Aus der Abteilung für Klinische Pharmakologie

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Medizinische Klinik und Poliklinik IV

Klinikum der Universität

Ludwig-Maximilians-Universität München

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Regulation of $\alpha_4\beta_7$ on naïve T cells upon viral infection

Dissertation

zum Erwerb des Doktorgrades der Naturwissenschaften

an der Medizinischen Fakultät

der Ludwig-Maximilians-Universität München

vorgelegt von

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aus Aachen

2012

Dedicated to my parents, Barbara and I Made Suhartha

and to my siblings,

Anja, André and Robin

Gedruckt mit Genehmigung der Medizinischen Fakultät

der Ludwig-Maximilians-Universität München

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Tag der mündlichen Prüfung: 13.12.2013

Erklärung

Diese Dissertation wurde im Sinne von § 6 Abs. 1 der Promotionsordnung vom 16. Juli 2010 von Frau Prof. Dr. Dr. Carole Bourquin betreut.

Ehrenwörtliche Versicherung

Diese Dissertation wurde selbstständig, ohne unerlaubte Hilfe erarbeitet.

München, am 19. Dezember 2012

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1 Summary

The elimination of virus-infected cells to block viral spread is substantially conveyed by effector $CD8^+$ T cells. Trafficking of naïve T cells into the lymph node is a crucial step for their activation to effector T cells. The entry into the lymph nodes is mediated by several cell adhesion molecules that are highly specific for given lymph nodes. For the ingress into the gut-associated lymphoid tissue (GALT) such as mesenteric lymph nodes and Peyer's patches, the integrin $\alpha_4\beta_7$ is the major homing receptor that interacts with its main ligand MAdCAM-1. On naïve T cells, $\alpha_4\beta_7$ is expressed at low level, however upon activation in the GALT its expression is significantly upregulated on effector T cells. Consequently these effector T cells migrate into the gastrointestinal tract, where MAdCAM-1 is also expressed.

The migration patterns of virus-specific $CD8^+$ T cells upon viral infection have been studied in detail. In contrast, the migration of the major population of virus-unspecific $CD8^+$ T cells, i.e. bystander-activated T cells is poorly understood. In our study, we aimed to determine how trafficking of these non-cognate, bystander-activated T cells is affected upon viral infection.

We discovered that $\alpha_4\beta_7$ expression was negatively regulated on bystander-activated $CD8^+$ T cells upon injection of poly (I:C), which imitates innate immune activation upon viral infection. This effect was also observed for viral infections such as Sendai, EMCV and the mutant form of VSV virus. Furthermore, we scrutinized the direct role of IFN-α on T cells to exert $\alpha_4\beta_7$ modulation. In the case of EMCV infection, IL-6 played a dominant role in the alteration of $\alpha_4\beta_7$ expression. Finally, using an adoptive transfer model we could prove that the downregulation severely impacted the trafficking of T cells into the Peyer's patches and to a lesser extent into the mesenteric lymph nodes.

These findings demonstrate that a mechanism to regulate trafficking of bystander-activated T cells during viral infection exists and that this is controlled by the induction of cytokines such as IFN-α and IL-6. We hypothesize that the downregulation of $\alpha_4\beta_7$ on naïve T cells functions 1) to allow space for virus-specific effector T cells to expand in the GALT and 2) to exclude bystander-activated T cells

from the GALT in order to prevent mistrafficking, which could cause autoimmune diseases.

Zusammenfassung

T-Zellen spielen eine essentielle Rolle im Aufbau der adaptiven Immunität. Die Migration von naiven T-Zellen in die Lymphknoten ist ein notwendiger Schritt für ihre Aktivierung zu Effektor-T-Zellen. Der Eintritt in die Lymphknoten wird durch verschiedene Zelladhäsionsmoleküle vermittelt, die hochspezifisch für bestimmte Lymphknoten sind. Für den Zugang in das darmassoziierte lymphatische Gewebe (GALT), wie mesenteriale Lymphknoten und Peyer-Plaques, ist das Integrin $\alpha_4\beta_7$ der Hauptrezeptor, der mit seinem Ligand MAdCAM-1 interagiert. Auf naiven T-Zellen ist das Integrin $\alpha_4\beta_7$ schwach exprimiert, jedoch wird es bei einer T-Zell-Aktivierung in GALT hochreguliert. Folglich wandern die Effektor-T-Zellen in den gastrointestinalen Trakt, in dem MAdCAM-1 ebenfalls exprimiert ist. Die Eliminierung von virus-infizierten Zellen zur Hemmung der Ausbreitung von Viren wird wesentlich von Effektor-$CD8^+$-T-Zellen durchgeführt.

Während viele Studien über die Migration von virus-spezifischen $CD8^+$ T-Zellen bereits bekannt sind, wurde die Migration der überwiegend virus-unspezifischen aktivierten $CD8^+$ Population bisher nicht detailliert untersucht. In unserer Studie ist das Ziel die Feststellung, ob die Migration dieser unspezifisch-aktivierten T-Zellen während einer viralen Infektion verändert wird.

Durch unsere Experimente konnten wir zeigen, dass die $\alpha_4\beta_7$ Expression auf unspezifisch-aktivierten T-Zellen bei einer Stimulation mit poly (I:C), die virale Infektion nachahmt, negativ reguliert wird. Diesen Effekt konnten wir auch in anderen viralen Infektionen mit Sendai, EMCV und einem mutierten VSV-Stamm beobachten. Des Weiteren konnten wir beweisen, dass IFN-α auf die T-Zellen eine direkte Funktion zur Regulierung der $\alpha_4\beta_7$ Expression ausübt. Im Falle einer EMCV Infektion, spielt IL-6 eine beträchtliche Rolle in die Herabregulation der $\alpha_4\beta_7$ Expression. Letzendlich konnten wir durch ein Adoptiv-Transfer-Experiment bestätigen, dass die $\alpha_4\beta_7$ Runterregulation auf die Migration der unspezifisch-aktivierten T-Zellen in den Peyer-Plaques und zum Teil in den mesenterialen Lymphknoten eine gravierende Auswirkung hat.

Unsere Studie hat damit gezeigt, dass ein Mechanismus zur Regulierung der Migration von unspezifisch-aktivierten T-Zellen während einer viralen Infektion

existiert und diese durch induzierte Zytokine wie IFN-α und IL-6 beeinflusst wird. Wir vermuten, dass die $\alpha_4\beta_7$ Runterregulation auf naiven T-Zellen nachstehende Funktionen hat. Erstens, erlaubt es den virus-spezifischen Effektor-T-Zellen in den Lymphknoten zu expandieren. Zweitens, ist es möglicherweise eine Präventionsmaßnahme, damit die unspezifisch-aktivierten T-Zellen nicht versehentlich in den Darm geleitet werden, was zu einer Autoimmunerkrankung führen könnte.

2 Introduction

2.1 Aim of thesis

The integrin $\alpha_4\beta_7$ is an essential cell adhesion molecule that allows naïve T cells to enter gut-associated lymphoid organs (GALT) such as Peyer's patches and mesenteric lymph nodes. Moreover, it also plays an indispensable role in migration of effector T cells activated in the GALT into the gastrointestinal tract. Since $\alpha_4\beta_7$ is known to play a role in many diseases such as HIV infection (Arthos et al., 2008), and graft-versus-host-disease (Waldman et al., 2006) and inflammatory bowel diseases (Rivera-Nieves et al., 2005), it is crucial to understand how this integrin is regulated.

It has been established that upon activation by antigen-presenting cell in the GALT, the antigen-specific activated T cells increase the expression of $\alpha_4\beta_7$ in order to gain access into the gastrointestinal tract, where the antigen of the intruders comes from (Johansson-Lindbom et al., 2003). However, how migration of antigen-unspecific (*bystander*) activated T cells is governed has been poorly studied. The first evidence that migration of bystander-activated T cells is also controlled during bacterial infection, was delivered by our colleagues in the lab, Simon Heidegger, Sophie Kirchner and colleagues (Heidegger et al., submitted). They demonstrated that upon *Salmonella typhimurium* infection $\alpha_4\beta_7$ expression on naïve T cells is decreased. They could also show that this effect can be mimicked when using TLR ligands and that the downregulation interfered with the ability of naïve T cells to enter the GALT. Furthermore, upon presence of TLR ligands and ovalbumin (OVA) as specific antigen, they could prove that T cells recognizing OVA (OT-I T cells) in the GALT increased their $\alpha_4\beta_7$ expression as previously described, whereas wild-type T cells did not.

Whereas these studies have started to scrutinize the regulation of $\alpha_4\beta_7$ expression on naïve T cells during bacterial infection, we next questioned how $\alpha_4\beta_7$ is altered during viral infection. In the first part of this thesis, poly (I:C), a TLR3 ligand that imitates viral infection, was utilized to answer following questions:

1. Does $\alpha_4\beta_7$ expression on naïve T cells change after poly (I:C) injection?
2. Which cytokines play a role?
3. How is the migration of these naïve T cells impaired?

In the second part of this thesis we intended to overexpress $\alpha_4\beta_7$ on T cells. T cells are essential for the direct killing of tumor cells in a high-specificity manner. However, in tumor-bearing patients T cells are often incapable of conducting their function due to 1) immunosuppressive microenvironment in the tumor, 2) failing in recognition of the tumor cells or 3) the absence of their specific-directed migration into the tumor. In this case, one possibility is to genetically modify T cells of tumor patients *in vitro* and transferred them back into the patient, referred to as adoptive T cell therapy. Adoptive T cell therapy has been used for treating tumors, where the infiltration number of the effector T cells into the tumor typically correlates with the success of tumor eradication and prolonged survival (Galon et al., 2006). Our studies in gastric tumors have revealed that transferring T cells of immunized mice into tumor-bearing mice was effective to reduce subcutaneous tumor size correlating with a high infiltration of effector T cells. However, the T cells were not able to enter the gastric tumor of the same mice due to the microenvironment in the tumor. Hence, they failed to execute their function in the tumor (Bourquin et al., 2010). We therefore aimed to overexpress $\alpha_4\beta_7$ on T cells to improve the targeting into gastrointestinal tumors.

2.2 T cell migration

T cells are one of the most motile cells in the body that constantly recirculate into secondary lymphoid organs. They belong like B cells to the secondary defense system, the adaptive immunity, which is linked to the activation of the innate immunity, the primary defense system. T cells have broad functions and tasks including elimination of virus-infected cells and tumor cells, potentiating immune response and activation of B cells. To exert their functions, T cells firstly need to be activated by an antigen-presenting cell (APC), which comprise the innate immune cells such as dendritic cells (DC) and macrophages, but also B cells. These cells are capable of presenting chopped short peptide fragments of intruders such as bacteria and virus (antigen) on their cell surface with the help of the majorhistocompatibility proteins (MHC class I, II). These peptides within the MHC proteins are recognized by T cells, which possess the right T cell receptor (TCR).

T cells are generated in the bone marrow as immature cells termed thymocytes. The maturation takes place in the thymus in which they migrate to. Following sequential selection mechanisms, cells that pass the negative and positive selection process, differentiate either into $CD4^+$ or $CD8^+$ T cells. At this time point, these mature T cells are referred to as naïve T cells because they have not yet encountered their specific antigen. In adults, naïve T cells consist of 25 million to 100 million distinct clones (Arstila et al., 1999). After their maturation in the thymus, these naïve T cells begin to traffic into the blood stream and enter the secondary lymphoid organs, where they eventually meet their cognate APC with the specific antigen. However, only a very small percentage of these naïve T cells (several thousand T cells) will obtain the opportunity to recognize their individual antigen (Von Andrian and Mackay, 2000).

The entry from blood vessels into the secondary lymphoid organs requires multistep adhesion cascades that are tightly governed by a plethora of distinct cell adhesion molecules (Springer, 1994; von Andrian and Mackay, 2000). The preferential migration into specific tissues is referred to as homing (Butcher and Picker, 1996).

2.2.1 Homing of naïve T cells into secondary lymphoid organs is mediated by multistep adhesion cascades

Unlike the migration into the spleen, the homing of T cells from blood vessels into the lymphoid organs such as peripheral lymph nodes, gut-draining mesenteric lymph nodes and Peyer's patches (lymph nodes along intestine), is an active process that requires the interaction between cell adhesion molecules and their ligands. These ligands, also termed *adressins*, are constitutively expressed on specialized endothelial venules, the so-called high endothelial venules (HEV) that function as the gateway into the lymphoid organs (Girard and Springer, 1995). Most cell adhesion molecules and adressins belong to four families of proteins: the selectin family, the mucin-like family, the immunoglobulin superfamily and the integrin family (Elangbam et al., 1997).

To enter the lymph nodes, T cells must initiate the adhesion into the HEVs (Table 1). This process is called *tethering and rolling* (step 1). Receptors responsible for tethering and rolling are also referred to as *homing receptors* and their ligands are termed *vascular addressins* (Berg et al., 1989). For the tethering onto the HEVs, T cells engage the constituvely expressed L-selectin that binds to peripheral-node addressin on HEV of peripheral lymph nodes (Berg et al., 1992; Kansas, 1992). However, the adhesion bond between L-selectin and peripheral-node addressin is non-permanent and keeps disassociating and reassociating upon pressure of flowing blood causing the rolling motion.

To stop rolling, T cells need to receive signals to activate their secondary receptors (*activation*, step 2). These receptors belong to the integrin family, which are characterized as heterodimeric non-covalently-linked proteins consisting of an α and a β chain. To date, eighteen α subunits and eight β subunits have been characterized forming 24 integrins in vertebrates (Zhang and Wang, 2012). Integrin members expressed on leukocytes include two β_2 integrins (LFA-1 or $\alpha_L\beta_2$ and $\alpha_M\beta_2$) and two α_4 integrins ($\alpha_4\beta_1$ and $\alpha_4\beta_7$) (Marelli-Berg et al., 2008). The ligands of the integrins are the members of immunoglobulin superfamily comprising mucosal addressin-cell adhesion molecule type 1 (MAdCAM-1); vascular-cell adhesion molecule 1; intracellular adhesion molecule 1 or 2 (Osborn et al., 1989; Kita et al., 1992; Briskin et al., 1993).

The signal activation for the integrins is provided by distinct chemokines immobilized on the endothelial cell surface that are capable to activate their corresponding chemokine receptors (Rot and Von Andrian, 2004). On the HEVs of secondary lymphoid organs, CCL19 and CCL21 are responsible for the interaction with CCR7 on T cells (Yoshida et al., 1997, 1998). The binding of the chemokine to its receptor induces a signal transmission through the so-called G-proteins causing conformational changes and higher affinity of the integrins to their ligands. As a consequence, a firm adhesion to the endothelium can be established, a process called *arrest* (step 3). For the arrest on HEVs of peripheral lymph nodes, LFA-1 on T cell binds to its ligand ICAM-1 or ICAM-2 (Marlin and Springer, 1987; Staunton et al., 1989). The last step is the *transendothelial migration* (step 4), which is the entry process of the T cell into the lymph node. This migration mainly occurs through endothelial cell junctions, also referred as to the paracellular route (Schoefl, 1972). An alternative way is the migration through the body of endothelium, the transcellular route (Cho and De Bruyn, 1981; Engelhardt and Wolburg, 2004).

In contrast to migration into peripheral lymph nodes, trafficking to the GALT comprising mesenteric lymph nodes and Peyer's patches is predominantly mediated by the integrin $\alpha_4\beta_7$ which is expressed on various type of immune cells (Postigo et al., 1993; Schweighoffer et al., 1993; Erle et al., 1994). $\alpha_4\beta_7$ mediates tethering, rolling and arrest by binding to its major ligand MAdCAM-1 expressed on HEVs of mesenteric lymph nodes and Peyer's patches (Nakache et al., 1989; Berlin et al., 1993). It has been demonstrated that formation of mesenteric lymph nodes, but mostly of Peyer's patches, is severely impaired in β_7-deficient mice due to the homing defect into these organs (Wagner et al., 1996). To note, it has also been reported that L-selectin can still mediate tethering and rolling into the mesenteric lymph nodes and to a lesser extent also into Peyer's patches by binding to the modified glycosylated MAdCAM-1 (Berg et al., 1993; Arbonés et al., 1994).

	Inductive site	
Target tissue	**Lymph node**	**Peyer's patches**
Tethering/rolling	L-selectin/PNAd (in PLN) $\alpha_4\beta_7$ /MAdCAM-1 (in MLN)	$\alpha_4\beta_7$ or L-selectin/ MAdCAM-1
Integrin activation	CCR7/CCL19, CCL21	CCR7/CCL19, CCL21
Firm adhesion	LFA-1/ICAM-1, ICAM-2	$\alpha_4\beta_7$ /MAdCAM-1 LFA-1/ICAM-1, ICAM-2

Table 1: Homing cascade that direct naïve T cells to lymph nodes (adapted from von Andrian and Mackay, 2000).

2.2.2 T cells activated in the GALT acquire gut-homing specificity

After homing into the GALT, T cells might eventually interact with an activated DC carrying antigens of the pathogen (Randolph et al., 2005). By chance, a few naïve T cells recognize the antigen presented by the DC leading to their activation and clonal expansion. Depending on the expression of CD4 or CD8 on the naïve T cells, the activated naïve T cells differentiate into T helper cells (T_h cells) or cytotoxic T cells (CTL), respectively. Whereas T_h cells are important in producing cytokines and activating other immune cells such as B cells, CTL and macrophages, CTLs are directed to eliminate infected cells or tumor cells that display the same antigen as the activating DC.

To exert their functions, the recently activated effector T cells need to egress from the lymphoid organs and move to the specific peripheral tissue where the antigen has been captured by the DC. These T cells acquire a new distinct repertoire of cell adhesion molecules depending upon where they are activated. For example, it has been shown that T cells activated in peripheral lymph nodes are imprinted to express E-selectin, P-selectin, CCR4 and CCR10, which are important for the homing to the skin (Picker et al., 1991; Campbell et al., 1999; Morales et al., 1999; Reiss et al., 2001; Mora and von Andrian, 2006).

In contrast, antigenic stimulation with DCs from Peyer's patches or mesenteric lymph nodes in the presence of antigen, adjuvant or anti-CD3 antibody enhances the

expression of $\alpha_4\beta_7$ and CCR9 on T cells (Stagg et al., 2002; Johansson-Lindbom et al., 2003; Mora et al., 2003). Consequently, these T cells are licensed to migrate into the gastrointestinal tract. Besides being expressed on the HEVs of Peyer's patches and mesenteric lymph nodes, the $\alpha_4\beta_7$ ligand MAdCAM-1 is also expressed on the intestinal and colonic lamina propria venules (Streeter et al., 1988; Nakache et al., 1989). In contrast, CCL25/TECK, the ligand of CCR9, is selectively expressed in the small intestine, but not in the colon, with highest expression in the duodenum (Stenstad et al., 2007).

The mechanism of how DCs from Peyer's patches and mesenteric lymph nodes elevate the expression of $\alpha_4\beta_7$ and CCR9 was elucidated by Iwata et al. (Iwata et al., 2004). They demonstrated that vitamin A-deficient mice have a markedly reduced $\alpha_4\beta_7^+$ $CD4^+$ T cells in their spleen, mesenteric lymph nodes and Peyer's patches. They could further show that all-*trans*-retinoic acid (RA), a vitamin A metabolite, is essential for the production of gut-specific $\alpha_4\beta_7^+$ T cells (Figure 2.1). *In vitro* incubation of T cells with all-*trans*-RA in presence of antigenic stimulation significantly increases the expression of $\alpha_4\beta_7$. This upregulation is due to the binding of RA to its receptor in the T cells, the heterodimer transcription factor RAR/RXR that subsequently drives transcriptions of various genes, including α_4 integrins (Kastner et al., 1995; Mangelsdorf and Evans, 1995; DeNucci et al., 2010). Furthermore, they discovered that DCs of Peyer's patches and mesenteric lymph nodes express not only alcohol dehydrogenases, but most importantly, also high levels of retinal dehyrogenases (Iwata et al., 2004). Whereas alcohol dehydrogenases convert vitamin A or retinol to retinal reversibly, retinal dehyrogenases catalyze the irreversible conversion from retinal to RA (Duester, 2000).

The capability to enhance $\alpha_4\beta_7$ expression on T cells and direct them to the gut is not only a feature of the GALT-DCs and the intestinal DCs. It has been recently discovered that liver-derived APC, namely the sinusoidal endothelial cells are able to induce $\alpha_4\beta_7$ expression on T cells (Neumann et al., 2012). Furthermore, intestinal enterocytes are also able to produce RA (Lampen et al., 2000).

The diverse potential sites for activation give benefits such as mounting a rapid immune response in a given area. However, they also bear the risks and potentials to develop improper immune reactions leading to autoimmune diseases.

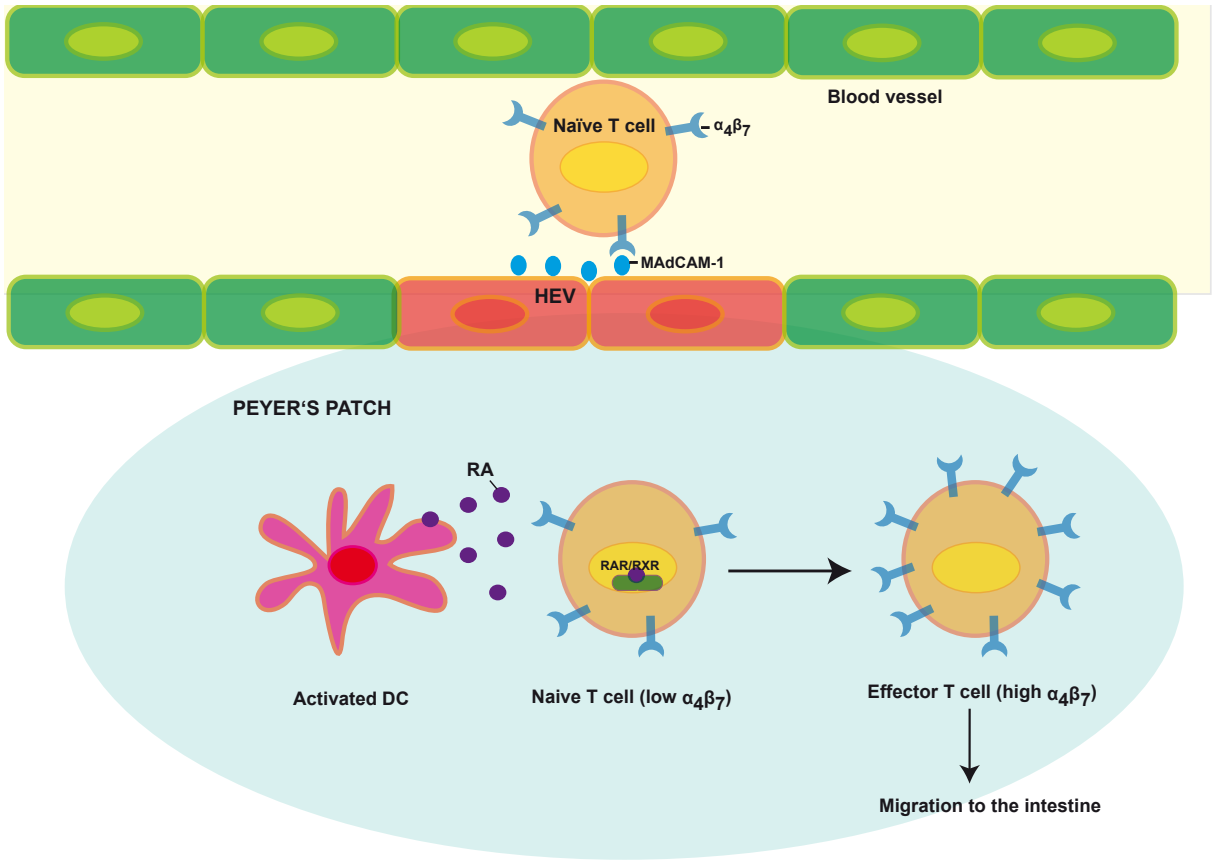

Figure 2.1 A model of T cell entry into the Peyer's patch mediated by interaction of $\alpha_4\beta_7$ with MAdCAM-1. Upon stimulation by activated dendritic cells producing retinoic acid, $\alpha_4\beta_7$ expression is upregulated, which leads to migration of effector T cells into the intestine.

