 48 hours after treatment, mice were sacrificed and secondary lymphoid organs were dissected. Activation level of local lymphocytes was reflected by their surface expression of CD69, a very sensitive activation marker that is rapidly up-regulated on most lymphocytes upon unspecific stimulation [Testi et al., 1989]. CD69 expression levels on T and B cells were examined by flow cytometry.

Naïve mice showed low expression levels of CD69 on $CD8^{pos}$ T cells in most examined SLO (Figure 3.1). In surprising contrast, cytotoxic T cells found in PP of these untreated mice showed moderate to high expression levels of the activation marker. Upon systemic stimulation with TLR7 and 9 ligands, $CD8^{pos}$ T cells showed markedly increased expression of CD69 in spleen, PLN and MLN. The immunostimulatory effect of CpG-ODN and RNA was comparable and both did not differ between lymph nodes

of the peripheral and intestinal compartment. However, cytotoxic T cells in PP which already had high expression levels of CD69 in naïve mice, showed no significant up-regulation of the activation marker upon systemic TLR stimulation. $CD8^{neg}$ T_H cells and B cells had similar *in vivo* expression patterns of CD69 upon systemic treatment with immunostimulatory oligonucleotides (data not shown).

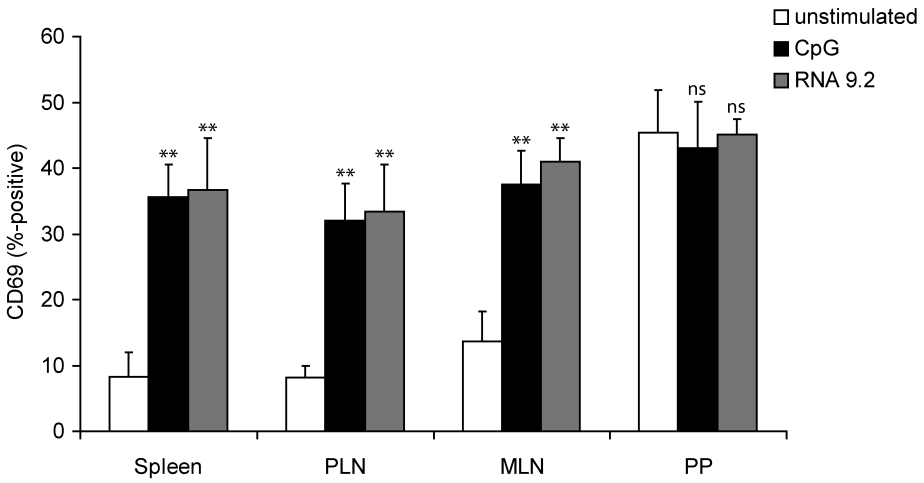

Figure 3.1: *In vivo* immunostimulatory effect of TLR7 and 9 ligands on $CD8^{pos}$ T cells in different secondary lymphoid organs. Mice were injected with 100 µg CpG-ODN subcutaneously or 20 µg RNA 9.2 intravenously. 48 hours after injection, lymphocyte populations from spleen, PLN, MLN and PP were isolated. CD69 surface expression of $CD8^{pos}$ T cells was examined by flow cytometry and is expressed as percentage of $CD69^{pos}$ cytotoxic T cells. Data show the mean values of individual mice (n = 3) ± SEM. Results are representative of 3 independent experiments. ***P* < 0.01; ns indicates not significant.

3.1.2 *In vivo* expression patterns of the gut-homing receptor $\alpha_4\beta_7$ upon systemic TLR stimulation

In a second step, effects of the most recently used oligonucleotides on lymphocytic expression of the gut-homing receptor $\alpha_4\beta_7$ *in vivo* were examined. With previous experiments showing increased expression of the co-stimulatory molecule CD69 on lymphocytes in both peripheral and intestinal SLO after systemic TLR7 and -9 stimulation, we investigated expression patterns of $\alpha_4\beta_7$ on T and B cells in the same lymphoid organs. Therefore, mice were again treated with CpG-ODN subcutaneously or RNA 9.2 intravenously. 48 hours later, $\alpha_4\beta_7$ surface expression was analyzed on lymphocytes from spleen, PLN, MLN and PP by flow cytometry.

As expected, naïve mice showed the highest percentage of $\alpha_4\beta_7^{pos}$ cytotoxic T cells in lymphoid organs of the GI-tract in a representative FACS analysis (Figure 3.2 A, gated on $CD8^{pos}$ T cells). $CD8^{pos}$ T cells expressing low levels of the homing receptor were

also found in spleen and PLN. Unexpectedly, when mice were treated with CpG-ODN or RNA, the proportion of $\alpha_4\beta_7^{pos}$ $CD8^{pos}$ T cells significantly decreased in all investigated SLO. The frequency of $\alpha_4\beta_7^{pos}$ cytotoxic T cells in MLN and PP reached levels similar to the PLN and the spleen. Combined data of repeated experiments showed that the reduced proportion of $\alpha_4\beta_7$-expressing $CD8^{pos}$ T cells was similar upon systemic stimulation with CpG-ODN and RNA (Figure 3.2 B). $CD8^{neg}$ T_H cells and B cells showed analog $\alpha_4\beta_7$ expression patterns after *in vivo* stimulation with the TLR7 and 9 ligands (Figure 3.2 C and D).

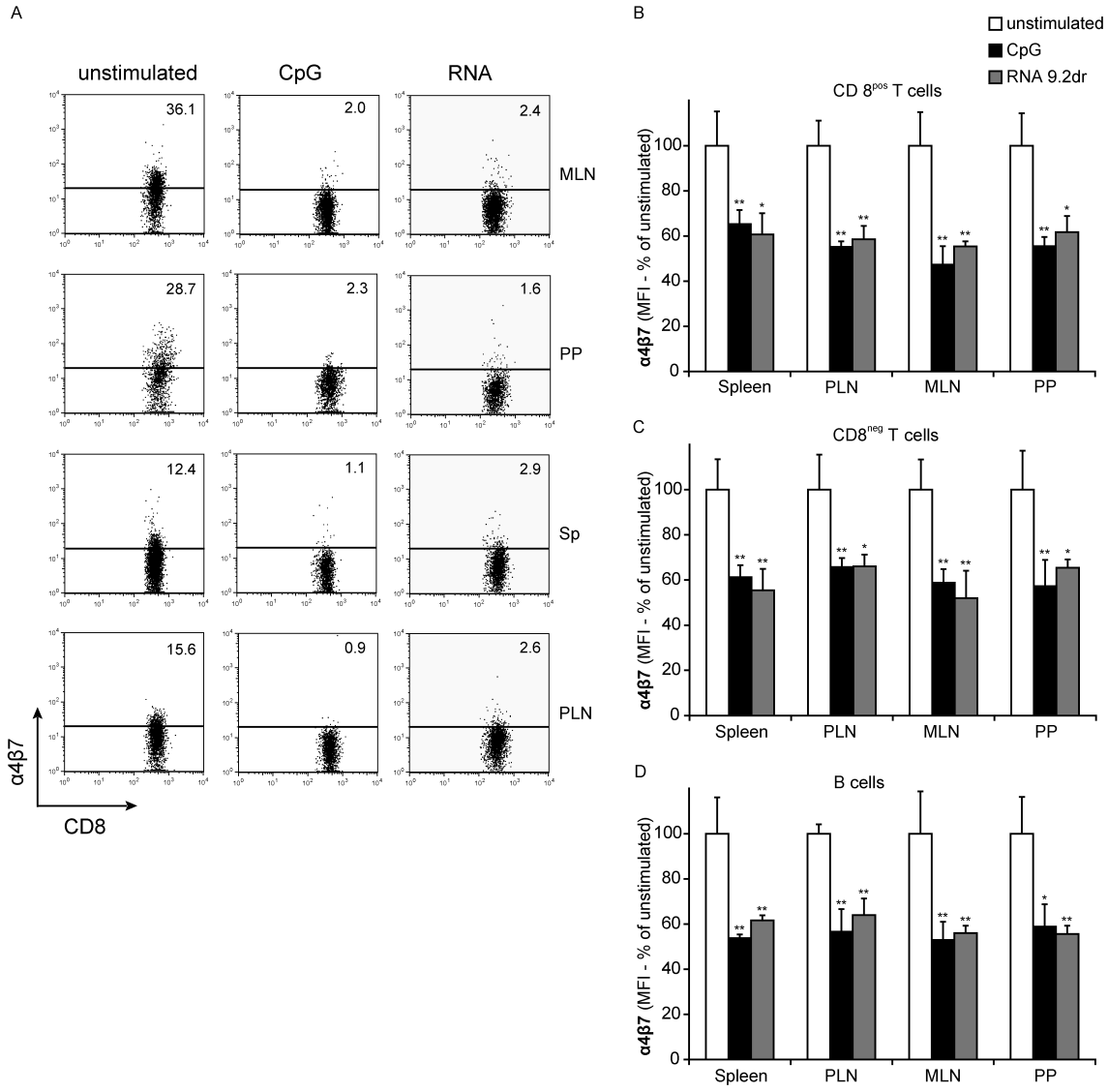

Figure 3.2: Effects of TLR7 and 9 ligands on expression patterns of $\alpha_4\beta_7$ *in vivo*. Mice were treated with 100 µg CpG-ODN subcutaneously or 20 µg RNA 9.2 intravenously. 48 hours later, lymphocyte populations from spleen, PLN, MLN and PP were isolated. $\alpha_4\beta_7$ surface expression was examined by flow cytometry. (A) FACS dot blots of one representative experiment are gated on cytotoxic T cells. Numbers indicate percentage of $\alpha_4\beta_7^{pos}$ cells. (B) Bar graphs show $\alpha_4\beta_7$ mean fluorescence intensity (MFI) in proportion to the unstimulated control group on different lymphocyte subsets. Data show the mean values of individual mice (n = 3) ± SEM. Results are representative of 2 independent experiments. *P < 0.05; **P < 0.01.

3.1.3 The role of different routes of CpG-administration for the expression of the gut-homing receptor $\alpha_4\beta_7$

With previous experiments showing *in vivo* downregulation of $\alpha_4\beta_7$ on lymphocytes in various SLO after subcutaneous CpG stimulation, we investigated which role the route of ODN administration played in regulation of the gut-homing receptor. Therefore, we treated mice with CpG-DNA intraperitoneally and intravenously. 48 hours after stimulation, mice were sacrificed and $\alpha_4\beta_7$ surface expression on lymphocytes from spleen, PLN, MLN and PP was analyzed by flow cytometry.

In vivo stimulation with CpG-DNA via intraperitoneal or intravenous injection showed downregulation of the gut homing receptor $\alpha_4\beta_7$ on T cells similar to subcutaneous application (Figure 3.3). However, intraperitoneal treatment resulted in slightly reduced downregulation of $\alpha_4\beta_7$ in non-intestinal SLO such as spleen and PLN. Although detectable, the surface expression of the homing receptor on $CD8^{pos}$ T cells was not significantly reduced when compared to T cells from unstimulated control mice. Taken together, these results implicate that the route of CpG *in vivo*-administration has no major effect on $\alpha_4\beta_7$ expression patterns at least in intestinal SLO

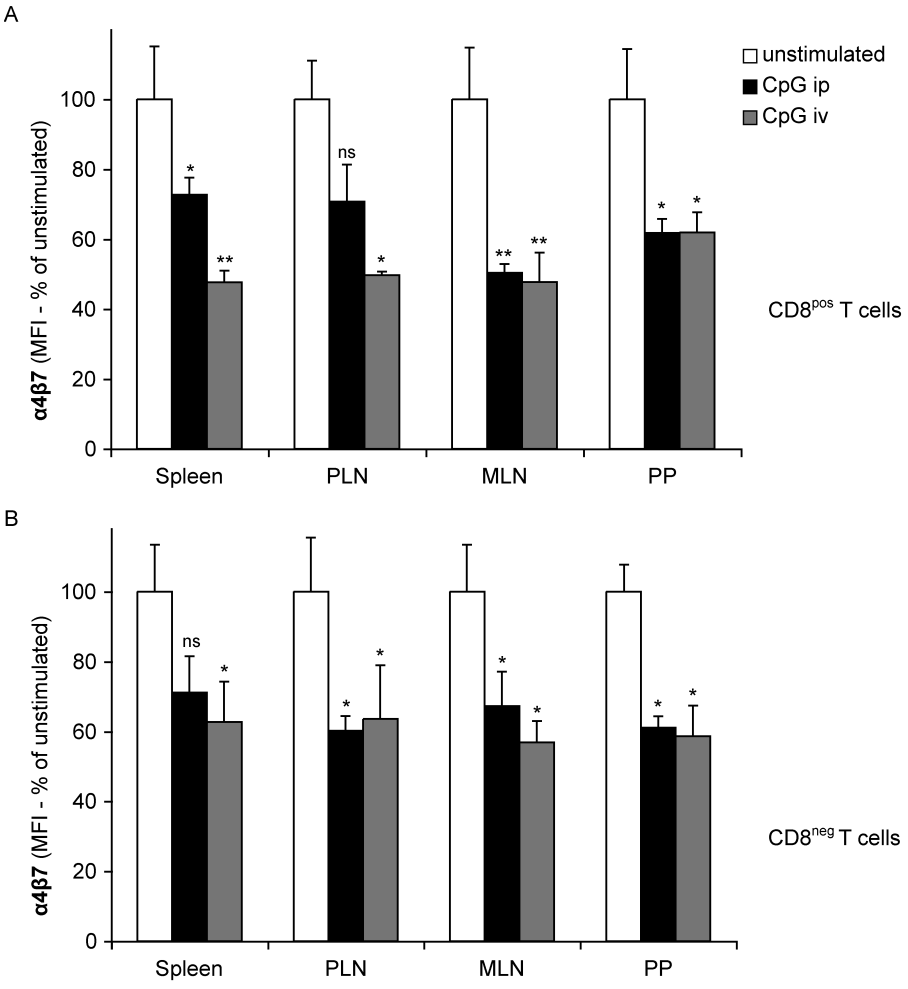

Figure 3.3: Role of the application route on the effect of immunostimulatory oligonucleotides *in vivo*. Mice were injected with CpG ODN 20 µg intraperitoneally or 20 µg complexed with DOTAP intravenously. 48h after injection, lymphocyte populations from different lymphoid organs were isolated. $\alpha_4\beta_7$ surface was examined by flow cytometry and is expressed as percentage of unstimulated control group for each sample. Data show the mean values of individual mice (n = 3) ± SEM. Results are representative of 2 independent experiments. *P < 0.05; **P < 0.01; ns indicates not significant.

3.2 Effects of TLR ligands on $\alpha_4\beta_7$ expression patterns of naïve lymphocytes

3.2.1 Kinetics and dose dependency of TLR7- and TLR9-mediated effects on $\alpha_4\beta_7$ expression *in vitro*

Previous experiments showed reduced frequency of $\alpha_4\beta_7$-positive lymphocytes in secondary lymphoid organs upon systemic TLR7 and TLR9 stimulation *in vivo*. We analyzed this effect in detail by further *in vitro*-experiments with special emphasis on dose dependency and kinetics. Murine spleen cells were generally stimulated with CpG ODN, and in addition in one experiment with CL097, a highly water-soluble derivative of the imidazoquinoline compound R848 and thus TLR7 and TLR8 agonist [Salio et al., 2007]. After 6 hours, cells were washed with PBS to remove free oligonucleotides and cultured. Surface expression of $\alpha_4\beta_7$ was examined on different lymphocytes subsets by flow cytometry at various time points.

Upon *in vitro*-stimulation with CpG-ODN as well as CL097 naïve splenic $CD8^{pos}$ T cells showed downregulation of the gut-homing receptor $\alpha_4\beta_7$. For both immunostimulatory oligonucleotides in this experiment a clear dependency between dose and subsequent impaired surface expression of $\alpha_4\beta_7$ on cytotoxic T cells was apparent (Figure 3.4 A). Both, T cells treated with CpG-ODN and those treated with CL097 showed mostly similar patterns of downregulation. As little as 0.1 µg/ml of the ON is sufficient to clearly reduce expression of $\alpha_4\beta_7$ on splenic $CD8^{pos}$ T cells *in vitro*. Whereas increased doses of stimulus indeed lower the expression of the homing molecule, the additional effect especially of dosages > 1.0 µg/ml is less dramatic. CpG-induced downregulation of $\alpha_4\beta_7$ *in vitro* showed a similar dose dependency for $CD8^{neg}$ T cells and B cells (data not shown).

Kinetics of $\alpha_4\beta_7$ surface expression showed temporally analog downregulation on $CD8^{pos}$ and $CD8^{neg}$ T cells as well as B cells upon CpG stimulation (Figure 3.4 B). Although more accentuated amongst B cells, $\alpha_4\beta_7$ was markedly reduced after 12 hours

of stimulated culture on all experimentally included lymphocyte subsets. After 42 hours, surface expression of the homing receptor reached a minimum on T and B cells and was then stable till the final measurement after 64 hours.

We thus show that the homing receptor $\alpha_4\beta_7$ is downregulated on splenic lymphocytes in a dose dependent manner after *in vitro*-stimulation with CpG-ODN or CL097, respectively. The CpG-induced downregulation is clearly detectable as early as 12 hours after stimulation and surface expression reaches a relative stable minimum after 42 hours.

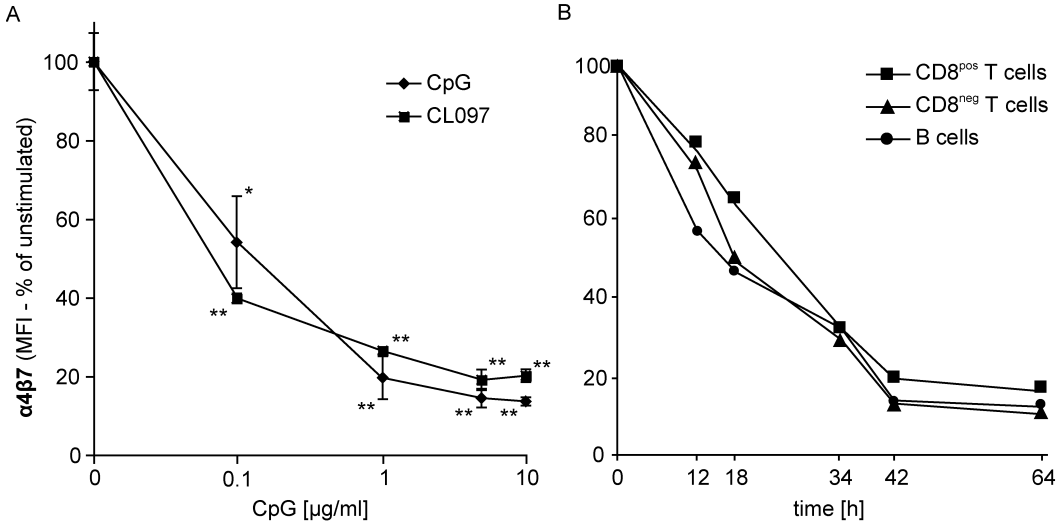

Figure 3.4: Dose dependency and kinetics of oligonucleotide-induced downregulation of $\alpha_4\beta_7$ expression *in vitro*. (A) Splenocytes were stimulated with CpG-ODN or CL097 (0.1, 1, 5 or 10 µg/ml) for 6 hours, then washed and cultured. 48 hours later, surface expression of $\alpha_4\beta_7$ on $CD8^{pos}$ T cells was examined by flow cytometry. Data show the mean values of triplicate samples ± SEM. Results are representative of 3 independent experiments. **P* < 0.05; ***P* < 0.01. (B) Spleen cells were stimulated with CpG-ODN (5 µg/ml) for 6 hours, then washed and cultured. $\alpha_4\beta_7$ expression was examined on lymphocyte subpopulations after 12, 18, 34, 42 and 62 hours.

3.2.2 Effects of different pattern-recognition receptor ligands on lymphocyte $\alpha_4\beta_7$ expression

To further evaluate regulation of lymphocytic $\alpha_4\beta_7$ surface expression we investigated the role of Toll-like receptors beyond TLR7 and 9 as well as other pattern-recognition receptors in the modulation of the gut-homing receptor. Hence, we stimulated murine spleen cells with PAM_3CSK_4 (a synthetic peptidoglycan stimulating TLR2) [Aliprantis et al., 1999], polyI:C (synthetic analog of dsRNA, TLR3) [Alexopoulou et al., 2001], LPS (bacterial cell wall component, TLR4), Flagellin (TLR5), CL097 (TLR7), CpG-DNA (TLR9), Curdlan, 3p-RNA 2.2 or low-dose polyI:C, the latter two complexed with

Lipofectamin. Curdan is a bacterial β-glycan and ligand for Dectin-1, a TLR-independent but synergistic PRR [Yoshitomi et al., 2005]. 3p-RNA 2.2 and low-dose polyI:C both complexed with the transfection agent Lipofectamin are ligands for the intracellular, cytoplasmatic RNA-helicases RIG-I and MDA5, respectively [Akira et al., 2006]. After 48 hours of stimulated culture, $\alpha_4\beta_7$ surface expression on lymphocytes was measured by flow cytometry.

In a representative FACS analysis, the vast majority of untreated splenic $CD8^{pos}$ T cells expressed low levels of $\alpha_4\beta_7$ after being cultured for 48 hours (Figure 3.5 A, dot blots gated on $CD8^{pos}$ T cells). However, the formerly prevailing fraction of $\alpha_4\beta_7^{pos}$ cytotoxic T cells was clearly diminished after treatment with all investigated PRR ligands. Compatibly, integrated data of three independent experiments (Figure 3.5 B) also showed clearly impaired surface expression of the gut-homing receptor on naïve splenic $CD8^{pos}$ T cells. Downregulation of $\alpha_4\beta_7$ on cytotoxic T cells was most impressive after stimulation by TLR4, 7 and 9 ligands, respectively. Stimulation with ligands of the intracellular helicases Rig-I and MDA5 as well as membrane-bound PRR Dectin-1 resulted in intermediate downregulation of $\alpha_4\beta_7$ on naïve $CD8^{pos}$ T cells where as activation of TLR3 and 5 lead to a less dramatic decrease in surface expression. Further experiments showed equivalent downregulation of the gut-homing receptor $\alpha_4\beta_7$ on naïve splenic $CD8^{neg}$ T_H cells and B cells (data not shown).

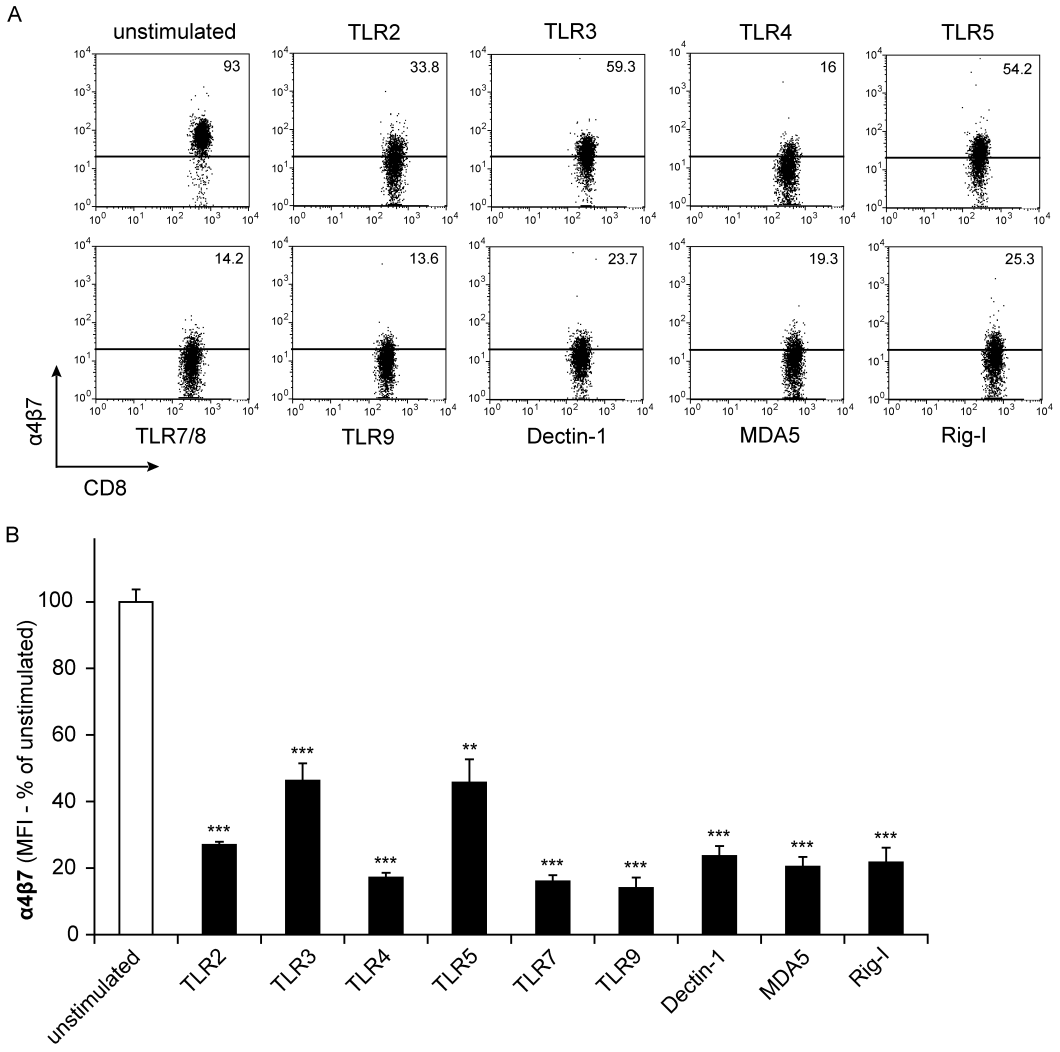

Figure 3.5: Effects of various pattern-recognition receptors on $\alpha_4\beta_7$ expression *in vitro*. Splenocytes were stimulated with Pam_3CSK_4 (1 µg/ml), polyI:C (180 µg/ml), LPS (5 µg/ml), Flagellin (2 µg/ml), CL097 (5 µ g/ml), CpG-ODN (5 µg/ml), Curdlan (100 µg/ml), 3p-RNA 2.2 (1 µg/ml) or polyI:C (1 µg/ml), the latter two complexed with Lipofectamin 2000. 48 hours later, surface expression of $\alpha 4\beta 7$ on cytotoxic T cells was examined by flow cytometry. (A) FACS dot blots of one representative experiment are gated on $CD8^{pos}$ T cells and numbers indicate percentage of $\alpha_4\beta_7^{pos}$ cells. (B) The bar graph shows $\alpha_4\beta_7$ mean fluorescence intensity in proportion to the unstimulated control group. Data show the mean values of triplicate samples ± SEM. Results are representative of 3 independent experiments. ***P* < 0.01; ****P* < 0.00.1.