2.2.3 Implication of $\alpha_4\beta_7$ in diseases

As a crucial homing receptor into the gastrointestinal tract, it is well established that $\alpha_4\beta_7$ is involved in the incurrence of several diseases such as HIV infection, graft–versus- host-disease, primary sclerosing cholangitis and inflammatory bowel diseases. Studies done by Arthos et al. demonstrate that $\alpha_4\beta_7^+$ $CD4^+$ T cells in genital mucosa are highly prone to infection by HIV virus due to high binding affinity of the viral envelope protein gp120 to $\alpha_4\beta_7$, which consists of a highly conserved tripeptide that mimics the tripeptide structure of MAdCAM-1 (Arthos et al., 2008). The binding of gp120 to $\alpha_4\beta_7$ initiates the formation of virological synapses that facilitate efficient cell-to-cell spreading of the virus, hence massive depletion of $CD4^+$ T cells.

In an acute graft-versus-hose-disease, a serious and often fatal complication following an allogeneic stem cell transplantation, recipient's tissues such as gastrointestinal tract, liver and skin are attacked by donor-derived CTLs.

The donor derived CTLs utilize L-selectin and $\alpha_4\beta_7$ to migrate to mesenteric lymph nodes, where they are activated by APCs presenting host antigens. The absence of both homing receptors on donor T cells has been shown to markedly ameliorate the acute colitis caused by the graft-versus-hose-disease due to significant reduction in early donor T-cell homing into the mesenteric lymph nodes (Dutt et al., 2005; Waldman et al., 2006).

The role of $\alpha_4\beta_7$ in inflammatory bowel diseases has also been exploited. Inflammatory bowel disease is a chronic inflammatory disorder in the gastrointestinal tissues that includes Crohn's disease and ulcerative colitis. One of the hallmarks of inflammatory bowel disease is the uncontrolled recruitment of leukocytes into the sites of inflammation, where they provoke cytokine-mediated tissue injury (Luster et al., 2005; Eksteen et al., 2008). Several studies using animal models of inflammatory bowel disease have demonstrated that administering antibodies specific for α_4 and $\alpha_4\beta_7$, but also for MAdCAM-1, ameliorate disease symptoms (Podolsky et al., 1993; Hesterberg et al., 1996; Picarella et al., 1997). However, depending upon which inflammatory bowel disease models are used, some studies have been yielded contradicting results. Colitis models utilizing $CD4^+$ T cell effector function suggest that $\alpha_4\beta_7$ is not required for T lymphocyte localization to the intestine and for the colitis pathogenesis. Moreover, the adoptive transfer of β_7-deficient T cells delays the onset of the disease over 9 weeks, however recipient mice still develope colitis at 25 weeks (Sydora et al., 2002).

Another gastrointestinal disease implicating $\alpha_4\beta_7^+$ T cells is the primary sclerosing cholangitis. It is a chronic liver disease triggered by progressive inflammation that affects the biliary tract causing obliteration of the bile ducts, hence cholestasis (Ponsioen, 2012). The occurrence of primary sclerosing cholangitis has been associated with inflammatory bowel disease as extraintestinal manifestations (Fausa et al., 1991; Olsson et al., 1991). Abberant MAdCAM-1 expression on the liver endothelium has been shown to be responsible for uncontrolled recruitment of $\alpha_4\beta_7^+$ T cells (Grant et al., 2001).

Whereas aberrant recruitment of $\alpha_4\beta_7^+$ T cells can lead to the aforementioned diseases, a proper trafficking of $\alpha_4\beta_7^+$ T cells to clear infection is also required. An infection study using recombinant virus (vesicular stomatitis virus) encoding

ovalbumin (OVA) demonstrates the critical role of $\alpha_4\beta_7$ for the migration of activated $CD8^+$ T cells into the MLN, PP and intestinal mucosa (Lefrançois et al., 1999). Furthermore, a study done by Kelly et al. has shown that the trafficking of $\alpha_4\beta_7^+$ CLA^+ effector T cells to the reproductive tract tissue is crucial during *chlamydia trachomatis* infection (Kelly et al., 2009).

Dysfunction of the recruitment of $\alpha_4\beta_7^+$ T cells has been also pointed out as a major problem to treat gastrointestinal tumors. Enarsson et al. demonstrated that in human gastric tumors the frequency of $\alpha_4\beta_7^+$ T cells was significantly decreased and this correlated with the decrease expression of MAdCAM-1 in the blood vessels in the tumor (Enarsson et al., 2006). Supporting this finding, a recent study in colorectal cancer could also reveal a reduced frequency of $\alpha_4\beta_7^+$ $CD4^+$ T cells and decreased expression of MAdCAM-1 in the tumor tissues compared to unaffected tissues (Svensson et al., 2012).

These numerous evidences of $\alpha_4\beta_7$ involvement in diseases emphasize the substantial importance of a proper control of T cell trafficking into the gastrointestinal tract.

2.2.4 Control of gut migration

Whereas the migration of effector T cells to certain peripheral tissues, such as skin or gut, has been studied in great detail (Mora et al., 2008), the trafficking pattern of naïve T cells during antigen-nonspecific immune (bystander) activation has been poorly investigated. Since only about one of 10^5 naïve T cells receives the chance to develop into an effector T cell during activation (Abbas and Janeway, 2000), it would be a high risk for the organism not to control the migration of the remaining naïve T cells, that are also in an activated but antigen-unspecific state (bystander activation) (Bangs et al., 2006). The fact that the gut is the largest peripheral tissue with tremendous amount of antigens derived from food and commensal bacteria, underlines the importance of possessing a tight surveillance for incoming T cell residents into the gut, in order to prevent unnecessary immune response causing autoimmune diseases.

Studies in our group have shown that upon *Salmonella typhimurium* but also *Escherichia coli* bacterial infection, the expression of $\alpha_4\beta_7$ on naïve T cells is decreased. Moreover, this decrease is also observed when T cells, cultured as splenocytes, are incubated with Toll-like receptors (TLR) ligands. The TLRs are receptors for conserved bacterial compounds that reside on the cell surface and in the endosome (Barton and Kagan, 2009; McGettrick and O'Neill, 2010). Furthermore, the downregulation was abrogated when using MyD88-deficient splenocytes. MyD88 is a crucial adaptor in TLR signaling (Medzhitov et al., 1998).

An elegant study in our group using an adoptive transfer model demonstrated that upon immune activation by CpG, the TLR9 ligand, and the presence of OVA, an antigen model, wild-type T cells in the MLN and PP decreased their $\alpha_4\beta_7$ expression leading to their impaired migration into the GALT. In contrast, the OT-I T cells, a transgenic $CD8^+$ T cells specific for OVA, increased their $\alpha_4\beta_7$ expression as previously described (Heidegger et al., submitted). The increased $\alpha_4\beta_7$ expression on OT-I T cells upon presence of TLR ligands and antigen confirmed an existing previous study (Johansson-Lindbom et al., 2003).

Whereas these studies have started to elucidate trafficking pattern of naïve T cells during bacterial infection, the migration of naïve T cells during viral infection still needs to be clarified.

2.3 Type I interferon signaling

One powerful weapon to fight viral invasion is the secretion of interferons (IFNs), a family of structurally related cytokines with antiviral activity, which can be expressed by almost every cell type, including leukocytes, fibroblasts and endothelial cells (González-Navajas et al., 2012). One of their functions is to trigger activation of genes that block viral replication in infected cells and to prevent apoptosis. Furthermore, they activate various types of innate and adaptive immune cells such as macrophages, NK, DC, B cells and $CD8^+$ T cells, promote neutrophil survival and the polarization of effector $CD4^+$ T cells into the Th1 type (Wang and Fish, 2012). Th1 cells produce certain cytokines enabling the immune system to clear viral and intracellular bacterial infections such as IFN-γ (Mosmann and Coffman, 1989; Zhu

and Paul, 2008). Based on their structural and functional properties, IFNs can be classified as type I, type II and type III IFNs (Takaoka and Yanai, 2006). As we are focusing on type I IFN, the next chapter will describe more specifically its induction, signaling and role in diseases and therapeutic settings.

2.3.1 Induction of type I IFNs

In humans and mice, the type I IFN family consists of 16 members including 12 IFN-α subtypes, IFN-β, IFN-ε, IFN-ω, IFN-κ (González-Navajas et al., 2012). The best-studied members are IFN-α and IFN-β.

The induction of type I IFN is initiated by recognition of microbe-specific pathogen-associated molecular patterns (PAMPS) by some members of the TLR family on the cell surface and in endosomes such as TLR3, TLR4, TLR7 and TLR9. These TLRs detect dsRNA, lipopolysaccharide (LPS), ssRNA and bacterial unmethylated CpG-containing dsDNA, respectively (Takeda et al., 2003; Heil et al., 2004) (Figure 2.2). Upon recognition of foreign nucleic acids in the endosome, TLR7 or TLR9 recruits the *adaptor myeloid differentiation primary-response protein 88* (MyD88). After a sequential recruitment process of other proteins, the signaling leads to the activation of among others the transcription factors NF-κB and AP1 which initiate the transcription of numerous proinflammatory cytokines, but also of type I IFN (Monroe et al., 2010; Desmet and Ishii, 2012).

In contrast, the activation of TLR3 in the endosome engages another adaptor protein termed TRIF (TIR-domain-containing adaptor protein) inducing mainly the transcription of type I IFN. TLR4 is among TLRs unique since it is compatible to both adaptors (Yamamoto et al., 2003). Whereas type I IFN induction by TLR3 and TLR4 via TRIF takes place in broad range of cell types, TLR7- or TLR9- mediated type I IFN secretion occurs mainly in DCs, particulary plasmacytoid DC (González-Navajas et al., 2012).

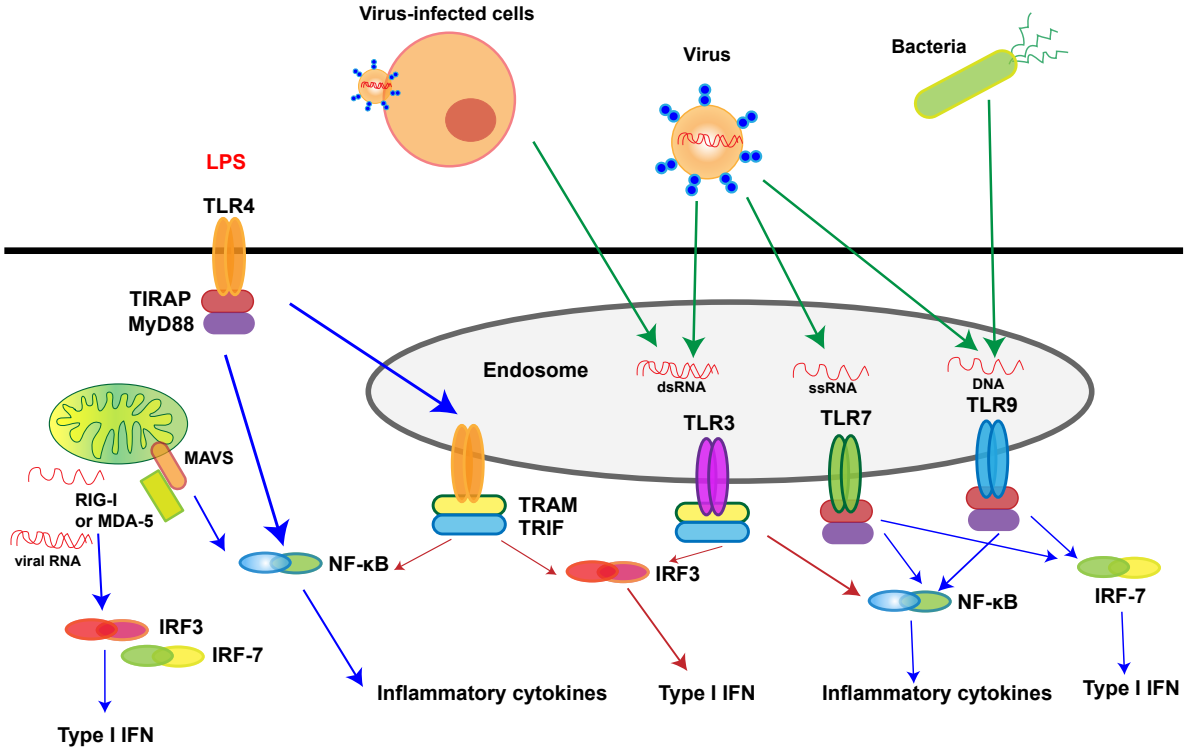

Figure 2.2: A simplified model of TLR and RIG-I-like-receptors (RLRs) signaling. Figure was modified from Yoneyama and Fujita (2010), Kawai and Akira (2010).

Another safeguard mechanism for viral offence that has evaded the TLR recognition is provided by the cytosolic sensors retinoic-acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA-5) that are capable of identifying viral RNA (Barral et al., 2009). Additionally, other receptors such as the stimulator of IFN-genes (STING), an endoplasmic reticulum-associated protein, and DAI (DNA-dependent activator of IRFs) have also been described to induce type I IFNs in response to cytosolic DNA (Takaoka et al., 2007; Ishikawa and Barber, 2008). Like TLR signaling, detection of foreign nucleic acids in the cytosol by RIG-I and MDA-5 elicits signaling cascades activating the transcription factors NF-κB and IRF3 and IRF7 mediated by the adaptor *mitochondrial antiviral signaling protein* (MAVS) on the mitochondrias (Yoneyama and Fujita, 2007).

2.3.2 Signaling pathways activated by type I IFNs

After the initiation of type I IFN induction induced by PAMPs recognition, secreted proinflammatory cytokines and type I IFN, mainly IFN-α and IFN-β, provoke a secondary signaling cascade by binding to their receptors (Figure 2.3). Recognition of IFN-α and IFN-β is conveyed by the cognate IFN receptor complex, IFNAR1 and IFNAR2 (Novick et al., 1994; Uzé et al., 2007). IFNAR1 and IFNAR2 are associated with the Janus protein tyrosine kinases (Jak PTKs), Tyk2 and Jak1, respectively. The binding of both types of IFNs leads to cross-activation of these JAK PTKs leading to specific tyrosine phosphorylation of their target substrates, Stat1 and Stat2 ("***s****ignal* ***t****ransducer and* ***a****ctivators of* ***t****ranscription*") (Darnell et al., 1994; Ihle and Kerr, 1995; Schindler and Darnell, 1995; Stark et al., 1998). Subsequently, the phosphorylation allows these two Stats to form a transcription factor with the DNA-binding subunit IRF-9 termed IFN-stimulated gene factor 3 (ISGF3) (Darnell et al., 1994; Haque and Williams, 1994; Bluyssen et al., 1996). Finally, the complex ISGF3 then translocates into the nucleus and initiates the transcription of numerous IFN-stimulated genes (ISGs) by binding to the IFN-stimulated response elements (ISREs) at the promoter site (Qureshi et al., 1995). The ISGs are responsible for the antiviral and immunomodulatory properties of the IFNs. To note, although the above-mentioned pathway downstream of IFNAR1/2-complex is the most prominent pathway activated by type I IFN, there are other type-I-IFN-dependent signaling pathways described (Takaoka and Yanai, 2006; González-Navajas et al., 2012).

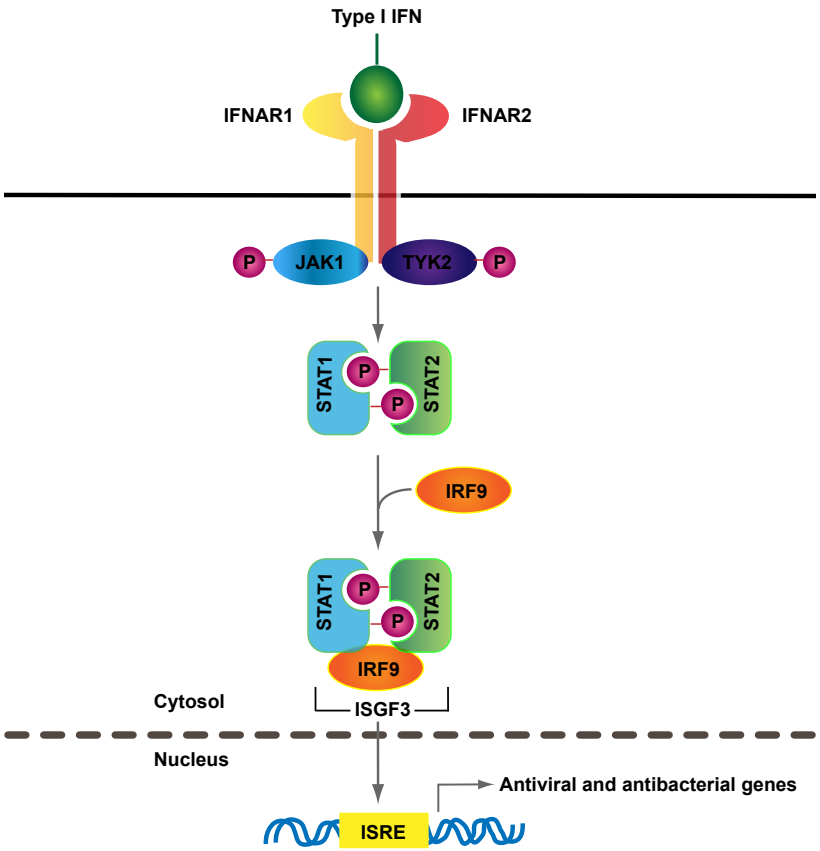

Figure 2.3: A simplified model of type I IFN signaling. Figure was modified from González-Navajas et al., 2012.

2.3.3 Implication in diseases and therapeutic use of type I IFN

Despite its beneficial effects in commencing the immune response during viral or bacterial infection, type I IFN has also been connected to several autoimmune and inflammatory disorders such as systemic lupus erythematosus, coeliac disease and psoriasis.

Systemic lupus erythematosus is a systemic autoimmune disease in which autoreactive T cells assault the body's own cells and tissues leading to inflammation and tissue damage. It has been shown that systemic lupus erythematosus patients display elevated levels of IFN-α in their blood that is produced mainly by plasmacytoid DCs (Ytterberg and Schnitzer, 1982; Bengtsson et al., 2000; Rönnblom, 2011). Similar to systemic lupus erythematosus, coeliac disease and psoriasis affecting the small intestine and skin respectively, are caused by uncontrolled overproduction of type I IFN (Funk et al., 1991; Schmid et al., 1994;

Monteleone et al., 2001). Nonetheless, type I IFN has also been used to treat diseases such as multiple sclerosis (an inflammatory disease affecting the brain), inflammatory bowel diseases and cancer (Ann Marrie and Rudick, 2006; Ferrantini et al., 2007; Musch et al., 2007).

2.4 Adoptive T cell therapy

As it has been mentioned in chapter 2.2, the trafficking into peripheral tissue is an essential step for cytotoxic $CD8^+$ T cells (CTL) to actuate their function in the tissue, i.e. to eliminate virus-infected cells by inducing programmed cell death apoptosis (Berke, 1995). Furthermore, CTLs are able to recognize and to eradicate tumor cells due to the presentation of tumor-antigens on MHC class I, which ideally represent parts of mutated proteins in the tumor cells (Cerottini et al., 1992; Boon et al., 1994).

Adoptive T cell transfer in tumor immunotherapy is based on the fact that CTL can kill tumor cells and administering *ex vivo* expanded T cells into the body can reconstitute the immunity, which has been lost in tumor patients due to tumor tolerance (Restifo et al., 2012). Numerous studies have demonstrated that infiltration by Th1 cells (IFN-γ-producing $CD4^+$ T cells) and CTL into the tumor correlates with tumor reduction and prolonged survival of patients (Galon et al., 2006).

However, in many cases these tumor-infiltrating lymphocytes are paralysed and incapable of tumor elimination since the tumor has evolved strategies to overcome immune recognition by establishing an immunosuppressive microenvironment. This includes the downregulation of MHC or co-stimulatory molecules, secretion of factors limiting CTL cytotoxic activity, expression of inhibitory ligands and decrease of homing signals for CTLs (Ngo et al., 2011). Circumventing these obstacles is still one of the major challenges in the immunotherapy research field.

Another issue is the restoration of tumor-infiltrating lymphocytes function. Although it has been shown that tumor-infiltrating lymphocytes outside the tumor microenvironment can exert their specific functions, some studies have also shown that most of the tumor-specific T cells have low-affinity TCRs to the tumor antigens. This is due to the fact that tumor antigens are mostly self-antigens and therefore T cells recognizing these antigens are negative selected during maturation.

Enhancing the TCR affinity to tumor antigens by introducing transgenic TCRs using integrating retroviral, lentiviral or plasmids is therefore one of strategies to improve clinical outcome (Park et al., 2011).

A further drawback is the blockade of T cells to enter the tumor due to the obstruction of homing signals. In our study, we could show that administering T cells from immunized mice can induce efficient eradication of subcutaneous gastric tumors in mice. However, in the same mice T cells are retained in the periphery of an autochtonous gastric tumor. This retention can also be seen in other studies (Galon et al., 2006; Mrass et al., 2006; Boissonnas et al., 2007). The enhanced expression of a homing signal such as MAdCAM-1 and the generation of tumor-antigen-specific CTLs with increased homing receptor $\alpha_4\beta_7$ might therefore be a promising strategy to direct T cells into the gastrointestinal tumors.

3 Materials and Methods

3.1 Materials

3.1.1 Technical equipment

Alpha Imager (gel documentation)	Alpha Innotech, San Leandro, USA
Balance (LP 6209)	Sartorius, Göttingen, Germany
BD FACS Canto II	BD Biosciences, San Diego, USA
Cell culture CO2 incubator (BD 6220)	Heraeus, Hanau, Germany
Cell culture laminar flow	Heraeus, Hanau, Germany
Cell strainer (40 and 70 μm, nylon mesh)	Fisher Scientific, Leicestershire, UK
Centrifuge 5424	Eppendorf, Hamburg, Germany
Centrifuge 5417R	Eppendorf, Hampburg, Germany
DynaMag 15/50 magnet	Invitrogen Dynal, Carlsbad, USA
Fluo-link (UV irradiation)	Vilber Lourmat, Marne La Vallée, France
Gel electrophoresis system	Bio-rad, Munich, Germany
MACSQuant Analyzer	Miltenyi Biotec, Bergisch Gladbach, Germany
Microscope Axiovert 25	Zeiss, Jena, Germany
Mithras LB940 multilabel plate reader	Berthold, Bad Wildbach, Germany
Multifuge 3L-R	Heraeus, Hanau, Germany
Nanodrop ND-1000	NanoDrop, Wilmington, USA
Neubauer hemocytometer	Optik Labor Frischknecht, Balgach, Germany
pH meter 3505	Jenway, Staffordshire, UK
Power Supply 200/2.0	Biorad, Munich, Germany
Refrigerators (4°C, -20°C, -80°)	Thermo scientific, Waltham, USA
Thermocycler T3	Biometra, Göttingen, Germany
Thermomixer	Eppendorf, Hamburg, Germany
IKA® Vortex 3	IKA, Staufen, Germany

3.1.2 Chemical, reagents and buffer

Acetic acid	Fisher Chemical, Leicestershire, UK
Ampicillin	Fisher Scientific, Leicestershire, UK
Agarose LE	Biozym, Hess. Oldendorf, Germany
Aqua ad injectabilia	Braun Melsungen AG, Melsungen, Germany
BD FACS lysing solution (10x)	BD Biosciences, San Diego, USA
BD Pharm Lyse (10x)	BD Biosciences, San Diego, USA
Bovine serum albumine (BSA)	Sigma Aldrich, Steinheim, Germany
Bromophenol blue, sodium salt	Promega, Wisconsin, USA
Calcium chloride	Acros Organics, Geel, Belgium
Dimethyl sulfoxide (DMSO)	Sigma Aldrich, Steinheim, Germany
Dulbecco's PBS (1x)	PAA, Pasching, Germany
Ethanol	Amresco, Solon, USA
Ethidium bromide	Sigma Aldrich, Steinheim, Germany
Ethylendiaminetetraacetic acid (EDTA)	Sigma Aldrich, Steinheim, Germany
FACSFlow, FACSSafe	Becton Dickinson, Heidelberg, Germany
Glycerol	Fisher Chemical, Leicestershire, UK
Isofluorane (Forene®)	Abbott, Zug, Switzerland
Isopropanol (70 Vol%)	Apotheke Innenstadt, LMU Munich
LB broth (Lennox)	Condalab, Madrid, Spain
LB agar (Lennox)	Condalab, Madrid, Spain
2-Mercaptoethanol	Acros Organics, Geel, Belgium
PharmLyse (10x) RBC lysis	Becton Dickinson, Heidelberg, Germany
Potassium chloride	Fisher Chemical, Leicestershire, UK
Saponin	Serva Electrophoresis GmbH, Heidelberg, Germany
Sodium carbonate	Sigma Aldrich, Steinheim, Germany
Sodium chloride	Sigma Aldrich, Steinheim, Germany
Sodium hydrogenphosphate	Fisher Chemical, Leicestershire, UK
Sodium phosphate	Sigma Aldrich, Steinheim, Germany
Sulfuric acid (H_2SO_4, 2N)	Apotheke Innenstadt, LMU Munich
TMB Substrate Reagent Set	Becton Dickinson, Heidelberg, Germany

Tris(hydroxymethyl)amino methane	Biosolve BV, Valkenswaard, Netherlands
Tween 20	Fisher Scientific, Leicestershire, UK

Buffer 1 (for T cell isolation)

1x PBS (without Ca2+ and Mg2+)
0.1% BSA
2 mM EDTA
pH 7.4

2% BSA

Dissolve 1 g BSA in 50 ml sterile water
Sterile filtrate with 0.2 μm filter

Buffer to wash Dynabeads® mouse T cell activator

1x PBS
0.1 % BSA
2 mM EDTA
pH 7.4

Blasticidin (5 mg/ml)

Dissolve 50 mg Blasticidin in 10 ml sterile PBS, aliquot to 1 ml, freeze at -20°C

ELISA coating buffer 1

0.2 M Sodium phosphate
in water
pH 6.5

ELISA coating buffer 2

0.1 mM Sodium carbonate
in water
pH 9.5

ELISA assay diluent

10% FCS
in PBS
pH 7.0

ELISA wash buffer

0.05% Tween
in PBS

6x loading dye

0.05 mg Bromophenol blue
2.8 ml ddH_2O
6 ml Glycerin

20X Saponin

20% (w/v) saponin in 1x PBS

50x TAE

to 900 ml ddH_2O

242.3 g Tris base

57 ml Acetic acid

18.6 g EDTA

Fill to 1 L

Transfection buffer

1.6 g NaCl

74 mg KCl

50 mg Na_2HPO_4

1 g HEPES

add 100 ml ddH_2O, pH 6.76

$CaCl_2$ 2M

Dissolve 11 g CaCl2 in 50 ml sterile ddH2O

Sterile filtrate with 0.2 μm filter

Puromycin (10 mg/ml)

Dissolve 100 mg with 10 ml sterile PBS

Aliquot 50 μl, store at -20°C

3.1.3 Kits

Cell Proliferation Dye eFluor® 670	eBiosciences, San Diego, USA
Cell Trace CFSE cell proliferation kit	Invitrogen/Molecular Probes, Eugene, USA
Dynal® Mouse T cell negative isolation Kit	Invitrogen/Molecular Probes, Eugene, USA
JETSTAR Plasmid purification MAXI kit	Genomed, Florida, USA
AxyPrep™ plasmid miniprep kit	Axygen biosciences, California, USA
Wizard® SV Gel and PCR Clean-Up System	Promega, Wisconsin, USA

Cytokine ELISA sets

IL-6 murine	BD Biosciences, 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

3.1.4 Cell culture reagents and media

Blasticidin	PAA, Pasching, Austria
Chloroquine	LabForce, Nunningen, Switzerland
β-Mercaptoethanol	Sigma Aldrich, Steinheim, Germany
Dulbecco's modified Eagle's medium (DMEM), high glucose	PAA, Pasching, Austria
Fetal calf serum (FCS)	GibcoBRL (invitrogen), Karlsruhe, Germany
L-Glutamine 200 mM	PAA, Pasching, Austria
HEPES	PAA, Pasching, Austria
MEM-NEAA (non-essential amino acids)	GibcoBRL (Invitrogen), Karlsruhe, Germany
Phosphate buffered saline (PBS)	PAA, Pasching, Austria
Penicillin/Streptomycin (100x)	PAA, Pasching, Austria
Protamine sulfate	Merck, New Jersey, USA
Puromycin	PAA, Pasching, Austria
Retronectin	Takara, Shiga, Japan
Roswell Park Memorial Institute (RPMI) 1640 medium	PAA, Pasching, Austria
Sodium pyruvate	PAA, Pasching, Austria
Trypan Blue solution	Eurobio, Cortaboeuf, France
Trypsin EDTA (1x)	PAA, Pasching, Austria
VLE RPMI 1640 medium (very low endotoxon)	Biochrom, Berlin, Germany

T cell medium	**Plat-E medium**
RPMI 1640	DMEM high glucose
10% FCS	10% FCS
1% L-Glutamine	1% Glutamine
1mM HEPES	100 IU Penicillin, Streptomycin

1 mM Sodium Pyruvate
100 IU/ml penicillin, streptomycin
50 μM 2-mercaptoethanol
10 μg/ml Blasticidin
1 μg/ml Puromycin

Plat-E hunger medium

DMEM high glucose
3% FCS
1% Glutamine
100 IU Penicillin, Streptomycin
10 μg/ml Blasticidin
1μg/ml Puromycin

Freezing medium for Plat-E and RF33 cells

500 μl 100% FCS
40 μl DMSO
Mix well
Add to 5 millions cells/ 400 μl in a cryo tubes
Freeze immediately to -80°C

Cytokines and growth factors:

LEAF™ Purified anti-mouse CD3ε Antibody (clone: 145-2C11)	Biolegend, San Diego, USA
LEAF™ Purified anti-mouse CD28 Antibody (clone: 37.51)	Biolegend, San Diego, USA
Dynabeads® Mouse T-Activator CD3/CD28 for Cell Expansion and Activation	Invitrogen, Eugene, USA
IFN-α, mouse, recombinant	Miltenyi Biotec, Bergisch Gladbach, Germany
Poly I:C	Invitrogen/Molecular Probes, Eugene, USA
CpG 1826 (CpG) (5'-TCCATGA*C*GTTCCTGA*C*GTT-3')	Coley Pharmaceuticals, Langenfeld, Germany
Recombinant murine IL-2	Peprotech, Rocky Hill, USA
Recombinant mouse IL-15 (carrier-free)	Biolegend, San Diego, USA
Resiquimod (R848)	Alexis Biochemicals, Lausen, Switzerland

Blocking antibodies:

Description	Isotype	Clone	Distributor
LEAF™ purified anti-mouse IL-6 antibody	Rat IgG1, κ	MP5-20F3	BioLegend
LEAF™ purified anti-mouse IFNAR-1 antibody	Mouse IgG1, κ	MAR1-5A3	BioLegend

Viruses:

The encephalomyocarditis virus (EMCV), vesicular stomatitis virus (VSV) and a mutant form of VSV as well as Sendai virus were kindly provided from the group of Prof. Simon Rothenfußer, Division of Clinical Pharmacology, University of Munich.

3.1.5 FACS antibodies

Description	Isotype	Clone	Distributor
anti-CD3	Armenian Hamster IgG	145-2C11	BioLegend
anti-CD4	Rat IgG2a, κ	RM4-5	BioLegend
anti-CD8a	Rat IgG2a, κ	52.67	BioLegend
anti-CD45R/B220	Rat IgG2a, κ	RA3-6B2	BioLegend
anti-CD69	Armenian Hamster IgG	HI-2F3	BioLegend
anti-LPAM-1 ($\alpha_4\beta_7$)	Rat IgG2a, κ	DATK32	BioLegend
anti-CD29	Armenian Hamster IgG	HMß1-1	BioLegend
anti-CD49d	Rat IgG2b, κ	R1-2	BioLegend
anti-β7	Rat IgG2a, κ	FIB504	BioLegend
anti-LFA1	Rat IgG1, κ	H155-78	BioLegend
anti-CCR7	Rat IgG2a, κ	4B12	BioLegend
anti-CCR9	Rat IgG2b	242503	R&D Systems
anti-CD62L	Rat IgG2a, κ	MEL-14	BioLegend

anti-CD44	Rat IgG2b, κ	IM7	BioLegend
anti-CD31	Rat IgG2a, κ	MEC13.3	BioLegend
FITC Armenian Hamster IgG Isotype Ctrl Antibody	Armenian Hamster IgG	HTK88	BioLegend
FITC Rat IgG2b, κ Isotype Ctrl Antibody	Rat IgG2b, κ	MRG2b-85	BioLegend
FITC Rat IgG2a, κ Isotype Ctrl Antibody	Rat IgG2a, κ	RTK2758	BioLegend

Plastic materials for cell culture experiments were purchased from Becton Dickinson (Heidelberg Germany), Brunschwig (Basel, Switzerland), Corning (Corning, USA), Eppendorf (Hamburg, Germany), Fisher Scientific (Leicestershire, UK) and Sarstedt (Nümbrecht, Germany).

3.1.6 Molecular biology

Templates for PCR:

cDNAs	**Clone ID**	**Catalog number**	**Company**
ITGA4	30093248	MMM1013-98478859	Thermo Scientific, Leicestershire, UK
ITGB1	5721348	MMM1013-9497715	Thermo Scientific, Leicestershire, UK
ITGB7	4187781	MMM1013-65317	Thermo Scientific, Leicestershire, UK

Primers for PCR:

Primers were ordered from Eurofins MWG Operon (Ebersberg, Germany) with following sequences:

Name of primer	Sequence (5' – 3')
ITGA4 forward	ATAAGAATGCGGCCGCATGGCTGCGGAACG
ITGA4 reverse	CCGGAATTCTCAGTCATCATTGCTTTTGC
ITGB1 forward	ATAAGAATGCGGCCGCATGAATTTGCAACTGGTTTCCTGG
ITGB1 reverse	CCGGAATTCCCCTCATACTTCGGATTGACCAC
ITGB7 forward	ATAAGAATGCGGCCGCATGGTGGATTCATCAACTGTTCTC
ITGB7 reverse	CCGGAATTCTCAGTCTGCTTCCCTGGTCAGAG

Table 2. Sequence of primers used for amplification of the genes ITGA4 (integrin α_4), ITGB7 (integrin β_7), ITGB1 (integrin β_1). Sequences marked with green or red indicate the restriction site of NotI and EcoRI, respectively.

Primers for sequencing:

Primers were ordered from Eurofins MWG Operon (Ebersberg, Germany) with following sequences:

Name of primer	Sequence (5' – 3')
ITGA4 primer 1	GCTTTTCCTATCTGTTCGTG
ITGA4 primer 2	GGCTACTCAGTTGGAGCTGGAC
ITGA4 primer 3	TGTGTTTCTCATATAAAGGC
ITGA4 primer 4	GACCAAGTGAGGGACAACAG
ITGB1 primer 1	CCACCAAGTTTCCCATCTCCA
ITGB1 primer 2	AAGGTGGCTTTGATGCAATC
ITGB1 primer 3	AGACATGGACGCTTACTGCAG
ITGB7 primer 1	GTTCCTGGCAGAGGGCAGCC
ITGB7 primer 2	TTTCACCACGTGCTGTCCCTCAC
ITGB7 primer 3	AGTGATGGACAGGGGGACCTTC
ITGB7 primer 4	GCTGCAGGAGGTCACACATTCTGTGC

Table 3. Sequence of primers for sequencing

Vector:

The retroviral vector pMP71 was kindly provided by Prof. Dr. Baum, Department of Experimental Haematology, Medizinische Hochschule Hannover.

Reagents and cells for cloning:

Pfu DNA Polymerase (native)	Fermentas, St. Leon-Rot, Germany
T4 DNA Ligase	Fermentas, St. Leon-Rot, Germany
NotI	Fermentas, St. Leon-Rot, Germany
Buffer O (orange)	Fermentas, St. Leon-Rot, Germany
EcoRI	Fermentas, St. Leon-Rot, Germany
GeneRuler™ 1kb DNA Ladder	Fermentas, St. Leon-Rot, Germany
1 Kb Ldder DNA Marker	Axygen Biosciences, California, USA
Max Efficiency DH5a Competent Cells	Invitrogen, Eugene, USA

3.1.7 Software

Adobe Illustrator CS4	Adobe System, San Jose, USA
Adobe Photoshop CS4	Adobe System, San Jose, USA
FlowJo	Tree Star, Ashland, USA
GraphPad Prism5 Software	GraphPad Software inc., La Jolla, USA
Lasergene® SeqMan Pro™	DNASTAR Inc., Wisconsin, USA
Microsoft Office	Microsoft, Redmond, USA
NCBI Entrez Gene	www.ncbi.nlm.nih.gov/sites/entrez?db=gene
Zotero	Roy Rosenzweig Center, Virginia, USA

3.2 Cell culture

3.2.1 General cell culture and cell viability testing

All cells were cultured in tissue culture flasks at 37°C, 5% CO2 and 95% humidity. All manipulations were conducted in a sterile condition under laminar flow hood. To check cell viability and determine cell concentration Trypan blue staining was used. Live cells are excluded from staining with Trypan blue, since it is not able to cross into live cells with intact cell membrane. However, it can traverse into dead cells. Thus, dead cells can be observed as blue-colored cells under the microscope. Cell suspensions were mixed well in a 0.25% Trypan blue in PBS at appropriate dilution (generally at a ratio of 1:10 or 1:20) and subsequently counted in a Neubauer hemocytometer under microscope. The cell concentration is calculated as followed:

Cell concentration (cells/ml) = number of cells counted x dilution factor x 10^4

3.2.2 *In vitro* stimulation

3.2.2.1 With TLR ligands

In a 96-well plate, splenocytes and T cells (4. 10^5 – 5. 10^5 cells/well) were stimulated with CpG (5 μg/ml), poly I:C (200 μg/ml), R848 (0.1 μg/ml) for 24 h prior flow cytometry analysis.

3.2.2.2 With recombinant mouse IFN-α

In a 96-well plate, splenocytes and T cells (4. 10^5 – 5. 10^5 cells/well) were stimulated with recombinant mouse IFN-α (10^3 U/ml) for 24 h prior to FACS analysis.

3.2.2.3 With interleukins

In a 96-well plate, splenocytes and T cells (4. 10^5 – 5. 10^5 cells/well) were stimulated with a mixture of interleukins IL-6 (100 ng/ml), IL-1β (100 ng/ml) and IL-12 (100 ng/ml) in presence or absence of IFN-α (10^3 U/ml) for 24 h prior to FACS analysis.

3.2.2.4 With viruses

In a 96-well plate, splenocytes and T cells (4. 10^5 – 5. 10^5 cells/well) were incubated with EMCV virus (10^7 pfu/ml), Sendai virus (100 or 250 U/ml), VSV virus (10^7 pfu/ml) and VSVmut virus (10^7 pfu/ml) for 3 h in a FCS-free T cell medium. Afterwards, cells were centrifuged and the supernatants were discarded and replaced with FCS-containing T cell medium. After 24 h of incubation, cells were analyzed by flow cytometry.

3.2.3 T cell purification

To purify T cells from splenocytes, a Dynal mouse T cell negative isolation kit from Invitrogen was used. The principle of the purification kit is as following: splenocytes are incubated with a mixture of monoclonal antibodies against non-T-cell markers such as antigens present on B cells, monocytes/macrophages, erythrocytes, granulocytes, dendritic cells, NK cells. Dynabeads, which are uniform, superparamagnetic polystyrene beads coated with polyclonal sheep-anti rat IgG antibody, are added to the suspension and are ideally attached to the mixture antibodies that have bound their target cells. The bead-bound cells are separated by a magnet from the T cells in the solution. Figure 3.1 depicts an example of percentage of T cells obtained after purification.

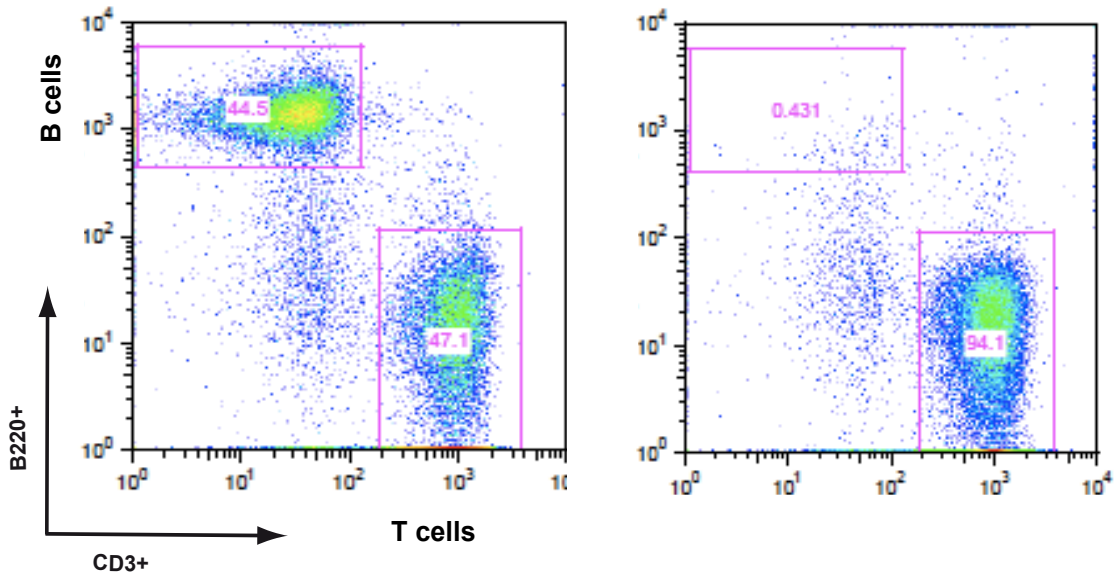

Figure 3.1: Comparison of percentage of T cells before and after purification. Splenocytes were stained for B220$^+$, a marker for B cells and CD3$^+$ before purification (left panel) and after purification (right panel).

3.2.4 Blocking antibodies

Splenocytes (4. 10^5 cells/well) were cultured in a 96-well plate in FCS-free T cell medium with CpG (5 μg/ml), EMCV (10^7 pfu/ml) or Sendai virus (100 U/ml) for 3 h. Triplicates were done for each of the groups. Afterwards, cells were centrifuged at 400g for 7 min to wash out CpG and the virus. The supernatants were discarded and FCS-containing T cell medium (200 μl) was added to the cells. After 24 h, cells were pelleted by centrifugation (400g, 7 min). The supernatants were pipetted into a new 96-well plate and cells were checked by flow cytometry for CD3, CD8 and $\alpha_4\beta_7$ to assure downregulation of $\alpha_4\beta_7$ expression on CD3$^+$CD8$^+$ cells. The remaining viruses in the supernatants were inactivated by UV-light (3 J/m^2). The supernatants were then added to purified T cells (250.000 cells/well, triplicates for each group). Additionally, to each group either no antibodies or antibodies against mouse-anti IL-6 (5 μg/ml), mouse-anti IFNAR-1 (1.5 μg/ml) or both were added to the wells. Cells were incubated overnight (24 h) prior to flow cytometry analysis.

3.2.5 Transfection

The retroviral packaging cell line Platinum-E (Plat-E) was kindly provided by Prof. Wolfgang Uckert, Max-Delbrück Center for Medical Medicine. Plat-E cells have been shown to induce high titers of retroviruses (Morita et al., 2000).

Briefly, $8x10^5$ – $1x\ 10^6$ Plat-E cells in 3 ml culture medium were transferred into a tissue-culture treated 6-well plate to yield 60-70% in the next day. On second day, the adherent Plat-E cells were incubated with 3 ml hunger medium (with 3% FCS) for one hour prior to transfection. This step is important to reduce the amount of serum proteins which could lead to increased particle size due to aggregation, thus minimizing transfection efficiency (Welzel et al., 2004).

150 µl Calcium chloride solution containing the vector was prepared as followed:

Reagents	End concentration	Volume (V_{end}=300 µl*)
100 mM Chloroquine	126.7 µM	0.38 µl
2M $CaCl_2$	100 mM	15 µl
Plasmid DNA	18 µg	18 µg or more
Sterile ddH_2O		add to 150 µl

Table 4. Preparation of calcium chloride solution. * The amount of reagents in µl (last column) was calculated for 300 µl calcium phosphate transfection solution (V_{end}=150 µl calcium chloride solution + 150 µl transfection buffer.

Subsequently, the solution was added drop-wise to the 150 µl transfection buffer (in a polystyrene tube) while vortexing the tube constantly. After incubation for 30 min at RT, visible precipitation (DNA-calcium phosphate complex) could be observed. The 300 µl precipitate-containing DNA solution was added slowly (drop-wise) to the Plat-E cells and incubated for six hours in the incubator (37°C, 5% CO_2) to allow uptake of DNA-calcium-phosphate complex. Thereafter, the medium was exchanged with 3 ml normal Plat-E medium (with 10% FCS) and cells were cultured for 48 hours in a incubator (37°C, 5% CO_2).

The calcium phosphate precipitation method was firstly described in 1973 (Graham and Van der Eb, 1973). Interaction of calcium cations with the negatively-charged phosphate backbone of the DNA and addition of phosphate-buffered solutions lead to

formation of precipitation that can be taken up by the cells via endocytosis. Addition of chloroquine can lead to enhanced transfection efficiency by inhibiting the acidification of the lysosomes, hence preventing the degradation of transfecting DNA by lysosomal hydrolases (Luthman and Magnusson, 1983). Thereafter, the transfecting DNA may be incorporated into the genome. However, the mechanism is not completely understood.

3.2.6 Transduction of the T-cell line RF33.70

Transduction of the T cell line RF33.70 was performed as following. A 24-well plate was incubated with 400 µl (12.5 µg/ml) RetroNectin for 2 h at RT. RetroNectin is a recombinant human fibronectin fragment with three functional domains that are able to interact with integrin on target cells and the virus particles. After incubation, the plate was blocked with 2% BSA for 30 min at 37°C, before washed once with 2 ml 1x PBS 25 mM HEPES. Thereafter, 10^5 cells (in 1 ml) of RF33.70 cell line and 1 ml filtered viral supernatant of Plat-E cells (through 0.45 µm filter) together with 4 µg/ml protamine sulfate and 1 % HEPES were added to each well and the plate was centrifuged at 32°C for 1.5 hours prior to incubation overnight at 37°C, 5% CO2. One day after, cells were pelleted, replated with 3 ml medium on a 6-well plate and incubated for 3 days. On the 8^{th} day of experiment, transduction efficiency was analyzed via FACS.

3.2.7 Transduction of primary T cell line

Transduction of primary T cells was done similarly to RF33.70 cell line. One day prior to transduction, spleen was isolated and single cell suspension was performed as described in 3.4.3.1. 2x 10^6 cells/ml were stimulated with 10 IU/ml recombinant IL-2, 1 µg/ml anti-mouse CD3 and 0.1 µg/ml anti-mouse CD28 overnight in a 6-well plate (4 ml/well).

On the transduction day, a 24-well plate was incubated with 400 µl (12.5 µg/ml) RetroNectin for 2 h at RT. After incubation, the plate was blocked with 2% BSA for 30 min at 37°C, before washed once with 2 ml 1x PBS 25 mM HEPES. Filtered viral

supernatant (1 ml) was added to the well and centrifuged at 2700 g, 4°C for 1.5 h. Thereafter, viral supernatant was discarded and 1x 10^6 cells (in 1 ml medium containing 10 IU/ml IL-2 and 4x10^5 Dynabeads® mouse T-activator CD3/CD28) were added to each well. The plate was centrifuged at 800g, 32°C for 30 min prior to incubation overnight at 37°C, 5% CO2. One day after, cells were again transduced with 1 ml filtered viral supernatant per well at 800g, 90 min, 32°C. After 6 hours incubation at 37°C, 5°C CO_2, 1 ml of supernatant was replaced with 1 ml fresh medium (+ 10 IU/ml IL-2).

One day after, cells were pelleted, replated with 2 ml medium containing 50 ng/ml recombinant IL-15 (1x 10^6 cells/ml) on a 12-well plate and incubated for two days. On the 8th day of experiment, transduction efficiency was analyzed via FACS.

3.3 Immunological methods

3.3.1 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is an antibody-based method to detect antigen in a sample. To detect cytokines one can utilize the so-called sandwich ELISA where two antibodies recognize the same antigen but two different epitopes. The method functions as following: the first antibody (capture antibody) is immobilized on a plate surface followed by blocking non-specific binding sites on surface before adding the samples. To detect the antigen, a biotinylated second antibody (detecting antibody) and subsequently a streptavidin-horseradish peroxidase conjugate are added to the antigen that is bound to the capture antibody. In presence of hydrogen peroxide, an oxidative reaction of the substrate 3,3',5,5' – tetramethylbenzidine (TMB) catalyzed by the enzyme horseradish peroxidase leads to blue color chromogen. This enzymatic reaction can be halted by adding sulfuric acid that causes a color change of the blue chromogen into yellow, which can be read on a spectrometer at 450 nm.