3.2.3 Role of dendritic cells in TLR-mediated downregulation of $\alpha_4\beta_7$ on naïve lymphocytes

In preceding experiments we had learned that stimulation of murine spleen cells with ligands of Toll-like and other pattern-recognition receptors induced downregulation of the gut-homing receptor $\alpha_4\beta_7$ on $CD8^{pos}$ and $CD8^{neg}$ T cells as well as B cells. Previous studies had described the expression of TLR2, 3, 5, and 9 on T cells; recently reviewed in 2007 by Kabelitz et al. However, both direct and indirect mechanisms of TLR-

mediated activation of T cells were heavily debated and there are still controversies whether T cells do have functional TLR and are therefore actually capable of sensing pathogen-associated molecular patterns themselves [Kranzer et al., 2000; Lipford et al., 2000; Verthelyi et al., 2001]. Hence, we wanted to investigate whether T and B cells respond directly to CpG-ODN with altered expression patterns of gut-homing molecules or whether the observed downregulation of $\alpha_4\beta_7$ on lymphocytes is mediated by another cell population upon TLR stimulation.

In a first step to investigate a possible role of other cell types in regulation of $\alpha_4\beta_7$ expression, we examined the lymphocytes' capability to directly respond to innate stimuli with altered expression patterns of the gut-homing receptor. Therefore, naïve murine splenic $CD8^{pos}$ T cells and B cells were isolated by magnetic-activated cell sorting. The purified cells were stimulated with CpG-DNA for 6 hours, then washed to remove free ODN and cultured. After 48 hours, their $\alpha_4\beta_7$ surface expression was examined by flow cytometry.

These experiments demonstrated that both, purified naïve splenic $CD8^{pos}$ T cells and B cells had no significantly different $\alpha_4\beta_7$ surface expression after CpG-stimulation compared to an untreated control group (Figure 3.6 A). When strictly isolated, these lymphocyte populations did not have the capability to directly respond to a TLR9 stimulus with previously described downregulation of $\alpha_4\beta_7$. With these findings, we concluded the existence of a stimulus-mediating cell type thereby indirectly controling the homing receptor on T and B cells.

With their central role in innate immunity with not only sensing, processing and presenting antigen but also local environmental factors to cells of adaptive immunity thereby imprinting tissue-specific homing, we suspected dendritic cells to also be a key player in the latter findings of indirectly controlled $\alpha_4\beta_7$ expression on naïve lymphocytes after TLR stimulation. To determine the role of DC in regulating $\alpha_4\beta_7$ expression on naïve T and B cells, murine splenocytes were depleted of DC by magnetic-activated cell sorting. These spleen cells were then stimulated with CpG-ODN for 6 hours, washed and cultured. $\alpha_4\beta_7$ surface expression was examined after 48 hours by flow cytometry.

Compatible to our previous experiments, CpG-stimulation of complete splenocytes, that is without depletion of DC, lead to a distinct downregulation of $\alpha_4\beta_7$ on both naïve T and B cells (Figure 3.6 B, left columns). Hereby, reduced expression levels of the homing

receptor after TLR9 stimulation were comparable on cytotoxic and helper T cells as well as B cells. In contrast, when DC were depleted from spleen cells, neither $CD8^{pos}$ and $CD8^{neg}$ T cells nor B cells showed significant reduction of $\alpha_4\beta_7$ expression following CpG-stimulation when compared to an untreated control group (Figure 3.6 B, right columns). These findings demonstrated that naïve T and B cells within splenic cells but in the absence of DC do not downregulate $\alpha_4\beta_7$ upon TLR stimulation, thus suggesting that DC are a vital component in regulating the homing receptor on naïve lymphocytes.

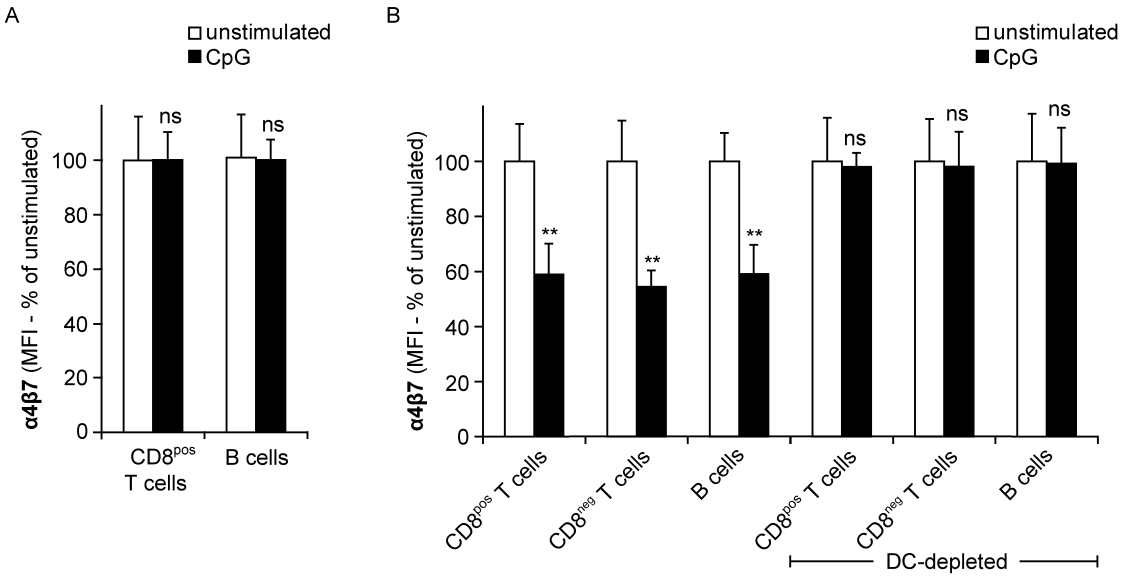

Figure 3.6: Relevance of direct versus indirect effects in mediating downregulation of $\alpha_4\beta_7$ surface expression on naïve lymphocytes upon CpG-DNA stimulation. A) Splenic $CD8^{pos}$ T cells and B cells were isolated by magnetic-activated cell sorting, stimulated with CpG-DNA (3 µg/ml) for 6 hours, then washed and cultured. (B) Spleen cells were depleted of dendritic cells, stimulated with CpG-ODN (3 µg/ml) for 6 hours, then washed and cultured. After 48 hours, $\alpha_4\beta_7$ surface expression was examined by flow cytometry. Data show the mean values of triplicate samples ± SEM. Results are representative of at least 3 independent experiments. $^{**}P < 0.01$; ns indicates not significant.

On the one hand, there are several additional cell populations like antigen-presenting macrophages and other mononuclear cells which also yield a great arsenal of different PRR and could therefore be able to mediate TLR signals and induce $\alpha_4\beta_7$ downregulation on lymphocytes. On the other hand, dendritic cells are an inhomogeneous family with members that differ in functions like cytokine production but which could – by their expression pattern of PRR – all be capable to communicate CpG-dependent regulation of the gut-homing receptor on naïve T and B cells. So, in a next step, we investigated whether other cell types played a role in this process or whether only dendritic cells alone were responsible for our findings. Simultaneously, different DC subsets were compared in their potency to induce downregulation of $\alpha_4\beta_7$

upon CpG stimulation. Therefore, different DC subtypes were raised from bone marrow precursors. For DC maturation either the two cytokines GM-CSF and interleukin 4 or the single factor Flt3-Ligand were used. Bone marrow cells cultured with GM-CSF (GM-DC) mature into myeloid DC only, where as Flt3L-derived DC (FL-DC) compromise both myeloid and plasmacytoid dendritic cells. The latter two were differentiated by MACS sorting with an anti-B220 antibody and then analyzed separately. Different DC subsets were stimulated with CpG-DNA for 6 hours, washed extensively to remove free ODN and were co-cultured with naïve splenocytes. After 48 hours of co-culture, $\alpha_4\beta_7$ expression was examined on splenic lymphocytes by flow cytometry.

When co-cultured with both, myeloid ($CD11c^{pos}$, $B220^{neg}$) or plasmacytoid ($CD11c^{pos}$, $B220^{pos}$) CpG-stimulated dendritic cells, naïve splenic lymphocytes showed clearly impaired surface expression of $\alpha_4\beta_7$ (Figure 3.7 A). This effect was similar on $CD8^{pos}$ and $CD8^{neg}$ T cells as well as B cells. There was no significant difference in downregulation of the homing receptor's surface expression between lymphocytes co-cultured with FL-DC and those co-cultured with GM-DC. These findings suggested that cell populations other than DC are not required for CpG-induced downregulation of $\alpha_4\beta_7$ on naïve splenic T and B cells. Furthermore, both bone marrow-derived mDC and pDC can alter the expression pattern of the gut-homing receptor upon a TLR9 stimulus.

In a last step, we excluded possible effects on $\alpha_4\beta_7$ regulation by cells other than DC which were present within the splenocytes but unstimulated during the previous experiment. We therefore purified splenic $CD8^{pos}$ T cells and B cells which had been shown previously not to respond with $\alpha_4\beta_7$ downregulation upon CpG-stimulation. The sorted cells were co-cultured with CpG-stimulated GM-DC for 48 hours. Then, $\alpha_4\beta_7$ expression on the lymphocytes was measured by flow cytometry.

When only co-cultured with CpG-activated dendritic cells but in the absence of other splenocytes, purified splenic $CD8^{pos}$ T cells reacted with significantly impaired $\alpha_4\beta_7$ surface expression (Figure 3.7 B). Equally co-cultured B cells showed downregulation of the homing receptor to a similar extent. Thus, we demonstrated that dendritic cells alone are capable and sufficient to mediate CpG-DNA stimulation to naïve splenic T and B cells and hence induce downregulation of $\alpha_4\beta_7$. This effect could be shown for both plasmacytoid and myeloid DCs, independent from whether DC were matured with GM-CSF and interleukin 4 or Flt3-Ligand.

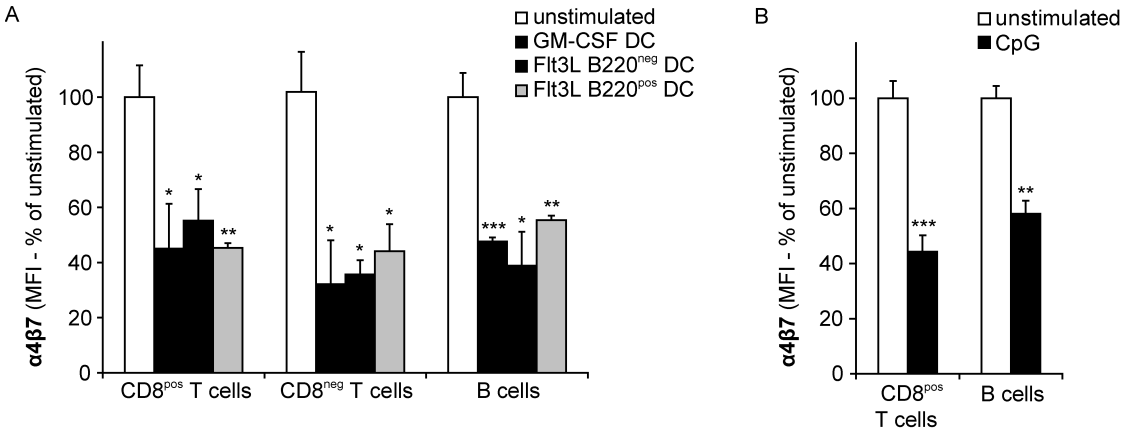

Figure 3.7 Role of dendritic cells in mediating downregulation of $\alpha_4\beta_7$ surface expression on naïve lymphocytes upon CpG stimulation. (A) GM-DC, $B220^{pos}$ FL-DC or $B220^{neg}$ FL-DC were stimulated with CpG-ODN (3 µg/ml) for 6 hours, then washed and co-cultured with naïve splenocytes. (B) GM-DC were stimulated with CpG-ODN (3 µg/ml) for 6 hours, then washed and co-cultured with magnetically purified naïve splenic $CD8^{pos}$ T cells and B cells. After 48 hours, $\alpha_4\beta_7$ surface expression was examined by flow cytometry. Data show the mean values of triplicate samples ± SEM. Results are representative of at least 3 independent experiments. *P < 0.05; **P < 0.01; ***P < 0.00.1.

3.2.4 Mechanism of DC-mediated downregulation of $\alpha_4\beta_7$ on naïve lymphocytes upon TLR stimulation

With previous experiments showing that DC are responsible for lymphocytic downregulation of $\alpha_4\beta_7$ following CpG stimulation, questions about the underlying mechanisms were raised. On the one hand, dendritic cells can stimulate T and B cells via direct receptor interaction, e.g. peptide - MHC complexes bind to the T cell receptor and subsequently activate naïve T cells. On the other hand, DC are potent producers of a wide arsenal of cytokines and other humoral components. On that account, the functional principle of the DC regulatory effect had to be differentiated between a cell-cell contact dependent or a soluble factor dominated mechanism.

Therefore, bone marrow-derived GM-DC were stimulated with CpG for 6 hours, washed and cultured. After 42 hours, the culture supernatant was gained. Then, splenic $CD8^{pos}$ T cells were purified by MACS and co-cultured either alone with CpG, CpG-activated and washed GM-DC or the previously gained supernatant from CpG-stimulated GM-DC culture. In this experiment T cells and DC were cultured in a transwell system separated by a 0.02 µm membrane. These small pores are too small for DC and T cells to penetrate the membrane and thus prevent direct cell-cell contact between both experimental cell populations. However, the pore size allows humoral

factors to pass the barrier. After 48 hours of co-culture, $\alpha_4\beta_7$ surface expression was examined on T cells by flow cytometry.

As shown in previous experiments, purified splenic $CD8^{pos}$ T cells did not respond with altered expression patterns of $\alpha_4\beta_7$ when stimulated with CpG-DNA in the absence of DC (Figure 3.8, left columns). However, CpG-activated DC induced $\alpha_4\beta_7$ downregulation on co-cultured cytotoxic T cells even when being separated by a membrane preventing direct cell-cell interaction (Figure 3.8, middle columns). In the same way, only the supernatant from CpG-activated DC culture – supposedly comprising all DC produced cytokines – leads to a comparable reduction of the homing receptor on co-cultured T cells (Figure 3.8, right columns). Further equivalent experiments with purified, splenic B cells co-cultured with TLR9-stimulated DC or their supernatant, respectively, showed similar results of downregulated $\alpha_4\beta_7$ (data not shown). Thus, we demonstrated that DC do not need direct cell contact to lymphocytes but rather release one or multiple soluble factors to induce downregulation of their gut-homing receptor $\alpha_4\beta_7$.

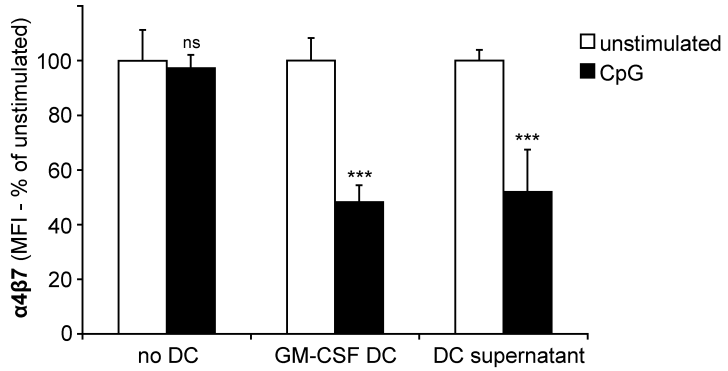

Figure 3.8: Role of direct cell-cell contacts versus humoral factors in CpG-induced and DC-mediated downregulation of $\alpha_4\beta_7$ surface expression on $CD8^{pos}$ T cells. Sorted splenic $CD8^{pos}$ T cells were cultured with or without CpG-activated (3 µ g/ml) GM-DC or the DC supernatant gained after 42 hours of CpG-stimulated DC culture. T cells and DC were separated in a Transwell system by a 0.02 µm membrane to prevent cell-cell contacts. After 48 hours, $\alpha_4\beta_7$ surface expression was examined on $CD8^{pos}$ T cells by flow cytometry. Data show the mean values of at least triplicate samples ± SEM. Results are representative of 3 independent experiments ****P* < 0.00.1; ns indicates not significant.

Knowing that humoral factors play a critical role in DC-induced downregulation of $\alpha_4\beta_7$ on lymphocytes, we assessed the regulatory capacity of various common DC-released cytokines, namely IL-6, IL-10, IL-12p40, IFN-α, TGF-β and TNF-α as well as T cell-derived IL-2. Therefore, bone marrow-derived GM-DC were stimulated with CpG-DNA and were co-cultured with purified splenic $CD8^{pos}$ T cells separated in a Transwell system by a 0.02 µm membrane to prevent direct cell-cell contact. Monoclonal

neutralizing antibodies targeting the cytokines mentioned above were added to the culture. These antibodies bind free cytokines and thereby competitively inhibit subsequent cytokine-receptor interactions. Thus, with appropriate levels of neutralizing antibody referring to common literature specifications, lymphocyte response to these DC-derived cytokines could be completely abrogated. After 48 hours of culture, $\alpha_4\beta_7$ surface expression on $CD8^{pos}$ T cells was measured by flow cytometry. An adequate degree of cytokine inhibition was assured by measuring culture levels of cytokines that escaped neutralization by blocking mAbs with an ELISA assay (data not shown).

Consistent with previous experiments, naïve cytotoxic T cells among complete splenocytes effectively downregulated $\alpha_4\beta_7$ surface expression when stimulated with CpG-DNA in absence of any neutralizing antibodies (Figure 3.9). Presence of anti-IL-6 antibody in the culture clearly diminished CpG-induced $\alpha_4\beta_7$ downregulation, resulting in higher $\alpha_4\beta_7$ surface expression on anti-IL-6-treated T cells when compared with T cells not treated with any neutralizing antibody. Nonetheless, $\alpha_4\beta_7$ expression levels of T cells treated with CpG and anti-IL-6 antibody were lower as those from naïve T cells. Hence, blocking the effect of interleukin 6 only partially abrogates CpG-induced downregulation of $\alpha_4\beta_7$ on naïve $CD8^{pos}$ T cells. Furthermore, expression of the homing receptor on CpG-stimulated T cells treated with anti-IL-10, anti-TGF-β or anti-TNF-α was slightly yet significantly reduced when compared with stimulated T cell not treated with any blocking antibody. There was no significant modification in downregulation of $\alpha_4\beta_7$ expression on CpG-activated T cells when being cultured with anti-IL-2, anti-IL-12p40 or anti-IFN-α antibodies. Analog experiments using mesenteric lymph node-originated intestinal DC instead of bone-marrow derived GM-DC showed similar effects of reduced downregulation of $\alpha_4\beta_7$ on naïve $CD8^{pos}$ T cells only with anti-IL-6 but no other neutralizing antibody (data not shown).

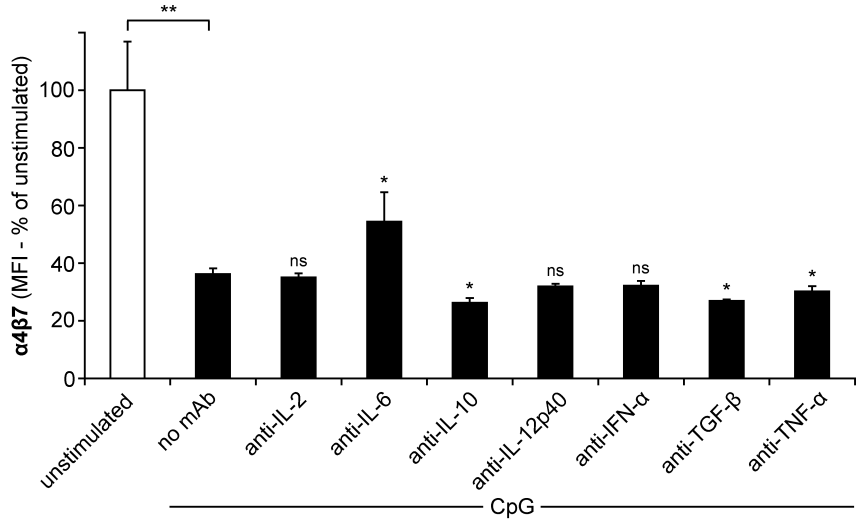

Figure 3.9: Effects of cytokine-neutralizing antibodies in CpG-induced downregulation of $\alpha_4\beta_7$ surface expression on CD8^{pos} T cells. Sorted splenic CD8^{pos} T cells were cultured with CpG-stimulated (3 µg/ml) GM-DC separated in a Transwell system by a 0.02 µm membrane to prevent direct cell-cell contact. Monoclonal blocking antibodies targeting different cytokines were added to the culture. After 48 hours, $\alpha_4\beta_7$ surface expression was examined on CD8^{pos} T cells by flow cytometry. Data show the mean values of at least triplicate samples ± SEM. Results are representative of 3 independent experiments. *P < 0.05; ns indicates not significant. The asterisk without brackets indicates comparison to the CpG-stimulated group that was not treated with any neutralizing antibody.

To confirm and further evaluate the data gained with the blocking antibody assay we rearranged the experimental setting using transgenic mice. We therefore obtained splenocytes from multiple knockout mice which lack certain pro-inflammatory transcription factors, cytokines or their receptors; namely IL-6, IL-10, IL-12, IFN-ɣ, NFκB-p50, IRF-3/7 and IFN-α receptor (IFNaR). NF-κB is a signaling molecule downstream of various PRR like TLR, cytoplasmatic RNA-sensing helicases or Dectin-1 and is compromised of the two heterodimers p50 and p65. It acts as a transcription factor which upon activation, translocates into the nucleus and leads to induction of pro-inflammatory genes. IRF-3 and 7 are signaling molecules in the activation cascade of TLR3, 4, 7 and 9 as well as the cytosolic RNA-helicases. In their function as a transcription factors, they are potent inducers of genes leading to production of type I interferons. The cytokine-deficient splenocytes were stimulated with CpG for 6 hours, then washed and cultured. After 48 hours, $\alpha_4\beta_7$ expression on lymphocytes was measured by flow cytometry.

Figure 3.10 shows $\alpha_4\beta_7$ downregulation on CpG-stimulated splenic CD8^{pos} T cells of knockout (KO) and corresponding wild type (WT) mice. $\alpha_4\beta_7$ surface expression is shown as percentage of unstimulated control groups. Varying levels of percentual $\alpha_4\beta_7$ downregulation amongst the experimental groups can be explained by the different strain backgrounds of the knockout mice. This is why, every KO mouse is individually compared to its corresponding WT with the same background.

Splenic CD8^{pos} T cells among complete splenocytes from IL-6-deficient mice stimulated with CpG downregulated $\alpha_4\beta_7$ to a significantly smaller extent as those T cells obtained from corresponding WT mice. This resulted in higher expression levels of the homing receptor on T cells of the KO group when compared to the WT. IRF-3/7 and IFNaR deficient CpG-stimulated splenocytes showed little but significantly increased downregulation of $\alpha_4\beta_7$ surface expression when compared to corresponding WT spleen cells. However, general levels of downregulation were rather low within these

strain backgrounds. There was no significant difference in $\alpha_4\beta_7$ expression upon CpG stimulation between KO and WT splenocytes in IL-10, IL-12, IFN-ɣ and NFκB-p50 groups. Further experiments showed equivalent data for $CD8^{neg}$ T cells and B cells with attenuated $\alpha_4\beta_7$ downregulation upon TLR9 activation only in IL-6 knockout mice (data not shown).

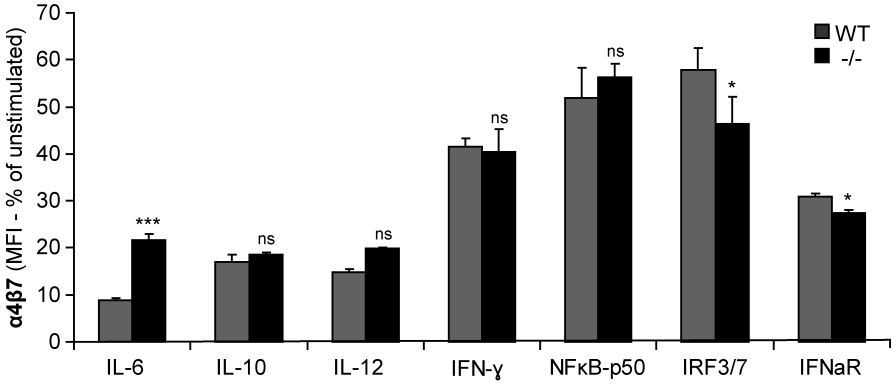

Figure 3.10: $\alpha_4\beta_7$ surface expression patterns of transgenic splenic $CD8^{pos}$ T cells deficient of cytokines or pro-inflammatory transcription factors following CpG stimulation. Splenocytes from different transgenic knockout mice were stimulated with CpG-ODN (3 µg/ml) and were cultured. After 48 hours, $\alpha_4\beta_7$ surface expression was examined on $CD8^{pos}$ T cells by flow cytometry. Data show the mean values of at least triplicate samples ± SEM. Results are representative of 3 independent experiments. $^*P < 0.05$; $^{***}P < 0.001$; ns indicates not significant. The asterisk indicates comparison to the corresponding CpG-stimulated wild type group.

These data strongly suggest that IL-6 plays an important role in DC-mediated downregulation of $\alpha_4\beta_7$ expression on lymphocytes upon CpG stimulation. IL-6-deficiency significantly impairs activated DC capability to induce downregulation of the homing receptor. In a next step, we tested whether these CpG-activated cells actually produce considerable amounts of IL-6 that could then induce downregulation of $\alpha_4\beta_7$ on co-cultured lymphocytes. Therefore, splenocytes were cultured in the presence of different TLR ligands, namely PAM_3CSK_4 (TLR2), LPS (TLR4), CL097 (TLR7) and CpG-DNA (TLR9). After 24 hours, the culture supernatant was obtained and IL-6 levels were determined by ELISA. Compatible with previous reports that murine DC secrete large amounts of different cytokines upon stimulation of TLR [Sparwasser et al., 1998], activated splenocytes produced an abundance of IL-6 (Figure 3.11). The highest secretion of IL-6 by splenocytes was observed after stimulation with CpG-DNA. But other TLR ligands also induced considerable amounts of IL-6 and these differences could simply resemble unbalanced activation levels due to differing TLR ligand activity.

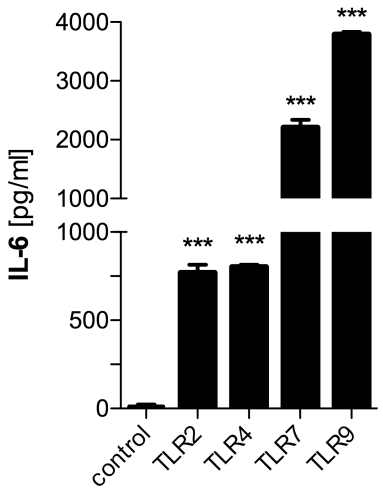

Figure 3.11: IL-6 secretion of murine splenocytes following TLR stimulation. Splenocytes were stimulated either with Pam_3CSK_4 (1 µg/ml), LPS (1 µg/ml), CL097 (5 µg/ml) or CpG-ODN (3 µg/ml) and were then cultured. After 24 hours, IL-6 in the culture supernatant was measured by ELISA. Data show the mean values of triplicate samples ± SEM. Results are representative of 2 independent experiments. ****P* < 0.001.