3.3.1.1 Measurement of IL-6

For the measurement of IL-6 concentration, samples were diluted 1:10 and the detection was performed according to the protocol from BD OptEIA™ (mouse IL-6

ELISA set) with some modifications. Briefly, a 96-well plate was coated with 50 µl/well diluted capture antibody (1:250, in coating buffer pH 9.5) and incubated overnight. After three times washing (150-200 µl ELISA wash buffer), unspecific binding sites on the plates were blocked with 150 µl/well assay diluent for 1 h at RT. After three times washing, 50 µl of the standards (highest was 1000 pg/ml) and samples were added into the wells and incubated for 2 h at RT. The plate was then washed five times and 50 µl of diluted detection antibody (1:500) and streptavidin-conjugated horseradish peroxidase (1:250) were added to each well for 1 h at RT. Subsequently, the plate was washed five times and 50 µl/well of substrate solution (two solutions, mixed in 1:1 ratio) was added to each well and incubated for 30 min at RT in the dark. The reaction was stopped by addition of 25 µl of 2N H_2SO_4. All measurements were read at 450 nm with a wavelength correction substraction at 590 nm.

3.3.1.2 Measurement of IFN-α

For the detection of IFN-α, plates were coated overnight at 4°C with 50 µl/well capture antibody in coating buffer (1 µg/ml). To block unspecific binding sites on the plates, plates were blocked with 150 µl/well assay diluent for 3 h at RT. After washing the plates for three times undiluted samples and standard (highest standard was 10^5 IU/ml) were applied on the plates and incubated at 4°C for overnight. After washing steps, 50 µl of detection antibody (625 ng/ml) was added for 3 h at RT. Subsequently, after washing the plates ten times, horseradish peroxidase (HRP)-conjugated F(ab')2 fragments (15 µg/ml) were added to the well and incubated for 3 h at RT. Afterwards, 50 µl/well substrate solution was added for approximately 30 min at RT in the dark. The reaction was halted by adding 25 µl 2N H_2SO_4. All measurements were read at 450 nm with a wavelength correction substraction at 590 nm.

3.3.2 Flow cytometry

Flow cytometry, also referred as "fluorescence-activated cell sorting" (FACS), is a method commonly used to measure and analyze physical characteristics of a single

particles such as cells, microorganisms and DNA. The measured characteristics can be the relative size (up to 0.2 to 150 μm), relative granularity or fluorescence intensity of a particle. A flow cytometer is built on three main systems: fluidics, optics and electronics. The fluidics system performs the transport of the particles in a stream to the laser (interrogation point). The optics system consists of 1) a laser, which illuminates the particles at the interrogation point and 2) optical filters that direct light signals to appropriate detectors. There are two types of light scattering: 1) the forward-scattered light (FSC), which is proportional to the size of a particle and detected on the axis of the laser beam in the forward direction by a photodiode, and 2) the side-scattered light (SSC), which reflects the cell granularity or internal complexity and is collected at an angle of 90°C by collection lens. It is therefore possible for example to distinguish subpopulations of leukocytes. However, to distinguish more defined subpopulations (e.g. $CD4^+$ and $CD8^+$ T cells), fluorochrome-conjugated antibodies are powerful tools that bind to specific antigens (e.g. CD4 and CD8) on target cells. The fluorochromes can be excited by laser beams and they emit light which then is passed through different selective optical filters (e.g. bandpass filter) and detected by different detectors (e.g. photomultiplier tubes, PMT). The last system of a flow cytometry is the electronics system. This system is tasked to convert light signals into electronic signals that can be processed by a computer.

In this thesis, experiments related to the revealing of IFN-α effect on $CD8^+$ T cells were conducted with BD FACSCanto™ II. The machine is equipped with three different lasers (405-, 488-, 633-nm), which can excite different fluorochromes, and three detector arrays that detect emitted lights. Experiments related to transfection and transduction were perfomed with a MACSQuant Analyzer (MACS Miltenyi Biotec) that also possesses three different lasers.

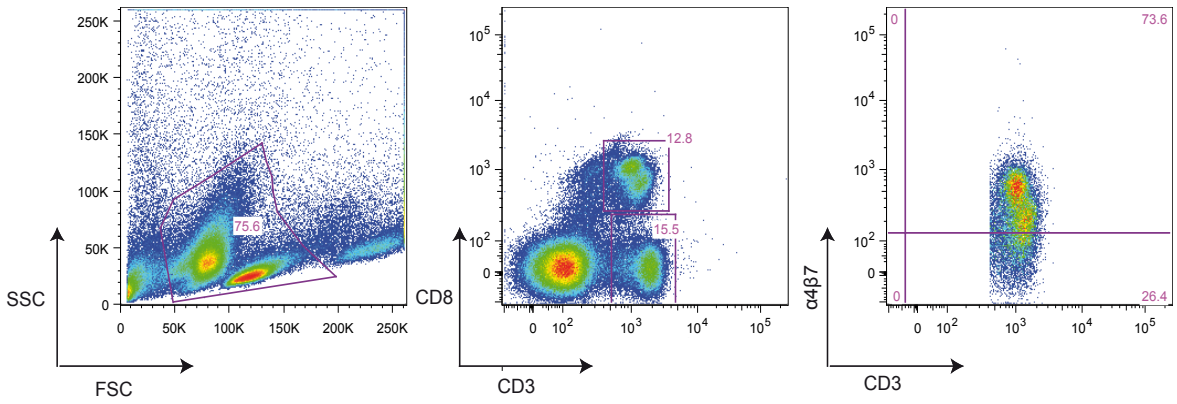

Figure 3.2: Lymphocyte gating strategy. Lymphocytes were gated based on their size and granularity in FSC-SSC-plot (left panel). In the next plot cells were gated based on their CD3 and CD8 expression (middle panel). $CD3^+$ $CD8^+$ cells were analyzed for $\alpha_4\beta_7$ expression (right panel). The value of median fluorescence intensity (MFI) was utilized to measure the shift in fluorescence intensity in the treated cell populations.

3.3.2.1 Analysis of cell surface expression molecules

To analyze the expression of cell surface antigens, cell suspensions ($2x10^5$ – $2x10^6$/200 µl in 1x PBS) were stained with fluorochrome-conjugated monoclonal antibodies (0.5 µl/antibody) against target antigens for 30 min at 4°C. Subsequently, the cells were washed by adding 2-3 ml 1x PBS the cells and centrifuged at 400g, 4°C for 5-7 min. Afterwards, this washing step was conducted one more time prior to flow cytometry acquisition. Data analysis was performed utilizing the software FlowJo.

3.3.2.2 Analysis of intracellular integrins

To analyze the percentage of produced integrins in the cytosol, intracellular staining was performed as following:

200 µl of each cultured cells in a well (Plat-E, RF33.70 or primary T cells) were transferred to 96-well plate. After centrifugation (2200 rpm, 2 min, RT), medium was discarded and cells were gently vortexed. For fixation, cells were incubated with 100 µl of 1x BD FACS™ lysing solution (diluted in ddH_2O) for 8 min at RT. Thereafter, cells were washed with 100 µl 1x saponin (diluted in sterile PBS) and centrifuged as above. Cells were again washed and permeabilized with 150 µl 1x saponin. Subsequently, cells were centrifuged before adding 50 µl antibody-mix

against α_4, β_1 and β_7 (1:400, diluted in 1x saponin). After 20 min incubation at 4°C, cells were washed with 150 µl 1x saponin and centrifuged. Afterwards, cells were washed with 150 µl 1x PBS. For measurement, cells were resuspended in 150 µl 1x PBS.

The permeabilization of the cell membrane by saponin is due to the reversible complexation of saponin with chloresterol in the cell membrane allowing forming of pores. It is therefore important to 1) fixate the cells, to avoid leakage of cytosolic proteins out of the cells and 2) add saponin to all steps (except the last washing steps).

3.3.2.3 CFSE and eFluor® 670 staining

To track the *ex vivo* IFN-α-stimulated splenocytes in the mice, we performed a CFSE and eFluor® 670 staining. CFSE, or carboxyfluorescein diacetate succinimidyl ester, is a molecule that can diffuse passively into cells and is primarly colorless and non-fluorescent. When intracellular esterases start to cleave the acetate group, the molecule becomes highly fluorescent and reactive and binds to intracellular amine. This binding is irreversible and causes the stable retaining of the fluorescent conjugates. Excess unconjugated products and by-products passively diffuse back into the medium. The fact that the fluorescent conjugates can be inherited by daughter cells and they can not be transferred to adjacent cells, makes CFSE to a powerful method for tracing cell proliferation *in vivo*. CFSE is excited by a 488-nm laser and emit light at 517 nm. Similar to CFSE, the cell proliferation dye eFluor® 670 (eBioscience) can also be used for tracing cells in vivo. This dye is excited with a 633-nm laser and its emission peak is at 670 nm.

5 µl of 5 mM CFSE was diluted with 45 µl 1x PBS to obtain a concentration of 0.5 mM. Cells (max. 1 x 10^8 cells) were resuspended in 1 ml 1x PBS and labeled with 25 µl of 0.5 mM diluted CFSE (end concentration 12.5 µM). The cells were then incubated at 37°C (in incubator) for 15 min and washed twice with 49 ml 1x PBS prior to injection into mice.

For the staining with eFluor® 670, cells were resuspended to a concentration of 20 x 10^6 /ml. eFluor® 670 (10 µM) was mixed in 1x PBS into the same volume as cell suspension and added to the cell suspension (final concentration 5 µM). The cells

were then incubated for 10 min at 37°C (in incubator). To stop the reaction, 5 ml of 1x PBS was added and the cells were incubated for 5 min on ice. PBS was added to the cells and the cells were centrifuged at 400g for 7 min. This washing step was repeated one more time.

3.4 Animal experimentation

3.4.1 Animals

Female C57BL/6 mice (8-12 weeks old) were obtained from Harlan-Winkelmann (Borchen, Germany). IFN-I receptor (IFNAR)-deficient mice on C57BL/6 background were kindly provided by Dr. Z. Waibler (Paul-Ehrlich Institute, Langen, Germany). All mice were anesthetized with isofluorane when transferring splenocytes retroorbitally. Animal experiments were approved by the local regulatory agency (Regierung von Oberbayern, Munich, Germany).

3.4.2 *In vivo* stimulation

3.4.2.1 In vivo stimulation with poly I:C

To investigate the effect of poly I:C on $\alpha_4\beta_7$ expression *in vivo*, five mice were sequentially injected with 250 μg poly I:C (Invitrogen) 120 h and 48 h prior to organ isolation.

3.4.2.2 In vivo stimulation with recombinant mouse IFN-α

To examine the function of IFN-α in vivo, three mice were injected for three days with 10 μg recombinant mouse IFN-α (kindly provided by Dr. Martin Schlapschy, from the chair of Biological Chemistry at the Technical University of Munich). On the fourth day secondary lymphoid organs were isolated.

3.4.3 Organ preparation

3.4.3.1 Spleen cell isolation

Mice were killed by cervical dislocation under anesthesia and the spleens were than passed through a 40 μm cell strainer to obtain single cell suspensions. Afterwards, cell suspensions were centrifuged for 400g, 7 min and erythrocytes were lysed with 1 ml 1x red blood lysis buffer for 1-2 minutes. To stop the lysis reaction, cell suspensions were diluted with 1x PBS (falcon filled to 50 ml). After centrifugation, cells were resuspended in 10 ml 1x PBS, counted with Neubauer hemocytometer and either kept on ice or used immediately for cell culture experiments or FACS staining.

3.4.3.2 Isolation of peripheral and mesenteric lymph nodes

Mice were killed by cervical dislocation under anesthesia. Peripheral (inguinal) lymph nodes were harvested and passed through a 40 μm cell strainer. After centrifugation (400g, 7 min) cells were resuspended in 1x PBS and either kept on ice or immediately used for *in vitro* cell culture experiments.

3.4.3.3 Isolation of mesenteric lymph nodes

Mice were killed by cervical dislocation under anesthesia. Mesenteric lymph nodes were harvested and passed through a 40 μm cell strainer. After centrifugation (400g, 7 min) remaining erythrocytes were lysed in 1x red blood lysis buffer. To stop the reaction, 1x PBS were added to dilute the lysis buffer. After centrifugation (400g, 7 min) the cells were resuspended in 1x PBS and either kept on ice or immediately used for *in vitro* cell culture experiments.

3.4.3.4 Isolation of Peyer's patches

Mice were killed by cervical dislocation under anesthesia. Peyer's patches along the small intestine were collected and passed through a 40 μm cell strainer. After centrifugation (400g, 7 min) cells were resuspended in 1x PBS and either kept on ice or immediately used for *in vitro* cell culture experiments.

3.4.4 Lymphocyte *in vivo* migration assay

To assess how the migration of splenocytes is affected by IFN-α treatment, splenocytes were incubated for 48 h with IFN-α (10^3 U/ml). After 48 h, untreated and IFN-α-stimulated splenocytes were labeled with eFluor® 670 and CFSE, respectively, (section 3.3.2.3) and mixed in a 1:1 ratio (each group 1-1.5 x 10^7 cells /75 µl). 150 µl of the mixed population were injected retro-orbitally into mice under anesthesia. An aliquot was saved as input ratio for the homing index. 18-24 h post injection, secondary lymphoid organs (spleen, peripheral and mesenteric lymph nodes and Peyer's patches) were harvested and single cell suspensions were obtained as described in earlier sections. The percentage of transferred splenocytes was examined by flow cytometry and the ratio of IFN-α-stimulated splenocytes (CFSE-labeled) to untreated splenocytes (eFluor® 670-labeled) in each organ was calculated.

For the calculation of the homing index, the ratio from each organ was divided by the ratio of the inputs:

$$\text{Homing index} = \frac{[(\text{IFN-}\alpha\text{-stimulated splenocytes})_{organ} / (\text{untreated splenocytes})_{organ})]}{[(\text{IFN-}\alpha\text{-stimulated splenocytes})_{input} / (\text{untreated splenocytes})_{input})]}$$

3.5 Molecular biology

3.5.1 Primer design

To amplify the gene of the integrins, primers for the specific gene were designed by using following criteria:

1. For the forward primer, the initial sequence at 5'-end (including bases encoding start codon) was selected
2. The length should be at least 20-22 nucleotides
3. The 3'-end should terminate with several guanosins (G) or cytidins (C) for efficient priming
4. Addition of restriction site for EcoRI and NotI

5. Addition of overhangs on 5'-end specific for EcoRI or NotI to allow efficient cut of PCR product. The list of overhangs for specific restriction enzymes can be found in New England BioLabs Inc. (http://www.neb.com/nebecomm/tech_reference/restriction_enzymes/cleavage_olignucleotides_old.asp#.UEC_hFShOOf)
6. For the reverse primer, last sequence in 3'-end (including bases encoding stop codon) was selected and reversed into its complementary sequence by utilizing a reverse complement tool: http://bioinformatics.org/sms/rev_comp.html.

3.5.2 Polymerase chain reaction

To amplify the gene, the polymerase chain reaction (PCR) was performed. Briefly, template double-stranded DNA is separated using high temperature (denaturation step). After DNA melting, two short oligonucleotides (primers) anneal to the the specific site at 5' and 3' end (annealing step). Subsequently, DNA polymerase can elongate the primer sequence. The elongated sequence is complementary to the template DNA (elongation step). These steps are repeated several times and an exponential amplification of the gene can be achieved.

After proceeding with the PCR at different temperatures to find the optimal condition, the following program and reaction mix were chosen to amplify the gene of integrins α_4, $\beta_7$and β_1:

Step	Temperature	Time
1	95°C	2 min
2	95°C	30 sec
3	65°C	1 min
4	72°C	4 min
5	repeat step 2 to 4 for 35x	
6	72°C	10 min
7	4°C	pause

Reagents	**1x (50 µl)**
10x *Pfu* buffer + $MgSO_4$	5 µl
dNTPs 2 mM	5 µl
Primer forward (ITGA4/ITGB1/ITGB7) (10 pmol/µl)	2.5 µl
Primer reverse (ITGA4/ITGB1/ITGB7) (10 pmol/µl)	2.5 µl
Template (cDNAs)	≤ 1 µg
Pfu polymerase (2.5 u/µl)	0.5 µl
ddH_2O	Add to 50 µl

3.5.3 Gelelectrophoresis

To analyze PCR results, PCR products were loaded on 1% agarose gel. Briefly, 2 g agarose were dissolved in 200 ml 1x TAE by boiling for 4-5 min and poured into a chamber with addition of 10 µl ethidium bromide (stock concentration: 10 mg/ml). To 50 µl PCR product, 8.3 µl 6x loading dye was added and the geleletrophoresis was performed for 45 min at 100 volts.

3.5.4 Digestion

PCR products of the genes were cut and purified with Wizard® SV Gel and PCR Clean-Up System (Promega) as decribed an their protocol and eluted in 50 µl ddH_2O. After DNA measurement (~ 15-20 ng/µl), PCR products were digested as followed:

Digestion mix (50 µl) for PCR products:

43 µl PCR product
5 µl 10x Buffer Orange (O)
1 µl EcoRI (10 u/µl)
1 µl NotI (10 u/µl)

The digestion with both enzymes leads to formation of sticky ends. Digestion mixes were incubated at 37°C for 2 hours before they were purified again with Wizard® SV Gel and PCR Clean-Up System.

The vector pMP71-GFP was digested with EcoRI and NotI to cut out GFP and generate compatible sticky ends for the PCR products.

Digestion mix (50 µl) for cutting vector:

2 µg pMP71-GFP
2 µl 10x Buffer Orange (O)
1 µl EcoRI (10 u/µl)
1 µl NotI (10 u/µl)
Add ddH_2O to 20 µl

After incubation at 37°C for 2 hours, to separate GFP from the digested pMP71 vector, 1% agarose gel was run as described in chapter 0. To purify the vector, the band in the gel corresponding to the size of the digested vector, was cut and purified using Wizard® SV Gel and PCR Clean-Up System.

3.5.5 Ligation

To ligate the digested PCR product of the genes and linearized pMP71 vector, an amount-ratio of 1:3 and 1:5 (vector:insert) with consideration of size ratio (in kilo base pairs, kbp) was calculated as followed:

Calculation for the gene α4:

1:3 (100 ng vector : 300 ng insert)

pMP71 size : gene α4 size = ~6 kbp : 3 kbp = 2 : 1

Amout of PCR product for gene α4 needed: 300 ng/ 2 = 150 ng

1:5 (100 ng vector : 500 ng insert)

Amout of PCR product for gene α4 needed: 500 ng/ 2 = 250 ng

Same calculation was done for the ligation of the genes β_1 and β_7:

1:3 (100 ng vector : 300 ng insert)

pMP71 size : gene β_1 or β_7 size = ~6 kbp : 2.4 kbp = 2.5 : 1

Amout of PCR product for gene β_1 or β_7 needed: 300 ng/ 2.5 = 120 ng

1:5 (100 ng vector : 500 ng insert)

Amout of PCR product for gene β_1 or β_7 needed: 500 ng/ 2.5 = 200 ng

Ligation mix (25 µl):

Reagents	**1:3**	**1:5**
Insert (PCR products)	150 ng* or 120 ng**	250* ng or 200** ng
Linearized vector pMP71	100 ng	100 ng
10x T4 DNA ligase buffer	2.5 µl	2.5 µl
T4 DNA ligase (5 u/µl)	1 µl	1 µl
ddH_2O	add to 25 µl	add to 25 µl

Table 5: Pipetting scheme for ligation mix. * Amount PCR product needed for ligation of the gene α4. ** PCR product needed for ligation of the genes β_1 or β_7 into the pMP71 vector

Ligation mix was incubated for one hour at RT prior to transformation into heat-shock competent *E.coli* cells.

3.5.6 Transformation

After thawing heat-competent DH5α *E.coli* cells (Invitrogen) on ice, 10 µl of ligation mix were added to the *E.coli* and incubated for two minutes on ice. Subsequently, cells were incubated at 42°C for 1 min (without shaking) before adding 1 ml LB medium (without ampicillin) and shaked at 37°C, 650 rpm for one hour. Thereafter, cells were centrifuged for 1500 rpm, 2 min. After discarding medium, cells were resuspended in remaining medium and plated on LB agar containing ampicillin (stock solution 100 mg/ml, diluted 1:1000) for overnight at 37°C. The vector pMP71 possesses an ampicillin resistance gene, which encodes the ampicillin-degrading enzyme beta-lactamase.

3.5.7 Colony PCR

To check which colonies possess the pMP71 vector containing the integrin genes, some colonies were numbered, picked with a tip and plated on new LB agars containing ampicillin, which were labeled with same numbers. The same tip was dipped in a PCR tube containing the following reaction mix:

Reagents	**1x (30 µl)**
10x *Pfu* buffer + $MgSO_4$	3 µl
dNTPs 2 mM	5 µl
Primer forward (ITGA4/ITGB1/ITGB7) (10 pmol/µl)	2.5 µl
Primer reverse (ITGA4/ITGB1/ITGB7) (10 pmol/µl)	2.5 µl
Pfu polymerase (2.5 u/µl)	0.5 µl
ddH_2O	Add to 30 µl

The same PCR program and analysis of PCR products as in chapter 3.5.2 and 3.5.3 were utilized.

3.5.8 Plasmid purification

E.coli clones, which were positive for the vector containing the integrin genes in colony PCR were grown in 3 ml liquid LB medium with ampicillin (diluted 1:1000, stock concentration 100 mg/ml) overnight (not more than 16 hours to prevent overcrowding of cells thus cell lysis). For long-term storage of the clones, 150 µl of *E.coli* cell suspension were mixed with 850 µl glycerol and freezed at -80°C. Mini plasmid purification was proceeded as described in the protocol of the AxyPrep™ plasmid miniprep kit (chapter 3.1.3).

To obtain a higher quantity of the vector for transfection purpose, maxi plasmid purification was done using JETSTAR Plasmid purification MAXI kit (chapter 3.1.3). Briefly, 1 ml LB medium with ampicillin with one tip of E.coli from agar plate or glycerol stock was cultured for around 6-8 hours. Thereafter, preculture was added into 500 ml LB medium with ampicillin (in a 2L Erlenmeyer flask covered with aluminium foil). The culture was incubated at 37°C, 180 rpm for maximum 16 hours before proceeding with the plasmid purification.

3.5.9 Sequencing

Purified pMP71 vectors containing the integrin genes were sent to sequecing (Eurofins MWG Operon, Ebersberg, Germany). For each sequencing primer, sequencing mix was done as followed:

Sequencing mix

DNA	50 ng/µl
Sequencing primer	2 pmol/µl
ddH_2O	add to 15 µl

3.5.10 Analysis of gene sequence

After obtaining the sequencing results from Eurofins MWG Operon, analysis was perfomed using the software Lasergene® SeqMan Pro™ to verify compliance of the sequence with the cDNA sequence of the integrins obtained in the PubMed database. Alternatively, DNA sequence was translated into protein sequence (http://web.expasy.org/translate/) and standard protein blast at NCBI website was performed.

3.6 Statistical analysis

Utilizing GraphPad Prism5 (GraphPad software) statistical significance was determined by unpaired, one-way analysis of variace (ANOVA) with Bonferroni's multiple comparison test (for more than two groups) or with independent two-tailed student's t-test (for two groups). Significance was set at $P < 0.05$; 0.01; 0.001 and were indicated with an asterix (*, **; ***) respectively. All data are expressed as mean ± SEM. Graphical design was conducted with Adobe Illustrator.

4 Results

4.1 $\alpha_4\beta_7$ Downregulation on CD8$^+$ T cells is a unique event upon poly (I:C) recognition

It has been shown in our laboratory that upon poly (I:C) treatment *in vitro* CD8$^+$ T cells exhibit a marked decreased expression of $\alpha_4\beta_7$ expression. As poly (I:C) is known to trigger the production of type I interferon upon signaling through TLR3, we further assessed the role of type I interferon in poly (I:C)-mediated $\alpha_4\beta_7$ downregulation. To examine this, we utilized splenocytes from IFNAR-1-deficient mice. IFNAR-1-deficient splenocytes lack one of the receptors for type I interferon that leads to dysfunction in transmitting the IFN signal into the cell. We cultured wild-type and IFNAR-1-deficient splenocytes with poly (I:C) 24 h and analyzed the expression of $\alpha_4\beta_7$ and CD69 on CD8$^+$ T cells by flow cytometry. Additionally, incubation of splenocytes with other TLR stimuli such as CpG type B and R848, TLR9 and TLR7 agonists, was also performed as positive controls.

Result of the *in vitro* experiment demonstrated that all TLR ligands are able to induce $\alpha_4\beta_7$ downregulation on CD8$^+$ T cells in splenocyte culture (Figure 4.1, left panel). In contrast, in IFNAR-1-deficient splenocytes this downregulation was markedly reduced upon stimulation with poly (I:C) and R848 (Figure 4.1, shaded area in the left panel). Furthermore, CpG impaired $\alpha_4\beta_7$ expression CD8$^+$ T cells in IFNAR-1-deficient splenocytes similarly as in the wild-type splenocytes. CpG type B has been shown to poorly induce IFN. Moreover, Heidegger et al. (submitted) has demonstrated IL-6 is essential in CpG-mediated $\alpha_4\beta_7$ downregulation.