Our data show that CpG-activated splenocytes produce high amounts of IL-6. However, expression levels of $\alpha_4\beta_7$ even on IL-6-deficient splenocytes stimulated with CpG-DNA are clearly reduced when compared to unstimulated spleen cells. Hence, other DC-released factors may contribute to the regulation of $\alpha_4\beta_7$. To investigate whether IL-6 alone is sufficient to induce downregulation of $\alpha_4\beta_7$ on T cells or whether other cytokines may act synergistically with IL-6, we cultured splenocytes in the presence of recombinant cytokines. After 48 hours, $CD8^{pos}$ T cells were analyzed for $\alpha_4\beta_7$ surface expression by flow cytometry. Very interestingly, treatment of splenocytes with recombinant IL-6 did not result in significantly reduced expression levels of $\alpha_4\beta_7$ on $CD8^{pos}$ T cells (Figure 3.12). Similarly, other common DC-released cytokines did not induce downregulation of the homing receptor. These data suggest that IL-6 alone is not sufficient to mediate the DC-induced downregulation of $\alpha_4\beta_7$ on T cells after CpG stimulation.

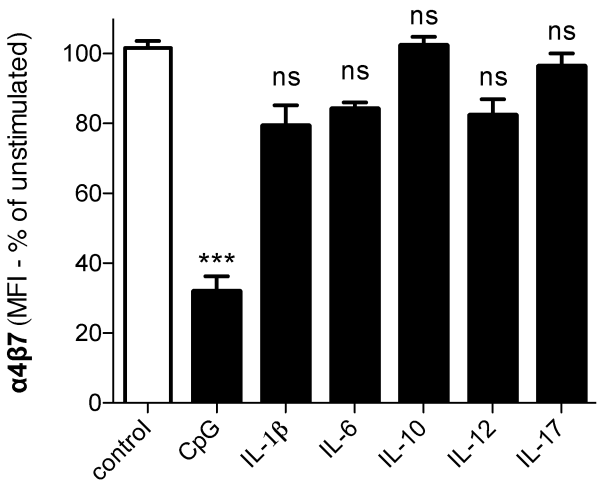

Figure 3.12: Effect of recombinant cytokines on $\alpha_4\beta_7$ expression of splenic CD8^{pos} T cells. Splenocytes were cultured in the presence of CpG-DNA (3 µg/ml) or different recombinant cytokines, namely IL-1β, IL-6, IL-10, IL-12p40 or IL-17 (each with 250 ng/ml). After 48 hours, $\alpha_4\beta_7$ expression of CD8^{pos} T cells was measured by flow cytometry. Data show the mean values of triplicate samples ± SEM. Results are representative of 2 independent experiments. ****P* < 0.001, ns indicates not significant.

3.2.5 Differences in $\alpha_4\beta_7$ regulation of antigen-specific and -unspecific naïve CD8^{pos} T cells after TLR4 stimulation *in vivo*

Previous studies have proven that tissue-specific homing properties are imprinted on lymphocytes during activation of their antigen-specific receptors by DC in secondary lymphoid organs [Johansson-Lindbom et al., 2003; Mora et al., 2005]. DC from MLN [Stagg et al., 2002] or PP [Mora et al., 2003] but not PLN are capable to imprint gut-homing specificity on activated T cells in the presence of TLR ligands. Our data now show that T cells from different SLO downregulate the expression of $\alpha_4\beta_7$ upon stimulation with different TLR ligands mediated by DC.

Treatment of immune cells with activators of the innate immune system such as CpG-DNA or other TLR ligands triggers stimulation of T cells without requiring detection of a nominal antigen by the Tcell receptor. This cytokine-driven antigen non-specific T cell activation is mediated mainly by DC and has been referred to as ‚bystander activation' [Kamath et al., 2005]. This bystander activation has two specified components: On the one hand, there is a strongly upregulated expression of the phenotypic activation marker CD69 on naïve and memory CD8^{pos} T cells, reported after the application of either polyI:C (TLR3), LPS (TLR4) or CpG-DNA (TLR9) in mice. On the other hand, there is division of memory CD8^{pos} T cells [Tough et al., 1996; Tough et al., 1997; Sun et al., 1998]. Bystander activated T cells have been reported to show increased responsiveness to subsequent stimulation via the TCR [Ramanathan et al., 2008].

We hypothesized that the expression of the gut-homing receptor $\alpha_4\beta_7$ in response to stimuli of the innate immune system is differentially regulated on antigen-specifically activated versus non-specifically activated, bystander lymphocytes. To investigate this question we utilized OT-I transgenic mice in an adoptive transfer model. In these animals, all $CD8^{pos}$ T cells have the same T cell receptor that recognizes the model antigen ovalbumin. This common model allows for the analysis of antigen-specific T cell responses.

$CD8^{pos}$ T cells were purified by magnetic sorting from splenocyte single cell suspensions of OT-I transgenic and strain matched wild type (WT) mice. OT-I $CD8^{pos}$ T cells were fluorescently labeled with CFSE while WT $CD8^{pos}$ T cells were marked with the fluorochrome Cell Tracker Violet. Both $CD8^{pos}$ T cell preparations were mixed at equal numbers and 5 - 10 x 10^6 labeled cells were injected intravenously into naïve WT recipient mice. One day after the adoptive T cell transfer, mice were immunized intraperitoneally with 100 µg LPS and 500 µg endotoxin-free ovalbumin. Naïve mice of the control group did not receive an adoptive T cell transfer and were treated with 150 µl of PBS intraperitoneally. Two days after immunization, mice were killed and secondary lymphoid organs were obtained. The surface expression of $\alpha_4\beta_7$ on transferred antigen-specifically activated OT-I and unspecific WT $CD8^{pos}$ T cells as well as endogenous $CD8^{pos}$ T cells of naïve WT mice was analyzed by flow cytometry.

As reported in previous studies [Johansson-Lindbom et al., 2003], $CD8^{pos}$ OT-I T cells that are activated in the MLN by their cognate antigen and LPS show a gut-homing phenotype with very strong upregulation of $\alpha_4\beta_7$ (Figure 3.13 right panel). Consistent with earlier findings [Agace, 2006], OT-I T cells activated in the spleen show intermediate expression of $\alpha_4\beta_7$ while OT-I cells activated in PLN do not upregulate the gut-homing receptor (Figure 3.13 left and middle panel). At the same time, transferred WT $CD8^{pos}$ T cells, that in generally do not recognize ovalbumin and are therefore unspecifically LPS-activated bystander cells, showed strongly reduced expression of $\alpha_4\beta_7$ when compared to $CD8^{pos}$ T cells of naïve WT mice. Endogenous $CD8^{pos}$ T cells of recipient mice showed downregulation of $\alpha_4\beta_7$ upon stimulation with LPS and ovalbumin comparable to previously transferred WT $CD8^{pos}$ T cells (data not shown). The downregulation of $\alpha_4\beta_7$ on WT $CD8^{pos}$ T cells in response to TLR4 stimulation seems to be a broad reaction pattern as it could be observed in all analyzed secondary organs. In summary, these data strongly suggest that antigen-specifically activated $CD8^{pos}$ T cells show diverse $\alpha_4\beta_7$ regulatory mechanisms in different SLO while

unspecifically LPS-activated bystander T cells respond with a uniform pattern of downregulation of the gut-homing receptor $\alpha_4\beta_7$.

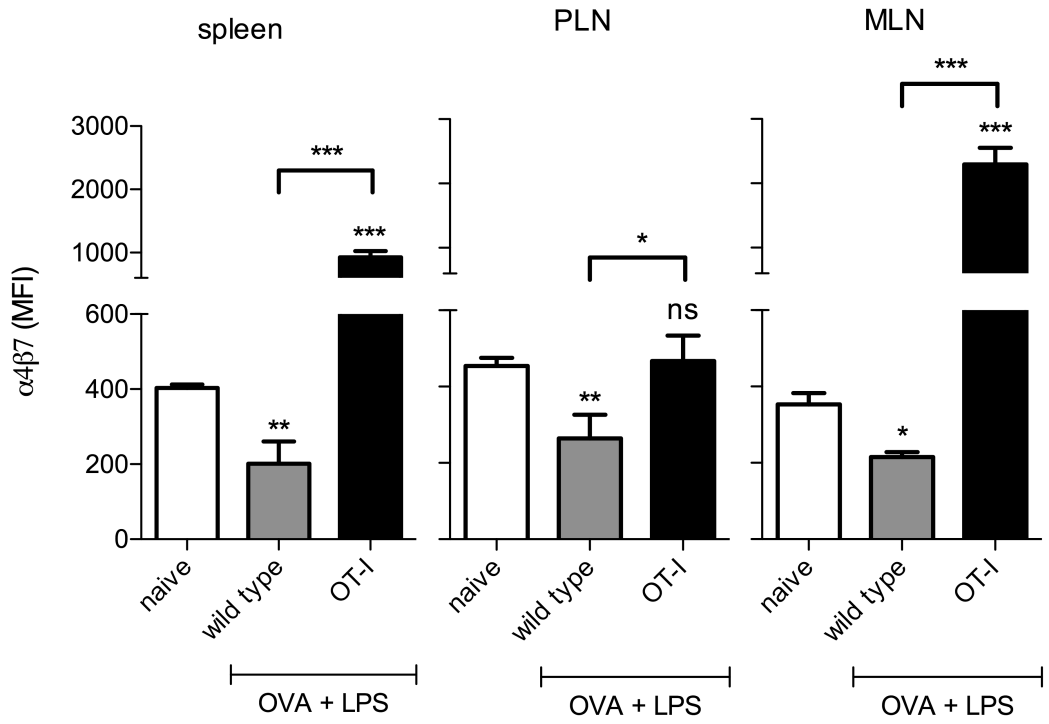

Figure 3.13: Differences in $\alpha_4\beta_7$ regulation of antigen-specific and -unspecific CD8^{pos} T cells after TLR4 stimulation in vivo. CD8^{pos} T cells were sorted out of splenocytes from wild type or OT-I transgenic mice by MACS. The two CD8^{pos} T cell preparations were stained with either CFSE or the Celltracker Violet, mixed at equal numbers and were adoptively transferred into naïve wild type recipient mice. 24 hours after the transfer of 5-10 x 10^6 T cells, mice were immunized with 500 µg ovalbumin and 100 µg LPS ip. Naïve mice in the control group did not receive an adoptive T cell transfer and were treated with 150 µl PBS ip. 48 hours after this immunization, $\alpha_4\beta_7$ expression was analyzed on transferred wild type and OT-I transgenic CD8^{pos} T cells as well as endogenous CD8^{pos} T cells of naïve control mice in different SLO. Data show the mean values of duplicate samples per mouse (n=4) ± SEM. Results are representative of 2 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ns indicates not significant. An asterisk without brackets indicates comparison to CD8^{pos} T cells of unstimulated naïve mice.

3.3 Imprinting of a gut-homing phenotype

3.3.1 Toll-like receptor effects on T cell gut imprinting

Our previous experiments have shown that bystanding naïve lymphocytes activated by DC-derived cytokines upon TLR stimulation in the absence of their cognate antigen subsequently downregulate the gut-homing receptor $\alpha_4\beta_7$. Now we wanted to investigate the effect of TLR stimulation on the process of imprinting gut-homing

specificity on T cells itself during antigen-specific T cell receptor simulation by dendritic cells.

Therefore, single-cell suspensions gained from peripheral and mesenteric lymph nodes were labeled with the fluorescent marker CFSE to later visualize cell proliferation. After extensive washing, complete cells were stimulated with CpG-DNA and cultured. Soluble anti-CD3ε antibody was added to the culture to simulate T cell receptor stimulation (see below). After five to eight days, proliferation and $\alpha_4\beta_7$ expression was measured on different T cell subsets by flow cytometry.

The αβ T cell receptor binds its cognate peptide-MHC complex on antigen-presenting cells but is not able to signal to the cell that antigen is actually bound. The T cell receptor is associated with four signaling chains (two ε, one ɣ, one δ) collectively called CD3 which link binding of extracellular antigen to intracellular signaling. A monoclonal antibody against the ε chain (anti-CD3ε) activates the signal transducer and subsequent intracellular signaling thereby resembling T cell receptor stimulation in the absence of the cell's cognate peptide-MHC complex. The soluble anti-CD3ε used in this work - unlike its plate-bound counterpart - is dependent on accessory cells like DC to be presented to T cells [Stagg et al., 2002]. The use of an accessory cell-dependent stimulus maximizes the potential for modification of the response by accessory cells such as DCs during the interaction with T cells. Therefore anti-CD3ε is a DC-dependent *pan*-T cell stimulus resembling antigen-specific T cell receptor activation which is a prerequisite for tissue-specific T cell imprinting.

Compatible to previous studies [Stagg et al., 2002], the proportion of $CD8^{pos}$ T cells expressing $\alpha_4\beta_7$ was consistently higher in cultured MLN than PLN cell solutions. For both cell populations, expression of the homing receptor was progressively upregulated with simultaneously decreasing CFSE-intensity which represents the inversely increasing numbers of T cell divisions (Figure 3.14 A, showing FACS data of one of three representative experiments, gated on $CD8^{pos}$ T cells). As the dye's fluorescent conjugates are retained by T cells throughout maturation and thus are inherited to daughter cells during mitotic cell division, proliferating T cell generations could be traced by linearly decreasing CFSE intensity. As described previously by Stagg et al. in 2002, these findings concluded that expression of the gut-homing integrin $\alpha_4\beta_7$ was enhanced when T cells are activated in the presence of DC derived from MLN compared with activation in the presence of DC from non-mucosal peripheral lymph nodes. The proportion of dividing cells expressing $\alpha_4\beta_7$ was consistently greater for

MLN than for PLN while the homing receptor was only upregulated on cells that had divided and expression progressively increased with the number of cell divisions.

When the single-cell solutions from MLN and PLN were additionally stimulated with CpG-DNA, the percentage of $\alpha_4\beta_7$ expressing $CD8^{pos}$ T cells was clearly reduced when compared to not CpG-stimulated cell cultures. Cytotoxic T cells activated with anti-CD3ε by CpG-stimulated MLN-DC among other cells showed strong proliferation. However, these T cells failed to acquire expression levels of $\alpha_4\beta_7$ comparable to T cells activated by MLN-DC that were not CpG-stimulated. Thus, these findings suggest that TLR9 stimulation attenuated intestinal DC capability to imprint gut-homing specificity on cytotoxic T cell during T cell receptor activation. Accordingly, the proportion of anti-CD3ε-activated $CD8^{pos}$ T cells with upregulated $\alpha_4\beta_7$ in PLN was slightly reduced after CpG stimulation when compared to T cells in the non-CpG PLN group. However, this reduction was not significant.

Figures 3.14 B to D show integrated data of at least three experiments for surface expression of $\alpha_4\beta_7$ on anti-CD3ε-activated T cell subsets cultured within MLN or PLN single-cell solutions. Expression of the gut-homing receptor is shown for every T cell generation as fold increase compared to base-level $\alpha_4\beta_7$ expression on non-responding cells. Again, for both PLN and MLN single-cell cultures the proportion of proliferating $CD8^{pos}$ T cells expressing $\alpha_4\beta_7$ integrin increased with the number of cell divisions (Figure 3.14 B). The difference between PLN and MLN cultures was maintained over the full range of cell divisions. Cytotoxic T cells as well as $CD8^{neg}$ T_H cells in MLN cell suspension clearly upregulated $\alpha_4\beta_7$ with every cell generation while additional CpG stimulation diminished this effect (Figure 3.14 C). There was no significant difference in expression of the homing receptor on $CD8^{pos}$ or $CD8^{neg}$ T cells within PLN cell suspension when comparing the CpG-stimulated with the unstimulated group. Recent studies demonstrated that regulatory T cells similar to other T cell subset are imprinted by intestinal DC with a gut-specific phenotype [Siewert et al., 2007]. Our findings confirmed that MLN-derived DC are potent inducers of $\alpha_4\beta_7$ also on regulatory T cells as T_{regs} showed predominant upregulation of the gut-homing receptor when cultured with MLN- but not PLN-derived cell solutions. Similar to cytotoxic and helper T cells, anti-CD3ε-activated proliferating T_{regs} showed significantly impaired expression levels of $\alpha_4\beta_7$ when co-cultured MLN-derived DC were stimulated with CpG-DNA. However, the failure to upregulate $\alpha_4\beta_7$ after CpG-stimulation of MLN-DC was less dramatic among T_{regs} than the other T cell subsets.

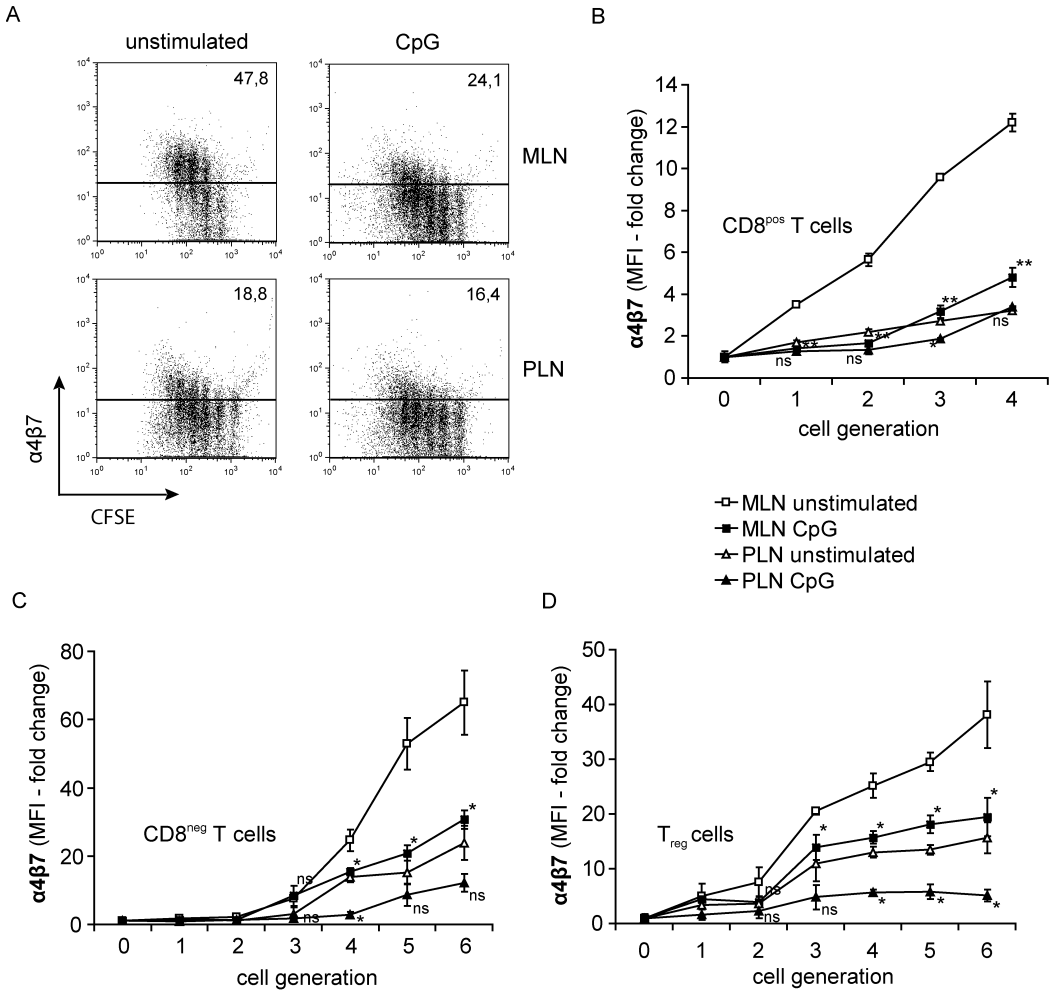

Figure 3.14: Effects of CpG-DNA on anti-CD3ε-activated MLN and PLN cell cultures. Single-cell suspensions from PLN and MLN were labeled with CFSE and stimulated with CpG-ODN (4 µg/ml). After extensive washing, cells were cultured in the presence of soluble anti-CD3ε antibody for 5 to 8 days. $\alpha_4\beta_7$ surface expression on each T cell generation was then measured by flow cytometry. A) FACS dot blots from one representative experiment out of three are gated on $CD8^{pos}$ T cells and numbers indicate percentage of $\alpha_4\beta_7$ expressing cells. (B) Data show the mean values of triplicate samples ± SEM. Results are representative of at least 3 independent experiments. The asterisk without brackets indicates comparison to the CpG-unstimulated group for each tissue culture. $*P < 0.05$; $**P < 0.01$; ns indicates not significant.

In a next step, we excluded possible side-effects on T cell gut-imprinting by cells other than DC. Not less important, we wanted to rule out the source of T cells as a potential parameter in different $\alpha_4\beta_7$-imprinting patterns upon CpG stimulation. Hence, we purified dendritic cells from PLN (PLN-DC) and MLN (MLN-DC) using MACS technique. Pure DC were stimulated with CpG-DNA and co-cultured with MACS-sorted splenic $CD8^{pos}$ T cells in the presence of soluble anti-CD3ε antibody. After 4 to 6 days, $\alpha_4\beta_7$ surface expression was measured on T cells by flow cytometry.

Consistent with our previous experiments and studies by Stagg et al. in 2002, purified anti-CD3ε-activated $CD8^{pos}$ T cells also showed significantly higher levels of $\alpha_4\beta_7$

surface expression when co-cultured with sorted MLN-DC compared to PLN-DC (Figure 3.15). Additionally, splenic $CD8^{pos}$ T cells showed expression levels of the homing receptor similar to MLN-originated cytotoxic T cells when co-cultured with intestinal DC. Upregulation of the homing receptor on $CD8^{pos}$ T cells was clearly diminished when MLN-DC were stimulated with CpG-ODN. T cells co-cultured with PLN-DC showed no significant difference in $\alpha_4\beta_7$ expression levels when DC were stimulated with CpG-DNA.

Thus, we demonstrated that CpG-ODN clearly influenced DC-mediated imprinting of T cells. MLN-DC which induced high expression levels of $\alpha_4\beta_7$ on co-cultured cytotoxic, helper and regulatory T cells, to a great extent lost this ability after CpG stimulation. PLN-DC did not induce high levels of the homing receptor on T cells, therefore CpG-stimulation lead to no significant differences in $\alpha_4\beta_7$ expression levels on co-cultured T cells. Imprinting effects by dendritic cells were independent from the source of responding T cells.

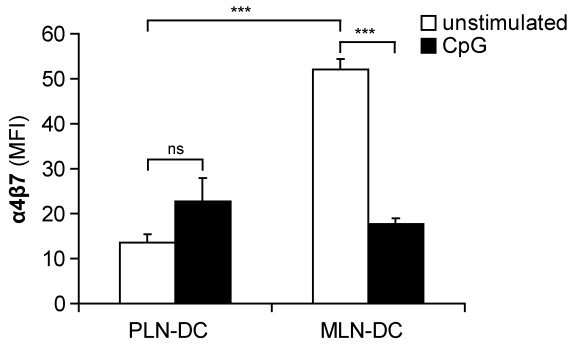

Figure 3.15: Effects of CpG-ODN stimulation on T cell imprinting. $CD11c^{pos}$ DC were purified from PLN or MLN, stimulated with CpG-ODN (4 µg/ml) and co-cultured with sorted splenic $CD8^{pos}$ T cells in the presence of soluble anti-CD3ε antibody. After 4 to 6 days, $\alpha_4\beta_7$ surface expression was measured on responding T cells by flow cytometry. Data show the mean values of triplicate samples ± SEM. Results are representative of at least 3 independent experiments. ***$P < 0.001$; ns indicates not significant.

3.3.2 Effects of TLR ligands on the retinoic acid metabolism of dendritic cells

Previous studies implicate a central role of the vitamin A metabolite retinoic acid in imprinting a gut-homing phenotype on lymphocytes as vitamin A deficiency affects $\alpha_4\beta_7$ expression of T and B cells. Compatibly, vitamin A-deficient mice have a dramatic reduction in the number of T cells specifically in the gut mucosa [Iwata et al., 2004]. Indeed, vitamin A-derived retinoic acid specifically enhances the expression of the gut-homing molecules $\alpha_4\beta_7$ and CCR9 on naïve T_H cells upon immobilized antibodies to CD3 and CD28; even in the absence of dendritic cells. RA-treated T cells migrate into

small-intestinal tissues more efficiently than control cell indicating that RA imprints T cells with gut-homing specificity when T cells are activated through the T cell receptor/CD3 complex [Iwata et al., 2004]. Iwata et al. also found that RA is produced by intestinal DC but not DC from PLN or spleen. Antagonists of the retinoic acid receptor suppress the capacity of intestinal DC to induce $\alpha_4\beta_7$ expression on T cells, indicating that RA is the physiologic and essential 'imprinter' for gut-homing specificity on lymphocytes [Iwata, 2009]. With our findings, that stimulation with TLR9 ligand CpG-DNA attenuates MLN-derived DC capability to imprint gut-homing specificity on T cells, we wanted to investigate the effect of TLR9 stimulation on the production of retinoic acid by dendritic cells.

RA biosynthesis from vitamin A occurs locally where it is required. The key step, an irreversible conversion of retinal to RA is catalyzed by retinal dehydrogenases encoded by the *Aldh1a* gene family, a subfamily of class I aldehyddehydrogenases, which are expressed in limited cell types [Napoli, 1999]. Furthermore, expression of these retinol aldehyd dehydrogenases (RALDH) show a tissue-restricted pattern and hence may be at least partially responsible for the differential regulation of homing molecules in intestinal and non-intestinal lymph nodes. In this regard, especially RALDH2 mRNA was strongly expressed in MLN-derived DC but only detected at low levels in PLN DC [Johansson-Lindbom and Agace, 2007]. Thus, to determine the effect of TLR9 stimulation on production of retinoic acid by dendritic cells, we wanted to investigate the expression patterns of RALDH2 in intestinal and non-intestinal dendritic cells after CpG-DNA stimulation by the determination of RALDH2 mRNA levels.

Therefore, mice were injected with Flt3-L-secreting melanoma cells to enhance dendritic cell frequency in secondary lymphoid organs (see chapter 2.2.4). Twelve to 14 days later, mice were sacrificed and DC were purified from PLN and MLN using the MACS technique. Sorted DC as well as additionally preparated hepatocytes were stimulated with CpG-ODN and cultured. After 18 hours, DC and hepatocytes were lysed and complete RNA was extracted and reversely transcribed into cDNA. Then, RALDH2 mRNA levels were measured using quantitative real-time polymerase chain reaction (qRT-PCR). RALDH2 mRNA levels were normalized to mRNA levels of ubiquitary expressed hypoxanthin-phosphoribosyl-transferase (HPRT).

Immature dendritic cells originated from both mesenteric and peripheral lymph nodes as well as complete hepatocytes all expressed measurable levels of RALDH2 mRNA. As described by Johansson-Lindbom and Agace in 2007, RALDH2 mRNA levels were

most prominent among MLN-derived DCs while PLN-DC and hepatocytes expressed only low levels of the enzyme's mRNA (Figure 3.16). However, when stimulated with CpG-ODN, both DC populations significantly upregulated RALDH2 mRNA levels when compared to constitutively expressed mRNA of the housekeeping gene HPRT. After CpG stimulation, both intestinal and non-intestinal DC showed similarly high expression of RALDH2 mRNA. Hepatocytes also increased expression of RALDH2 mRNA after TLR9 stimulation. However, the extend of upregulation was less intensive on hepatocytes as compared to dendritic cells.

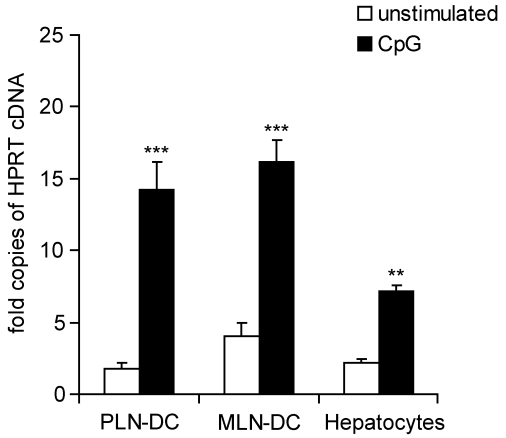

Figure 3.16: Effect on CpG-DNA stimulation on RALDH2 mRNA levels in dendritic cells and hepatocytes. Hepatocytes and sorted CD11c^{pos} DC from PLN and MLN were stimulated with CpG-ODN (4 µg/ml) and cultured for 18 hours. Cells were then lysed and complete RNA was extracted and reversely transcribed into cDNA. Finally, RALDH2 mRNA levels were measured by quantitative real-time PCR and are expressed relative to HPRT mRNA levels for each sample. Data show the mean values of triplicate samples ± SEM. Results are representative of two independent experiments. $^{**}P < 0.01$; $^{***}P < 0.001$.

3.3.3 Opposing effects of retinoic acid and TLR ligands on T cell gut-homing specificity

With conflicting findings that CpG-DNA stimulation attenuates intestinal DC gut imprinting capacity which is known to be RA-dependent but simultaneously enhances their expression of RALDH mRNA – the key enzyme in RA synthesis – we wanted to investigate the effect of CpG-stimulated dendritic cells on RA-induced upregulation of $\alpha_4\beta_7$ on lymphocytes.

According to protocols from previous studies [Iwata et al., 2004], complete splenocytes were therefore activated with anti-CD3ε and additional anti-CD28. CD28 is expressed on T cells in association with their antigen-specific Tell receptor. CD28 binds to co-stimulatory molecules on antigen-presenting cells and thereby delivers the essential

second signal alongside T cell receptor-peptide-MHC complex interaction. Thus CD28 signaling drives activation and proliferation of T cells that encounter specific antigen presented by dendritic cells or other professional APC. Combined activation of CD3ε and CD28 results in a *pan*-T cell stimulus and proliferation independent of accessory cells such as DC. Such activated T cells were cultured with different concentrations of retinoic acid to induce a gut-homing phenotype. CpG-DNA was added to the culture to stimulate dendritic cells. Three to four days later, $\alpha_4\beta_7$ surface expression on T cells was measured by flow cytometry.

Compatible with findings of Iwata et al. in 2004, retinoic acid induced high expression of the gut-homing integrin $\alpha_4\beta_7$ on $CD8^{pos}$ and $CD8^{neg}$ T cells that were activated by anti-CD3ε and anti-CD28 (Figure 3.17). Upregulation of the homing receptor was observed with RA concentrations as low as 0.1 nM and grew in prominence with increasing culture concentration of the vitamin A-metabolite. As described previously, the induction of $\alpha_4\beta_7$ on anti-CD3ε/CD28-activated T cells was independent of dendritic cells as DC-depleted splenocyte cultures showed unaffected upregulation of the gut-homing molecule (data not shown). Splenocytes that were cultured in the presence of additional CpG-DNA, showed impaired induction of $\alpha_4\beta_7$ on cytotoxic and helper T cells. Culture concentrations of the TLR9 ligand of 0.3 µg/ml did not result in significantly altered expression levels of the gut-homing receptor on T cells, while CpG concentrations of 3.0 µg/ml entailed impaired upregulation of $\alpha_4\beta_7$ on T lymphocytes. Even in the presence of CpG-DNA, retinoic acid induced $\alpha_4\beta_7$ on T cells in a dose-dependent manner although this induction was clearly decreased in its magnitude.

Thus we demonstrated that CpG stimulation of spleen cells significantly reduces RA-mediated DC-independent upregulation of $\alpha_4\beta_7$ on anti-CD3ε/CD28-activated T cells amongst splenocytes. CpG-DNA concentrations of 3.0 µg/ml are sufficient to attenuate induction of the gut-homing receptor by RA but fail to totally abrogate imprinting effects of retinoic acid concentrations > 1 nM.

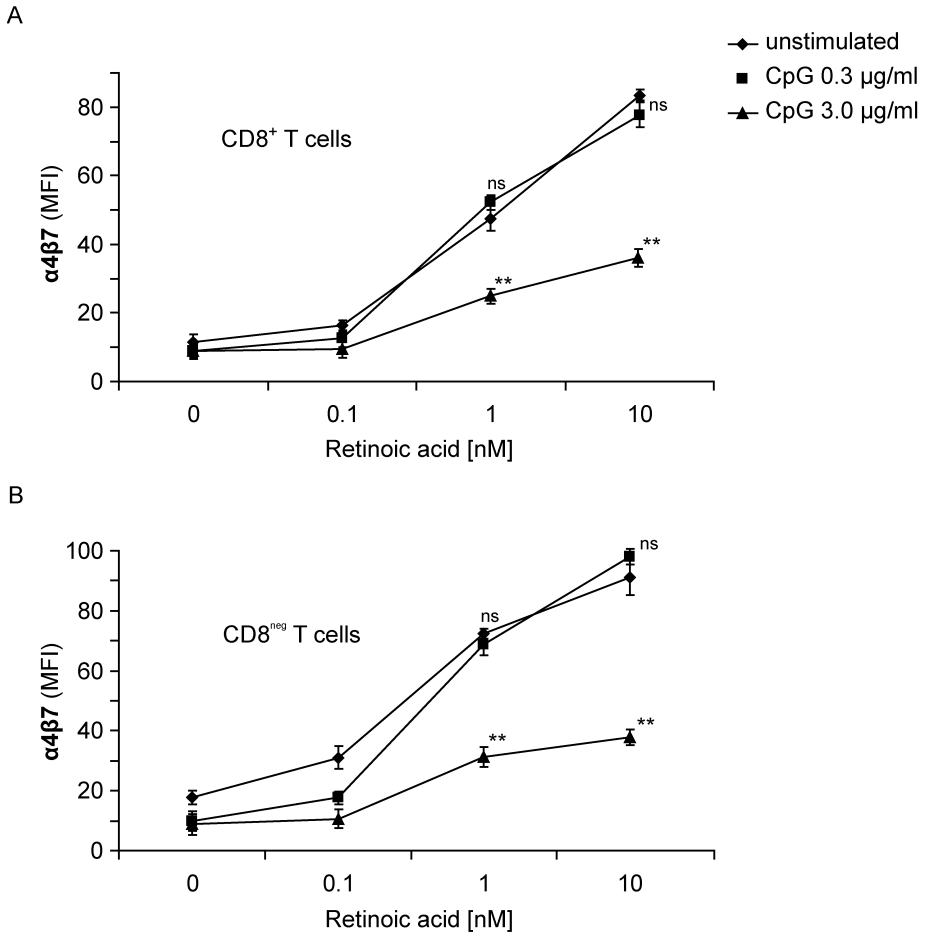

Figure 3.17: Competitive effects of retinoic acid and TLR ligands on T cell gut-homing specificity. Splenocytes were activated with anti-CD3ε and anti-CD28 antibodies and cultured in the presence of different concentrations of retinoic acid. Variable amounts of CpG-DNA were added to the cultures. After three to four days, $\alpha_4\beta_7$ surface expression on T cells was measured by flow cytometry. Data show the mean values of quadruplicate samples ± SEM. Results are representative of two independent experiments. The asterisk indicates comparison to the CpG-unstimulated group for each RA concentration. $^{**}P < 0.01$; ns indicates not significant.

3.4 Effects of TLR ligands on lymphocyte homing *in vivo*

Previous experiments have shown that after stimulation with Toll-like and other pattern-recognition receptors, dendritic cells release IL-6 amongst other cytokines inducing significant downregulation of the gut-homing receptor $\alpha_4\beta_7$ on naïve lymphocytes. In a next step, we investigated whether this downregulation of $\alpha_4\beta_7$ has functional consequences and results in altered migration patterns of responding T and B cells *in vivo*. Therefore, we utilized short term homing experiments as used previously to investigate lymphocyte migration patterns *in vivo* [Mora et al., 2003; Iwata et al., 2004].

Splenocytes were cultured with or without CpG-ODN for 48 hours. Both cell preparations were labeled with the fluorescent marker CFSE. Two different CFSE concentrations were used to later distinguish the two experimental groups. This resulted in two clearly distinct CFSE labeling intensities with CpG-stimulated splenocytes being CFSE highly positive ($CFSE^{high}$) and naive spleen cells showing low CFSE fluorescence intensity ($CFSE^{low}$). After extensive washing, both cell preparations were mixed an injected intravenously into naïve recipient mice. An aliquot was saved to assess the input ratio ($[CFSE^{high}]_{input}$ / $[CFSE^{low}]_{input}$). Four to eight hours after the adoptive transfer, mice were sacrificed and preparated organs screened for the injected splenocytes by flow cytometry. For all adoptively transferred lymphocyte subpopulations $CFSE^{high}$ / $CFSE^{low}$ ratios were measured in each tissue preparation and normalized to the input ration. Therefore, the homing index (HI) was calculated as the ration of $[CFSE^{high}]_{tissue}$ / $[CFSE^{low}]_{tissue}$ to $[CFSE^{high}]_{input}$ / $[CFSE^{low}]_{input}$.

With a homing index close to 1.0, $CD8^{pos}$ T cells showed no altered migration to spleen and peripheral lymph nodes after CpG-stimulation in *in vivo*-short term homing experiments (Figure 3.18). In MLN and PP, secondary lymphatic organs of the gastro-intestinal tract, the HI of cytotoxic T cells was significantly reduced after TLR9 activation. In the same way, CD^{8pos} T cell homing to small bowl and colon was clearly diminished after CpG-stimulation. T cell migration to liver and lung showed no relation to the innate stimulus. $CD8^{neg}$ T cells showed similar migratory. However, migration of $CD8^{neg}$ T cells to gut lymphatic and effector organs was reduced to a less extent after CpG-stimulation when compared to $CD8^{pos}$ T cells. Regulatory T cells too, showed reduced migration to MLN and PP after CpG stimulation. Small bowl and colon as well as the lung could not be analyzed for T_{regs} due to technical reasons. Notably, T_{regs} showed increased migration to PLN when stimulated with CpG-ODN. B cell migration patterns were basically similar to those of $CD8^{pos}$ T cells.

Thus, we demonstrated that the observed downregulation of $\alpha_4\beta_7$ on lymphocytes mediated by DC after CpG-stimulation results in altered migration patterns *in vivo*. When adoptively transferred after CpG-stimulation T and B cells migrated significantly less to the GI-tract and associated secondary lymphoid organs. CpG-ODN has no significant effect on migratory behavior to spleen, PLN, liver and lung except for T_{regs} which show increased migration to PLN after CpG stimulation.

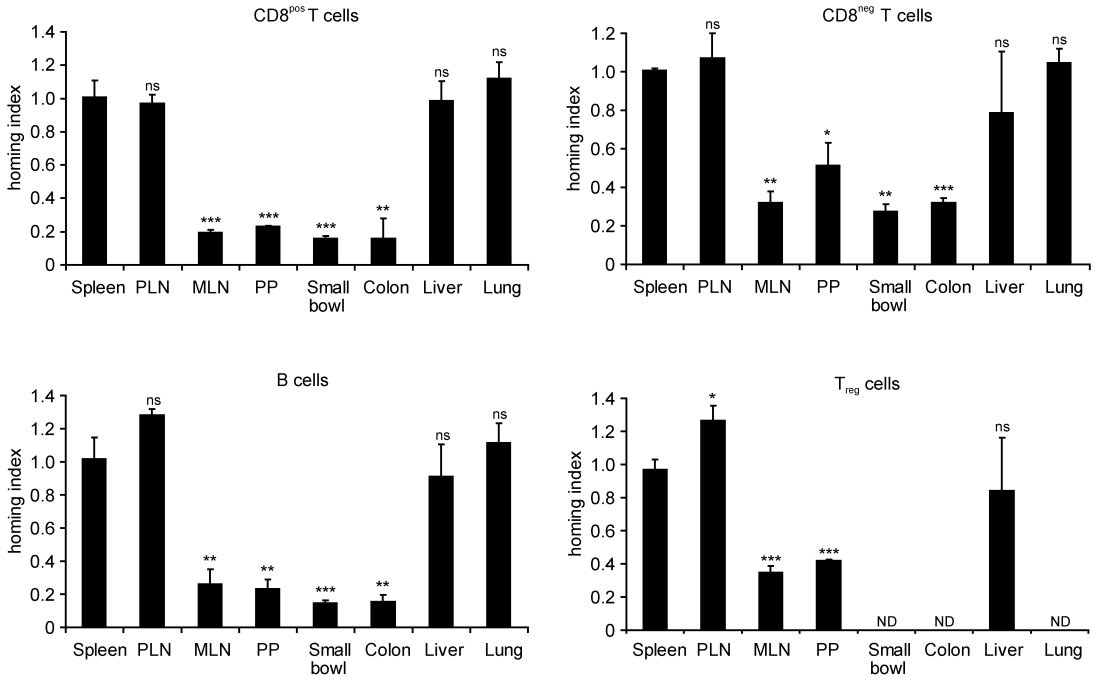

Figure 3.18: CpG-ODN stimulation has an effect on lymphocyte gut-homing *in vivo*. Splenocytes were cultured with or without CpG-ODN (4 µg/ml) for 48 hours and stained with CFSE (1mM or 0.1 mM). Both cell preparations were mixed and injected intravenously into recipient mice. After 4 hours adoptive transfer, organs were analyzed for the injected splenocytes. The homing index (HI, the ratio of [stimulated $CFSE^{high}$ cells] / [unstimulated $CFSE^{low}$ cells] corrected to the input ration) in recipient tissues was determined by flow cytometry. Data show the mean values of individual recipient mice (n = 3) ± SEM. Results are representative of 3 independent experiments. The asterisk indicates comparison to HI for spleen homing. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; ns indicates not significant.

4 DISCUSSION

4.1 TLR stimulation of dendritic cells affects gut-tropic homing of both naïve and antigen-experienced bystander lymphocytes

During their recirculation, blood-borne lymphocytes make a series of interactions with endothelial cells. Naïve lymphocytes preferentially leave the blood stream to organized lymphoid tissues where they edge their way through the vessel wall and enter the tissue stroma to scavenge the connective tissue network in search for their specific antigen [von Andrian and Mempel, 2003]. If a lymphocyte does not find its antigen, it leaves the lymphatic organ via efferent lymphatic vessels and reenters the systemic blood circulation. The then still naïve lymphocyte is free to continue its journey to any other secondary lymphoid organ. But in the rare event that a naïve T or B cell finds its cognate antigen in the lymphoid organ, its migratory pattern changes profoundly. The lymphocyte starts to rapidly proliferate and differentiate and its numerous descendents then enter the blood circulation via the efferent lymphatic vessels. However, these activated effector and memory cells – now imprinted in specific tissue microenvironment – preferentially leave the blood stream in the same type of lymphatic tissue in which their ancestry has initially been activated. Even more fundamentally, they have acquired new properties in terms of homing molecules which allow them to leave the blood stream in non-lymphoid peripheral tissue which is associated with and mainly draining into the lymph node where tissue-specific imprinting took place. Here they exert their effector function in fighting pathogens or transformed host cells.

Intestinal homing involves recirculation to organized lymphatic tissues (such as MLN and PP) and to the non-lymphoid lamina propria of the intestinal wall. Some of the lymphocytes that enter the lamina propria are further attracted to the epithelium of the gut where they constitute the unique population of intraepithelial lymphocytes; a characteristic feature shared by all mucosal sites [Salmi and Jalkanen, 2005]. MLN and PP serve as primary target organs for the entry of naïve lymphocytes while lamina propria and associated epithelium function as the ‘peripheral’ effector target organs for activated gut-specific lymphocytes.

4.1.1 The gut-specific integrin $\alpha_4\beta_7$ has a special status amongst homing receptors

As described above, efficient homing to lymphoid and peripheral sites requires organ-specific homing receptors interacting with endothelial adhesion molecules. Although numerous such molecules are involved in lymphocyte interaction with vascular endothelium at different steps of the adhesion cascade, only a few of them guarantee tissue specificity of the homing process. These homing receptors are best characterized for the dichotomous systems of skin and intestinal homing. The integrin $\alpha_4\beta_7$, the gut-specific molecule besides CCR9 and questionable CCR10, has an outstanding position amongst the inhomogeneous family of homing molecules. Uncommonly, $\alpha_4\beta_7$ is highly involved in lymphocyte recirculation to both operational units of the intestinal immune compartment: lymphoid tissues and peripheral effector sites [Butcher and Picker, 1996]. Furthermore, the $\alpha_4\beta_7$ integrin facilitates – in contrast to all other homing molecules – several steps of the adhesion cascade.

Key players in lymphocyte homing to the skin are CCR4, CCR10 and the cutaneous lymphocyte antigen (CLA). CLA is a carbohydrate modification of P-Selectin glycoprotein ligand-1 that binds to P- and E-Selectin which are constitutively expressed on endothelium of skin venules [Fuhlbrigge et al., 1997]. Therefore, CLA is vital for lymphocyte recirculation to the skin. However, other sets of homing molecules mediate the initial migration of T and B cells to skin-draining peripheral lymph nodes where they may meet their cognate antigen and be imprinted with skin-specificity. In contrast, the integrin $\alpha_4\beta_7$ not only plays a critical role in both recirculation of antigen-experienced cells to the intestinal lamina propria (their peripheral effector organ) and of naïve lymphocytes to intestinal lymphatic tissue but also is important in more than one step of both these extravasation processes [Hamann et al., 1994]. Thus, while initial interactions like tethering and rolling of naïve lymphocytes in high endothelial venules of peripheral lymph nodes are exclusively mediated by interaction of L-Selectin with the endothelial adhesion molecule PNAd, HEV in mesenteric lymph nodes and PP also express MAdCAM-1 which binds $\alpha_4\beta_7$ on incoming lymphocytes [Berlin et al., 1993]. After chemokine activation, $\alpha_4\beta_7$-MAdCAM-1 interaction also facilitates final adhesion to the endothelium and allows subsequent diapedesis. As in gut lymphoid organs, tissue-specific recirculation of antigen-experienced effector lymphocytes to the intestinal lamina propria is dependent of this integrin for several steps: $\alpha_4\beta_7$-MAdCAM-1 interaction facilitates initial tethering and rolling as well as gut-specific adhesion of lymphocytes to the endothelium in lamina propria venules [Berlin et al., 1995].

Thus, $\alpha_4\beta_7$ has a special role amongst homing receptors as it mediates tissue-specific recirculation of lymphocytes to both lymphoid and effector organs of the intestinal compartment. Hence, transgenic mice that lack the β_7-unit of the heterodimeric integrin, not only have hypocellular PP, but also significantly reduced numbers of lymphocytes in the intestinal lamina propria [Wagner et al., 1996]. Furthermore, in short-term *in vivo* assays, these β_7-deficient mice showed reduced lymphocyte migration to lamina propria and PP. However, efficient recirculation to PP required both $\alpha_4\beta_7$ and the 'standard' lymph node receptor L-Selectin, as double-knockout mice showed more severly decreased PP-homing than mice which lacked only one of the homing receptors.

4.1.2 Interleukin-6 release by dendritic cells upon CpG stimulation affects expression of the gut-homing receptor $\alpha_4\beta_7$ on bystander lymphocytes

The $\alpha_4\beta_7$ integrin is expressed at low levels on naïve T and B cells which is enough to allow them to enter and scan MLN and PP in search of their specific antigen [Bargatze et al., 1995]. Due to the simultaneous expression of further homing molecules such as L-Selectin, the same naïve lymphocytes can also leave the blood stream to peripheral non-mucosal lymph nodes. It is therefore coincidence which lymph node is scanned by a single lymphocyte and the spreading of the entirety of naïve lymphocytes among the lymphoid tissues of different immune compartments is a matter of random statistical distribution.

In the present work we show that dendritic cells induce strongly reduced expression of the gut-homing receptor $\alpha_4\beta_7$ on lymphocytes upon stimulation of various PRR. This downregulation seems to be a broad reaction pattern on T and B cells following different stimuli of the innate immune system. However, this regulatory effect was limited to antigen-unspecifically activated bystander cells. While several reports have characterized the expression patterns of $\alpha_4\beta_7$ on antigen-specifically activated lymphocytes [Johansson-Lindbom et al., 2003; Mora et al., 2005], we give evidence for the first time, that bystander activated $CD8^{pos}$ T cells in different SLO show downregulation of the gut-homing integrin $\alpha_4\beta_7$. Because of the very nature of the transgenic OT-I mouse model with only $CD8^{pos}$ T cells having the unique TCR to recognize the model antigen ovalbumin, we could not investigate the regulatory mechanisms of $\alpha_4\beta_7$ on antigen-specific $CD4^{pos}$ T cells and B cells.

In this context, we demonstrated that DC-produced IL-6 was partly responsible for reduced surface expression of $\alpha_4\beta_7$ on T and B cells. However, lymphocytes from transgenic mice which lacked functional IL-6 still showed distinct downregulation of $\alpha_4\beta_7$ upon stimulation with CpG-DNA. Furthermore, $CD8^{pos}$ T cells did not respond with significantly impaired expression levels of the homing receptor when stimulated in vitro with recombinant IL-6 only. We hypothesize that DC-produced IL-6 alone is not potent in mediating the downregulation of $\alpha_4\beta_7$ on lymphocytes upon CpG stimulation by itself. TLR stimulation of DC results in a broad array of secreted cytokines. Together with at least one additional yet unknown factor, IL-6 might synergistically downregulate the surface expression of $\alpha_4\beta_7$ on bystander T and B cells. However, other factors that synergize with IL-6 in the regulation of $\alpha_4\beta_7$ still have to be identified in further studies.

IL-6 has a wide spectrum of biological activities that help to coordinate a systemic response to infection. IL-6 is released by dendritic cells and macrophages after engagement of their pathogen-recognition receptors with microbial or viral PAMP. Additional minor sources of IL-6 are endothelial and epithelial cells as well as T_H cells. Locally, the interleukin activates T and B cells. Systemic effects of IL-6 are the release of acute phase proteins by hepatocytes, mobilization of neutrophilic granulocytes from the bone marrow and an increased body temperature facilitated by protein and energy mobilization in fat and muscle tissue [Horn et al., 2000]. With decreased viral and bacterial replication at elevated body temperatures, all these effects aim to efficiently eliminate infections.

Additional factors released by DC upon TLR stimulation that cooperated with IL-6 to induce downregulation of $\alpha_4\beta_7$ on bystanding naïve lymphocytes could not yet be identified. Other cytokines commonly produced by DC such as IL-10 or IL-12 as well as T cell-derived IL-2 and IFN-γ proved not to be involved in $\alpha_4\beta_7$ surface expression levels. IFN-α, TNF-α and TGF-β seemed rather to attenuate but not to facilitate downregulation of the gut-homing integrin upon TLR stimulation. We also investigated CpG-DNA stimulation of splenocytes that were deficient for one or several transcription factors involved in TLR9 signaling. Both the NF-κB p50-subunit as final transcription factor in the MyD88-dependent pathway as well as IRF-3 and 7 in the TRIF-dependent signaling were not absolutely required for DC to respond to CpG-DNA with release of cytokines that induced downregulation of $\alpha_4\beta_7$ on lymphocytes. These findings may be caused by overlapping functions of the different signaling pathways in production of IL-6 and other pro-inflammatory cytokines. Furthermore, additional yet unidentified

DNA-receptors have been suggested that may contribute in this setting [Ishii and Akira, 2006].

4.1.3 Functional aspects of TLR-induced downregulation of $\alpha_4\beta_7$ in short-term homing experiments

4.1.3.1 Lymphocyte recirculation to the gut is effectively reduced after TLR9 stimulation

Studies using neutralizing antibodies specific for the $\alpha_4\beta_7$-integrin or knockout mice have shown the important role of this homing receptor in mediating the entry of T and B cells into intestinal lymphoid organs as well as into the lamina propria of small and large bowel [Hamann et al., 1994; Lefrancois et al., 1999]. However, $CD8^{pos}$ cytotoxic T cells that were deficient for the β_7-subunit could enter the mouse small intestine and provide immunity to infection with rotavirus, indicating that the $\alpha_4\beta_7$-integrin was not absolutely required for T-cell entry into the intestinal mucosa [Kuklin et al., 2000]. In this work we now demonstrate that stimulation of splenic DC with CpG-ODN results in downregulation of $\alpha_4\beta_7$ surface expression on co-cultured T and B lymphocytes. In subsequent short-term homing experiments these spleen-originated lymphocytes were significantly impaired in their ability to enter intestinal sites. Upon DC-stimulation with the TLR9 ligand, naïve bystander lymphocytes showed unchanged homing to the spleen and PLN while they failed to efficiently recirculate to MLN and PP when compared to unstimulated control cells.

Antigen-experienced lymphocytes are more diverse than naïve T and B cells with respect to their migratory properties and are hence subdivided into central memory, effector memory and effector cells [Sallusto et al., 1999]. The distinct patterns of recirculation of these subgroups represent different levels of immune surveillance. Central memory lymphocytes maintain L-Selectin and, like naïve T and B cells, can migrate into secondary lymphoid organs where they rapidly respond upon reactivation. In contrast, effector and long-lived effector memory cells do not express L-Selectin and cannot recirculate to SLO. However, they efficiently migrate to non-lymphoid peripheral tissues where they exert their immune function. With the short-term homing experiments in this work, we demonstrate that adoptively transferred splenic T and B cells fail to home to the small or large bowel after *in vitro*-pretreatment with CpG-ODN. In contrast, untreated donor lymphocytes, even though in low absolute numbers, efficiently relocate to the small and large intestine. As naïve T and B cells are generally excluded from non-lymphoid organs [Johansson-Lindbom and Agace, 2007], the

transferred lymphocytes that recirculated to the small and large intestine supposedly represent effector memory T and B cells. Indeed, previous studies demonstrated that approximately 29 % of spleen-derived memory T cells in naïve mice show a gut-specific phenotype with high expression of $\alpha_4\beta_7$ [Campbell and Butcher, 2002]. Taken together, these findings suggest that not only naïve bystander but also antigen-experienced bystander effector memory lymphocytes downregulate $\alpha_4\beta_7$ surface expression and show impaired ability to recirculate to intestinal sites when pre-treated with CpG-ODN and adoptively transferred into naïve recipient mice.