As control for proper immune activation in the experiment, splenocytes were also stained for CD69 (Figure 4.1, right panel), which is an early activation marker that is rapidly upregulated in various type of immune cells upon type I IFN-dependent immune activation (Kamphuis et al., 2006). The flow cytometry analysis demonstrated that poly (I:C) and R848 induced a significant upregulation of CD69 on CD8$^+$ T cells in wild-type splenocytes whereas this upregulation was entirely abolished on CD8$^+$ T cells in IFNAR-1-deficient splenocytes, which confirmed the

dysfunction of IFN signaling in these splenocytes. As expected, CD69 on T cells was not upregulated upon stimulation with CpG in wild-type and IFNAR-1-deficient splenocytes. It is however interesting to note that the upregulation of CD69 on T cells was not a prerequisite for the changes in $\alpha_4\beta_7$ expression.

In summary, although decrease of $\alpha_4\beta_7$ on $CD8^+$ T cells in IFNAR-1-deficient splenocytes was not entirely abrogated, this experiment indicates that poly (I:C) and R848-mediated $\alpha_4\beta_7$ downregulation is IFN-dependent.

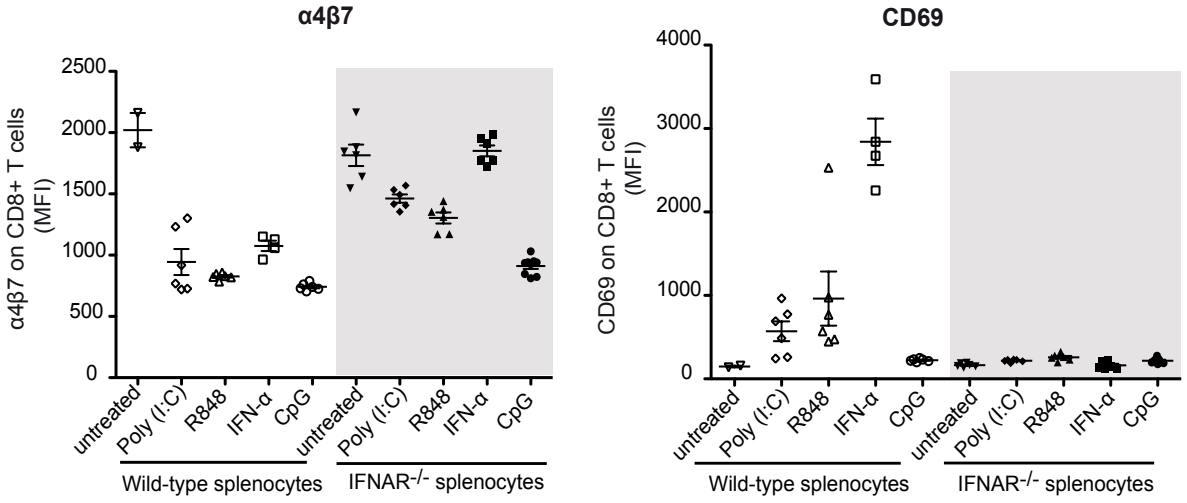

Figure 4.1: $\alpha_4\beta_7$ downregulation following poly (I:C) and R848 *in vitro* stimulation is dependent on IFN-α (left panel). Splenocytes from wild-type (C57BL/6) and IFNAR-1-deficient mice were treated for 24 h with different TLR ligands. The expression of the early activation marker CD69 (right panel) and $\alpha_4\beta_7$ were measured on $CD8^+$ T cells by flow cytometry.

4.1.1 Recombinant IFN-α modulates $\alpha_4\beta_7$ expression in a dose-dependent manner

To further evaluate the potential of type I IFN to modulate surface expression of $\alpha_4\beta_7$, we next stimulated splenocytes for 24 h with different concentrations of recombinant mouse-IFN-α and analyzed subsequently $\alpha_4\beta_7$ expression on $CD8^+$ T cells by flow cytometry.

Flow cytometry analysis of $CD8^+$ T cells displayed an IFN-α-dose-dependent downregulation of $\alpha_4\beta_7$ expression on $CD8^+$ T cells in splenocyte culture. A marked decrease of $\alpha_4\beta_7$ expression emerged at a dose of 10^2 U/ml. This decrease reached

its peak at 10^3 U/ml (Figure 4.2, panel C). At this dose, the $\alpha_4\beta_7$ expression level on $CD8^+$ T cells has declined approximately 50% in comparison with the untreated splenocytes. Interestingly, a higher concentration than 10^3 U/ml did not lead to a definite further downregulation of $\alpha_4\beta_7$ expression indicating a maximum signaling at 10^3 U/ml. Moreover, higher IFN-α concentration correlated with a higher CD69 expression on $CD8^+$ T cells with the highest MFI at 10^5 U/ml (data not shown).

We next performed a time-course experiment to determine whether longer incubation of splenocytes with recombinant mouse-IFN-α leads to more impairment of $\alpha_4\beta_7$ expression. Splenocytes were cultured with IFN-α (10^3 U/ml) for 24 h and 48 h prior to flow cytometry analysis.

The result of the experiment demonstrated that longer incubation time than 24 h indeed induced further downmodulation of $\alpha_4\beta_7$ expression on $CD8^+$ T cells (Figure 4.2, panel D). However, it seemed that the untreated $CD8^+$ T cells lost their $\alpha_4\beta_7$ expression after 48 h. To conclude, we discovered that recombinant IFN-α, when cultured on splenocytes, is able to downregulate $\alpha_4\beta_7$ expression on $CD8^+$ T cells in a dose-and time-dependent manner.

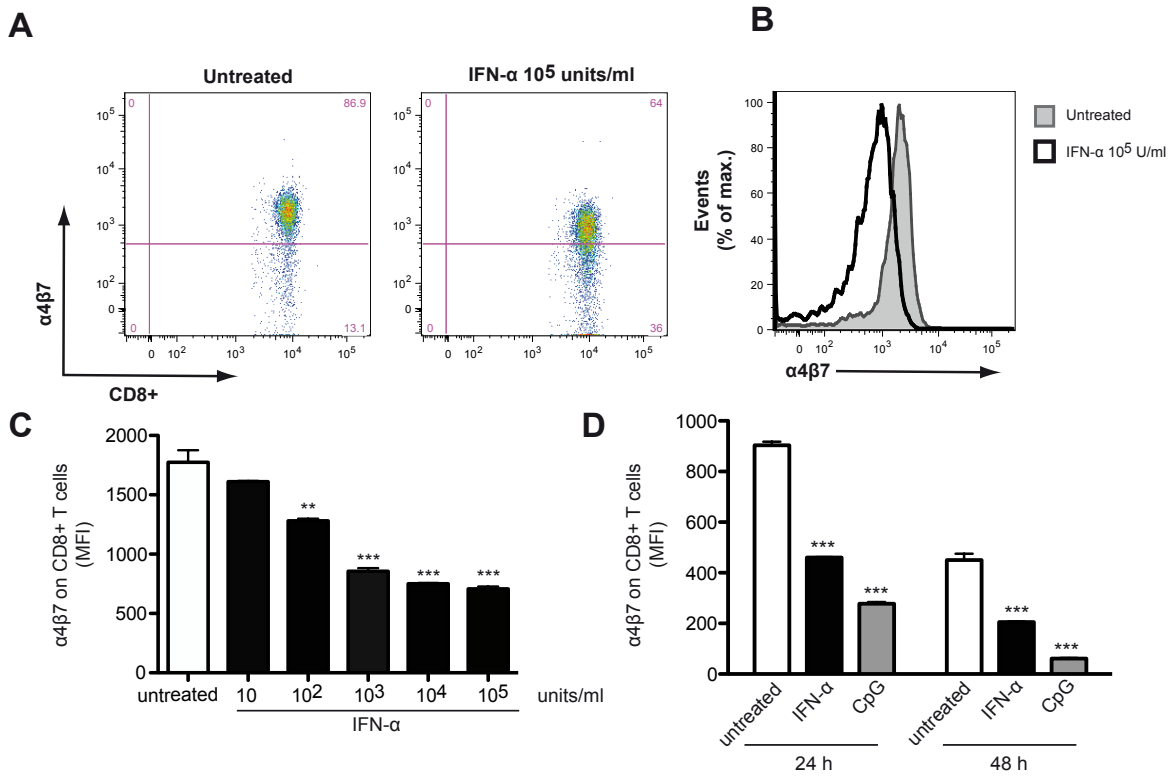

Figure 4.2: Recombinant IFN-α decreases $\alpha_4\beta_7$ expression on CD8+ T cells in a dose-dependent manner. Splenocytes were incubated with different concentrations of recombinant mouse IFN-α for 24 h and analyzed by flow cytometry. Expression of $\alpha_4\beta_7$ after IFN-α treatment (A, B) was dose-dependent (C). A time-course experiment showed a downregulation of $\alpha_4\beta_7$ expression after 24 h and 48 h (D). CpG, a TLR9 agonist, was used as positive control for $\alpha_4\beta_7$ downregulation.

4.1.2 The cytokines IL-12, IL-1β and IL-6 do not synergize with IFN-α in regulation of $\alpha_4\beta_7$ expression

The next question to be examined was whether other cytokines contribute or synergize with IFN-α in controlling $\alpha_4\beta_7$ expression. To examine this, splenocytes were co-cultured with the cytokines IL-1β, IL-12 and IL-6 in presence or absence of IFN-α. Additionally, CpG was used as positive control in the experiment.

In the absence of IFN-α the incubation of the splenocytes with the cytokines IL-1β, IL-12, IL-6 did not alter $\alpha_4\beta_7$ expression (Figure 4.3). However, this stood in contrast to previous *in vitro* studies in our laboratory that pointed out the role of IL-6 in CpG-mediated $\alpha_4\beta_7$ downregulation.

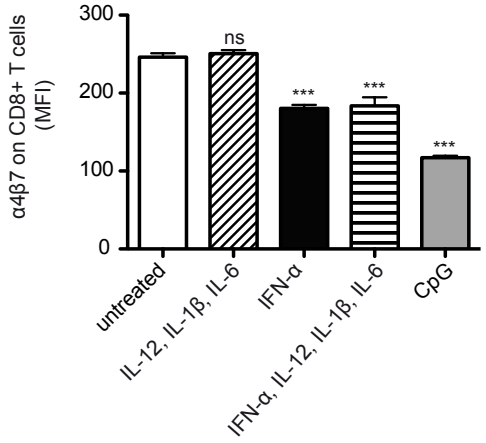

Figure 4.3: The cytokines IL-12, IL-1β and IL-6 do not alter $\alpha_4\beta_7$ expression. Splenocytes were incubated for 24 h with IFN-α or with a combination of proinflammatory cytokines (100 ng/ml). CpG, a TLR9 agonist, was used as positive control for $\alpha_4\beta_7$ downregulation.

4.1.3 Decrease of $\alpha_4\beta_7$ expression on CD8$^+$ T cells upon IFN-α stimulation is most pronounced among other cell adhesion molecules

Previous experiments in the last sections have revealed that $\alpha_4\beta_7$ expression is impaired upon stimulation by IFN-α. In the next study, we wanted to characterize further whether the downmodulation of $\alpha_4\beta_7$ expression is a unique event among other cell adhesion molecules upon immune activation by IFN-α. We examined several cell adhesion molecules that are known to be expressed on CD8$^+$ T cells.

CD62L or L-selectin is an important cell adhesion molecule for lymphocyte homing into the peripheral lymph nodes that is highly expressed on naïve T and B cells. LFA-1, also known as $\alpha_L\beta_2$, is an integrin that has been shown to be essential in CD8$^+$ T cell activation (Li et al., 2009). CD31 is a glycoprotein expressed on many types of cells including naïve CD8$^+$ T cells that induces integrin-mediated adhesion. CCR7 is a chemokine receptor that is expressed on naïve as well as on central memory but not effector memory T cells and it has been described as an important molecule to migrate into the lymph nodes and into the splenic white pulp region (Forster et al., 1999). CCR9 is a chemokine receptor important for the homing to the small intestine and is expressed mainly on naïve CD8$^+$ T cells and gut-homing T cells, but barely on naïve CD4$^+$ T cells (Carramolino et al., 2001). CD44 is a glycoprotein that is highly expressed on memory T cells and at a low level on naïve T cells. CD29, a β_1 integrin, can pair with α_4 and plays a role in the migration of T cells

into the bone marrow. Cutaneous lymphocyte-associated antigen (CLA) is a glycoprotein expressed preferentially in skin-homing memory T cells.

We cultured splenocytes with IFN-α for 24 h and performed subsequently flow cytometry analysis of the expression of the cell adhesion molecules CD62L, LFA-1, CD31, CCR7, CCR9, CD44, CLA, CD29 on $CD8^+$ T cells.

Whereas no changes in expression of CCR7, CD44, CLA could be observed, flow cytometry analysis showed that CD62L expression is slightly decreased upon activation by IFN-α (Figure 4.4). This finding corresponds with the result of Hamman A. et al (2000). They reported a rapid downregulation of CD62L upon activation by anti-CD3 followed by a decreased migration into the lymph nodes. A very modest decrease in CD29 expression and slight increase in CD31 and LFA-1 on $CD8^+$ T cells were observed. However, the most pronounced alteration in surface expression was measured for $\alpha_4\beta_7$ expression. Interestingly, CCR9, which is a chemokine receptor that is important for the migration into the small intestine, especially to duodenum, exhibited no alteration in its expression after stimulation with IFN-α. This experiment indicated that the downregulation of $\alpha_4\beta_7$ expression on naïve $CD8^+$ T cells upon IFN-α stimulation is a unique event among cell adhesion molecules expressed on T cells.

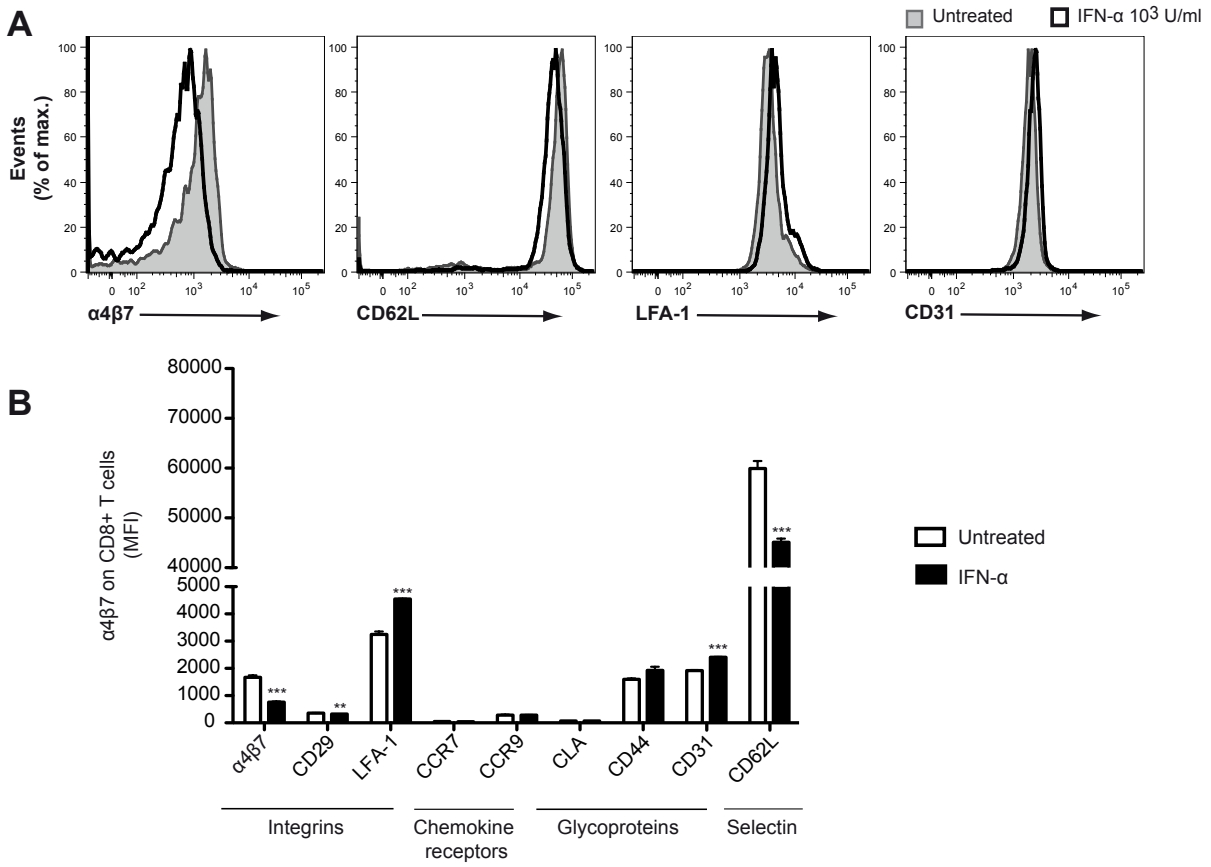

Figure 4.4: No decrease in expression of other cell adhesion molecules on CD8$^+$ T cells after IFN-α treatment. Splenocytes were incubated with IFN-α for 24 h prior to analysis by flow cytometry for $\alpha_4\beta_7$, CCR9, CD44, CD29, CD62L, CCR7, CD31, LFA-1 and CLA expression on CD8$^+$ T cells. Histograms of several examined cell adhesion molecules with expression changes after IFN-α treatment are shown in panel A (grey solid area is the untreated splenocytes and black line is the IFN-α treated splenocytes). Comparison of all analyzed cell adhesion molecules is depicted in panel B.

4.1.4 IFN-α induces $\alpha_4\beta_7$ downregulation on purified T cells

We next investigated whether the downregulation is a direct effect or mediated by different cell types. CD3$^+$ cells were sorted with a negative isolation kit and cultured with IFN-α for 24 h prior to flow cytometry analysis. As a positive control, we also cultured whole splenocytes with IFN-α and CpG for 24 h. Previous experiments in our laboratory showed that CpG was able to induce downregulation of $\alpha_4\beta_7$ expression on CD8$^+$ T cells in splenocyte culture, however it failed to do so in a culture of purified T cells. This *in vitro* finding suggested that naïve T cells lack

functional TLR9, despite the report by Cottalorda A. et al. (2006) that mRNAs of TLR9 can be detected in naïve T cells.

Confirming previous experiments, flow cytometry analysis showed that CpG led to a decrease of $\alpha_4\beta_7$ expression on $CD8^+$ T cells in splenocyte culture (Figure 4.5), whereas this effect was totally abrogated in purified T cells. In contrast, in the presence of IFN-α, $\alpha_4\beta_7$ expression on purified $CD8^+$ T cells remained as low as on $CD8^+$ T cells in splenocyte culture. Hence, IFN-α could act directly on T cells to trigger $\alpha_4\beta_7$ downregulation and this could take place without participation of other cell populations.

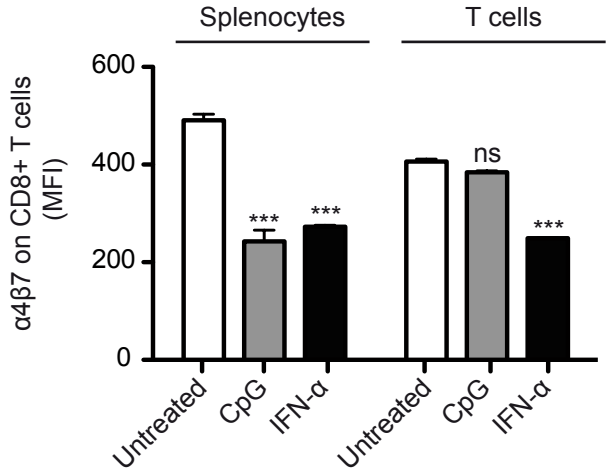

Figure 4.5: IFN-α downregulates $\alpha_4\beta_7$ on $CD8^+$ T cells in a direct manner. Splenocytes and purified T cells were incubated with IFN-α (10^3 U/ml) for 24 h prior to analysis by flow cytometry for $\alpha_4\beta_7$ expression on $CD8^+$ T cells. The percentage of T cell purity is 92.7% (gated on $CD3^+$).

4.1.5 Poly (I:C) decreases $\alpha_4\beta_7$ expression on $CD8^+$ T cells *in vivo*

To investigate the role of poly (I:C) *in vivo* in modulating $\alpha_4\beta_7$ expression, we treated mice two times intraperitoneally with poly (I:C) on the first and third day of the experiment. On the fifth day secondary lymphoid organs such as spleen, peripheral and mesenteric lymph nodes, were isolated and cells were stained with fluorescence-coupled antibodies against CD3, CD8, and $\alpha_4\beta_7$.

We observed that untreated $CD8^+$ T cells in mesenteric lymph nodes displayed a higher $\alpha_4\beta_7$ expression compared with $CD8^+$ T cells in peripheral lymph nodes and spleen. Moreover, in mice treated with poly (I:C) $\alpha_4\beta_7$ expression on $CD8^+$ T cells was significantly diminished in spleen, as well as in peripheral and mesenteric lymph nodes (Figure 4.6). This supported previous *in vitro* findings in our laboratory. This downregulation could also be seen in $CD4^+$ T cells (data not shown).

To summarize, our experiment showed that poly (I:C) recognition *in vivo* also led to $\alpha_4\beta_7$ downregulation on naïve T cells.

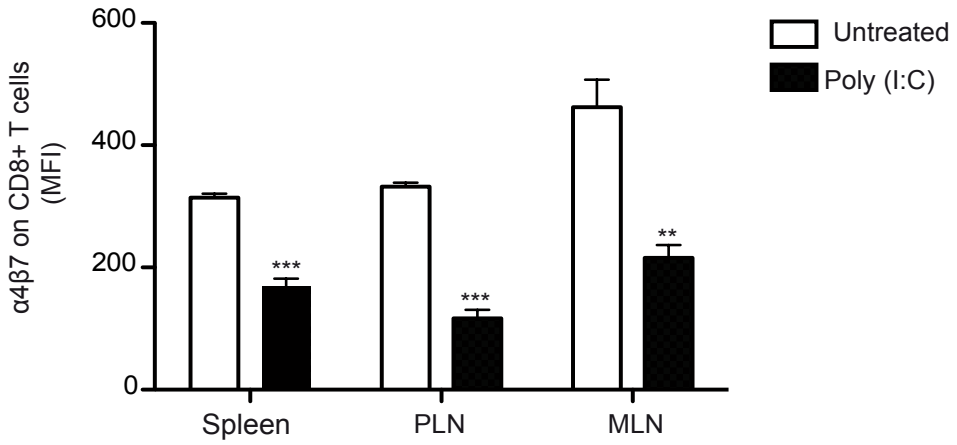

Figure 4.6: Poly (I:C) decreases $\alpha_4\beta_7$ expression on $CD8^+$ T cells *in vivo*. Mice (n=5/group) were treated intraperitoneally with poly (I:C) (2x 230 μg; 5 days and 2 days prior to isolation). Spleen, peripheral (PLN) and mesenteric lymph nodes (MLN) were analyzed by flow cytometry for $\alpha_4\beta_7$ expression on $CD8^+$ T cells.

Our *in vitro* finding with the IFNAR-1-deficient splenocytes encouraged us to further explore the role of type I interferon in modulating $\alpha_4\beta_7$ expression on $CD8^+$ T cells *in vivo*. To investigate this, wild-type and IFNAR-1-deficient mice were treated intraperitoneally with poly (I:C) on the first and third day of the experiment. On the fifth day, spleen, peripheral lymph nodes, mesenteric lymph nodes and Peyer's patches were isolated and we subsequently analyzed $\alpha_4\beta_7$ expression on $CD8^+$ T cells via flow cytometry.

Consistent to the result in Figure 4.6, we observed a nearly two-fold decrease of $\alpha_4\beta_7$ expression on $CD8^+$ T cells in peripheral and mesenteric lymph nodes of the

wild-type mice upon treatment with poly (I:C) (Figure 4.7). Though less pronounced, we also noticed a marked decrease of $\alpha_4\beta_7$ expression on $CD8^+$ T cells in the Peyer's patches of the wild-type mice. By contrast, $\alpha_4\beta_7$ expression on $CD8^+$ T cells was not altered in the secondary lymphoid organs of the IFNAR-1-deficient mice treated with poly (I:C) compared with untreated IFNAR-1-deficient and wild-type mice. This stood somewhat in contrast to the previous *in vitro* experiment (Figure 4.1). Hence, this experiment demonstrated that *in vivo* the impaired $\alpha_4\beta_7$ surface expression appeared to be totally dependent on IFN-signaling, whereas *in vitro* other signaling pathways might contribute to $\alpha_4\beta_7$ regulation.