4.1.3.2 Homing to the colon: TLR9 affects segregated recirculation to this organ

While the small bowel and the colon share the importance of $\alpha_4\beta_7$-MAdCAM-1 interactions for efficient lymphocyte homing to these effector organs of intestinal immunity [Lefrancois et al., 1999], there seem to be additional still unknown factors that mediate recirculation to the large bowel. Despite the fact that PP-originated DC induce high levels of $\alpha_4\beta_7$ surface expression on $CD8^{pos}$ T cells, these *in vitro*-activated T cells have the capacity to enter the small intestinal lamina propria but show poor migration to the colon mucosa [Mora et al., 2003]. Furthermore, the colon lacks $CCR9^{pos}$ cells and its ligand CCL25 is nearly exclusively expressed in the small intestine [Papadakis et al., 2000]. Consistently, CCR9 blockade decreases adhesion of T lymphocytes in the small but not in the large bowel [Hosoe et al., 2004]. Notably, CCL28 is highly expressed by colonic epithelial cells, although its corresponding receptor CCR10 has been reported only on mucosal B but not T cells [Kunkel et al., 2003].

Thus, it seems that lymphocyte homing to the large bowel depends on additional homing molecules besides $\alpha_4\beta_7$ that are not sufficiently induced by DC from PP. Furthermore, other chemoattractant pathways than CCR9/CCL25 may play a role in directing lymphocyte migration to the colon mucosa. It remains to be determined whether T and B cells primed *in vitro* by DC from colon-draining lymph nodes efficiently home to the colonic mucosa. Notably, in competitive homing experiments, $CD8^{pos}$ T cells activated with appendix-DC migrated very inefficiently to the colon (Mora and von Andrian, unpublished data). In our short-term homing experiments we now demonstrated that DC-released cytokines upon TLR9 stimulation not only impaired the capability of adoptively transferred lymphocytes to enter the small intestine but also to enter the colon. But as lymphocyte recirculation to the colon, too, is highly dependent of the $\alpha_4\beta_7$-integrin, our findings could not give evidence for TLR-induced regulation of the yet unknown colon-homing molecule(s).

4.1.3.3 Steady state versus inflammation: impact on current research models

In current short-term *in vivo* research models, lymphocytes are pretreated *in vitro*, then are fluorescently or radioactively labeled and adoptively transferred into naïve mice. After varying periods of several hours to few a days, recipient mice are sacrificed and the localization of transferred cells is determined to analyze their homing patterns. But lymphocyte recirculation to lymphoid and peripheral effector organs is not only dependent on their expression of homing receptors but also on the endothelium's equipment of adhesion molecules. These vascular addressins are very differently regulated and expressed during inflammation, which results in significant changes between homeostatic and inflammatory states in the relative potency of these molecules to mediate lymphocyte recruitment to specific compartments.

Inflammation-induced chemokines and endothelial adhesion molecules play a role in mediating lymphocyte trafficking to the inflamed gut. Although expression of both CCL25 [Hosoe et al., 2004] and MAdCAM-1 [Briskin et al., 1997] enhances with inflammation, the effects of the interaction with their ligands CCR9 and $\alpha_4\beta_7$ appear to be less important compared to the now rapidly increasing involvement of inflammation-induced pathways including vascular cell adhesion molecule-1 (VCAM-1) and vascular adhesion protein-1 (VAP-1) [Salmi et al., 1993; Sans et al., 1999]. These adhesion molecules are not tissue-specific and are expressed at low levels in vessels of non-inflamed gut tissue but are induced with ongoing inflammation. As inflammation develops, signals that drive organ-specific recirculation may become diluted by generic inflammation-associated processes; an important concept to consider when the impact of certain homing molecules is examined in steady-state experimental settings [Eksteen et al., 2008]. Although a definite role of homeostatic homing molecules $\alpha_4\beta_7$ and CCR9 has been shown in lymphocyte recirculation during ongoing inflammation [Picarella et al., 1997; Petrovic et al., 2004], it will be important to distinguish the relative contribution of tissue-specific versus non-specific traffic molecules to lymphocyte homing in different inflammatory settings.

4.2 Imprinting tissue tropism by dendritic cells and the role of Toll-like receptor ligands

4.2.1 The different effects of TLR ligands on T cell gut-imprinting *in vitro* and *in vivo*

Dendritic cells mediate the selective generation of gut- and skin-tropic lymphocytes during antigen presentation. This process depends on soluble signals derived from regionally imprinted DC. Expression of the gut-specific $\alpha_4\beta_7$-integrin and the skin-tropic ligands of P- and E-Selectin are induced at low levels on T cells activated by a wide range of DC such as those derived from the spleen; this can be considered as a kind of 'default' setting [Agace, 2006]. This default expression of homing molecules can be divergently regulated by soluble factors selectively released by intestinal and skin-derived DC. Intestinal DC produce soluble factors that enhance the expression of $\alpha_4\beta_7$ and CCR9 while suppressing skin-homing molecules. This seems to be – at least in part – mediated by retinoic acid. On the other hand, skin-derived DC and Langerhans cells produce soluble factors that enhance the expression of ligands for P- and E-Selectin and suppress the expression of $\alpha_4\beta_7$ and CCR9. Several studies support these findings as murine TCR-transgenic T cells stimulated either with antigen-pulsed DC isolated from intestinal lymph nodes or anti-CD3 antibody in the presence of these DC expressed high levels of $\alpha_4\beta_7$ and CCR9 [Stagg et al., 2002; Johansson-Lindbom et al., 2003; Mora et al., 2003]. DC were necessary and sufficient for *in vitro*-induction of these gut-homing molecules as DC-depleted mesenteric lymph node-cells failed to imprint gut-specificity on co-cultured cytotoxic T cells [Johansson-Lindbom et al., 2003].

In this work we demonstrate that stimulation of DC with a TLR9 ligand attenuated their ability to imprint gut-specificity upon T cells *in vitro*. T lymphocytes activated with soluble anti-CD3ε antibody and co-cultured with DC showed significantly impaired surface expression of the gut-homing integrin $\alpha_4\beta_7$ if DC were previously stimulated with CpG-DNA. These findings applied for PLN-derived as well as MLN-derived DC. The unorthodox fact that DC from peripheral non-mucosal lymph node can imprint low levels of gut-homing molecules *in vitro* after all has been described previously [Dudda et al., 2005]. Recent studies suggest that splenic DC possess the enzymatic machinery to generate functional ligands of the retinoic acid-receptor at concentrations sufficiently high to induce low expression of $\alpha_4\beta_7$ *in vitro.* However, T cells activated by TLR4 ligands *in vivo* in the splenic environment failed to express $\alpha_4\beta_7$. Thus, additional mediators within non-mucosal environments appeared to suppress $\alpha_4\beta_7$ induction [Svensson et al., 2008].

Further reports showed that the expression of intestinal and cutaneous homing receptors is induced on T cells following their priming in gut- and skin-associated lymph nodes *in vivo.* After intraperitoneal administration of a model antigen TCR-transgenic T cells activated in MLN upregulated their expression of $\alpha_4\beta_7$ and CCR9, whereas priming in peripheral lymph nodes resulted in induction of the skin-tropic homing molecules P- and E-Selectin ligands [Campbell and Butcher, 2002; Johansson-Lindbom et al., 2003; Stenstad et al., 2006]. Factors that influenced the efficiency of tissue-specific T cell generation *in vivo* included the route of antigen administration and the use of adjuvant. In an ovalbumin-specific TCR-transgenic model, oral administration of SIINFEKL - a peptide epitope of ovalbumin - led to an efficient upregulation of $\alpha_4\beta_7$ and CCR9 on responding adoptively transferred transgenic T cells in MLN while intraperitoneal administration of antigenic peptide induced comparable expression levels only after co-administration of adjuvant [Johansson-Lindbom et al., 2003]. In these experiments suitable adjuvants for enhancing gut-specific imprinting of adoptively transferred T cells upon intraperitoneal antigen administration were alum, the TLR3-ligand polyI:C and the TLR4-ligand LPS.

These *in vivo*-effects of systemic TLR3/4 stimulation to enhance antigen-specific imprinting of a gut-phenotype on responding T cells in MLN are in contrast with our findings that CpG-DNA impairs gut-imprinting by MLN-derived DC *in vitro.* However, effects of systemic TLR9 instead of TLR3/4 stimulation on T-cell imprinting in mesenteric lymph nodes have not been described yet. Others have shown for humoral but not cellular immune responses that subcutaneously delivered vaccines containing antigen and polyI:C or LPS stimulated the induction of both systemic and mucosal immune responses with high levels of specific serum-IgG and fecal-IgA [Enioutina et al., 2008]. In contrast, vaccines containing TLR9 ligands could induce systemic immune responses but failed to induce mucosal humoral immunity. Furthermore, the TLR3/4 adjuvant-dependent augmentation of a gut-homing phenotype on T cells in MLN in these experiments might be caused by altered DC-migratory behavior and not maturation-induced direct effects upon the imprinting process itself. Turnbull and colleagues have shown in rats that systemic administration of the TLR4 ligand LPS resulted in dramatically enhanced DC emigration from the intestinal lamina propria and the liver into draining lymph nodes [Turnbull et al., 2005]. Unlike their counterparts from spleen, accumulated DC in mesenteric lymph nodes did not express the maturation markers CD80/86 but could have allowed for enhanced gut-imprinting of transferred T cells in MLN upon systemic TLR stimulation in the reports described above just by their increased frequency. In contrast, systemic LPS had no effect on DC numbers and distribution in PP. Thus, further studies will need to

address the divergent effects of TLR-adjuvants on tissue-specific T cell imprinting *in vivo*.

Generally speaking, requirements for imprinting a tissue-specific phenotype upon T and B lymphocytes by DC *in vivo* differ from those of *in vitro*-experiments. First of all, unlike the *in vivo*-situation, DC do not need a maturation stimulus for effective induction of tissue-specificity on T and B cells *in vitro*. However, that might be explained by the fact that DC used for *in vitro*-experiments often have already undergone maturation during the preceding isolation procedure [Johansson-Lindbom et al., 2003]. Furthermore, recent studies have shown that *in vitro*-activation by intestinal DC or addition of retinoic acid is sufficient to instruct expression of $\alpha_4\beta_7$ and CCR9 on lymphocytes whereas *in vivo* tissue-specific stroma cells, but not DC alone, allowed the mesenteric lymph nodes to induce the generation of gut tropism [Hammerschmidt et al., 2008]. Peripheral lymph nodes transplanted into the gut mesenteries failed to facilitate the generation of gut-homing T cells, even though gut-derived DC entered the transplants and primed T cells. In contrast PLN-derived DC that failed to induce $\alpha_4\beta_7$ and CCR9 *in vitro* readily induced these factors *in vivo* upon injection into MLN-afferent lymphatics. The group concluded that *in vivo*, gut-homing T cells could only be generated in a permissive lymph node environment that was determined by resident stroma cells and DC. These resident stroma cells might produce negative signals that impair the generation of gut-homing T cells in transplanted and *in situ* PLN.

In addition, Svensson et al. demonstrated that antigen dose not only played a central role in regulating various aspects of T cell differentiation including cytokine production and cytolytic activity but also in the induction of tissue-specific homing receptors [Svensson et al., 2008]. Activation of TCR-transgenic T cells by MLN-derived DC pulsed with a low antigen dose or administration of a low antigen dose *in vivo* led to efficient generation of $\alpha_4\beta_7^{pos}$ $CCR9^{pos}$ T cells, whereas pulsing of MLN-DC or *in vivo* administration of a high antigen dose resulted in reduced $\alpha_4\beta_7$ and CCR9 surface expression levels. Together, these results suggested that a negative signal is generated at higher antigen doses that could inhibit RA-induced expression of $\alpha_4\beta_7$ and CCR9 downstream of retinoic acid receptor signaling. Similar dose-dependent regulatory effects of maturational stimuli such as TLR ligands upon DC-imprinting properties are conceivable but remain to be determined.

4.2.2 The role of retinoic acid in TLR-attenuated T cell imprinting of gut specificity

The mechanisms by which dendritic cells from different anatomical sites generate tissue-specific T and B cells are currently the subject of intensive investigation. A breakthrough was made by Iwata et. al in 2004, when they found that the ability of intestinal DC to generate $\alpha_4\beta_7^{pos}$ $CCR9^{pos}$ gut-specific T cells lies in their selective ability to produce retinoic acid. We have demonstrated that following activation with CpG-DNA, dendritic cells from peripheral and intestinal lymph nodes show increased intracellular levels of RALDH2 mRNA, a transcript coding for the key enzyme in RA synthesis. These findings are unexpected as CpG stimulation at the same time attenuates the capability of these cells to imprint a gut phenotype upon co-cultured T cells. Little is known about the regulation of the enzyme in dendritic cells and other cell populations. Uematsu and colleagues recently reported that RALDH2 mRNA expression in lamina propria-derived DC was enhanced upon TLR5 stimulation while TLR4 activation failed to induce the enzyme in splenic DC [Uematsu et al., 2008]. It was beyond the scope of this work to analyze the levels of RA produced by dendritic cells or to assess the formation and release of metabolites after CpG stimulation.

In subsequent experiments in this work, we demonstrate that CpG-activated dendritic cells significantly reduced DC-independent upregulation of $\alpha_4\beta_7$ on anti-CD3ε/CD28-activated T cells. These findings suggested that in response to TLR9 stimulation, DC produce an additional factor that inhibited RA-mediated upregulation of the gut-homing receptor $\alpha_4\beta_7$ on co-cultured T cells. Compatible with these findings, Svensson et al. suggested a model in which competing signals generated during DC/T cell interactions play a central role in regulating tissue-homing receptor expression on responding T cells, and that the relative intensity of these signals is dependent on the DC source and antigen dose [Svensson et al., 2008]. Dudda and colleagues expanded this model to a point that tissue-selective homing receptor expression on T cells is governed by inductive as well as suppressive signals from both DC and tissue microenvironments at the site of antigen-priming [Dudda et al., 2005]. So far retinoic acid and the vitamin D-metabolite calciferol have been identified as positive or negative signals, respectively, for imprinting a gut-specific phenotype upon T cells. Calciferol is generated at uniquely high concentrations in keratinocytes, macrophages and dendritic cells in the skin [Sigmundsdottir et al., 2007] and therefore is highly unlikely to account for impaired gut-imprinting ability of *in vitro*-CpG-stimulated MLN-derived DC in our experiments. Other factors have been proposed to influence tissue-selective imprinting of lymphocytes. *In vivo* neutralization of the co-stimulatory molecule CD134L (also called OX40L), which

was expressed by a subset of intestinal DC, inhibited the expression of $\alpha_4\beta_7$ by MLN-primed T cells in a transfer model of murine colitis [Malmstrom et al., 2001]. In addition, others recently described a surprising capacity of intestinal DC to store and carry RA, suggesting that DC deliver epithelial-derived RA to the MLN rather than to produce it by themselves [Saurer et al., 2007]. However, doubt has been raised about this study's experimental settings and further experiments are required to assess the proposed 'RA-storing capacity' of DCs [Iwata, 2009].

The instrinsic factors that regulate the very expression of $\alpha_4\beta_7$ on T cells remain poorly understood. Recently, DeNucci and colleagues have raised a very interesting aspect in this concern. For the $\alpha_4\beta_7$-integrin, the α_4-subunit pairs with β_7. But α_4 can also form heterodimers with β_1. The group now demonstrated, that β_1 regulates the expression of $\alpha_4\beta_7$ through preferential pairing with the α_4-subunit in murine $CD4^{pos}$ T cells [DeNucci et al., 2010]. β_1 dominates the excessively expressed β_7 for α_4-pairing, thus controlling the amount of unpaired α_4 that could form $\alpha_4\beta_7$ heterodimers. Increasing the abundance of the α_4-subunit in relation to β_1 is crucial in the RA-mediated upregulation of $\alpha_4\beta_7$ on T cells. Furthermore, they demonstrate that high abundance of the β_1-subunit can potently suppress RA-induced upreglation of $\alpha_4\beta_7$. Our data suggest that upon CpG-stimulation in vitro, DC produce additional soluble factors that inhibit RA-mediated upregulation of $\alpha_4\beta_7$ on T cells. We now hypothesize that CpG-activated DC might induce β_1 upregulation in T cells, thereby impeding the formation of the $\alpha_4\beta_7$-heterodimer. Further experiments will need to verify this hypothesis. Other recent reports have highlighted the role of metalloproteinase-dependent shedding in the regulation of surface homing molecules like L-Selectin after TLR stimulation [Morrison et al., 2010]. It is hence possible that the diminished RA-mediated upregulation of $\alpha_4\beta_7$ on T cells upon CpG stimulation is not regulated on the RA production or $\alpha_4\beta_7$-synthesis level but by the turnover and degradation of the gut-homing integrin on the cell surface.

Thus, the identification and role of different soluble factors produced by dendritic cells or the tissue microenvironment of induction sites that drive T cell homing and the molecular mechanisms, especially in our setting of impaired gut-specific imprinting after CpG-DNA stimulation, still have to be defined in detail. However, in our case the search can be focused on DC-released factors as imprinting was also diminished in CpG-ODN-activated co-cultures of purified MLN-DC and T cells; that is to the exclusion of MLN microenvironment such as stroma cells and matrix components.

4.2.3 The imprinting of dendritic cells

Although DC targeting for enhanced antigen delivery and presentation has become a prominent focus of both infectious and cancer vaccine research, few studies have systematically examined the potential of manipulating peripheral DC for the purpose of vaccine-induced mucosal immunity. Based on the well-recognized plasticity of the DC system, such studies ought to investigate whether DC could be modulated toward "mucosa-type" DC and whether mucosa-borne DC maintain this phenotype during immunotherapy or vaccine/adjuvant administration, respectively. In this context, it will be fundamental to understand how dendritic cells are themselves imprinted with the ability to subsequently induce different tissue-specific homing phenotypes on T and B lymphocytes.

So far, it was demonstrated that based on conventional DC markers like CD8α, CD11b, CD11c and B220, there seem to be few differences in the ability of DC subsets from a given lymphoid tissue to generate tissue-tropic effector cells [Agace, 2006]. Johansson-Lindbom and colleagues demonstrated that DC can be imprinted with the ability to generate tissue-specific T cells before entering a lymph node. Furthermore, MLN-DC that express CD103, which make up about 40% of dendritic cells in mesenteric lymph nodes, are more potent to induce $\alpha_4\beta_7$ and CCR9 on responding T cells *in vitro* when compared to $CD103^{neg}$ MLN-DC [Johansson-Lindbom et al., 2005]. These $CD103^{pos}$ MLN-DC seem to originate from the intestinal lamina propria and have migrated to the MLN upon antigen uptake where they make up the T cell gut-imprinting DC population [Jang et al., 2006]. These reports indicate that the tissue-microenvironment where dendritic cells have encountered antigen and from where they afterwards have migrated rather than the identity of the draining lymph node have a role in imprinting DC with the ability to generate tissue-tropic T and B cells. However, as mentioned above, Hammerschidt et. al reported that *in vivo* stroma cells, but not DC, allow the MLN to induce the generation of gut tropism as PLN transplanted into the gut mesenteries failed to support the generation of gut-homing T cells, even though lamina propria-derived DC entered the transplants and primed T cells; furthermore DC that failed to induce $\alpha_4\beta_7$-integrin and CCR9 *in vitro* readily induce these factors *in vivo* upon injection into MLN afferent lymphatics. The authors suggested that under physiological conditions, stroma cells and DC may cooperate in shaping a lymph node environment that in the MLN is characterized by high levels of RA, thereby favoring the induction of gut-homing molecules.

Taken together, these results clearly disprove the formerly mentioned possibility that subsets of pre-imprinted DC precursors from the bone marrow selectively localize to distinct peripheral tissue and thereby facilitate T cell tissue-tropism but rather demonstrate that the ability of DC to imprint a distinct tissue-specific phenotype upon T and B cells is regulated dynamically and can be reconditioned during their lifetime. Regardless of whether dendritic cells are imprinted with the ability to induce tissue tropism at the site of antigen uptake or in the draining lymph nodes by stroma cells, the factors used in this process remain to be determined. In a recent porcine study, monocyte-derived non-mucosal DC were analyzed for their capacity to imprint mucosal homing receptor expression on lymphocytes following targeting with selected immunomodulatory and adjuvant factors. Saurer et. al demonstrated that these DC when pre-treated with RA promoted up-regulation of $\alpha_4\beta_7$-integrin and CCR9 mRNA expression on activated lymphocytes *in vitro* [Saurer et al., 2007]. They proposed a novel role for RA as mucosal immune modulator with epithelia cells being the possible source of RA in the intestinal mucosa. However, these RA-treated DC did not totally the function of resemble intestinal-borne DC and thus further studies will be needed to deepen our knowledge about the process of DC imprinting.

4.3 TLR ligands influence future perspectives for vaccine design, cancer immunotherapy and HIV treatment

As our understanding of the mechanisms that control the generation of tissue-tropic T and B lymphocytes steadily grows, the potential for using this knowledge for therapeutic purposes becomes evident. At the onset of this work, vaccination and cancer immunotherapy were two of several scenarios in which manipulating the tissue-homing receptor profile of lymphocytes could have clinical application and might be influenced by already utilized TLR-adjuvants.

Many pathogenic bacteria such as *Salmonella enterica subsp. enterica serovar typhimurium* and *Vibrio cholerea* but also viruses like HIV invade our body at mucosal surfaces and therefore protective mucosal immune responses to vaccinations are often desired [Neutra and Kozlowski, 2006]. Today, most vaccines are still administered intramuscularly or subcutaneously, delivering the vaccine antigen to non-mucosal sites. Adjuvants that boost the antigen-specific immune response but also induce the expression of mucosal homing receptors on responding T and B cells at the site of vaccination might enhance vaccine efficacy for such pathogens. Conversely, vaccines

that target those distinct DC subsets such as CD0103^{pos} DC in the intestinal lamina propria, that are physiologically able to generate gut-tropic lymphocytes, could be used to increase lymphocyte localization to desired intestinal sites of infection.

Furthermore, the $\alpha_4\beta_7$-integrin has recently been implicated to play a vital role in HIV pathogenesis. Several reports have demonstrated before that HIV infection leads to the destruction of most CD4^{pos} T cells in gut-associated lymphoid tissues, leaving a damaged and thus compromised tissue in its wake [Haase, 2005; Picker, 2006]. Arthos and colleagues have now shown that HIV-1 binds to the $\alpha_4\beta_7$-integrin through an interaction with the viral envelope glycoprotein gp120, which enhances infection of T cells by the virus [Arthos et al., 2008]. Thus, pharmacological modulation of the expression of the $\alpha_4\beta_7$ integrin by TLR ligands may in the future serve as a novel therapeutic approach in the treatment of HIV.

Two main pillars of cancer immunotherapy are DC immunization and T cell immunotherapy where ex vivo antigen-loaded DC or primed tumor-specific T cells, respectively, are adoptively transferred. Regarding DC immunization, the route of DC administration appears crucial in dictating the homing receptor expression pattern of responding lymphocytes. Only DC that were injected intraperitoneally were able to generate gut-tropic T cell responses, regardless of the origin of adoptively transferred DC [Dudda et al., 2004; Dudda et al., 2005]. As mentioned above, together these reports indicate that DC are re-imprinted either at the site of immunization or by dominant soluble factors and/or stroma cells in the draining lymph nodes at the time of T cell priming. Identification of these DC imprinting factors may allow effective conditioning of the immunization site before DC transfer or even ex vivo generation of 'intestinal' DC. In T cell immunotherapy we are facing problems by the inefficiency of *in vitro*-primed and expanded T cells to localize to the relevant tumor site and associated draining lymph nodes [Dudley and Rosenberg, 2003]. The identification of the factors including TLR adjuvants that regulate the induction of homing receptors on lymphocytes may thus allow for the *in vitro*-generation of tumor-specific T cells with an enhanced ability to target distinct tumors; in our case especially tumors of the intestinal tract.

In this work we demonstrate that immunostimulants like TLR ligands that have been widely and successfully used in immunotherapy, can have significant impact on the tissue-tropism of a subsequently triggered immune response. Thus, stimulation of TLR, here shown for TLR9 ligands, can result in downregulation of the gut-homing receptors

$\alpha_4\beta_7$-integrin and CCR9 on bystander naïve lymphocytes as well as on memory T and B cells with afterwards impaired ability to enter intestinal lymphoid tissues and lamina propria in short-term homing experiments. The process of imprinting tissue-tropism is affected as well, as DC stimulated with TLR9 ligands failed to induce high levels of $\alpha_4\beta_7$-integrin on responding T cells *in vitro*. These findings point out the difficulty to mount effective mucosal immune responses by using these adjuvants and could explain why we face inefficient therapy results in treating intestinal tumors with TLR9 adjuvant while subcutaneous marker tumors responded satisfyingly to the same regimen. However, in other experimental settings, TLR ligands proved more than once that they can be powerful tools as vaccine adjuvants and in antitumor therapy and are too valuable to be given up for intestinal/mucosal immunotherapy.

Although many homing receptors that are involved in T cell tissue tropism have been identified, future studies need to address the cellular and molecular mechanisms that regulate their *in vivo* expression. Solving the following outstanding issues will be central to utilize our new insights in tissue-specific homing of lymphocytes for immunotherapy and to overcome the obstacles illustrated for intestinal immunotherapy in this work:

(1) First and foremost, the current understanding of the mechanisms that drive generation of tissue-tropic T and B cells as well as our findings are based on murine models. Confirmation that similar mechanisms apply for humans are urgently required.

(2) It is essential to identify the anatomical sites where DC themselves are imprinted with the ability to induce tissue specificity on lymphocytes, the molecular mechanism underlying this process and the particular influence of commonly used adjuvants like TLR ligands.

(3) It is important to require a better understanding of the molecular signals generated by dendritic cells that regulate expression of the homing receptors on responding T and B cells. Then, undesired signals could be overwritten and lymphocytes reprogrammed to designated target tissues.

(4) We still need to investigate whether the ability to generate tissue-specific lymphocyte subsets is restricted to the intestine and the skin or is a property of all lymph nodes.

(5) Last, it will be necessary to determine crucial effects of inflammation and systemic infection on site-specific induction and function of tissue-homing receptor expression.

4.4 TLR ligands in inflammatory bowel disease

Inflammatory bowel diseases (IBD) are a group of idiopathic disorders of chronic inflammation of the GI tract supposedly due to inappropriate mucosal immune responses to normal intestinal microbes. IBD are traditionally divided into two subtypes, ulcerative colitis (UC) and Crohn's disease (CD), which share the common pathway of uncontrolled intestinal inflammation. Hereby, CD primarily affects the small intestine and the terminal ileum while UC is mainly limited to the large intestine. A key element in the pathogenesis of both UC and CD is enhanced leukocyte recruitment from the blood circulation which provides a potential target for therapeutical inhibition.

4.4.1 Aberrant homing in inflammatory bowel diseases

The exact pathogenesis of IBD is still poorly understood. Common theories share the importance of mutations of innate PRR like NOD2 that lead to defective microbial sensing by epithelial and antigen-presenting cells [Kobayashi et al., 2005]. Failure to clear commensal microbes through such innate immune mechanisms leads to a compromised barrier function, bacterial colonization of the epithelium and subsequent activation of the acquired immune response [Eksteen et al., 2008]. The resulting inflammation consists of T cells and macrophages in CD and is strongly polarized towards a T_H1/ T_H17 immune response with production of pro-inflammatory cytokines like TNF-α, IL-12/IL-23 and IFN-ɣ/IL-17 [Fuss et al., 2006]. UC, on the other hand, is characterized by an infiltrate of neutrophils in addition to T cells and an excess of IL-13 and IL-5 resembling a modified T_H2 immune response [Fuss et al., 2004].

Regardless of the underlying cause, both UC and CD are associated with massive, inappropriate influx and accumulation of immune cells into the gut. The gut-specific endothelial adhesion molecules MAdCAM-1 [Briskin et al., 1997] and CCL25 [Papadakis et al., 2001] are upregulated on mucosal vessels in IBD resulting in sustained recruitment of lymphocytes. As the inflammation establishes, signals that drive organ specificity become diluted by generic inflammation-associated processes [Eksteen et al., 2008]. Dysregulated immune responses to commensal bacteria, an abundance of TLR ligands and a sustained overproduction of pro-inflammatory cytokines up-regulate the expression of additional adhesion molecules such as ICAM-1 and VCAM-1 on the endothelium of mucosal venules, thereby promoting continuous leukocyte recruitment into the inflamed tissue [Nakamura et al., 1993]. The increased local secretion of cytokines changes the microenvironment of the gut in favor of maturation and activation of dendritic cells. DC then emigrate into intestinal lymphoid

tissues, creating a positive feedback loop between gut tissue and lymphoid organs by redirecting additional lymphocytes to the intestinal compartment [Bell et al., 2001].

An additional feature of IBD is the capacity of chronic gut inflammation to trigger inflammation in extra-intestinal sites. These extra-intestinal manifestations of IBD occur when lymphocytes infiltrate the joints, eyes, skin, lungs or liver. It has been suggested that extra-intestinal complications arise because chronic intestinal inflammation leads to the generation of activated circulating gut-effector lymphocytes that somehow bind to tissue vessels outside the GI tract. Indeed, patients with IBD-associated autoimmune hepatitis and/or primary sclerosing cholangitis (PSC) showed anomalous expression of MAdCAM-1 and CCL25 in liver endothelium [Hillan et al., 1999]. Accordingly, 20 % of intrahepatic T cells in PSC patients had a mucosal phenotype with high expression of $\alpha_4\beta_7$ and CCR9 [Eksteen et al., 2004]. Recruitment of these lymphocytes to the liver in response to aberrantly expressed gut-homing molecules, the possible encounter with their cognate antigen and their subsequent activation results in hepatitis and liver injury. However, the signals responsible for inducing the expression of MAdCAM-1 and CCL25 in the liver of patients with IBD are currently unknown. Extension of this model of aberrant lymphocyte recirculation to other extra-intestinal manifestations of IBD could explain their tissue distributions as well as point to new therapies that are based on modulating tissue-specific lymphocyte homing [Eksteen et al., 2008].

4.4.2 Anti-adhesion molecule therapy in inflammatory bowel disease

The crucial role of adhesion molecules in inflammatory disorders makes them interesting targets for drug development. Several therapeutic compounds directed against trafficking of lymphocytes towards the gut mucosa have been designed recently, and are being further developed as a novel class of drugs that modulate lymphocyte migration and hopefully thereby alter course of the local inflammatory reaction in the intestinal wall of patients with Crohn's disease or ulcerative colitis. Several experimental and first clinical studies have shown promising results of the new class of therapeutics.

In IBD, anti-adhesion therapy focuses on two pathways: the $\alpha_4\beta_7$ integrin/MAdCAM-1 and the LFA-1/ICAM-1 interaction. In a murine model of spontaneous ileal inflammation, the SAMP1/Yit mouse, which shares histological features with human ileal CD, prophylactic antibody blockade of MAdCAM-1 prevented the development of ileitis [Matsuzaki et al., 2005]. In ongoing clinical studies, a specific $\alpha_4\beta_7$ integrin

antibody, LDP02, showed success in treatment of patients with clinically present IBD [Marshall, 2001; van Assche and Rutgeerts, 2002]. Natalizumab, a humanized monoclonal α_4-integrin IgG4 antibody that not only blocks the $\alpha_4\beta_7$/MAdCAM-1 interaction but also inhibits the inflammation-induced $\alpha_4\beta_1$-VCAM-1 binding, proved efficient in therapy of patients with active CD [Ghosh et al., 2003]. ICAM-1 is another endothelial adhesion molecule that is upregulated and hence gains importance during inflammation. In the murine dextran sulphate sodium-induced colitis model, inhibition of ICAM-1 with antibodies or small inhibitory RNA (siRNA) reduced experimental colitis [Bennett et al., 1997; Taniguchi et al., 1998]. SiRNAs are antisense oligonucleotides that hybridize to a sequence-specific target RNA molecule, most often mRNA, and consecutively prevent the translation of the protein encoded by this RNA. SiRNA targeting ICAM-1 has shown efficacy in open-label studies in patients with UC and chronically active CD [Eksteen et al., 2008]. However, other studies could not convincingly show efficiency of anti-ICAM antisense oligonucleotides [van Assche and Rutgeerts, 2002]. Taken together, these studies suggest that anti-adhesion molecule therapy is an attractive concept for the treatment of IBD.

4.4.3 Murine colitis models and CpG therapy

Several recent studies investigated the role of immunostimulatory CpG-DNA motifs in the pathogenesis and treatment of IBD. As impaired mucosal barriers, dysregulated intestinal immune responses against commensal bacteria and overproduction of pro-inflammatory cytokines were postulated to contribute in the pathogenesis of IBD, Rachmilewitz and colleagues speculated that known cell-survival-promoting effects and immunostimulatory properties of CpG-DNA could inhibit damage to the colonic mucosa and limit exposure of subepithelial tissue to commensal bacteria and inflammatory products. These combined effects could thereby result in the inhibition of chronic intestinal inflammation [Rachmilewitz et al., 2002]. Indeed, they could demonstrate that subcutaneously or intragastrically injected CpG-DNA could prevent – when given prophylactically – and ameliorate – when given after onset of the disease – clinical and chemical signs of colitis in a murine model. These results were reproducible in different models of chemically-induced colitis (dextran sulphate sodium-induced and hapten-induced colitis) as well as in an IL-10 knockout model of spontaneous colitis. Treatment with CpG-DNA resulted in lower levels of colonic pro-inflammatory cytokines such as IL-6, IL-1β and TNF-α, pro-apoptotic caspase-3 and 9, decreased epithelial cell death and preserved colonic mucosal architecture. These effects were independent of the nature of the associated $CD4^{pos}$ T cell response as the different involved murine

colitis models were driven by both T_H1 and T_H2-weigthed adaptive immune responses. Hence the authors concluded that survival-promoting and immunostimulatory effects on innate immunity by CpG-DNA limits mucosal damage and invasion of commensal bacteria and thereby reduces the subsequent mucosal inflammation driven by adaptive immunity [Rachmilewitz et al., 2002].

Later, it was demonstrated that the beneficial effects in IBD patients of treatment with probiotic bacteria [Hart et al., 2003] is mediated by their immunomodulating DNA [Rachmilewitz et al., 2004]. Mice with experimental colitis treated with probiotics showed significant improvement in their symptoms. This effect was totally abrogated in TLR9-deficient mice while mice lacking functional TLR2 and 4 showed normal response to the treatment. Administration of ɣ-irradiated probiotics ameliorated the experimental colitis as did the viable probiotics making it unlikely that bacterial metabolites or their competitive inhibition of commensal microbes were responsible for their protective effects on the colonic mucosa. Subcutaneous and intragastric but not rectal administration of irradiated probiotics was effective in ameliorating the experimental colitis indicating that probiotic DNA acts systemically and was mainly absorbed in the small intestine. Rachmilewitz et al. concluded that immunomodulating DNA motifs from commensal bacteria may play a physiological role in the maintenance of mucosal homeostasis, limit bacterial invasion into colonic tissue and reduce resulted inflammation [Rachmilewitz et al., 2004]. Subsequent studies identified type I IFN as mediators of the anti-inflammatory effect by CpG-DNA in murine models of experimental colitis [Katakura et al., 2005; Lee et al., 2006].

These promising results raised hope that the immunomodulating properties of CpG-DNA could soon be facilitated in clinical trials [Bradbury, 2002] and were further augmented by reports that CpG-DNA inhibited the ex vivo production of the pro-inflammatory cytokines TNF-α and IL-1β by inflamed colonic mucosa of patients with active ulcerative colitis [Rachmilewitz et al., 2006].

In contrast, Obermeier et al. found divergent effects of immunostimulatory oligonucleotides in a chemically-induced model of murine colitis. When CpG-DNA was injected intraperitoneally, only prophylactic treatment could ameliorate the colitis while treatment in a chronic state of disease aggravated its symptoms [Obermeier et al., 2002; Obermeier et al., 2003]. Pretreatment with CpG-DNA but not LPS reduced levels of IFN-ɣ, IL-6 and IL-12 in MLN during the experimental colitis while these pro-inflammatory cytokines were elevated by CpG-treatment after the onset of acute

colitis. The authors hypothesized that the commensal bacterial DNA-induced immune response perpetuates established murine experimental colitis and that pre-treatment with CpG-DNA may have lead to 'desensitization' with suppressed production of inflammatory cytokines as well as IL-10-induced tolerance towards bacterial DNA. Further studies consolidated Obermeier and colleagues' theory that CpG-DNA derived from luminal bacteria contributes to the perpetuation of chronic late-stage intestinal inflammation. They demonstrated that eight weeks after the onset of dextran sulfate sodium-induced colitis in TLR9-deficient mice, intestinal inflammation was significantly lower and pro-inflammatory cytokine production was markedly reduced when compared to colitis in wild-type mice [Obermeier et al., 2005]. Treatment of wild-type mice suffering from chronic chemically-induced colitis with adenoviral oligonucleotide that are known to block CpG DNA effects, resulted in a significant amelioration of disease with a reduced production of IFN-ɣ but stable levels of TGF-β and IL-10 in colonic tissue.

More recent studies by the same group provided data supporting the concept that bacterial CpG DNA derived from the normal gut flora may contribute essentially to the homeostasis between effector and regulatory immune mechanisms in healthy individuals to protect them from chronic intestinal inflammation [Obermeier et al., 2005]. They used the murine SCID transfer model in which colitis was induced by transfer of naïve splenic T_H cells into recipients with severe combined immunodeficiency (SCID) that are characterized by an absolute absence of functional T cells. In contrast, T_H cells from CpG-ODN treated donors did not induce significant intestinal inflammation in SCID recipients. Additionally, T_H cells from TLR9-deficient mice induced markedly more severe colitis in SCID recipients than cells from wild-type controls, suggesting similar protective role of 'endogenous' bacterial DNA leading to a less 'aggressive' phenotype of these cells. Cotransfer of CpG-treated and naïve TH cells protected recipient animals from colitis, indicating regulatory activity. Attenuated, CpG-treated cells showed reduced proliferation and produced less IFN-ɣ, IL-5 and IL-6 after anti-CD3 stimulation, but there was no difference in regulatory T cell surface markers between 'aggressive' and attenuated cell pools. Recent reports showed that these protective properties of CpG-DNA were independent from a pre-existing bacterial flora indicating non-antigen specific effects and that TGF-β and type I IFNs as well as IL-10 independent mechanism were involved [Bleich et al., 2009].

Taken together, these findings point out the critical role of bacterial CpG-DNA in the pathogenesis of chronic intestinal inflammation. Constant exposure of a healthy

organism to commensal bacterial DNA may contribute to the homeostasis of the intestinal immune system regulating the delicate balance between effector and regulatory mechanisms. To which extent these CpG-DNA motifs perpetuate established intestinal inflammation and when is the critical time point in the course of diseases that CpG-ODNs turn from friend to foe, remains to be clarified. However, this demonstrates the multifaceted host response toward CpG motifs of bacterial DNA and how a single TLR stimulus may change the susceptibility to complex disorders as IBD [Obermeier et al., 2005].

Different theories of the mechanism how CpG-DNA realizes its protective effect in intestinal inflammation have been postulated. Rachmilewitz et al. suggest diverse immunomodulatory effects of CpG-DNA, on the one hand enhancing innate immunity and its epithelia barrier function to prevent pathogens from reaching deeper layers of the intestinal mucosa and on the other hand reducing production of pro-inflammatory cytokines in the colonic mucosa [Katakura et al., 2005; Rachmilewitz et al., 2006]. Obermeier and colleagues hypothesize that bacterial DNA-motifs might induced regulatory properties in T cell subsets [Obermeier et al., 2005; Bleich et al., 2009]. In this work we demonstrate that CpG-DNA exposure results in reduced surface expression of the gut-homing integrin $\alpha_4\beta_7$ on T cells *in vivo* and *in vitro.* Short-term homing experiments confirmed poor migration to intestinal lymphoid tissue, the small intestine and the colon by these lymphocytes. Although previous studies with murine models of experimental colitis did not address expression patterns of gut-specific adhesion molecules on lymphocytes nor their distribution and/or accumulation in the inflamed colon, it is tempting to speculate that the impaired capability of T cells to migrate to the intestinal tract may have contributed to the protective effect of CpG-DNA in murine experimental colitis. Further studies will be needed to elucidate the role of lymphocyte migratory behavior in amelioration of murine colitis after CpG-treatment. Nevertheless, these finding emphasize the potential of CpG-DNA motifs as novel compounds in the treatment of IBD.

4.5 Summary

Effective immune surveillance requires that immune cells are recruited and positioned in specific tissue compartments where they are able to carry out their functions. This is achieved for lymphocytes primarily through the selective expression of adhesion molecules and chemokine receptors, which allow them to bind to their endothelial ligands in vascular beds of particular effector organs. Among these migratory molecules, also called homing receptors, are the $\alpha_4\beta_7$ integrin and the chemokine receptor CCR9 that mediate lymphocyte homing to the gastrointestinal tract. Expression of the correct access code of homing receptors by T cells is crucial for efficient tissue-specific immune responses but also induces autoimmune diseases and allergy. Thus, interfering with T cell homing is emerging as a promising therapeutic strategy.

In recent years, the factors involved in the regulation of T cell homing have been the subject of intense research. In the first part of this study, we focused on the effect of innate immune activation by Toll-like receptor agonists, such as immunostimulatory CpG DNA, on the expression patterns of the gut-homing receptor $\alpha_4\beta_7$ on naïve lymphocytes. We demonstrate that *in vivo* stimulation of mice with CpG DNA leads to a decrease of approximately 60 % in surface expression of $\alpha_4\beta_7$ on lymphocytes in peripheral and mesenteric lymph nodes. This effect was not dependent on the route of CpG administration. *In vitro* experiments showed that downregulation of the gut adhesion molecule on lymphocytes is mediated by dendritic cell (DC)-released cytokines including interleukin-6 and is not dependent on direct cell-cell interaction. In subsequent short-term homing experiments these CpG-stimulated lymphocytes were markedly impaired in their ability to enter intestinal sites as they failed to efficiently recirculate to mesenteric lymph nodes and Peyer's patches.

Several reports have shown that signals derived from DC contribute to the selective generation of tissue-tropic T cells in secondary lymphoid organs. A key factor in this process appears to be the DC-delivered vitamin A metabolite, retinoic acid. However, it remains unclear how DC themselves are imprinted in their local environment with the ability to induce different tissue-specific homing phenotypes on T and B lymphocytes. In the second part of this study we demonstrate that stimulation of mesenteric lymph node-derived DC with the TLR9 ligand CpG attenuates their ability to imprint gut specificity upon antigen-specific T cells. Unexpectedly, following activation with CpG DNA, DC from peripheral and intestinal lymph nodes show increased intracellular

levels of RALDH2 mRNA, a transcript coding for the key enzyme in retinoic acid synthesis. Furthermore, these CpG-stimulated DC reduced upregulation of $\alpha_4\beta_7$ on activated T cells, suggesting that in response to unspecific innate immune activation, DC produce an additional factor that inhibits retinoic acid-mediated upregulation of the gut-homing receptor $\alpha_4\beta_7$ on T cells.

Systemic administration of the TLR9 ligand CpG-DNA induces a state of systemic inflammation. Our data now show that in this inflammatory state, downregulation of the $\alpha_4\beta_7$ integrin critically alters migration patterns of lymphocytes. Naïve T cells are then impaired in their capacity to enter intestinal secondary lymphoid organs and imprinting of gut-homing specificity on intestinal antigen-experienced T cells by dendritic cells is markedly attenuated. Thus, during systemic inflammation, the gut is deprived of locally activated T cells and further lymphocyte influx. As the largest peripheral immune compartment, the gastrointestinal system constitutes a huge lymphocyte reservoir. Thus, we hypothesize that it may provide an enormous pool of lymphocytes that are recruited during infection.

Future studies will need to specify the cellular and molecular mechanisms that regulate the *in vivo* expression of the homing receptor $\alpha_4\beta_7$. Solving this key issue should increase our understanding of tissue-specific immune and inflammatory processes in the gastrointestinal tract. Therapeutic modulation of organ-selective trafficking patterns of lymphocytes will allow tissue targeting of immune responses against local tumors and therapeutic re-routing of pathogenic T and B cells in gut-specific autoimmune and inflammatory diseases such as ulcerative colitis and Crohn's disease. Furthermore, recent studies have identified the integrin $\alpha_4\beta_7$ as a coreceptor of HIV and modulating $\alpha_4\beta_7$ expression may serve as a novel therapeutic approach in this disease.

4.6 Zusammenfassung

Eine effektive Immunüberwachung setzt voraus, dass Abwehrzellen in spezifische Gewebskompartimente rekrutiert und dort positioniert werden, wo sie ihre spezielle Funktion ausüben können. Für Lymphozyten wird dies in erster Linie durch die selektive Expression von Adhäsionsmolekülen und Chemokinrezeptoren gewährleistet. Diese ermöglichen den Lymphozyten ihren endothelialen Liganden in den Gefäßbetten bestimmter Effektororgane zu binden und in diese einzuwandern. Innerhalb dieser Migrationsmoleküle, auch *Homing*-Rezeptoren genannt, vermitteln das $\alpha_4\beta_7$ Integrin und der Chemokinrezeptor CCR9 die gezielte Migration von Lymphozyten in den Gastrointestinaltrakt. Die Expression des richtigen Musters an *Homing*-Rezeptoren auf T-Zellen entscheidet sowohl über das Zustandekommen einer effizienten gewebsspezifischen Immunantwort als auch von Autoimmunerkrankungen und Allergien. Dementsprechend zeichnet sich die Beeinflussung der zielgerichteten T-Zell-Migration als ein vielversprechender zukünftiger therapeutischer Ansatz ab.

In den letzten Jahren waren die Faktoren, die an der Regulation des Migrationsverhaltens von T-Zellen beteiligt sind, Gegenstand intensiver Forschung. Im ersten Teil dieser Arbeit untersuchten wir, wie eine Aktivierung des angeborenen Immunsystems durch *Toll-like*-Rezeptor-Agonisten, wie zum Beispiel immunstimulatorische CpG-DNA, das Expressionsmuster des gastrointestinalen *Homing*-Rezeptors $\alpha_4\beta_7$ auf naïven Lymphozyten beeinflusst. Wir zeigen, dass *in vivo*-Stimulation mit CpG-DNA bei Mäusen zu einer 60-prozentigen Abnahme der Oberflächenexpression von $\alpha_4\beta_7$ auf Lymphozyten in peripheren und mesenterialen Lymphknoten führt. Dieser Effekt war nicht abhängig vom Applikationsweg für CpG-DNA. Fortführende *in vitro* Experimente haben gezeigt, dass die Runterregulation des Adhäsionsmoleküls auf Lymphozyten durch Zytokine vermittelt wird, die von dendritischen Zellen (DC) sezerniert werden. Dieser Prozess involviert Interleukin-6 und ist nicht abhängig von direktem Zell-Zell-Kontakt. In anschließenden Migrations-Experimenten waren CpG-aktivierte Lymphozyten stark in ihrer Fähigkeit eingeschränkt, in intestinales Gewebe einzuwandern, da sie nicht in der Lage waren, effizient zu den mesenterialen Lymphknoten oder Peyer'schen Plaques zu migrieren.

Mehrere Arbeiten haben gezeigt, dass von DC stammende Signale zur selektiven Bildung von gewebsspezifischen T-Zellen in sekundär-lymphatischen Organen beitragen. Ein Schlüsselfaktor in diesem Prozess scheint der von DC bereitgestellte Vitamin-A-Metabolit Retinolsäure zu sein. Jedoch bleibt es unklar, wie DC ihrerseits in

ihrer lokalen Umgebung die Fähigkeit erlangen, verschiedene gewebsspezifische Migrations-Phänotypen auf T- und B-Zellen zu prägen. Im zweiten Teil dieser Arbeit zeigen wir, dass die Stimulation von DC aus mesenterialen Lymphknoten mit *Toll-like*-Rezeptor-Liganden deren Fähigkeit einschränkt, Darm-Spezifität auf T-Zellen einzuprägen. Unerwarteterweise reagieren DC auf eine Aktivierung durch CpG-DNA mit einem gesteigerten intrazellulären Level an RALDH2 messenger RNA; einem Transkript, das für das Schlüsselenzym der Retinolsäure-Synthese kodiert. Weiterhin verringern diese CpG-stimulierten DC die Hochregulation von $\alpha_4\beta_7$ auf aktivierten T Zellen. Dies legt nahe, dass DC in Reaktion auf einen unspezifischen Stimulus des angeborenen Immunsystems, einen weiteren Faktor sezernieren, der die durch Retinolsäure vermittelte Hochregulation von $\alpha_4\beta_7$ auf T-Zellen blockiert.

Systemische Anwendung des *Toll-like*-Rezeptor-9-Liganden CpG-DNA induziert einen Zustand systemischer Entzündung. Unsere Daten legen nun nahe, dass während diesem entzündlichen Zustand durch die Runterregulation des $\alpha_4\beta_7$ Integrins, die Migrationsmuster von Lymphozyten entscheidend verändert werden. Naive T-Zellen sind nun in ihrer Fähigkeit eingeschränkt in intestinale sekundärlymphatische Organe einzuwandern und das Prägen von Darm-Spezifität auf antigenerfahrenen T-Zellen durch lokale DC ist deutlich vermindert. Dementsprechend ist das Magen-Darm-System während der systemischen Entzündungsphase von lokal aktivierten als auch neu eingewanderten T-Zellen abgeschnitten. Da das gastrointestinale System das größte periphere Immunkompartiment darstellt und auf diese Weise einen enormen Pool an Lymphozyten bereithält, nehmen wir an, dass diese während einer systemischen Infektion rekrutiert werden können.

Zukünftige Arbeiten werden die zellulären und molekularen Mechanismen präzisieren müssen, die die Expression des *Homing*-Rezeptors $\alpha_4\beta_7$ regulieren. Die Lösung dieser Schlüsselfrage wird unser Verständnis von gewebsspezifischen Immun- und Entzündungsvorgängen im Magen-Darm-System entscheidend verbessern. Die therapeutische Modulation organspezifischer Migrationsmuster wird uns die Erzeugung zielgerichteter Immunantworten gegen lokale Infektionen und Tumoren sowie die therapeutische Umleitung von pathogenen Lymphozyten in chronisch-entzündlichen Erkrankungen wie Colitis ulcerosa und Morbus Crohn erlauben. Des Weiteren wurde in aktuellen Studien $\alpha_4\beta_7$ Integrin als Korezeptor des HI-Virus identifiziert, so dass die Modulation der $\alpha_4\beta_7$ Expression als neuartiger therapeutischer Ansatz bei der Bekämpfung von HIV dienen könnte.

5 REFERENCE LIST

Agace, W. W. 2006. Tissue-tropic effector T cells: generation and targeting opportunities. *Nat Rev Immunol.* 6(9): 682-692.

Akira, S., S. Uematsu and O. Takeuchi. 2006. Pathogen recognition and innate immunity. *Cell.* 124(4): 783-801.

Alexopoulou, L., A. C. Holt, R. Medzhitov and R. A. Flavell. 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature.* 413(6857): 732-738.

Aliprantis, A. O., R. B. Yang, M. R. Mark, S. Suggett, B. Devaux, J. D. Radolf, G. R. Klimpel, P. Godowski and A. Zychlinsky. 1999. Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. *Science.* 285(5428): 736-739.

Arstila, T. P., A. Casrouge, V. Baron, J. Even, J. Kanellopoulos and P. Kourilsky. 1999. A direct estimate of the human alphabeta T cell receptor diversity. *Science.* 286(5441): 958-961.

Arthos, J., C. Cicala, K. Martinelli, D. Van Ryk, D. Wei, E. Chung, K. Reitano, S. Kottilil, D. Goode and A. Fauci. 2008. HIV-1 envelope protein binds to and signals through integrin alpha4beta7, the gut mucosal homing receptor for peripheral T cells. *Nat Immunol.* 9(3): 225-227.

Baldridge, J. R., P. McGowan, J. T. Evans, C. Cluff, S. Mossman, D. Johnson and D. Persing. 2004. Taking a Toll on human disease: Toll-like receptor 4 agonists as vaccine adjuvants and monotherapeutic agents. *Expert Opin Biol Ther.* 4(7): 1129-1138.

Bargatze, R. F., M. A. Jutila and E. C. Butcher. 1995. Distinct roles of L-selectin and integrins alpha 4 beta 7 and LFA-1 in lymphocyte homing to Peyer's patch-HEV in situ: the multistep model confirmed and refined. *Immunity.* 3(1): 99-108.

Bell, S. J., R. Rigby, N. English, S. D. Mann, S. C. Knight, M. A. Kamm and A. J. Stagg. 2001. Migration and maturation of human colonic dendritic cells. *J Immunol.* 166(8): 4958-4967.

Bendelac, A., M. Bonneville and J. F. Kearney. 2001. Autoreactivity by design: innate B and T lymphocytes. *Nat Rev Immunol.* 1(3): 177-186.

Bennett, C. F., D. Kornbrust, S. Henry, K. Stecker, R. Howard, S. Cooper, S. Dutson, W. Hall and H. I. Jacoby. 1997. An ICAM-1 antisense oligonucleotide prevents and reverses dextran sulfate sodium-induced colitis in mice. *J Pharmacol Exp Ther.* 280(2): 988-1000.

Berlin, C., R. F. Bargatze, J. J. Campbell, U. H. von Andrian, M. C. Szabo, S. R. Hasslen, R. D. Nelson, E. L. Berg, S. L. Erlandsen and E. C. Butcher. 1995. alpha 4 integrins mediate lymphocyte attachment and rolling under physiologic flow. *Cell.* 80(3): 413-422.

Berlin, C., E. L. Berg, M. J. Briskin, D. P. Andrew, P. J. Kilshaw, B. Holzmann, I. L. Weissman, A. Hamann and E. C. Butcher. 1993. Alpha 4 beta 7 integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. *Cell.* 74(1): 185-195.

Beutner, K. R., S. K. Tyring, K. F. Trofatter, Jr., J. M. Douglas, Jr., S. Spruance, M. L. Owens, T. L. Fox, A. J. Hougham and K. A. Schmitt. 1998. Imiquimod, a patient-applied immune-response modifier for treatment of external genital warts. *Antimicrob Agents Chemother.* 42(4): 789-794.