To summarize, our *in vivo* experiment revealed that upon poly (I:C) injection, $\alpha_4\beta_7$ expression on naïve $CD8^+$ T cells is downreregulated and this absolutely requires an intact type I IFN signaling.

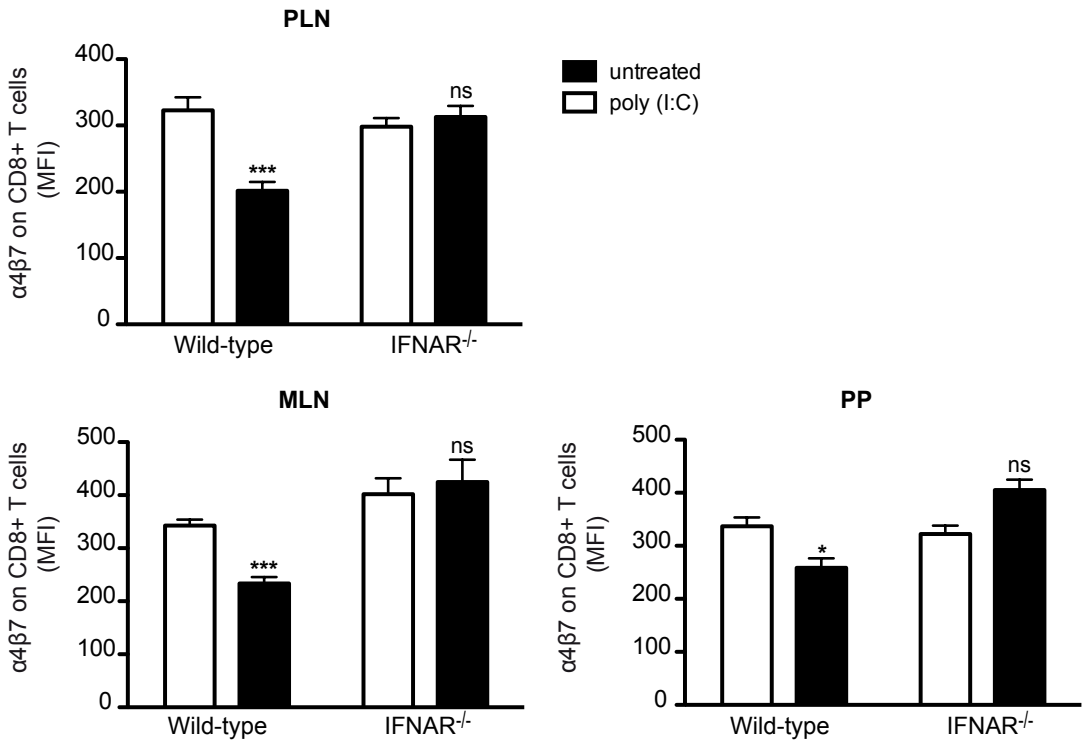

Figure 4.7: $\alpha_4\beta_7$ downregulation is abolished in IFNAR-1-deficient mice following poly (I:C) injection. Mice (n=5/group) were treated intraperitoneally with poly (I:C) (2x 230 μg; 5 days and 2 days prior to isolation). Spleen, peripheral lymph nodes (PLN), mesenteric lymph nodes (MLN) and Peyer's patches (PP) were analyzed by flow cytometry for $\alpha_4\beta_7$ expression on $CD8^+$ T cells.

4.1.6 $\alpha_4\beta_7$ expression on CD8$^+$ T cells is downregulated in mice treated with IFN-α

To confirm the conducted *in vitro* studies and the *in vivo* role of IFN-α in poly (I:C)-mediated $\alpha_4\beta_7$ expression, we next investigated whether IFN-α impairs $\alpha_4\beta_7$ expression *in vivo* as was seen with poly (I:C) (Figure 4.6, Figure 4.7). We challenged mice for three days with 10 μg recombinant IFN-α intraperitoneally and analyzed $\alpha_4\beta_7$ expression on CD8$^+$ T cells on the fourth day.

Flow cytometry analysis demonstrated that in all examined secondary lymphoid organs of mice treated with IFN-α, $\alpha_4\beta_7$ expression levels on CD8$^+$ T cells decreased at least two-fold compared to untreated mice (Figure 4.8, left panel). As a positive control of immune activation, CD69 expression on CD8$^+$ T cells was analyzed (Figure 4.8, right panel). IFN-α induced a significant upregulation of CD69 on CD8$^+$ T cells in spleen, mesenteric and peripheral lymph nodes, confirming that the recombinant IFN-α was functional and the application route was sufficient to induce immune activation. Interestingly, untreated splenic CD8$^+$ T cells had already a higher CD69 expression than CD8$^+$ T cells in the peripheral and mesenteric lymph nodes.

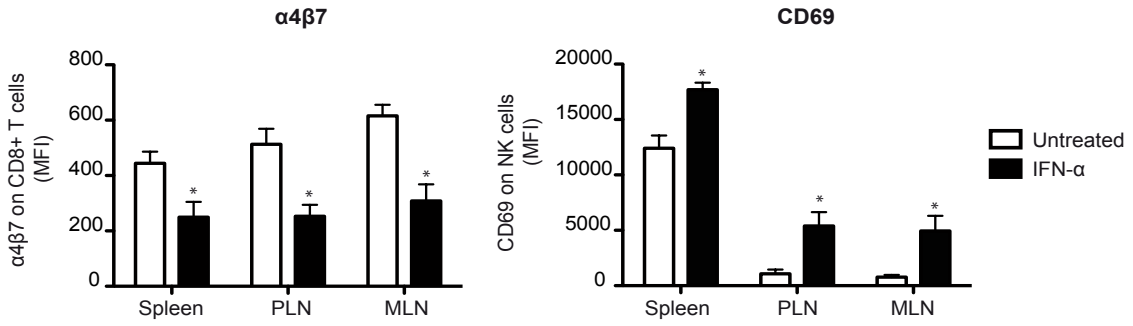

Figure 4.8: IFN-α induces $\alpha_4\beta_7$ downregulation on CD8+ T cells *in vivo* (left panel). Mice (n=3/group) were treated for 3 days intraperitoneally with recombinant mouse IFN-α (10 μg/day). Spleen, peripheral lymph nodes (PLN), mesenteric lymph nodes (MLN) were taken out 24 h after the last injection and analyzed by flow cytometry for $\alpha_4\beta_7$ expression on CD8$^+$ T cells. CD69, an early activation marker, was used as positive control for immune activation by IFN-α (right panel).

4.2 Regulation of $\alpha_4\beta_7$ expression on $CD8^+$ T cells upon virus recognition

After scrutinizing the role of recombinant IFN-α in modulating $\alpha_4\beta_7$ expression, we were interested to further evaluate whether viral infection would also lead to alteration in $\alpha_4\beta_7$ expression. Upon viral infection, type I IFN is an essential factor to alarm neighbouring cells and to prevent other cells to be infected. To examine this, we used different viruses: Sendai, enchephalomyocarditis (EMCV), wild-type and mutant form of vesicular stomatitis (VSV) virus. The VSV DNA is known to encode the so-called matrix protein or “M protein” which blocks the host transcription by inhibiting the initiation of RNA polymerase. Moreover, it can also interfere with the nucleocytoplasmic transport. The mutant form of VSV (VSVmut) used in this experiment has a mutation in the M protein leading to its disability to inhibit the host gene transcriptions and nucleocytoplasmic transport.

We incubated splenocytes with different concentrations of the viruses. After 3 h, the viruses were washed out of the cell culture and the splenocytes were incubated for 24 h prior to flow cytometry analysis. As positive control for $\alpha_4\beta_7$ expression, we also treated splenocytes with CpG and recombinant mouse IFN-α.

Flow cytometry analysis of $\alpha_4\beta_7$ expression depicted in Figure 4.9 (upper panel) revealed that incubation of splenocytes with Sendai virus caused a similar decrease in expression of $\alpha_4\beta_7$ on $CD8^+$ T cells as with recombinant IFN-α. Interestingly, the downregulation was not dependent on the virus concentration. Furthermore, EMCV induced downregulation of $\alpha_4\beta_7$ expression on $CD8^+$ T cells and in contrast to Sendai virus, the effect was even more pronounced when higher virus titer was used in the splenocyte culture. Incubation of splenocytes with VSV virus led to a very modest decrease of $\alpha_4\beta_7$ expression by VSV. This might be due to the ability of M protein to inhibit nucleocytoplasmic transport, thus leading to transcriptional repression of cytokines. In contrast, incubation of splenocytes with the VSVmut led to significant alteration in $\alpha_4\beta_7$ expression in a dose-dependent manner.

Additionally, expression of CD69, an IFN-dependent early activation marker, was examined in the same samples (Figure 4.9, lower panel). Incubation of splenocytes with Sendai virus led to a higher upregulation of CD69 than with recombinant IFN-α.

This might be due to higher IFN-α levels produced in splenocytes incubated with Sendai virus. Furthermore, EMCV and VSV-treated splenocytes, like CpG-treated splenocytes showed no or only modest CD69 upregulation on $CD8^+$ T cells. In contrast, in splenocyte culture incubated with VSVmut (10^6 and 10^7 pfu/ml) CD69 expression on $CD8^+$ T cells was upregulated in dose-dependent manner.

To summarize, we demonstrated that viruses such as Sendai, EMCV and VSVmut could induce the downregulation of $\alpha_4\beta_7$ expression on naïve $CD8^+$ T cells in splenocyte culture.

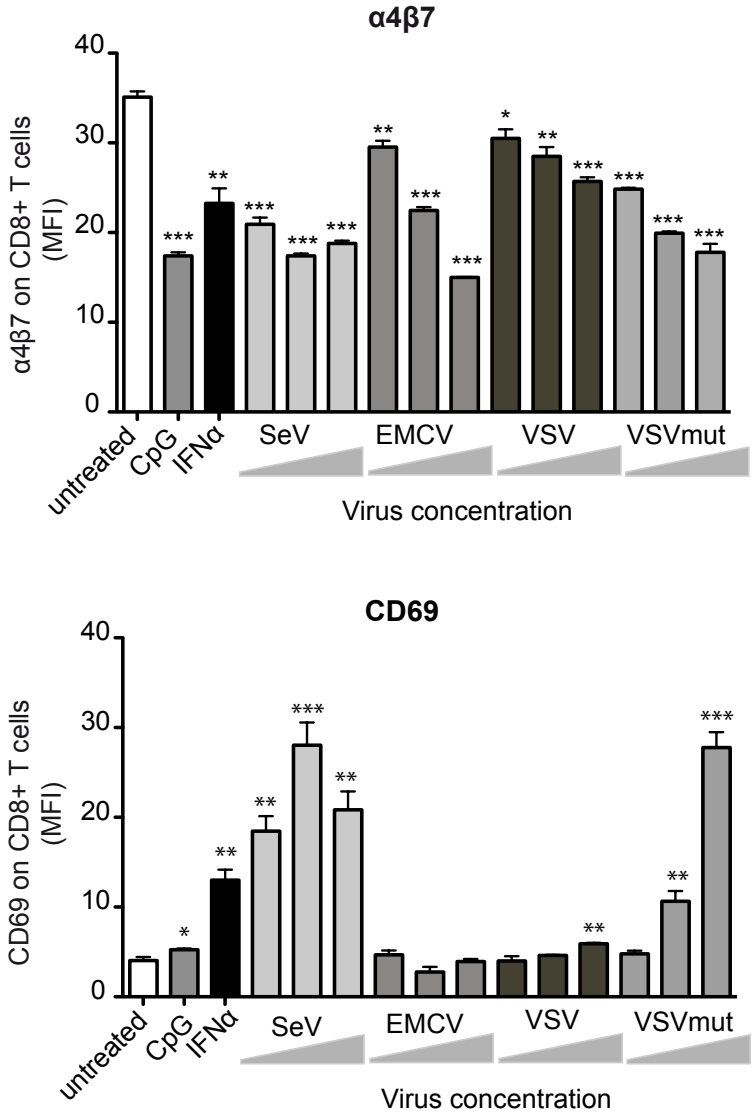

Figure 4.9: $\alpha_4\beta_7$ is downregulated on $CD8^+$ T cells upon incubation with virus (upper panel). Splenocytes were incubated for 24 h with different concentrations of virus (EMCV: 10^5, 10^6, 10^7 pfu/ml; SeV: 10, 100, 250 U/ml; VSV: 10^5, 10^6, 10^7 pfu/ml; VSVmut M31R: 10^5, 10^6, 10^7 pfu/ml). CpG and IFN-α were used as positive controls. CD69, an IFN-α-dependent early activation marker, was used as control for immune reaction (lower panel).

4.2.1 Virus-induced $\alpha_4\beta_7$ downregulation is less pronounced on purified T cells

In the next study, we assessed the impact of the viruses on purified T cells. Splenocytes and purified T cells were incubated with Sendai virus, EMCV, VSV and VSVmut for 3 h before the viruses were washed out of the cell culture. After 24 h of incubation, the $\alpha_4\beta_7$ expression on $CD8^+$ T cells was measured by flow cytometry. As positive control for the downregulation, we cultured splenocytes and purified T cells with IFN-α. Additionally, we stimulated the splenocytes and T cells with CpG as a positive and negative control, respectively.

Consistent with previous result described in Figure 4.9, in splenocyte culture $\alpha_4\beta_7$ expression on $CD8^+$ T cells was decreased in the presence of EMCV, Sendai and VSVmut, whereas incubation with VSV led to a modest decrease (Figure 4.10, upper panel). Strikingly, the decrease of $\alpha_4\beta_7$ expression on $CD8^+$ T cells was markedly less pronounced on purified T cells incubated with the viruses. As expected, stimulation with recombinant IFN-α inhibited $\alpha_4\beta_7$ expression on $CD8^+$ T cells in splenocyte culture as well as on purified T cell culture. Unexpectedly, stimulation of purified T cells with CpG still induced a decrease of $\alpha_4\beta_7$ expression, although less marked than in splenocyte culture. This might be possibly due to some cell populations (e.g. DCs) existing in the 94%-purified T cells that were still able to induce TLR9 signaling. In a better purified T cell population we would therefore expect that the downregulation of $\alpha_4\beta_7$ expression by the viruses would even be more insignificant.

Additionally, we performed flow cytometry analysis of CD69 expression (Figure 4.10, lower panel). Confirming the previous experiment, incubation with Sendai and VSVmut led to an upregulation of CD69 on $CD8^+$ T cells in splenocyte culture whereas EMCV and VSV did not induce CD69 expression. In contrast, CD69 expression on purified $CD8^+$ T cells did not significantly increase upon stimulation with Sendai virus. Furthermore, in purified T cells VSVmut failed to induce CD69 expression. Since the production of type I IFN was necessary to induce CD69 upregulation on T cells, this finding would suggest that the recognition of the VSVmut was mediated by other immune cells and other immune cells than T cells were responsible for the initiation of type I IFN production.

In summary, we observed a less pronouced $\alpha_4\beta_7$ downregulation on purified naïve $CD8^+$ T cells than in splenocyte culture.

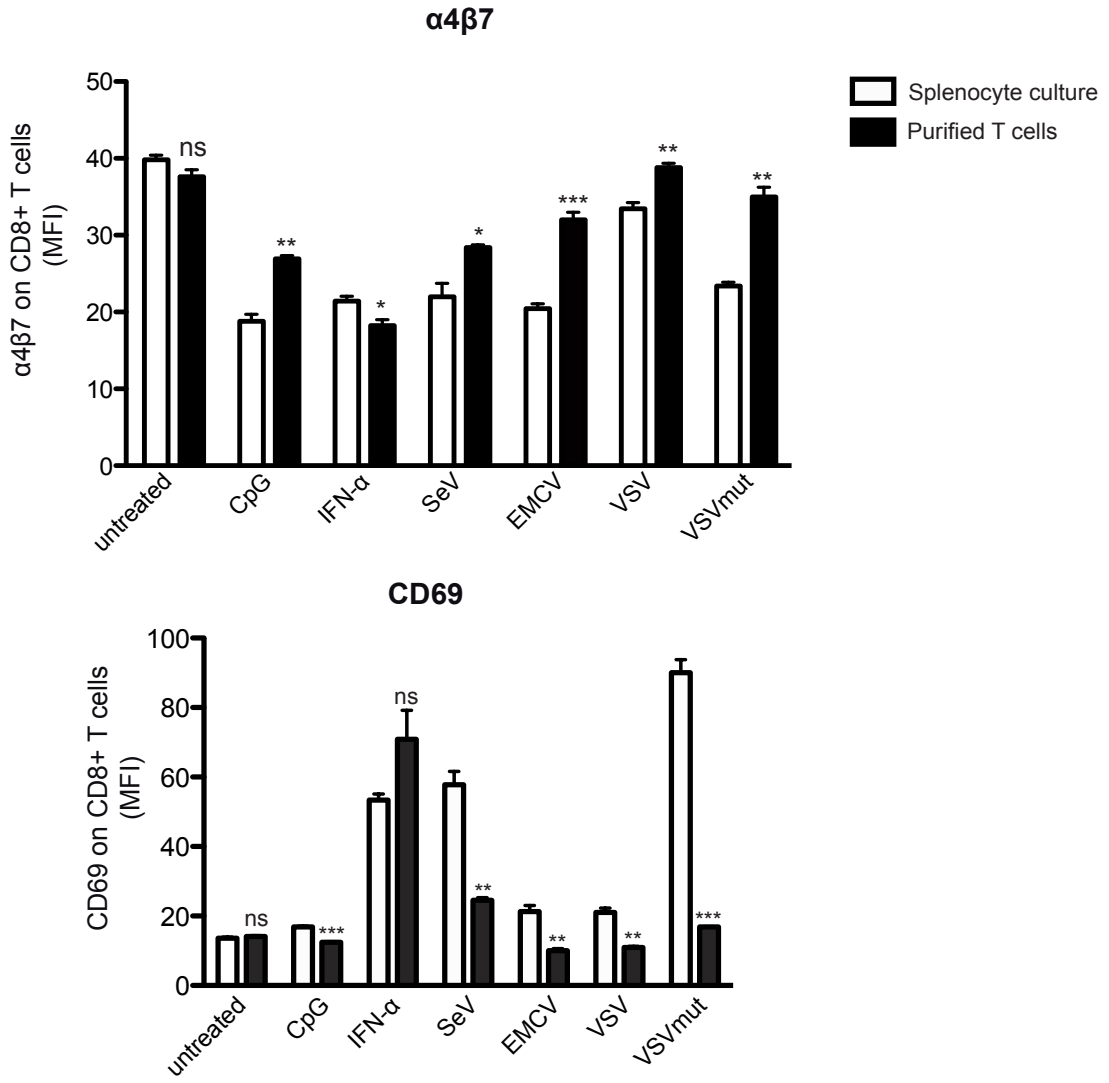

Figure 4.10: $\alpha_4\beta_7$ downregulation by virus is less pronounced on purified T cells (upper panel). Splenocytes and purified T cells (94.1 % after purification) were incubated for 24 h with viruses (SeV 250 U/ml, EMCV 10^7 pfu/ml, VSV 10^7 pfu/ml, VSVmut M31R 10^7 pfu/ml). Splenocytes and purified T cells were gated based on their expression of CD3, CD8 and $\alpha_4\beta_7$. CD69, an IFN-α-dependent early activation marker, was used as control for immune reaction (lower panel). Statistical analysis to compare a same group (e.g. CpG) in the two different conditions (splenocyte culture versus purified T cells) was perfomed using independent two-tailed student's t-test.

4.2.2 IFN-α and IL-6 are detected in supernatant of virus-treated splenocytes

To affirm the role of IFN-α in the virus-mediated downregulation of $\alpha_4\beta_7$ expression, we examined the presence of IFN-α in the supernatants of splenocyte culture incubated with the viruses. Additionally, IL-6 concentration was also measured.

The analysis of IL-6 ELISA data (Figure 4.11, left panel) showed that incubation of splenocytes with CpG induced a four-fold increase of IL-6 compared to the untreated. This was expected, since it is known that CpG trigger IL-6 production upon NF-κB activation. Interestingly, incubation of splenocytes with the highest viral titer of EMCV (10^7 U/ml EMCV in 4.10^5 splenocytes) led to a large production of IL-6 comparable to splenocytes incubated with CpG, whereas IL-6 level in supernatant of Sendai, VSV, and VSVmut-treated splenocytes remained as low as in the untreated condition.

Figure 4.11 (right panel) showed a significant large amount of IFN-α produced in the supernatant of splenocytes cultured with Sendai virus. Furthermore, supernatant cultured with VSVmut virus showed a correlation between increasing amount of viral titer and IFN-α production. In the supernatant of splenocytes treated with highest amount of EMCV virus, we could detect a slight increase of IFN-α production. In contrast, we could not detect IFN-α in the supernatant of splenocytes cultured with VSV. This could be correlated to our previous finding (Figure 4.9), in which we observed a less pronounced $\alpha_4\beta_7$ downregulation on $CD8^+$ T cells in splenocyte culture treated with VSV virus. The inhibition of nucleocytoplasmic transportation by M protein might be the cause of the absence of IFN-α production.

To summarize, in the supernatant of splenocytes treated with Sendai and VSVmut virus we could detect the presence of IFN-α, whereas in the supernatant of splenocytes incubated with EMCV a substantial amount IL-6 could be measured.

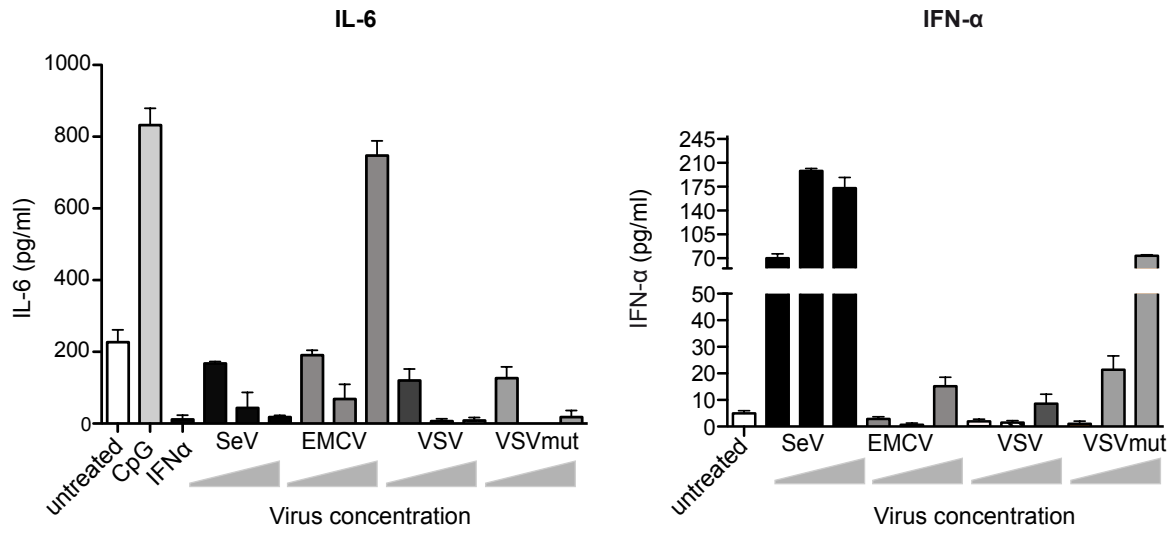

Figure 4.11: EMCV, SeV, VSVmut-treated splenocytes produce large amount of IL-6 and IFN-α, respectively. Supernatants of 24-hour with virus cultured splenocytes were analyzed by IL-6 (left panel) and IFN-α ELISA (right panel).

4.2.3 Blocking the IL-6 or type I IFN receptor inhibits $\alpha_4\beta_7$ downregulation

To confirm the role of IL-6 and IFN-α in virus-induced decrease of $\alpha_4\beta_7$ expression, we incubated purified T cells with supernatants of splenocytes incubated with EMCV and Sendai virus. We added blocking antibody against IL-6 or IFNAR-1 or a combination of both to the purified T cells. As negative control for $\alpha_4\beta_7$ downregulation, we added supernatant of untreated splenocytes to the purified T cells. As positive control for IL-6-mediated $\alpha_4\beta_7$ downregulation, purified T cells were cultured in the supernatant of splenocytes treated with CpG.

Supernatants of CpG and virus-cultured splenocytes without blocking antibodies induced a decrease of $\alpha_4\beta_7$ expression, supporting that soluble factors play a role in inducing changes in $\alpha_4\beta_7$ expression (Figure 4.12). The decrease was significantly reduced when IL-6 antibody was present in CpG and EMCV-treated conditions and addition of blocking antibody against IFNAR-1 did not improve the reduction in these conditions. In contrast, blocking antibody against IFNAR-1 but not against IL-6 significantly inhibited the downregulation of $\alpha_4\beta_7$ expression on $CD8^+$ T cells in Sendai-treated supernatant.