Bleich, A., L. M. Janus, A. Smoczek, A. M. Westendorf, U. Strauch, M. Mahler, H. J. Hedrich, S. Fichtner-Feigl, J. Scholmerich, W. Falk, C. Hofmann and F. Obermeier. 2009. CpG motifs of bacterial DNA exert protective effects in mouse models of IBD by antigen-independent tolerance induction. *Gastroenterology.* 136(1): 278-287.

Bourquin, C., D. Anz, K. Zwiorek, A. L. Lanz, S. Fuchs, S. Weigel, C. Wurzenberger, P. von der Borch, M. Golic, S. Moder, G. Winter, C. Coester and S. Endres. 2008. Targeting CpG oligonucleotides to the lymph node by nanoparticles elicits efficient antitumoral immunity. *J Immunol.* 181(5): 2990-2998.

Bourquin, C., L. Schmidt, V. Hornung, C. Wurzenberger, D. Anz, N. Sandholzer, S. Schreiber, A. Voelkl, G. Hartmann and S. Endres. 2007. Immunostimulatory RNA oligonucleotides trigger an antigen-specific cytotoxic T-cell and IgG2a response. *Blood.* 109(7): 2953-2960.

Bradbury, J. 2002. New treatment for inflammatory bowel disease coulnd soon enter clinical trials. *Lancet.* 359(1583): 11.

Briskin, M., D. Winsor-Hines, A. Shyjan, N. Cochran, S. Bloom, J. Wilson, L. M. McEvoy, E. C. Butcher, N. Kassam, C. R. Mackay, W. Newman and D. J. Ringler. 1997. Human mucosal addressin cell adhesion molecule-1 is preferentially expressed in intestinal tract and associated lymphoid tissue. *Am J Pathol.* 151(1): 97-110.

Brown, G. D., J. Herre, D. L. Williams, J. A. Willment, A. S. Marshall and S. Gordon. 2003. Dectin-1 mediates the biological effects of beta-glucans. *J Exp Med.* 197(9): 1119-1124.

Burckstummer, T., C. Baumann, S. Bluml, E. Dixit, G. Durnberger, H. Jahn, M. Planyavsky, M. Bilban, J. Colinge, K. L. Bennett and G. Superti-Furga. 2009. An orthogonal proteomic-genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome. *Nat Immunol.* 10(3): 266-272.

Butcher, E. C. and L. J. Picker. 1996. Lymphocyte homing and homeostasis. *Science.* 272(5258): 60-66.

Campbell, D. J. and E. C. Butcher. 2002. Rapid acquisition of tissue-specific homing phenotypes by CD4(+) T cells activated in cutaneous or mucosal lymphoid tissues. *J Exp Med.* 195(1): 135-141.

Chamaillard, M., M. Hashimoto, Y. Horie, J. Masumoto, S. Qiu, L. Saab, Y. Ogura, A. Kawasaki, K. Fukase, S. Kusumoto, M. A. Valvano, S. J. Foster, T. W. Mak, G. Nunez and N. Inohara. 2003. An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat Immunol.* 4(7): 702-707.

Coban, C., K. J. Ishii, T. Kawai, H. Hemmi, S. Sato, S. Uematsu, M. Yamamoto, O. Takeuchi, S. Itagaki, N. Kumar, T. Horii and S. Akira. 2005. Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. *J Exp Med.* 201(1): 19-25.

Coombes, J. L., K. R. Siddiqui, C. V. Arancibia-Carcamo, J. Hall, C. M. Sun, Y. Belkaid and F. Powrie. 2007. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med.* 204(8): 1757-1764.

Cooper, C. L., H. L. Davis, M. L. Morris, S. M. Efler, M. A. Adhami, A. M. Krieg, D. W. Cameron and J. Heathcote. 2004. CPG 7909, an immunostimulatory TLR9 agonist oligodeoxynucleotide, as adjuvant to Engerix-B HBV vaccine in healthy adults: a double-blind phase I/II study. *J Clin Immunol.* 24(6): 693-701.

Cooper, C. L., H. L. Davis, M. L. Morris, S. M. Efler, A. M. Krieg, Y. Li, C. Laframboise, M. J. Al Adhami, Y. Khaliq, I. Seguin and D. W. Cameron. 2004. Safety and immunogenicity of CPG 7909 injection as an adjuvant to Fluarix influenza vaccine. *Vaccine.* 22(23-24): 3136-3143.

Davenport, M. P., M. C. Grimm and A. R. Lloyd. 2000. A homing selection hypothesis for T-cell trafficking. *Immunol Today.* 21(7): 315-317.

den Haan, J. M., S. M. Lehar and M. J. Bevan. 2000. CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. *J Exp Med.* 192(12): 1685-1696.

Dennehy, K. M. and G. D. Brown. 2007. The role of the beta-glucan receptor Dectin-1 in control of fungal infection. *J Leukoc Biol.* 82(2): 253-258.

DeNucci, C. C., A. J. Pagan, J. S. Mitchell and Y. Shimizu. 2010. Control of alpha4beta7 integrin expression and CD4 T cell homing by the beta1 integrin subunit. *J Immunol.* 184(5): 2458-2467.

Dhodapkar, M. V., R. M. Steinman, J. Krasovsky, C. Munz and N. Bhardwaj. 2001. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. *J Exp Med.* 193(2): 233-238.

Doyle, S., S. Vaidya, R. O'Connell, H. Dadgostar, P. Dempsey, T. Wu, G. Rao, R. Sun, M. Haberland, R. Modlin and G. Cheng. 2002. IRF3 mediates a TLR3/TLR4-specific antiviral gene program. *Immunity.* 17(3): 251-263.

Dudda, J. C., A. Lembo, E. Bachtanian, J. Huehn, C. Siewert, A. Hamann, E. Kremmer, R. Forster and S. F. Martin. 2005. Dendritic cells govern induction and reprogramming of polarized tissue-selective homing receptor patterns of T cells: important roles for soluble factors and tissue microenvironments. *Eur J Immunol.* 35(4): 1056-1065.

Dudda, J. C., J. C. Simon and S. Martin. 2004. Dendritic cell immunization route determines CD8+ T cell trafficking to inflamed skin: role for tissue microenvironment and dendritic cells in establishment of T cell-homing subsets. *J Immunol.* 172(2): 857-863.

Dudley, M. E. and S. A. Rosenberg. 2003. Adoptive-cell-transfer therapy for the treatment of patients with cancer. *Nat Rev Cancer.* 3(9): 666-675.

Eksteen, B., A. J. Grant, A. Miles, S. M. Curbishley, P. F. Lalor, S. G. Hubscher, M. Briskin, M. Salmon and D. H. Adams. 2004. Hepatic endothelial CCL25 mediates the recruitment of CCR9+ gut-homing lymphocytes to the liver in primary sclerosing cholangitis. *J Exp Med.* 200(11): 1511-1517.

Eksteen, B., E. Liaskou and D. H. Adams. 2008. Lymphocyte homing and its role in the pathogenesis of IBD. *Inflamm Bowel Dis.* 14(9): 1298-1312.

Enioutina, E. Y., D. Bareyan and R. A. Daynes. 2008. TLR ligands that stimulate the metabolism of vitamin D3 in activated murine dendritic cells can function as effective mucosal adjuvants to subcutaneously administered vaccines. *Vaccine.* 26(5): 601-613.

Feng, N., M. C. Jaimes, N. H. Lazarus, D. Monak, C. Zhang, E. C. Butcher and H. B. Greenberg. 2006. Redundant role of chemokines CCL25/TECK and CCL28/MEC in IgA+ plasmablast recruitment to the intestinal lamina propria after rotavirus infection. *J Immunol.* 176(10): 5749-5759.

Fernandes-Alnemri, T., J. W. Yu, P. Datta, J. Wu and E. S. Alnemri. 2009. AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. *Nature.* 458(7237): 509-513.

Franchi, L., T. Eigenbrod, R. Munoz-Planillo and G. Nunez. 2009. The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. *Nat Immunol.* 10(3): 241-247.

Friedberg, J. W., H. Kim, M. McCauley, E. M. Hessel, P. Sims, D. C. Fisher, L. M. Nadler, R. L. Coffman and A. S. Freedman. 2005. Combination immunotherapy with a CpG oligonucleotide (1018 ISS) and rituximab in patients with non-Hodgkin lymphoma: increased interferon-alpha/beta-inducible gene expression, without significant toxicity. *Blood.* 105(2): 489-495.

Fuhlbrigge, R. C., J. D. Kieffer, D. Armerding and T. S. Kupper. 1997. Cutaneous lymphocyte antigen is a specialized form of PSGL-1 expressed on skin-homing T cells. *Nature.* 389(6654): 978-981.

Fuss, I. J., C. Becker, Z. Yang, C. Groden, R. L. Hornung, F. Heller, M. F. Neurath, W. Strober and P. J. Mannon. 2006. Both IL-12p70 and IL-23 are synthesized during active Crohn's disease and are down-regulated by treatment with anti-IL-12 p40 monoclonal antibody. *Inflamm Bowel Dis.* 12(1): 9-15.

Fuss, I. J., F. Heller, M. Boirivant, F. Leon, M. Yoshida, S. Fichtner-Feigl, Z. Yang, M. Exley, A. Kitani, R. S. Blumberg, P. Mannon and W. Strober. 2004. Nonclassical CD1d-restricted NK T cells that produce IL-13 characterize an atypical Th2 response in ulcerative colitis. *J Clin Invest.* 113(10): 1490-1497.

Gallucci, S., M. Lolkema and P. Matzinger. 1999. Natural adjuvants: endogenous activators of dendritic cells. *Nat Med.* 5(11): 1249-1255.

Gantner, B. N., R. M. Simmons, S. J. Canavera, S. Akira and D. M. Underhill. 2003. Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *J Exp Med.* 197(9): 1107-1117.

Geisse, J., I. Caro, J. Lindholm, L. Golitz, P. Stampone and M. Owens. 2004. Imiquimod 5% cream for the treatment of superficial basal cell carcinoma: results from two phase III, randomized, vehicle-controlled studies. *J Am Acad Dermatol.* 50(5): 722-733.

Ghosh, S., E. Goldin, F. H. Gordon, H. A. Malchow, J. Rask-Madsen, P. Rutgeerts, P. Vyhnalek, Z. Zadorova, T. Palmer and S. Donoghue. 2003. Natalizumab for active Crohn's disease. *N Engl J Med.* 348(1): 24-32.

Girardin, S. E., I. G. Boneca, L. A. Carneiro, A. Antignac, M. Jehanno, J. Viala, K. Tedin, M. K. Taha, A. Labigne, U. Zahringer, A. J. Coyle, P. S. DiStefano, J. Bertin, P. J. Sansonetti and D. J. Philpott. 2003. Nod1 detects a unique

muropeptide from gram-negative bacterial peptidoglycan. *Science.* 300(5625): 1584-1587.

Girardin, S. E., I. G. Boneca, J. Viala, M. Chamaillard, A. Labigne, G. Thomas, D. J. Philpott and P. J. Sansonetti. 2003. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J Biol Chem.* 278(11): 8869-8872.

Glimcher, L. H. and K. M. Murphy. 2000. Lineage commitment in the immune system: the T helper lymphocyte grows up. *Genes Dev.* 14(14): 1693-1711.

Groothuis, T. A. and J. Neefjes. 2005. The many roads to cross-presentation. *J Exp Med.* 202(10): 1313-1318.

Gross, O., A. Gewies, K. Finger, M. Schafer, T. Sparwasser, C. Peschel, I. Forster and J. Ruland. 2006. Card9 controls a non-TLR signalling pathway for innate anti-fungal immunity. *Nature.* 442(7103): 651-656.

Haase, A. 2005. Perils at mucosal front lines for HIV and SIV and their hosts. *Nat Rev Immunol.* 5(10): 783-792.

Hamann, A., D. P. Andrew, D. Jablonski-Westrich, B. Holzmann and E. C. Butcher. 1994. Role of alpha 4-integrins in lymphocyte homing to mucosal tissues in vivo. *J Immunol.* 152(7): 3282-3293.

Hammerschmidt, S. I., M. Ahrendt, U. Bode, B. Wahl, E. Kremmer, R. Forster and O. Pabst. 2008. Stromal mesenteric lymph node cells are essential for the generation of gut-homing T cells in vivo. *J Exp Med.* 205(11): 2483-2490.

Harrington, L. E., R. D. Hatton, P. R. Mangan, H. Turner, T. L. Murphy, K. M. Murphy and C. T. Weaver. 2005. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol.* 6(11): 1123-1132.

Hart, A. L., A. J. Stagg and M. A. Kamm. 2003. Use of probiotics in the treatment of inflammatory bowel disease. *J Clin Gastroenterol.* 36(2): 111-119.

Hayashi, F., K. D. Smith, A. Ozinsky, T. R. Hawn, E. C. Yi, D. R. Goodlett, J. K. Eng, S. Akira, D. M. Underhill and A. Aderem. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature.* 410(6832): 1099-1103.

Heil, F., H. Hemmi, H. Hochrein, F. Ampenberger, C. Kirschning, S. Akira, G. Lipford, H. Wagner and S. Bauer. 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science.* 303(5663): 1526-1529.

Hemmi, H., T. Kaisho, O. Takeuchi, S. Sato, H. Sanjo, K. Hoshino, T. Horiuchi, H. Tomizawa, K. Takeda and S. Akira. 2002. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat Immunol.* 3(2): 196-200.

Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature.* 408(6813): 740-745.

Hieshima, K., Y. Kawasaki, H. Hanamoto, T. Nakayama, D. Nagakubo, A. Kanamaru and O. Yoshie. 2004. CC chemokine ligands 25 and 28 play essential roles in

intestinal extravasation of IgA antibody-secreting cells. *J Immunol.* 173(6): 3668-3675.

Hillan, K. J., K. E. Hagler, R. N. MacSween, A. M. Ryan, M. E. Renz, H. H. Chiu, R. K. Ferrier, G. L. Bird, A. P. Dhillon, L. D. Ferrell and S. Fong. 1999. Expression of the mucosal vascular addressin, MAdCAM-1, in inflammatory liver disease. *Liver.* 19(6): 509-518.

Horn, F., C. Henze and K. Heidrich. 2000. Interleukin-6 signal transduction and lymphocyte function. *Immunobiology.* 202(2): 151-167.

Horng, T., G. M. Barton, R. A. Flavell and R. Medzhitov. 2002. The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. *Nature.* 420(6913): 329-333.

Hornung, V., A. Ablasser, M. Charrel-Dennis, F. Bauernfeind, G. Horvath, D. R. Caffrey, E. Latz and K. A. Fitzgerald. 2009. AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature.* 458(7237): 514-518.

Hornung, V., J. Ellegast, S. Kim, K. Brzozka, A. Jung, H. Kato, H. Poeck, S. Akira, K. K. Conzelmann, M. Schlee, S. Endres and G. Hartmann. 2006. 5'-Triphosphate RNA is the ligand for RIG-I. *Science.* 314(5801): 994-997.

Horsmans, Y., T. Berg, J. P. Desager, T. Mueller, E. Schott, S. P. Fletcher, K. R. Steffy, L. A. Bauman, B. M. Kerr and D. R. Averett. 2005. Isatoribine, an agonist of TLR7, reduces plasma virus concentration in chronic hepatitis C infection. *Hepatology.* 42(3): 724-731.

Hosoe, N., S. Miura, C. Watanabe, Y. Tsuzuki, R. Hokari, T. Oyama, Y. Fujiyama, H. Nagata and H. Ishii. 2004. Demonstration of functional role of TECK/CCL25 in T lymphocyte-endothelium interaction in inflamed and uninflamed intestinal mucosa. *Am J Physiol Gastrointest Liver Physiol.* 286(3): G458-466.

Huang, B., J. Zhao, H. Li, K. L. He, Y. Chen, S. H. Chen, L. Mayer, J. C. Unkeless and H. Xiong. 2005. Toll-like receptors on tumor cells facilitate evasion of immune surveillance. *Cancer Res.* 65(12): 5009-5014.

Ikeda, Y., Y. Adachi, T. Ishii, N. Miura, H. Tamura and N. Ohno. 2008. Dissociation of Toll-like receptor 2-mediated innate immune response to Zymosan by organic solvent-treatment without loss of Dectin-1 reactivity. *Biol Pharm Bull.* 31(1): 13-18.

Ishii, K. J. and S. Akira. 2006. Innate immune recognition of, and regulation by, DNA. *Trends Immunol.* 27(11): 525-532.

Ishii, K. J., T. Kawagoe, S. Koyama, K. Matsui, H. Kumar, T. Kawai, S. Uematsu, O. Takeuchi, F. Takeshita, C. Coban and S. Akira. 2008. TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines. *Nature.* 451(7179): 725-729.

Ishii, K. J., S. Uematsu and S. Akira. 2006. 'Toll' gates for future immunotherapy. *Curr Pharm Des.* 12(32): 4135-4142.

Iwata, M. 2009. Retinoic acid production by intestinal dendritic cells and its role in T-cell trafficking. *Semin Immunol.* 21(1): 8-13.

Iwata, M., A. Hirakiyama, Y. Eshima, H. Kagechika, C. Kato and S. Y. Song. 2004. Retinoic acid imprints gut-homing specificity on T cells. *Immunity.* 21(4): 527-538.

Janeway, C. A., Jr. 1989. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol.* 54 Pt 1(1-13.

Jang, M. H., N. Sougawa, T. Tanaka, T. Hirata, T. Hiroi, K. Tohya, Z. Guo, E. Umemoto, Y. Ebisuno, B. G. Yang, J. Y. Seoh, M. Lipp, H. Kiyono and M. Miyasaka. 2006. CCR7 is critically important for migration of dendritic cells in intestinal lamina propria to mesenteric lymph nodes. *J Immunol.* 176(2): 803-810.

Johansson-Lindbom, B. and W. W. Agace. 2007. Generation of gut-homing T cells and their localization to the small intestinal mucosa. *Immunol Rev.* 215(226-242.

Johansson-Lindbom, B., M. Svensson, O. Pabst, C. Palmqvist, G. Marquez, R. Forster and W. W. Agace. 2005. Functional specialization of gut CD103+ dendritic cells in the regulation of tissue-selective T cell homing. *J Exp Med.* 202(8): 1063-1073.

Johansson-Lindbom, B., M. Svensson, M. A. Wurbel, B. Malissen, G. Marquez and W. Agace. 2003. Selective generation of gut tropic T cells in gut-associated lymphoid tissue (GALT): requirement for GALT dendritic cells and adjuvant. *J Exp Med.* 198(6): 963-969.

Kabelitz, D. 2007. Expression and function of Toll-like receptors in T lymphocytes. *Curr Opin Immunol.* 19(1): 39-45.

Kadowaki, N., S. Ho, S. Antonenko, R. W. Malefyt, R. A. Kastelein, F. Bazan and Y. J. Liu. 2001. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J Exp Med.* 194(6): 863-869.

Kagan, J. C. and R. Medzhitov. 2006. Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. *Cell.* 125(5): 943-955.

Kagan, J. C., T. Su, T. Horng, A. Chow, S. Akira and R. Medzhitov. 2008. TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. *Nat Immunol.* 9(4): 361-368.

Kamath, A. T., C. E. Sheasby and D. F. Tough. 2005. Dendritic cells and NK cells stimulate bystander T cell activation in response to TLR agonists through secretion of IFN-alpha beta and IFN-gamma. *J Immunol.* 174(2): 767-776.

Katakura, K., J. Lee, D. Rachmilewitz, G. Li, L. Eckmann and E. Raz. 2005. Toll-like receptor 9-induced type I IFN protects mice from experimental colitis. *J Clin Invest.* 115(3): 695-702.

Kato, H., S. Sato, M. Yoneyama, M. Yamamoto, S. Uematsu, K. Matsui, T. Tsujimura, K. Takeda, T. Fujita, O. Takeuchi and S. Akira. 2005. Cell type-specific involvement of RIG-I in antiviral response. *Immunity.* 23(1): 19-28.

Kato, H., O. Takeuchi, E. Mikamo-Satoh, R. Hirai, T. Kawai, K. Matsushita, A. Hiiragi, T. S. Dermody, T. Fujita and S. Akira. 2008. Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. *J Exp Med.* 205(7): 1601-1610.

Kato, H., O. Takeuchi, S. Sato, M. Yoneyama, M. Yamamoto, K. Matsui, S. Uematsu, A. Jung, T. Kawai, K. J. Ishii, O. Yamaguchi, K. Otsu, T. Tsujimura, C. S. Koh, C. Reis e Sousa, Y. Matsuura, T. Fujita and S. Akira. 2006. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature.* 441(7089): 101-105.

Kawai, T. and S. Akira. 2006. Innate immune recognition of viral infection. *Nat Immunol.* 7(2): 131-137.

Kawai, T. and S. Akira. 2007. TLR signaling. *Semin Immunol.* 19(1): 24-32.

Kawai, T. and S. Akira. 2009. The roles of TLRs, RLRs and NLRs in pathogen recognition. *Int Immunol.* 21(4): 317-337.

Kelly, M. G., A. B. Alvero, R. Chen, D. A. Silasi, V. M. Abrahams, S. Chan, I. Visintin, T. Rutherford and G. Mor. 2006. TLR-4 signaling promotes tumor growth and paclitaxel chemoresistance in ovarian cancer. *Cancer Res.* 66(7): 3859-3868.

Kobayashi, K. S., M. Chamaillard, Y. Ogura, O. Henegariu, N. Inohara, G. Nunez and R. A. Flavell. 2005. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science.* 307(5710): 731-734.

Kranzer, K., M. Bauer, G. B. Lipford, K. Heeg, H. Wagner and R. Lang. 2000. CpG-oligodeoxynucleotides enhance T-cell receptor-triggered interferon-gamma production and up-regulation of CD69 via induction of antigen-presenting cell-derived interferon type I and interleukin-12. *Immunology.* 99(2): 170-178.

Krug, A., A. Towarowski, S. Britsch, S. Rothenfusser, V. Hornung, R. Bals, T. Giese, H. Engelmann, S. Endres, A. M. Krieg and G. Hartmann. 2001. Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high amounts of IL-12. *Eur J Immunol.* 31(10): 3026-3037.

Kuklin, N. A., L. Rott, J. Darling, J. J. Campbell, M. Franco, N. Feng, W. Muller, N. Wagner, J. Altman, E. C. Butcher and H. B. Greenberg. 2000. alpha(4)beta(7) independent pathway for CD8(+) T cell-mediated intestinal immunity to rotavirus. *J Clin Invest.* 106(12): 1541-1552.

Kunkel, E. J. and E. C. Butcher. 2002. Chemokines and the tissue-specific migration of lymphocytes. *Immunity.* 16(1): 1-4.

Kunkel, E. J., C. H. Kim, N. H. Lazarus, M. A. Vierra, D. Soler, E. P. Bowman and E. C. Butcher. 2003. CCR10 expression is a common feature of circulating and mucosal epithelial tissue IgA Ab-secreting cells. *J Clin Invest.* 111(7): 1001-1010.

Kurt-Jones, E. A., L. Popova, L. Kwinn, L. M. Haynes, L. P. Jones, R. A. Tripp, E. E. Walsh, M. W. Freeman, D. T. Golenbock, L. J. Anderson and R. W. Finberg. 2000. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat Immunol.* 1(5): 398-401.

Lee, J., D. Rachmilewitz and E. Raz. 2006. Homeostatic effects of TLR9 signaling in experimental colitis. *Ann N Y Acad Sci.* 1072(351-355.

Lefrancois, L., C. M. Parker, S. Olson, W. Muller, N. Wagner, M. P. Schon and L. Puddington. 1999. The role of beta7 integrins in CD8 T cell trafficking during an antiviral immune response. *J Exp Med.* 189(10): 1631-1638.

LeibundGut-Landmann, S., O. Gross, M. J. Robinson, F. Osorio, E. C. Slack, S. V. Tsoni, E. Schweighoffer, V. Tybulewicz, G. D. Brown, J. Ruland and C. Reis e Sousa. 2007. Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. *Nat Immunol.* 8(6): 630-638.

Lemaitre, B., E. Nicolas, L. Michaut, J. M. Reichhart and J. A. Hoffmann. 1996. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell.* 86(6): 973-983.

Lipford, G. B., S. Bendigs, K. Heeg and H. Wagner. 2000. Poly-guanosine motifs costimulate antigen-reactive CD8 T cells while bacterial CpG-DNA affect T-cell activation via antigen-presenting cell-derived cytokines. *Immunology.* 101(1): 46-52.

Lund, J., A. Sato, S. Akira, R. Medzhitov and A. Iwasaki. 2003. Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. *J Exp Med.* 198(3): 513-520.

Malmstrom, V., D. Shipton, B. Singh, A. Al-Shamkhani, M. J. Puklavec, A. N. Barclay and F. Powrie. 2001. CD134L expression on dendritic cells in the mesenteric lymph nodes drives colitis in T cell-restored SCID mice. *J Immunol.* 166(11): 6972-6981.

Maraskovsky, E., K. Brasel, M. Teepe, E. R. Roux, S. D. Lyman, K. Shortman and H. J. McKenna. 1996. Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. *J Exp Med.* 184(5): 1953-1962.

Marshall, J. K. 2001. LDP-02 (Millenium). *Curr Opin Investig Drugs.* 2(4): 502-504.

Martinon, F., K. Burns and J. Tschopp. 2002. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell.* 10(2): 417-426.

Matsuzaki, K., Y. Tsuzuki, H. Matsunaga, T. Inoue, J. Miyazaki, R. Hokari, Y. Okada, A. Kawaguchi, S. Nagao, K. Itoh, S. Matsumoto and S. Miura. 2005. In vivo demonstration of T lymphocyte migration and amelioration of ileitis in intestinal mucosa of SAMP1/Yit mice by the inhibition of MAdCAM-1. *Clin Exp Immunol.* 140(1): 22-31.

Mazzoni, A. and D. M. Segal. 2004. Controlling the Toll road to dendritic cell polarization. *J Leukoc Biol.* 75(5): 721-730.

Medzhitov, R. 2007. Recognition of microorganisms and activation of the immune response. *Nature.* 449(7164): 819-826.

Medzhitov, R. and C. A. Janeway, Jr. 1997. Innate immunity: impact on the adaptive immune response. *Curr Opin Immunol.* 9(1): 4-9.

Medzhitov, R., P. Preston-Hurlburt and C. A. Janeway, Jr. 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature.* 388(6640): 394-397.

Mora, J. R., M. R. Bono, N. Manjunath, W. Weninger, L. L. Cavanagh, M. Rosemblatt and U. H. Von Andrian. 2003. Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature.* 424(6944): 88-93.

Mora, J. R., G. Cheng, D. Picarella, M. Briskin, N. Buchanan and U. H. von Andrian. 2005. Reciprocal and dynamic control of CD8 T cell homing by dendritic cells from skin- and gut-associated lymphoid tissues. *J Exp Med.* 201(2): 303-316.

Mora, J. R., M. Iwata, B. Eksteen, S. Y. Song, T. Junt, B. Senman, K. L. Otipoby, A. Yokota, H. Takeuchi, P. Ricciardi-Castagnoli, K. Rajewsky, D. H. Adams and U. H. von Andrian. 2006. Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. *Science.* 314(5802): 1157-1160.

Morrison, V. L., T. A. Barr, S. Brown and D. Gray. 2010. TLR-mediated loss of CD62L focuses B cell traffic to the spleen during Salmonella typhimurium infection. *J Immunol.* 185(5): 2737-2746.

Nakamura, S., H. Ohtani, Y. Watanabe, K. Fukushima, T. Matsumoto, A. Kitano, K. Kobayashi and H. Nagura. 1993. In situ expression of the cell adhesion molecules in inflammatory bowel disease. Evidence of immunologic activation of vascular endothelial cells. *Lab Invest.* 69(1): 77-85.

Napoli, J. L. 1999. Interactions of retinoid binding proteins and enzymes in retinoid metabolism. *Biochim Biophys Acta.* 1440(2-3): 139-162.

Neutra, M. R. and P. A. Kozlowski. 2006. Mucosal vaccines: the promise and the challenge. *Nat Rev Immunol.* 6(2): 148-158.

Obermeier, F., N. Dunger, L. Deml, H. Herfarth, J. Scholmerich and W. Falk. 2002. CpG motifs of bacterial DNA exacerbate colitis of dextran sulfate sodium-treated mice. *Eur J Immunol.* 32(7): 2084-2092.

Obermeier, F., N. Dunger, U. G. Strauch, N. Grunwald, H. Herfarth, J. Scholmerich and W. Falk. 2003. Contrasting activity of cytosin-guanosin dinucleotide oligonucleotides in mice with experimental colitis. *Clin Exp Immunol.* 134(2): 217-224.

Obermeier, F., N. Dunger, U. G. Strauch, C. Hofmann, A. Bleich, N. Grunwald, H. J. Hedrich, E. Aschenbrenner, B. Schlegelberger, G. Rogler, J. Scholmerich and W. Falk. 2005. CpG motifs of bacterial DNA essentially contribute to the perpetuation of chronic intestinal inflammation. *Gastroenterology.* 129(3): 913-927.

Obermeier, F., U. G. Strauch, N. Dunger, N. Grunwald, H. C. Rath, H. Herfarth, J. Scholmerich and W. Falk. 2005. In vivo CpG DNA/toll-like receptor 9 interaction induces regulatory properties in CD4+CD62L+ T cells which prevent intestinal inflammation in the SCID transfer model of colitis. *Gut.* 54(10): 1428-1436.

Ozinsky, A., D. M. Underhill, J. D. Fontenot, A. M. Hajjar, K. D. Smith, C. B. Wilson, L. Schroeder and A. Aderem. 2000. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proc Natl Acad Sci U S A.* 97(25): 13766-13771.

Papadakis, K. A., J. Prehn, S. T. Moreno, L. Cheng, E. A. Kouroumalis, R. Deem, T. Breaverman, P. D. Ponath, D. P. Andrew, P. H. Green, M. R. Hodge, S. W. Binder and S. R. Targan. 2001. CCR9-positive lymphocytes and thymus-expressed chemokine distinguish small bowel from colonic Crohn's disease. *Gastroenterology.* 121(2): 246-254.

Papadakis, K. A., J. Prehn, V. Nelson, L. Cheng, S. W. Binder, P. D. Ponath, D. P. Andrew and S. R. Targan. 2000. The role of thymus-expressed chemokine and

its receptor CCR9 on lymphocytes in the regional specialization of the mucosal immune system. *J Immunol.* 165(9): 5069-5076.

Petrovic, A., O. Alpdogan, L. M. Willis, J. M. Eng, A. S. Greenberg, B. J. Kappel, C. Liu, G. J. Murphy, G. Heller and M. R. van den Brink. 2004. LPAM (alpha 4 beta 7 integrin) is an important homing integrin on alloreactive T cells in the development of intestinal graft-versus-host disease. *Blood.* 103(4): 1542-1547.

Picarella, D., P. Hurlbut, J. Rottman, X. Shi, E. Butcher and D. J. Ringler. 1997. Monoclonal antibodies specific for beta 7 integrin and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) reduce inflammation in the colon of scid mice reconstituted with CD45RBhigh CD4+ T cells. *J Immunol.* 158(5): 2099-2106.

Pichlmair, A., O. Schulz, C. P. Tan, T. I. Naslund, P. Liljestrom, F. Weber and C. Reis e Sousa. 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science.* 314(5801): 997-1001.

Picker, L. 2006. Immunopathogenesis of acute AIDS virus infection. *Curr Opin Immunol.* 18(4): 399-405.

Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton and B. Beutler. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science.* 282(5396): 2085-2088.

Rachmilewitz, D., F. Karmeli, S. Shteingart, J. Lee, K. Takabayashi and E. Raz. 2006. Immunostimulatory oligonucleotides inhibit colonic proinflammatory cytokine production in ulcerative colitis. *Inflamm Bowel Dis.* 12(5): 339-345.

Rachmilewitz, D., F. Karmeli, K. Takabayashi, T. Hayashi, L. Leider-Trejo, J. Lee, L. M. Leoni and E. Raz. 2002. Immunostimulatory DNA ameliorates experimental and spontaneous murine colitis. *Gastroenterology.* 122(5): 1428-1441.

Rachmilewitz, D., K. Katakura, F. Karmeli, T. Hayashi, C. Reinus, B. Rudensky, S. Akira, K. Takeda, J. Lee, K. Takabayashi and E. Raz. 2004. Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. *Gastroenterology.* 126(2): 520-528.

Ramanathan, S., J. Gagnon and S. Ilangumaran. 2008. Antigen-nonspecific activation of CD8+ T lymphocytes by cytokines: relevance to immunity, autoimmunity, and cancer. *Arch Immunol Ther Exp (Warsz).* 56(5): 311-323.

Reinhardt, R. L., S. J. Kang, H. E. Liang and R. M. Locksley. 2006. T helper cell effector fates--who, how and where? *Curr Opin Immunol.* 18(3): 271-277.

Robinson, M. J., D. Sancho, E. C. Slack, S. LeibundGut-Landmann and C. Reis e Sousa. 2006. Myeloid C-type lectins in innate immunity. *Nat Immunol.* 7(12): 1258-1265.

Romagne, F. 2007. Current and future drugs targeting one class of innate immunity receptors: the Toll-like receptors. *Drug Discov Today.* 12(1-2): 80-87.

Rossi, M. and J. W. Young. 2005. Human dendritic cells: potent antigen-presenting cells at the crossroads of innate and adaptive immunity. *J Immunol.* 175(3): 1373-1381.

Salio, M., A. O. Speak, D. Shepherd, P. Polzella, P. A. Illarionov, N. Veerapen, G. S. Besra, F. M. Platt and V. Cerundolo. 2007. Modulation of human natural killer T cell ligands on TLR-mediated antigen-presenting cell activation. *Proc Natl Acad Sci U S A.* 104(51): 20490-20495.

Sallusto, F., D. Lenig, R. Forster, M. Lipp and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature.* 401(6754): 708-712.

Salmi, M., K. Granfors, M. Leirisalo-Repo, M. Hamalainen, R. MacDermott, R. Leino, T. Havia and S. Jalkanen. 1992. Selective endothelial binding of interleukin-2-dependent human T-cell lines derived from different tissues. *Proc Natl Acad Sci U S A.* 89(23): 11436-11440.

Salmi, M. and S. Jalkanen. 2005. Lymphocyte homing to the gut: attraction, adhesion, and commitment. *Immunol Rev.* 206(100-113.

Salmi, M., K. Kalimo and S. Jalkanen. 1993. Induction and function of vascular adhesion protein-1 at sites of inflammation. *J Exp Med.* 178(6): 2255-2260.

Sans, M., J. Panes, E. Ardite, J. I. Elizalde, Y. Arce, M. Elena, A. Palacin, J. C. Fernandez-Checa, D. C. Anderson, R. Lobb and J. M. Pique. 1999. VCAM-1 and ICAM-1 mediate leukocyte-endothelial cell adhesion in rat experimental colitis. *Gastroenterology.* 116(4): 874-883.

Saurer, L., K. C. McCullough and A. Summerfield. 2007. In vitro induction of mucosa-type dendritic cells by all-trans retinoic acid. *J Immunol.* 179(6): 3504-3514.

Schmitz, F., A. Heit, S. Guggemoos, A. Krug, J. Mages, M. Schiemann, H. Adler, I. Drexler, T. Haas, R. Lang and H. Wagner. 2007. Interferon-regulatory-factor 1 controls Toll-like receptor 9-mediated IFN-beta production in myeloid dendritic cells. *Eur J Immunol.* 37(2): 315-327.

Schoenemeyer, A., B. J. Barnes, M. E. Mancl, E. Latz, N. Goutagny, P. M. Pitha, K. A. Fitzgerald and D. T. Golenbock. 2005. The interferon regulatory factor, IRF5, is a central mediator of toll-like receptor 7 signaling. *J Biol Chem.* 280(17): 17005-17012.

Shi, G. P., J. A. Villadangos, G. Dranoff, C. Small, L. Gu, K. J. Haley, R. Riese, H. L. Ploegh and H. A. Chapman. 1999. Cathepsin S required for normal MHC class II peptide loading and germinal center development. *Immunity.* 10(2): 197-206.

Shortman, K. and W. R. Heath. 2001. Immunity or tolerance? That is the question for dendritic cells. *Nat Immunol.* 2(11): 988-989.

Shortman, K. and Y. J. Liu. 2002. Mouse and human dendritic cell subtypes. *Nat Rev Immunol.* 2(3): 151-161.

Shurin, M. R., P. P. Pandharipande, T. D. Zorina, C. Haluszczak, V. M. Subbotin, O. Hunter, A. Brumfield, W. J. Storkus, E. Maraskovsky and M. T. Lotze. 1997. FLT3 ligand induces the generation of functionally active dendritic cells in mice. *Cell Immunol.* 179(2): 174-184.

Siewert, C., A. Menning, J. Dudda, K. Siegmund, U. Lauer, S. Floess, D. J. Campbell, A. Hamann and J. Huehn. 2007. Induction of organ-selective CD4+ regulatory T cell homing. *Eur J Immunol.* 37(4): 978-989.

Sigmundsdottir, H. and E. C. Butcher. 2008. Environmental cues, dendritic cells and the programming of tissue-selective lymphocyte trafficking. *Nat Immunol.* 9(9): 981-987.

Sigmundsdottir, H., J. Pan, G. F. Debes, C. Alt, A. Habtezion, D. Soler and E. C. Butcher. 2007. DCs metabolize sunlight-induced vitamin D3 to 'program' T cell attraction to the epidermal chemokine CCL27. *Nat Immunol.* 8(3): 285-293.

Sparwasser, T., E. S. Koch, R. M. Vabulas, K. Heeg, G. B. Lipford, J. W. Ellwart and H. Wagner. 1998. Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur J Immunol.* 28(6): 2045-2054.

Springer, T. A. 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell.* 76(2): 301-314.

Stagg, A. J., M. A. Kamm and S. C. Knight. 2002. Intestinal dendritic cells increase T cell expression of alpha4beta7 integrin. *Eur J Immunol.* 32(5): 1445-1454.

Steinman, R. M. and Z. A. Cohn. 1973. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med.* 137(5): 1142-1162.

Stenstad, H., A. Ericsson, B. Johansson-Lindbom, M. Svensson, J. Marsal, M. Mack, D. Picarella, D. Soler, G. Marquez, M. Briskin and W. W. Agace. 2006. Gut-associated lymphoid tissue-primed CD4+ T cells display CCR9-dependent and -independent homing to the small intestine. *Blood.* 107(9): 3447-3454.

Stetson, D. B. and R. Medzhitov. 2006. Type I interferons in host defense. *Immunity.* 25(3): 373-381.

Sun, S., X. Zhang, D. F. Tough and J. Sprent. 1998. Type I interferon-mediated stimulation of T cells by CpG DNA. *J Exp Med.* 188(12): 2335-2342.

Svensson, M., B. Johansson-Lindbom, F. Zapata, E. Jaensson, L. M. Austenaa, R. Blomhoff and W. W. Agace. 2008. Retinoic acid receptor signaling levels and antigen dose regulate gut homing receptor expression on CD8+ T cells. *Mucosal Immunol.* 1(1): 38-48.

Takaoka, A., Z. Wang, M. K. Choi, H. Yanai, H. Negishi, T. Ban, Y. Lu, M. Miyagishi, T. Kodama, K. Honda, Y. Ohba and T. Taniguchi. 2007. DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. *Nature.* 448(7152): 501-505.

Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda and S. Akira. 1999. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity.* 11(4): 443-451.

Takeuchi, O., T. Kawai, P. F. Muhlradt, M. Morr, J. D. Radolf, A. Zychlinsky, K. Takeda and S. Akira. 2001. Discrimination of bacterial lipoproteins by Toll-like receptor 6. *Int Immunol.* 13(7): 933-940.

Taniguchi, T., H. Tsukada, H. Nakamura, M. Kodama, K. Fukuda, T. Saito, M. Miyasaka and Y. Seino. 1998. Effects of the anti-ICAM-1 monoclonal antibody on dextran sodium sulphate-induced colitis in rats. *J Gastroenterol Hepatol.* 13(9): 945-949.

Tanimura, N., S. Saitoh, F. Matsumoto, S. Akashi-Takamura and K. Miyake. 2008. Roles for LPS-dependent interaction and relocation of TLR4 and TRAM in TRIF-signaling. *Biochem Biophys Res Commun.* 368(1): 94-99.

Testi, R., J. H. Phillips and L. L. Lanier. 1989. Leu 23 induction as an early marker of functional CD3/T cell antigen receptor triggering. Requirement for receptor cross-linking, prolonged elevation of intracellular [Ca++] and stimulation of protein kinase C. *J Immunol.* 142(6): 1854-1860.

Ting, J. P., R. C. Lovering, E. S. Alnemri, J. Bertin, J. M. Boss, B. K. Davis, R. A. Flavell, S. E. Girardin, A. Godzik, J. A. Harton, H. M. Hoffman, J. P. Hugot, N. Inohara, A. Mackenzie, L. J. Maltais, G. Nunez, Y. Ogura, L. A. Otten, D. Philpott, J. C. Reed, W. Reith, S. Schreiber, V. Steimle and P. A. Ward. 2008. The NLR gene family: a standard nomenclature. *Immunity.* 28(3): 285-287.

Tough, D. F., P. Borrow and J. Sprent. 1996. Induction of bystander T cell proliferation by viruses and type I interferon in vivo. *Science.* 272(5270): 1947-1950.

Tough, D. F., S. Sun and J. Sprent. 1997. T cell stimulation in vivo by lipopolysaccharide (LPS). *J Exp Med.* 185(12): 2089-2094.

Turnbull, E. L., U. Yrlid, C. D. Jenkins and G. G. Macpherson. 2005. Intestinal dendritic cell subsets: differential effects of systemic TLR4 stimulation on migratory fate and activation in vivo. *J Immunol.* 174(3): 1374-1384.

Uematsu, S., K. Fujimoto, M. H. Jang, B. G. Yang, Y. J. Jung, M. Nishiyama, S. Sato, T. Tsujimura, M. Yamamoto, Y. Yokota, H. Kiyono, M. Miyasaka, K. J. Ishii and S. Akira. 2008. Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5. *Nat Immunol.* 9(7): 769-776.

van Assche, G. and P. Rutgeerts. 2002. Antiadhesion molecule therapy in inflammatory bowel disease. *Inflamm Bowel Dis.* 8(4): 291-300.

Venkataraman, T., M. Valdes, R. Elsby, S. Kakuta, G. Caceres, S. Saijo, Y. Iwakura and G. N. Barber. 2007. Loss of DExD/H box RNA helicase LGP2 manifests disparate antiviral responses. *J Immunol.* 178(10): 6444-6455.

Verthelyi, D., K. J. Ishii, M. Gursel, F. Takeshita and D. M. Klinman. 2001. Human peripheral blood cells differentially recognize and respond to two distinct CPG motifs. *J Immunol.* 166(4): 2372-2377.

Vignali, D. A., L. W. Collison and C. J. Workman. 2008. How regulatory T cells work. *Nat Rev Immunol.* 8(7): 523-532.

von Andrian, U. H. and C. R. Mackay. 2000. T-cell function and migration. Two sides of the same coin. *N Engl J Med.* 343(14): 1020-1034.

von Andrian, U. H. and T. R. Mempel. 2003. Homing and cellular traffic in lymph nodes. *Nat Rev Immunol.* 3(11): 867-878.

Vremec, D., J. Pooley, H. Hochrein, L. Wu and K. Shortman. 2000. CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. *J Immunol.* 164(6): 2978-2986.

Wagner, N., J. Lohler, E. J. Kunkel, K. Ley, E. Leung, G. Krissansen, K. Rajewsky and W. Muller. 1996. Critical role for beta7 integrins in formation of the gut-associated lymphoid tissue. *Nature.* 382(6589): 366-370.

Warnock, R. A., S. Askari, E. C. Butcher and U. H. von Andrian. 1998. Molecular mechanisms of lymphocyte homing to peripheral lymph nodes. *J Exp Med.* 187(2): 205-216.

Wurzenberger, C., V. H. Koelzer, S. Schreiber, D. Anz, A. M. Vollmar, M. Schnurr, S. Endres and C. Bourquin. 2009. Short-term activation induces multifunctional dendritic cells that generate potent antitumor T-cell responses in vivo. *Cancer Immunol Immunother.* 58(6): 901-913.

Yamamoto, M., S. Sato, H. Hemmi, S. Uematsu, K. Hoshino, T. Kaisho, O. Takeuchi, K. Takeda and S. Akira. 2003. TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nat Immunol.* 4(11): 1144-1150.

Yanai, H., D. Savitsky, T. Tamura and T. Taniguchi. 2009. Regulation of the cytosolic DNA-sensing system in innate immunity: a current view. *Curr Opin Immunol.* 21(1): 17-22.

Yarovinsky, F., D. Zhang, J. F. Andersen, G. L. Bannenberg, C. N. Serhan, M. S. Hayden, S. Hieny, F. S. Sutterwala, R. A. Flavell, S. Ghosh and A. Sher. 2005. TLR11 activation of dendritic cells by a protozoan profilin-like protein. *Science.* 308(5728): 1626-1629.

Yoneyama, M. and T. Fujita. 2008. Structural mechanism of RNA recognition by the RIG-I-like receptors. *Immunity.* 29(2): 178-181.

Yoshitomi, H., N. Sakaguchi, K. Kobayashi, G. D. Brown, T. Tagami, T. Sakihama, K. Hirota, S. Tanaka, T. Nomura, I. Miki, S. Gordon, S. Akira, T. Nakamura and S. Sakaguchi. 2005. A role for fungal {beta}-glucans and their receptor Dectin-1 in the induction of autoimmune arthritis in genetically susceptible mice. *J Exp Med.* 201(6): 949-960.

Zabel, B. A., W. W. Agace, J. J. Campbell, H. M. Heath, D. Parent, A. I. Roberts, E. C. Ebert, N. Kassam, S. Qin, M. Zovko, G. J. LaRosa, L. L. Yang, D. Soler, E. C. Butcher, P. D. Ponath, C. M. Parker and D. P. Andrew. 1999. Human G protein-coupled receptor GPR-9-6/CC chemokine receptor 9 is selectively expressed on intestinal homing T lymphocytes, mucosal lymphocytes, and thymocytes and is required for thymus-expressed chemokine-mediated chemotaxis. *J Exp Med.* 190(9): 1241-1256.

Zhang, D., G. Zhang, M. S. Hayden, M. B. Greenblatt, C. Bussey, R. A. Flavell and S. Ghosh. 2004. A toll-like receptor that prevents infection by uropathogenic bacteria. *Science.* 303(5663): 1522-1526.

6 APPENDIX

6.1 Abbreviations

A

AP-1	Activated protein 1
APC	Allophycocyanin
APC	Antigen-presenting cell

B

B16-FL	B16 melanoma cell line expressing Flt3-L

C

Cardif	CARD adapter inducing IFNβ
CCR	Chemokine receptor
CD	Crohn’s disease
cDNA	Copy-desoxyribonucleic acid
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CLA	Cutaneous lymphocyte antigen
CpG	Oligonucleotide with cytosine-(phosphate)-guanine motifs

D

DC	Dendritic cell
DMEM	Dulbecco's modified Eagle´s medium
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleid acid
ds	Double-stranded

E

EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
E-Lig	E-selectin ligand

F

FACS	Fluorescent-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isocyanate

FL-DC	Flt3L-derived dendritic cells
Flt3-L	Fms-like tyrosine kinase 3 ligand
Foxp3	Forkhead box p3
FSC	Forward scatter

G

GI	Gastro-intestinal
GM-CSF	Granulocyte-macrophage colony stimulating factor
GM-DC	Dendritic cells differentiated with GM-CSF and IL-4

H

HBSS	Hank's balanced salt solution
HEV	High endothelial venules
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HRP	Horseradish peroxidase

I

IBD	Inflammatory bowel disease
IF	Interferon
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
IFNaR	Interferon-α receptor
ip	intraperitoneally
IPS-1	IFN-β promoter stimulator 1
IRF	Interferon regulatory factor

L

LFA-1	Lymphocyte function-associated antigen 1
LP	Lamina propria
LPAM-1	Leukocyte Peyer’s patches adhesion molecule 1
LPS	Lipopolysaccharid
LRR	Leucine-rich repeat

M

mAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MAdCAM-1	Mucosal addressin cell adhesion molecule-1

MAPK	Mitogen-activated protein kinase
MDA5	Melanoma differentiation associated gene 5
mDC	Myeloid dendritic cell
MLN	Mesenteric lymph node
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response gene 88

N

nd	Not determined
NEAA	Non-essential amino acids
NF-κB	Nuclear factor-κB
ns	Not significant

O

ODN	Oligodesoyxnucleotide
ON	Oligonucleotide

P

PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PLN	Peripheral lymph node
PP	Peyer's patches
PGN	Peptoglycans
PNAd	Peripheral lymph node addressin
PFA	Paraformaldehyde
PRR	Pattern-recognition receptor
PSC	Primary sclerosing cholangitis
PYD	Pyrin domain

Q

qRT-PCR	Quantitative real-time PCR

R

RA	Retinoic acid
RALDH	Retinol aldehyd dehydrogenase
RIG-I	Retinoic acid-inducible gene-I
RPMI	Roswell Park Memorial Institute
RNA	Ribonucleic acid
RT	Room temperature

S

SEM	Standard error of the mean
SLO	Secondary lymphoid organs
ss	Single-stranded
SSC	Sideward scatter

T

T_H cell	T-helper cell
TIR	Toll/IL-1 receptor
TIRAP	TIR domain-containing adapter molecule
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TGF	Tumor growth factor
TNF	Tumor necrosis factor
TRAM	TRIF-related adapter molecule
T_{reg} cell	Regulatory T cell
TRIF	TIR-containing adapter inducing IFNβ

U

UC	Ulcerative colitis

V

VLE	Very low endotoxin

6.2 Publications

6.2.1 Original publications

Bourquin C, Wurzenberger C, Heidegger S, Fuchs S, Anz D, Weigel S, Sandholzer N, Winter G, Coester C, Endres S
Delivery of Immunostimulatory RNA Oligonucleotides by Gelatin Nanoparticles Triggers an Efficient Antitumoral Response.
J Immunother. 2010. 33(9):935-44 *(JIF 3.203)*

Heidegger S, Stephan N, v. der Borch P, Anz D, Sandholzer N, Endres S, Bourquin C
Influence of immunotherapy on the gut-homing phenotype of lymphocytes.
Manuscript in preparation 2010

6.2.2 Oral presentations

Heidegger S
In vivo migration of dendritic cells
1st Annual Retreat, Graduiertenkolleg 1202, Frauenchiemsee, Germany, 2006

Heidegger S
Shaping T cell migration: regulation of gut-homing receptor LPAM-1 by TLR-Ligands
2nd Annual Retreat, Graduiertenkolleg 1202, Tutzing, Germany, 2007

6.2.3 Poster presentations

Wurzenberger C, Heidegger S, Schreiber S, Weigel S, Endres S, Bourquin C
Kinetics of DC activation by CpG oligonucleotides for the induction of CD8 T cell responses
5th International Meeting on Dendritic Cell Vaccination and Other Strategies to tip the Balance of the Immune System, Bamberg, Germany, 2007

Heidegger S, von der Borch P, Stephan N, Wurzenberger C, Sandholzer N, Endres S, Bourquin S
Tissue-specific migration: Effect of Toll-like receptor ligands on imprinting gut tropism upon murine lymphocytes
Berichtskolloquium, Graduiertenkolleg 1202, München, Germany, 2009

6.3 Curriculum vitae

Name	Simon Martin Heidegger
Geburtsdatum und -ort	15. Mai 1983 in Weingarten (Württ.)
Staatsangehörigkeit	Deutsch

Schulausbildung und Hochschulstudium

1993 bis 2002	Gymnasium St. Konrad in Ravensburg, Abitur (1,1)
2002 bis 2004	Vorklinischer Studienabschnitt Medizin an der LMU München
September 2004	Ärztliche Vorprüfung (1,0)
2004 bis 2009	Klinischer Studienabschnitt Medizin an der TU München
November 2009	Ärztliche Prüfung (1,5)

Klinische Ausbildung

März 2005	Famulatur; Innere Medizin, Immunologie, Rheumatologie; München-Bogenhausen
September 2005	Famulatur; Anästhesiologie; Deutsches Herzzentrum des Freistaat Bayern
März 2007	Famulatur; Abteilung für klinische Pharmakologie; Medizinische Klinik Innenstadt München
September 2007	Famulatur; Innere Medizin, Pulmologie; Dr. S. Englmeier
Februar bis April 2008	Ausbildung im Praktischen Jahr; Herzchirurgie; Deutsches Herzzentrum des Freistaat Bayern
April bis Juni 2008	Ausbildung im Praktischen Jahr; Allgemeinchirurgie; Addenbrooke's Hospital, Cambridge Medical School, UK
Juni bis Oktober 2008	Ausbildung im Praktischen Jahr; Anästhesiologie; Klinikum Rechts der Isar München
Oktober 2008 bis Februar 2009	Ausbildung im Praktischen Jahr; Innere Medizin; Weill Cornell Medical College, New York

Promotion

Seit November 2005	Doktorarbeit in der Abteilung für Klinische Pharmakologie Leiter: Prof. Dr. Stefan Endres Medizinische Klink Innenstadt der Universität München

Stipendien

Seit 2006	Promotionsstipendium im Rahmen des von der DFG geförderten Graduiertenkollegs 1202
Seit 2007	Förderung durch die Studienstiftung des Deutschen Volkes
2008	PJ-Stipendium, Kooperation zwischen TU München und dem Weill Cornell Medical College, New York

6.4 Acknowledgements

First and foremost, I would like to thank Prof. Stefan Endres for giving me the opportunity to work on my doctoral thesis in his Division of Clinical Pharmacology. His support for us students is exceptional. In this spirit, I would like to thank him for promoting my admission to the 'Studienstiftung des Deutschen Volkes' and the 'Graduiertenkolleg 1202 – Oligonukleotide in Zellbiologie und Therapie'.

I am very grateful to PD Dr. Dr. Carole Bourquin for providing me with such an interesting research project and letting me participate in planning and implementation. Her constant personal encouragement and scientific challenging were truly inspiring.

Last but not least, I thank my fellow doctoral students and co-workers for such a cordial atmosphere in the laboratory and their active support especially in times of need.