With this experiment we could confirm the role of IFN-α in modulating $\alpha_4\beta_7$ expression on $CD8^+$ T cells, when immune cells are challegend by Sendai and

VSVmut virus. Furthermore, we could also demonstrate that secretion of IL-6 by immune cells treated with EMCV virus is essential for $\alpha_4\beta_7$ downregulation on naïve $CD8^+$ T cells.

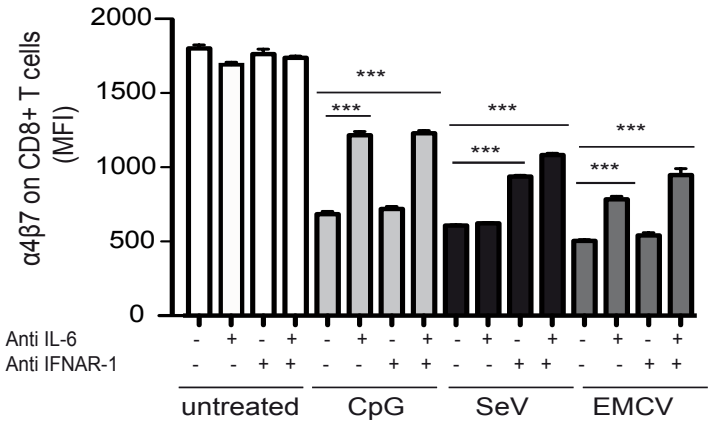

Figure 4.12: Blocking IL-6 and IFNAR-1 significantly inhibit virus-mediated $\alpha_4\beta_7$ downregulation on $CD8^+$ T cells. Purified T cells (250.000 cells/well, triplicate per group) were cultured for 24 h with supernatant of untreated splenocytes (untreated group, white bars) or with supernatant of virus-treated splenocytes in presence or absence of antibody against IL-6 (5 μg/ml) and IFN-α (1.5 μg/ml). CpG was used as positive control. Cells were stained for CD3, CD8 and $\alpha_4\beta_7$.

4.3 IFN-α-stimulated splenocytes have an impaired migration into the gut-associated lymphoid organs

After revealing the ability of IFN-α to modulate $\alpha_4\beta_7$ expression, our next question was whether the decrease of expression of $\alpha_4\beta_7$ alters the migration pattern of naïve T cells into the secondary lymphoid organs. To test this, splenocytes were cultured for 48 h in the presence and absence of IFN-α. We then labeled the untreated and IFN-α-treated splenocytes with eFluor670 and CFSE, respectively. Both are fluorescent cell staining dyes that bind cellular proteins and are equally distributed between daughter cells when the cells divide. The differently labeled splenocytes were then mixed in 1:1 ratio and transferred retroorbitally into nine mice. 24 h after adoptive transfer, spleen, Peyer's patches, peripheral and mesenteric lymph nodes were isolated from five mice. We then analyzed the percentage of the fluorescent-stained splenocytes by flow cytometry.

Flow cytometry analysis showed that 24 h after of adoptive transfer, homing of the differently labeled splenocytes into the spleen did not differ (Figure 4.13, panel A and B), confirming that the migration of immune cells into the spleen is not dependent on $\alpha_4\beta_7$ expression. Furthermore, migration of IFN-α-treated splenocytes into the peripheral lymph nodes was moderately reduced. By contrast, the trafficking of IFN-α-treated splenocytes into mesenteric lymph node was markedly reduced compared with untreated splenocytes. Most importantly, the effect of reduced migration of IFN-α-treated splenocytes was most pronounced in the Peyer's patches.

Four days after the adoptive transfer, flow cytometry analysis of the rest four mice showed that, the difference in migration pattern between IFN-α-treated and untreated splenocytes was abolished (Figure 4.13, panel C). This suggested that the reduced migration into the GALT is a transient mechanism upon immune stimulation by IFN-α.

In this experiment we demonstrated that IFN-α-mediated $\alpha_4\beta_7$ downregulation induced a markedly reduced migration of the splenocytes into the Peyer's patches and mesenteric lymph nodes and to a lesser extent into the peripheral lymph nodes.

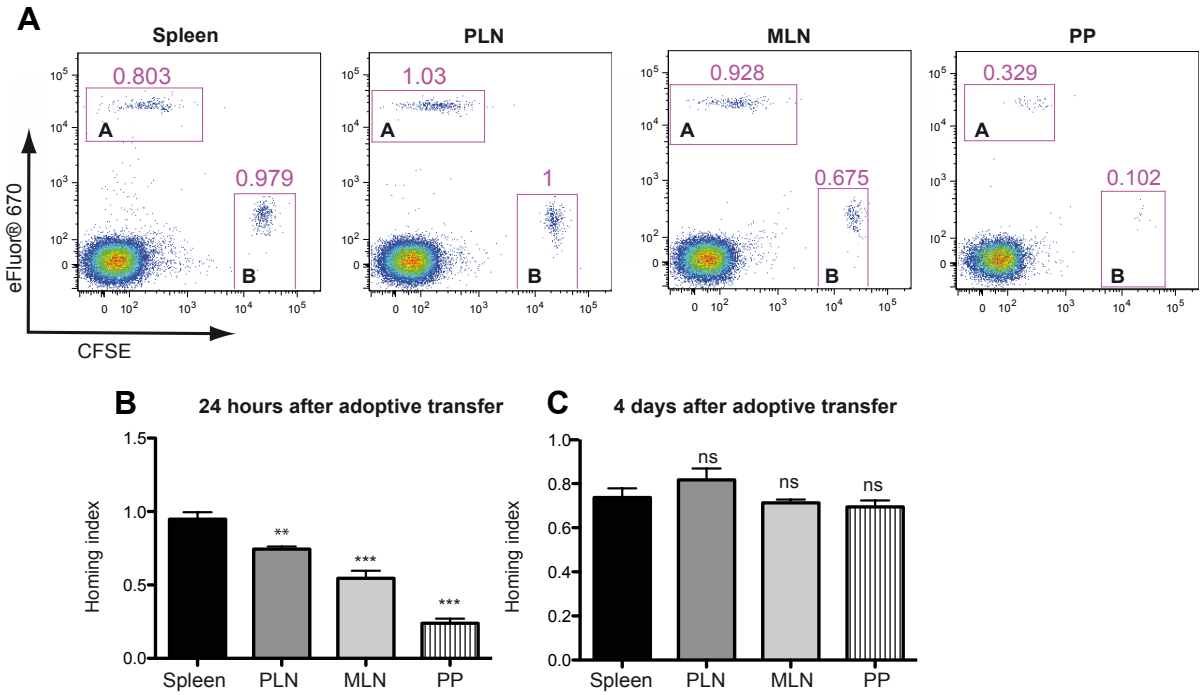

Figure 4.13: IFN-α-treated splenocytes have a temporary impaired migration into the mesenteric lymph nodes and Peyer's patches. Untreated and IFN-α treated splenocytes (10 millions cells each group) were labeled with eFluor670 and CFSE respectively and injected into mice (n=9) in 1:1 ratio. After 24 h, five mice were sacrificed and labeled splenocytes in the spleen, peripheral lymph nodes (PLN), mesenteric lymph nodes (MLN) and Peyer's patches (PP) were tracked by flow cytometry and analyzed by FlowJo software (A). Homing index was calculated and quantified with the statistical program GraphPad Prism5 (B). After four days, labeled splenocytes in the rest of other four mice were analyzed and homing index was calculated (C).

4.4 Cloning the integrin genes

To clone the genes into the retroviral vector pMP71, we firstly performed PCR to amplify the genes for the integrins. Figure 4.14 depicted the result of gel electrophoresis after PCR. The expected size for ITGB1 and ITGB7 (the genes for β1 and β7) was at 2.4 kilo basepairs and for ITGA4 (the gene for α4) was at 3 kilo basepairs. As clearly showed in the figure, we could amplify DNA sequences at the right size of the integrin genes.

We then performed digestion and ligation of the genes with the pMP71 vectors prior to transformation into *E.coli* to amplify the ligated vectors. *E.coli* resistant to ampicillin

were screened for the integrin genes using colony PCR before sending for sequencing.

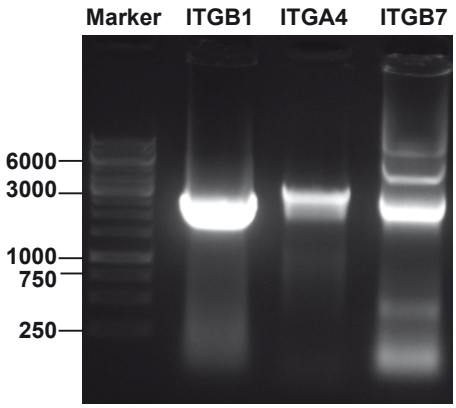

Figure 4.14: PCR product applied on gel.
1 Kb GeneRuler from Fermentas was used (in base pairs, bp).

4.4.1 Validation of insertion of the integrin genes in the vector pMP71

Figure 4.15 represents vectors, which were sent to sequencing and have shown to have the right sequence of the genes. To verify the insertion of the integrin genes, test digestion with EcoRI and NotI was performed and resulted in excision of the genes from the vector at the correct size (2.4 Kb for ITGB1 and ITGB7, 3 Kb for ITGA4). pMP71-GFP was used to verify correctness of the digestion. As expected the gene for GFP at 700 bp could be detected.

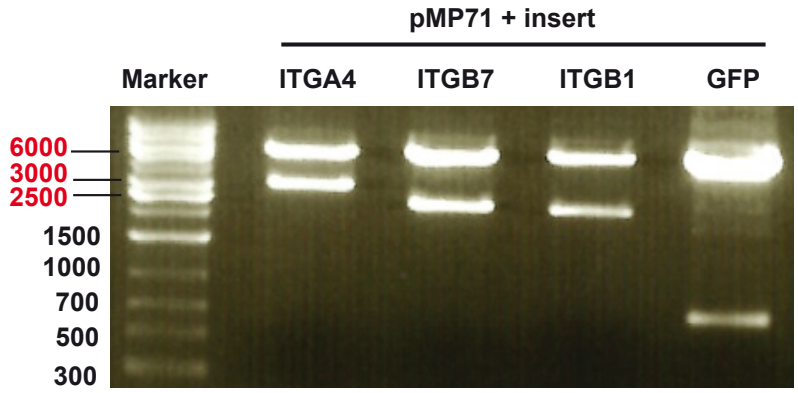

Figure 4.15: Image of gelelectrophoresis of vector digestion. 1 μg of vectors containing the integrin genes or GFP was digested with EcoRI and NotI.1 Kb ladder DNA marker from Axygen Biosciences was used (in base pairs).

4.4.2 Transfection and transduction of the integrin genes

Using calcium-phosphate transfection method, we could induce the expression of GFP, α_4 and β_1 in Plat-E cells. To note, we used 18 μg pMP71-GFP and 40 μg for pMP71-ITGA4 or pMP71-ITGB1. Untransfected Plat-E did not express detectable amount of the integrins α_4 and β_1 (data not shown). Transfection efficiency of GFP was 66.7 % whereas the integrins α_4 and β_1 show an upregulation of cell surface expression of 48.2 % and 11.9 %, respectively (Figure 4.16, panel A upper graph).

When we checked transduction efficiency, we obtained 81.9% GFP-positive primary T cells, whereas the integrins α_4 and β_1 are 12.3% and 37.8 % expressed on cell surface of the T cells (Figure 4.16, panel A lower graph). However, β_1 was already expressed (22.5%) in untransduced T cells (Figure 4.16, right graph on panel B).

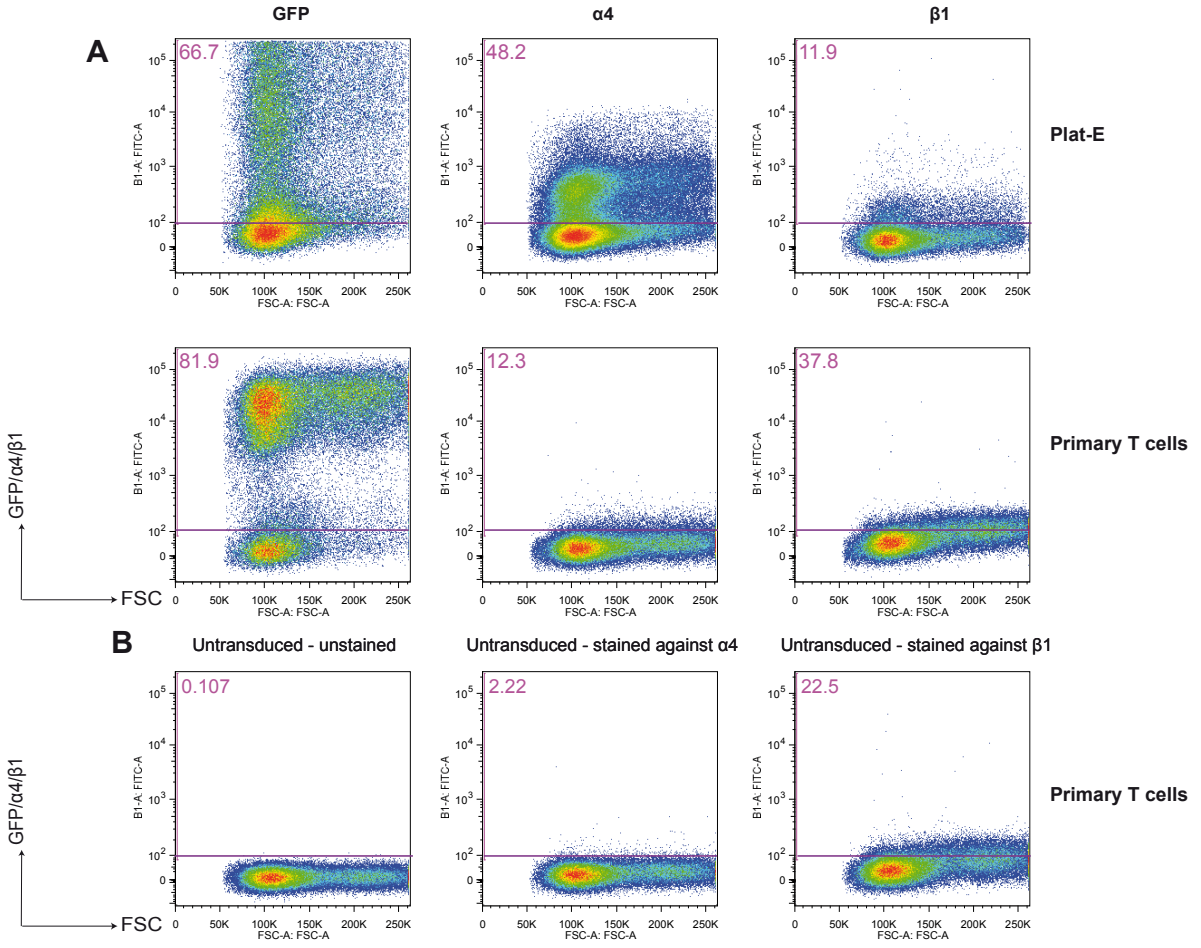

Figure 4.16: Transfection of Plat-E (panel A upper panel) with pMP71-GFP, pMP71-ITGA4 and pMP71-ITGB1 and transduction with viral supernatant of Plat-E (panel A lower panel). Results of transfection of 18 μg of pMP71-GFP and 40 μg pMP71-ITGA4 and pMP71-ITGB1 are shown in the figure. Untransduced primary T cells were stained with antibodies against α_4 and β_1 (panel B).

For β_7 integrin we firstly did not detect an increased of cell surface expression after transfection of Plat-E cells. However, when we performed intracellular staining, we could detect β_7 expression within the cells (Figure 4.17). This intracellular staining confirmed the functionality of the retroviral vector containing β_7. Unlike α_4 and β_1, it seemed that in Plat-E cells β_7 does not have pairing subunit (either α_4 or α_E), which hampers the transportation of β_7 onto the cell surface. Due to the low transfection efficiency, we could not detect expression of β_7 on the surface of primary T cells or intracellularly (data not shown).

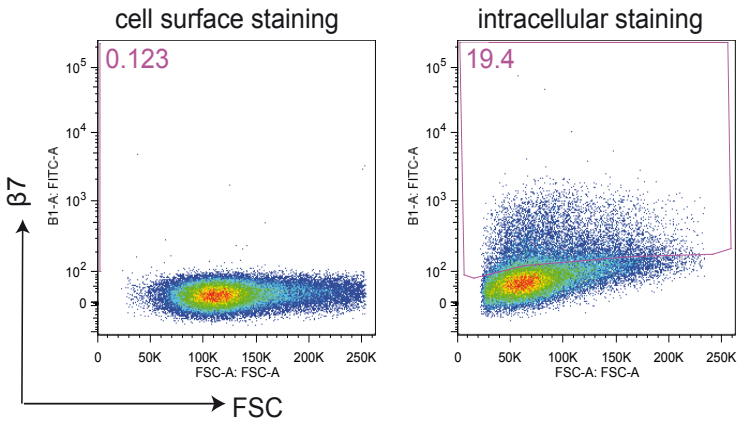

Figure 4.17: Transfection of Plat-E cells with retroviral vector containing β_7 integrin gene. Cell surface and intracellular staining of β_7 integrin are depicted in left and right panel, respectively.

To summarize, we demonstrate that the cloning of the integrin genes into the retroviral vector pMP71 results in a properly functioning retroviral vector expressing the integrin proteins in the retroviral packaging cell line Plat-E. We could also show that the transduction into primary T cells works best with the retroviral vector pMP71 containing the GFP gene and to a lesser extent α_4 and β_1 genes. The transduction of primary T cells with the integrin genes should therefore be optimized. Higher transfection efficiency could be reached using shorter time of incubation to form Ca^{2+} -DNA-phosphate complexes. Also, longer time of Plat-E incubation after transfection may help to produce higher titer of retroviruses, since the integrin genes are larger than GFP gene. Long-term cell culture of Plat-E or T cell lines may impact transfection and transduction efficiency, respectively. pH of transfection buffer should also be checked regularly. Furthermore, the use of different assays such as Western

blot to detect protein expression should be taken into consideration, since fixation process in the intracellular staining could alter protein structure and reduce binding affinity of the antibody.

5 Discussion

5.1 A novel mechanism to control migration of bystander-activated T cells during viral infection

Although much is known about the migration of effector T cells during viral infection, the migration of the bystander-activated T cells during this period remains elusive. These are T cells defined as antigen-unspecific T cells, which upon stimulation (e.g. bacterial or viral infection) undergo phenotypic and functional changes independent from the specific T cell receptor (TCR) stimulation (Bangs et al., 2006). These alterations, which can be mediated by cytokines, may facilitate the bypass of control checkpoints.

5.1.1 Bystander-activated T cells: implication in protective immunity and autoimmunity

The role of bystander-activated T cells in protective immunity or autoimmunity has not been yet completely understood. Supporting the protective immunity model, using LMCV-specific TCR transgenic mice in which CTL-mediated antiviral effector is exclusively mediated by contact-dependent perforin-mediated cytotoxicity, Ehl et al. showed that upon infection with a pathogen unrelated to LMCV, the nonspecific activated T cells are capable of providing an antiviral response (Ehl et al., 1997). They also demonstrated that bystander activation with unrelated virus is not sufficient to induce diabetes, whereas LMCV infection can promote diabetes in these transgenic mice.

However, there are also evidences for the role of bystander-activated T cells in autoimmune diseases. In fact, viral infections, which may induce nonspecific activation, have been related to the induction of autoimmune diseases (Kim et al., 2006). For example, a study by Nogai at al. reveals that activation of autoreactive T cell in TCR-transgenic mice upon LPS provoked the development of experimental autoimmune enchephalitis (EAE), a murine model for multiple sclerosis (Nogai et al.,

2005). This finding could be recently verified by another group, where they demonstrated that T cells deficient in TLR4, which is the receptor for LPS, were not able to promote EAE (Reynolds et al., 2012).

Considering that bacterial or viral infections are a common occurance, it is rather striking that autoimmune diseases are not the general pattern. Although some correlations to genetical and environmental factors have been described (Fasano and Shea-Donohue, 2005), which affect the severity of autoimmune diseases, we hypothesize that mechanisms for controlling migration of bystander-activated T cells during infection may exist.

5.1.2 The dual function of the integrin $\alpha_4\beta_7$

The migration of naïve T cells and skin-specific central memory T cells into the peripheral lymph nodes is governed by a set of cell adhesion molecules which is distinct from the set of cell adhesion molecules expressed on the skin-specific effector T cells. In contrast, intestinal-specific effector T cells and naïve T cells require the expression of the homing receptor $\alpha_4\beta_7$ to enter the gastrointestinal tract and the GALT. The difference in utilization of integrin $\alpha_4\beta_7$ relies exclusively on the state of the expression on naïve T cells (low level) and intestinal effector T cells (high level). Moreover, in comparison to other integrins, $\alpha_4\beta_7$ is unique due to its ability to mediate rolling and firm adhesion (Gorfu et al., 2009).

As a peripheral tissue with the largest surface area, the gastrointestinal tract is a home for a vast numbers of commensal bacteria and thus represents the largest source of non-self antigens, a strict regulation of $\alpha_4\beta_7$ expression is therefore vital to prevent mistrafficking.

5.1.3 Downregulation of $\alpha_4\beta_7$ upon viral infection is mediated by IFN-α or IL-6

Consistent with the bacterial infection study perfomed by Heidegger et al. (manuscript submitted), we observe that $\alpha_4\beta_7$ expression is negatively regulated on naïve $CD8^+$ T cells in mice receiving poly (I:C). Poly (I:C) is structurally similar to a double-stranded RNA and can be detected by TLR3 or the cytosolic MDA-5 that also

detect viral double-stranded RNA. Hence, poly (I:C) elicits the same signaling pathway as in viral infections, which makes it attractive for its utilization as viral infection model.

A similar downmodulation of $\alpha_4\beta_7$ was described in human T cell clones, either upon specific recognition of alloantigens, with immobilized anti-CD3 mAbs or soluble anti-CD43 (Hernández-Caselles et al., 1996). In their study, they demonstrated that the membrane expression of α_4 integrin can be regulated by TCR/CD3-dependent as well as independent signals. In agreement with their study, our observation could extend the knowledge of the $\alpha_4\beta_7$ regulation on primary naïve T cells upon immune stimulation in mice.

Interestingly, stimulation of splenocytes from IFNAR-deficient mice with poly (I:C) and R848 induced partial reduction of the observed $\alpha_4\beta_7$ downregulation in wild-type splenocytes, suggesting a significant role for type I IFN, whereas the effect of CpG does not seem to be type IFN-dependent. Indeed, Heidegger et al. could show that IL-6 plays a dominant role in $\alpha_4\beta_7$ regulation upon CpG recognition.

5.1.3.1 Absolute requirement of type I IFN signaling to induce $\alpha_4\beta_7$ downregulation in vivo

In contrast to our *in vitro* data, $\alpha_4\beta_7$ downregulation on T cells is completely inhibited in IFNAR knockout mice upon injection of poly (I:C), indicating an absolute requirement of type I IFN signaling in induction of $\alpha_4\beta_7$ modulation when examined *in vivo*. The difference may due to different response to poly (I:C) between splenic and peritoneal APCs. In the *in vivo* experimental setting, upon intraperitonal injection the uptake of poly (I:C) is perfomed by the peritoneal APCs, whereas *in vitro* splenic APCs are responsible for the detection. Indeed, several studies have shown that the splenic and peritoneal macrophages exhibit distinct alterations upon stimulations (Shortman and Wu, 2004; Stout and Suttles, 2005; Olsson and Sundler, 2006). Study conducted by Liu et al. demonstrates that splenic macrophages express higher co-stimulatory molecules and cytokines, whereas peritoneal macrophages express higher level of TLR2 and TLR4 and are superior in phagocytosis (Liu et al., 2006). Hence, depending upon which macrophages recognize poly (I:C), production of additional cytokines may be induced by splenic macrophages.

5.1.3.2 IFN-α directly induces $\alpha_4\beta_7$ downregulation on naïve CD8$^+$ T cells in a time and dose-dependent manner

In further experiments we validated the fact that IFN-α is able to modulate $\alpha_4\beta_7$ expression on splenic CD8$^+$ T cells *in vitro* in a time- and dose-dependent manner. Interestingly, higher dosage of IFN-α than 10^3 U/ml did not lead to an additional significant decrease of $\alpha_4\beta_7$ expression, although CD69 expression was steadily enhanced. When modulation of $\alpha_4\beta_7$ expression on purified T cells and T cells in splenocyte culture was compared, it is apparent that the level of decreased expression is quite similar, suggesting that intrinsic type I IFN signaling solely in T cells at a concentration threshold of 10^3 U/ml is sufficient to induce the alteration. Interestingly, $\alpha_4\beta_7$ expression on T cell surface could not be completely shut off with a higher dosage of IFN-α. One possibility would be that it has some functions during T cell activation.

Corresponding to the *in vitro* experiment, we could further demonstrated that IFN-α can lead to the modulation of $\alpha_4\beta_7$ expression on CD8$^+$ T cells *in vivo*, suggesting that the alteration is physiologically relevant. Downregulation of the α_4 subunit on primed T cells was described in the analysis of multiple sclerosis patients receiving IFN-β treatment (Muraro et al., 2000). However, in their study no changes in α_4 expression on unprimed naïve T cells was observed and whether β_7 expression is modulated on unprimed T cells was not shown in this study.

5.1.3.3 Changes in other cell adhesion molecules on naïve CD8$^+$ T cells upon IFN-α treatment

Analysis of other cell adhesion molecule expressions upon 24h-treatment with IFN-α revealed that there is a slight but marked decrease of L-selectin (CD62L) expression on CD8$^+$ T cells. However, this stood in contrast to a previous study by Kampuis et al., in which they demonstrated a minor increase of L-selectin on lymphocytes upon poly (I:C) injection into wild-type mice (Kamphuis et al., 2006). The difference could be explained by their analysis of L-selectin on total lymphocytes, which include B and T cells, whereas in our study we specifically surveyed the expression of L-selectin on CD8$^+$ T cells. Indeed, another study evinced that IFN-α induces an upregulation of L-selectin on human B lymphoid Daudi cell lines and in B cell subpopulations (Evans

et al., 1993). Hence, the increase expression of L-selectin on B cells might mask the decreased expression of L-selectin on activated naïve T cells.

In the same study by Kamphuis et al., they did not observe a downregulation of CD49d (α_4 subunit) or upregulation of LFA-1 (CD11a/CD18). However, like L-selectin, CD49d and LFA-1 surface expression analyses were perfomed on total lymphocytes. Thus, a "masking" effect as in case of L-selectin could be the reason for the difference. In contrast to their study, the substanstial increase of LFA-1 expression on $CD8^+$ T cells in presence of IFN-α corresponded the observation in a systemic infection study with LMCV that is known to induce robust type I IFN (Merigan et al., 1977; Andersson et al., 1994, 1995; Zhou et al., 2010).

Furthermore, a slight but significant increase of CD31 on T cells was also observed. CD31 is expressed on human naïve and $CD8^+$ T cells (Stockinger et al., 1992). It was shown that, when CD31 is transduced into human T cell clones lacking CD31, the T cell clones acquire a reduced proliferation capability due to a blockage in cell cycle progression in G_0/G_1 stage (Prager et al., 2001). The CD31 upregulation on naïve $CD8^+$ T cells upon IFN-α stimulation could therefore be a mechanism to inhibit proliferation of the bystander-activated cells. Suporting this idea, Dondi et al. demonstrated that resting naïve T cells delayed their entry into cell cycle after TCR triggering when treated with IFN-α (Dondi et al., 2003)

Our experiment demonstrates that other cell adhesion molecules on naïve $CD8^+$ T cells, such as L-selectin, LFA-1 and CD31, are also moderately regulated upon activation type I IFN signaling. Our observation also shows that deeper analysis of a specific subpopulation of lymphocytes (in our case naïve $CD8^+$ T cells) could reveal different results with previous studies on whole lymphocytes, since different immune cell populations within lymphocytes could act differently to a specific cytokine such as IFN-α. Though the modulation effect is not as strong as in the case of $\alpha_4\beta_7$, the observations on other cell adhesion molecules should not be disregarded, since minor alteration in their expression level on the cell surface could have an important function in fine-tuning how the immune cell should react to the environmental changes induced by IFN-α.

5.1.3.4 Alteration in $\alpha_4\beta_7$ expression upon incubation with different viruses

In our further extensive studies using several virus such as VSV, VSVmut, EMCV and Sendai, we could reproduce the same findings as with poly (I:C), in which we also observed downregulation of $\alpha_4\beta_7$ expression on naïve $CD8^+$ T cells. In the case of Sendai and VSVmut, the alteration of $\alpha_4\beta_7$ expression seemed to be predominantly dependent on IFN-α production. Interestingly, the modulation was not dose-dependent in Sendai infection, whereas increasing virus titer of VSVmut led to an enhanced inhibition of $\alpha_4\beta_7$ expression. Moreover, this correlated with the amount of produced IFN-α in both conditions. It seemed that low titer of Sendai virus in splenocyte culture was sufficient to induce robust IFN production, which corresponded to at least to the amount of control IFN-α (10^3 U/ml), since the downregulation was comparable. Indeed, IFN-producing Sendai virus was shown to be superior inducer when compared with influenza virus at the same MOI (multiplicity of infection) (López et al., 2003).

Similar to CpG, $\alpha_4\beta_7$ downregulation on $CD8^+$ T cells induced by EMCV is mainly mediated by IL-6. Consistent with the observed result with VSVmut virus, the downregulation seems to be dependent on the amount of virus infecting the splenocytes and this amount correlates with the production of IL-6 measured in the supernatant. Interestingly, CD69 upregulation on T cells seems not to be a prerequisite for the downregulation, since EMCV infection, similar to CpG, did not enhanced its expression, but the alteration in $\alpha_4\beta_7$ expression was still observed.

The results obtained using different viruses support the fact that our models for innate immune activation using CpG and poly (I:C) mirror the effect induced by EMCV and Sendai virus, respectively.

Whether the downregulation requires an active infection or only uptake was not adressed in our study. A study performed by López et al. with Sendai virus suggested that infection is needed for cytokine production by DCs, since incubation of DCs with UV-light-inactivated Sendai virus did not produce any cytokines (López et al., 2003). Supporting this, when purified T cells were incubated with virus, we could not observe cytokine production (data not shown) in the supernatant, indicating that other immune cells are the main cytokine producer upon virus challenge. Also, the upregulation of CD69 on purified T cells incubated with Sendai or VSVmut virus

was almost abolished, indicating that the production of type I IFN, at least in the initiating step, was due to the viral recognition of other immune cells, such as DCs. In the absence of cytokine production, the alteration of $\alpha_4\beta_7$ expression on the purified T cells upon viral infection was barely observed. However, in purified T cells cultured with Sendai and EMCV virus, $\alpha_4\beta_7$ expression was still moderately downregulated. This could be due to the few non-T cells (e.g. dendritic cells) in the purified T cell fraction, since the control CpG condition also showed a decrease of $\alpha_4\beta_7$ expression, which should be abrogated on purified T cells. Another possibility is that the virus can still infect T cells. Consistent with this, it has been reported that EMCV and Sendai virus are able to be detected directly by T cells since they express the cognate receptors RIG-I and MDA-5 (Anz et al., 2010).

Our blocking experiment could confirm the role of IFN-α and IL-6 in modulation of $\alpha_4\beta_7$ expression upon Sendai and EMCV infection, respectively. However, additional cytokines could play a role, since the downregulation is not completely inhibited.

5.1.4 Regulation of of $\alpha_4\beta_7$ expression

Our finding raised the question of how the modulation of $\alpha_4\beta_7$ expression is regulated. The reduced availability of proteins on the cell surface can be affected by several mechanisms: shedding, endocytosis, transcription downregulation and a higher rate of protein degradation by proteasomes. Shedding is a process in which the protein is cleaved at their juxta-membrane region by matrix metalloproteinases, leading to the release of the extracellular part of the proteins to the environment (Huovila et al., 2005). Whereas this process is known to disengage growth factors, cytokines and mediators from their membrane-bound precursors, it also functions to downregulate protein expression on the cell surface. A prominent example is the shedding of L-selectin, which rapidly occurs upon activation on T cells (Jung and Dailey, 1990) but also on other immune cells (Kishimoto et al., 1989; Morrison et al., 2010).

Another mechanism is the endocytosis, which leads to the engulfment of cell surface proteins into the cells. Subsequently, proteins can either be sent to lysosomal degradation or recycled back to the cell membrane. There are different pathways that

mediate this process, the most common are the clathrin and the caveolin-dependent pathways. It has been described that generally integrins are internalized via the endocytosis pathways and one integrin can utilize several endocytic routes (Caswell et al., 2009). Moreover, integrins are commonly not degraded upon internalization but are transported back to cell surface (Caswell and Norman, 2006, 2008; Jones et al., 2006; Pellinen and Ivaska, 2006).

In case of $\alpha_4\beta_7$, the endocytic route and re-transport to the cell surface is likely the possible mechanism since its downregulation is a transient state and the shedding mechanism would not present an efficient and energy-saving way for this recycling effect. Supporting this idea, Hernández-Caselles and coworkers demonstrate that T cell clones which recognize *Staphylococcus* enterotoxin B, downregulate $\alpha_4\beta_7$ expression and the decreased expression is abrogated in presence of an actin-depolymerizing agent cytochalasin D, indicating the inhibition of the receptor-mediated endocytosis (Hernández-Caselles et al., 1996; Lamaze et al., 1997).

Since IL-6 and IFN-α seem to be able to downregulate $\alpha_4\beta_7$ on naïve T cells directly, it would be interesting to scrutinize which factors in the signaling would induce endocytosis. Due to the fast transcriptional induction of interferon-stimulated genes (4-6 hours after type I IFN signaling activation) (Dondi et al., 2003), it is likely that endocytosis may be triggered by interferon-stimulated genes.

5.1.5 Transient blockade into the GALT and to lesser extent into peripheral lymph nodes

Supporting our finding in the alteration of $\alpha_4\beta_7$ expression upon IFN-α stimulation, in our adoptive transfer experiment we observe a severe defect in migration into the Peyer's patches. This supports the hypothesis that $\alpha_4\beta_7$ downregulation on naïve $CD8^+$ T cells influences their migration pattern. This correlates with the study of Wagner and coworkers, in which they demonstrated that the migration of $\beta_7^{-/-}$ lymphocytes into the Peyer's patches was almost abolished (Wagner et al., 1996) and this effect was even more pronounced when L-selectin/$\beta_7^{-/-}$ lymphocytes were utilized (Wagner et al., 1998). Furthermore, their migration study reveals a less dramatic impact for the trafficking of $\beta_7^{-/-}$ lymphocytes into MLN (49% reduction

compared to the wild-type lymphocytes). Consistently, in our study we observe that the migration of IFN-α-treated splenocytes into MLN is reduced by half compared to the wild type splenocytes.

However, the reduction of L-selectin on $CD8^+$ T cells upon IFN-α stimulation may also play a role in the observed result for MLN, since L-selectin act together with β_7 to promote lymphocyte trafficking into MLN, which was confirmed by migration studies using L-selectin/$\beta_7^{-/-}$ lymphocytes (Wagner et al., 1998; Dutt et al., 2005). Furthermore, IFN-α treatment causes a less significant reduced migration into the PLN, which is more likely due to the L-selectin downregulation (Warnock et al., 1998).

5.2 Physiologcal role of $\alpha_4\beta_7$ downregulation

It is tempting to speculate that $\alpha_4\beta_7$ downregulation is implicated in the regulation of T cell proliferation, as it has been shown for L-selectin. A study by Nishijima et al. demonstrates that T cell proliferation induced by immobilized anti-CD3 was enhanced in presence of antibody against L-selectin (Nishijima et al., 2005). It is therefore tempting to speculate that the downregulation of $\alpha_4\beta_7$ and L-selectin in presence of IFN-α would also be a mechanism to inhibit or reduce proliferation of the bystander-activated T cells. Supporting this idea, Marshall and coworkers demonstrate that proliferation of bystander-activated $CD8^+$ T cells was suppressed upon viral infection, specifically due to type I IFN. Moreover, they show that this was due to a delayed onset of division that was an intrinsic defect of T cells (Marshall et al., 2011). Consistently, Nishijima and coworkers further reveal that the expression level of p27 on T cells, a cyclin-dependent kinase inhibitor, was decreased in presence of antibodies against L-selectin. Even more interestingly, p27 is shown to be increased in human naïve T cells treated with IFN-α prior to T cell activation (Dondi et al., 2003). Since we discover that $\alpha_4\beta_7$ is downregulated upon IFN-α treatment, it would therefore be interesting to investigate whether the downmodulation of $\alpha_4\beta_7$ contributes to the cell cycle inhibition due to the increase expression of p27. One possibility is to utilize T cells overexpressing $\alpha_4\beta_7$ that do not downregulate $\alpha_4\beta_7$ upon IFN-α treatment and compare p27 expression with

unmodified T cells that are sensitive toward IFN-α.

The interpretation of the functional importance of the observed reduced migration is challenging. It could be that the reduced entry into the mesenteric lymph nodes and Peyer's patches for bystander-activated T cells would provide space for effector T cells in the GALT to expand. Moreover, bystander-activated T cells are known to have an increased responsiveness to subsequent stimulation via TCR, thus lowering the threshold of activation (Alves et al., 2005; Gagnon et al., 2008). The impaired homing into the GALT during viral infection may therefore prevent an inappropriate T cell activation for these pre-activated T cells, which could lead to recognition of self-antigen, hence causing autoimmune diseases.

Marshall and coworkers demonstrated that viral infection leads to a transient immune suppression and that this might contribute to the inability of a vaccine to elicit immunity (Marshall et al., 2011). A recent study showed that injection of poly (I:C) prior to antigen leads to a suppression of antigen-specific $CD8^+$ T cell expansion and reduction of effector differentiation (Ngoi et al., 2012). Furthermore, it was shown that this is due to the downregulation of the IL-33 receptor subunit ST2 affecting the production of IFN-γ by $CD8^+$ effector T cells. At the same time, IFN-γ and IL-33 might contribute to occurrence of inflammatory bowel diseases (Fasano and Shea-Donohue, 2005; Seidelin et al., 2011). Interestingly, using systemic lung injury model they could show that the pretreatment with poly (I:C) and blocking ST2 could reduce the systemic accumulation of effector T cells thus limiting damage in the periphery.

The impairment of T-cell effector differentiation by poly (I:C) pretreatment would explain on one hand the observation by Marshall and coworkers why a vaccine is not able to boost immunity when viral infection just occurred shortly prior to vaccination. On the other hand, the effect seemed to be a mechanism to prevent uncontrolled immune infiltration. Furthermore, the downregulation of $\alpha_4\beta_7$ might contribute to the observed defect of $CD8^+$ T cells. The transient blockade mechanism might prevent inappropriate activation bystander-activated T cells on early time point of infection but at the cost of excluding naïve $CD8^+$ T cells from the GALT that would recognize an antigen of an invading pathogen in this time window.

Both studies of Marshall and Ngoi also additionally showed that co-injection of antigen and virus (or poly (I:C)) leads to an adjuvant effect, in contrast to the sequential injection causing immune suppression or defect response of $CD8^+$ T cell. Since the adoptive-transfer study of Heidegger et al. was also performed by co-injecting OVA and CpG or LPS simultaneously, it would be therefore interesting to investigate if there is a delay in $\alpha_4\beta_7$ upregulation by OVA-specific T cells when sequential injection is performed.

5.3 Therapeutical approach of T cells overexpressing $\alpha_4\beta_7$

In the second part of this project, we have cloned the integrin genes α_4, β_1 and β_7 into retroviral vector to transduce them into primary T cells. De Nucci and coworkers demonstrate that overexpression of α_4 leads to higher expression of $\alpha_4\beta_7$ on the cell surface (DeNucci et al., 2010). The aim of our project is to increase $\alpha_4\beta_7$ expression on T cells to improve targeting into gastrointestinal tumors. T cells are important in tumor rejection since they are able to recognize tumor-associated antigens, which is however often impeded by tumor immune evasion. TCR engineering to improve avidity of T cells towards these antigens is one of the promising strategies in the tumor immunotherapy field to overcome this evasion.

However, a high efficacy of tumor eradication requires specific accumulation of these T cells in the tumor. Our study in SV40 tumor mouse model has demonstrated that adoptive transfer of tumor-specific T cells is highly effective in eradication of subcutaneous tumor, however they fail to eliminate autochtonous stomach tumor due to its immunosuppressive microenvironment (Bourquin et al., 2010). Therefore, breaking of the immunosuppression and selective homing of tumor-specific T cells into the tumor, thereby increasing accumulation of these T cells in the tumor, are crucial steps in refinement of tumor immunotherapy. Overexpression of cell adhesion molecules on T cells and also their ligands in the tumor would be strategies to enhance homing into the tumor.

In our future study, we plan to investigate whether the overexpression of $\alpha_4\beta_7$ on T cells would increase the infiltration of T cells into the SV40 autochtonous stomach tumor, in combination with increasing expression of MAdCAM-1 in the stomach tumor. Additionally, it would be interesting to analyze whether the stomach tumor

eradication is enhanced when $\alpha_4\beta_7$ is overexpressed on T cells from TCR transgene mice that recognize specifically SV40 antigen.

5.4 Conclusion and outlook

The migration of unspecifically activated naïve T cells in a viral infection has been poorly researched in contrast to the migration of virus-specific activated T cells. In our viral infection study utilizing poly (I:C), we discovered that the integrin $\alpha_4\beta_7$ expression on naïve $CD8^+$ T cells was negatively regulated upon recognition of poly (I:C). Furthermore, we demonstrated that this effect was mediated by IFN-α. Consistently, studies with Sendai and VSVmut virus confirmed the role of IFN-α in its ability to control $\alpha_4\beta_7$ expression. In the case of our study using EMCV virus, we demonstrated that IL-6 was the major cytokine that mediated the $\alpha_4\beta_7$ downmodulation. Finally, using an adoptive transfer model we demonstrated that migration of bystander-activated $CD8^+$ T cells into the GALT, especially into the Peyer's patches, was severely impaired upon exposure to IFN-α due to the downregulation of the $\alpha_4\beta_7$ expression.

The downregulation of $\alpha_4\beta_7$ expression on the naïve $CD8^+$ T cells and the subsequent reduced migration into the GALT might be a mechanism to prevent further inappropriate activation of the preactivated naïve $CD8^+$ T cells in the GALT, as indicated by the upregulation of the early activation marker CD69 on these T cells. Consequently, this action could be a safeguard mechanism to avoid uncontrolled influx of unspecific T cells into the intestine, hence minimizing the risk of developing autoimmune diseases.

In the second part of our project, we successfully cloned the integrin genes α_4, β_1 and β_7 into retroviral vectors. In transfection experiments we could further demonstrate that these retroviral vectors are functional, since we could detect higher expression of all integrins in the transfected retroviral packaging cell line (Plat-E cells). Finally, we could also observe more expression of α_4 and β_1 in the transduced primary T cells. However, transfection and transduction efficiency still need to be optimized.

In tumor patients, very often the T-cell receptors (TCR) are poorly reactive toward tumor antigen. Modifications such as TCR engineering to achieve high specificity toward tumor antigens and the development of a chimeric immune receptor, a fusion of antibody domain with TCR chain to overcome MHC downregulation often

observed in tumors, are promising strategies to optimize the effectiveness of adoptive T cell transfer for tumor patients. Selective migration of these T cells into the tumor could serve as an additional improvement in the adoptive T cell transfer therapy. Our retroviral vectors might therefore be a promising tool to investigate whether overexpression of $\alpha_4\beta_7$ enhances homing of tumor-specific T cells into gastrointestinal tumors and contributes to an improved tumor immunotherapy. Gastrointestinal tumor murine models such as the autochtonous SV40 stomach model and a pancreas tumor model expressing ovalbumin, are available in our laboratory for exploring the benefit of the $\alpha_4\beta_7$ overexpression in the adoptive transfer model to treat tumors. The overexpression of $\alpha_4\beta_7$ on T cells of existing transgenic mice, which specifically possess TCR against the antigen SV40 or ovalbumin, and the adoptive transfer of these T cells into the above-mentioned tumor murine models, are the next steps to verify the efficacy of this therapeutic strategy.

6 Reference List

Abbas, A.K., and Janeway, C.A., Jr (2000). Immunology: improving on nature in the twenty-first century. Cell *100*, 129–138.

Alves, N.L., Arosa, F.A., and Van Lier, R.A.W. (2005). IL-21 sustains CD28 expression on IL-15-activated human naive CD8+ T cells. J. Immunol. *175*, 755–762.

Andersson, E.C., Christensen, J.P., Marker, O., and Thomsen, A.R. (1994). Changes in cell adhesion molecule expression on T cells associated with systemic virus infection. J Immunol *152*, 1237–1245.

Andersson, E.C., Christensen, J.P., Scheynius, A., Marker, O., and Thomsen, A.R. (1995). Lymphocytic choriomeningitis virus infection is associated with long-standing perturbation of LFA-1 expression on CD8+ T cells. Scand. J. Immunol. *42*, 110–118.

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

Ann Marrie, R., and Rudick, R.A. (2006). Drug Insight: interferon treatment in multiple sclerosis. Nat Clin Pract Neurol *2*, 34–44.

Anz, D., Koelzer, V.H., Moder, S., Thaler, R., Schwerd, T., Lahl, K., Sparwasser, T., Besch, R., Poeck, H., Hornung, V., et al. (2010). Immunostimulatory RNA blocks suppression by regulatory T cells. J. Immunol. *184*, 939–946.

Arbonés, M.L., Ord, D.C., Ley, K., Ratech, H., Maynard-Curry, C., Otten, G., Capon, D.J., and Tedder, T.F. (1994). Lymphocyte homing and leukocyte rolling and migration are impaired in L-selectin-deficient mice. Immunity *1*, 247–260.

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

Arthos, J., Cicala, C., Martinelli, E., Macleod, K., Van Ryk, D., Wei, D., Xiao, Z., Veenstra, T.D., Conrad, T.P., Lempicki, R.A., et al. (2008). HIV-1 envelope protein binds to and signals through integrin alpha4beta7, the gut mucosal homing receptor for peripheral T cells. Nat. Immunol. *9*, 301–309.

Bangs, S.C., McMichael, A.J., and Xu, X.-N. (2006). Bystander T cell activation--implications for HIV infection and other diseases. Trends Immunol. *27*, 518–524.

Barral, P.M., Sarkar, D., Su, Z., Barber, G.N., DeSalle, R., Racaniello, V.R., and Fisher, P.B. (2009). Functions of the cytoplasmic RNA sensors RIG-I and MDA-5: key regulators of innate immunity. Pharmacol. Ther. *124*, 219–234.

Barton, G.M., and Kagan, J.C. (2009). A cell biological view of Toll-like receptor function: regulation through compartmentalization. Nat. Rev. Immunol. *9*, 535–542.

Bengtsson, A.A., Sturfelt, G., Truedsson, L., Blomberg, J., Alm, G., Vallin, H., and Rönnblom, L. (2000). Activation of type I interferon system in systemic lupus erythematosus correlates with disease activity but not with antiretroviral antibodies. Lupus *9*, 664–671.

Berg, E.L., Goldstein, L.A., Jutila, M.A., Nakache, M., Picker, L.J., Streeter, P.R., Wu, N.W., Zhou, D., and Butcher, E.C. (1989). Homing receptors and vascular addressins: cell adhesion molecules that direct lymphocyte traffic. Immunol. Rev. *108*, 5–18.

Berg, E.L., Magnani, J., Warnock, R.A., Robinson, M.K., and Butcher, E.C. (1992). Comparison of L-selectin and E-selectin ligand specificities: The L-selectin can bind the E-selectin ligands Sialyl Lex and Sialyl Lea. Biochemical and Biophysical Research Communications *184*, 1048–1055.

Berg, E.L., McEvoy, L.M., Berlin, C., Bargatze, R.F., and Butcher, E.C. (1993). L- selectin-mediated lymphocyte rolling on MAdCAM-1. Nature *366*, 695–698.

Berke, G. (1995). The CTL's kiss of death. Cell *81*, 9–12.

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

Bluyssen, A.R., Durbin, J.E., and Levy, D.E. (1996). ISGF3 gamma p48, a specificity switch for interferon activated transcription factors. Cytokine Growth Factor Rev. *7*, 11–17.

Boissonnas, A., Fetler, L., Zeelenberg, I.S., Hugues, S., and Amigorena, S. (2007). In vivo imaging of cytotoxic T cell infiltration and elimination of a solid tumor. J. Exp. Med. *204*, 345–356.

Boon, T., Cerottini, J.C., Van den Eynde, B., Van der Bruggen, P., and Van Pel, A. (1994). Tumor antigens recognized by T lymphocytes. Annu. Rev. Immunol. *12*, 337–365.

Bourquin, C., Von der Borch, P., Zoglmeier, C., Anz, D., Sandholzer, N., Suhartha, N., Wurzenberger, C., Denzel, A., Kammerer, R., Zimmermann, W., et al. (2010). Efficient eradication of subcutaneous but not of autochthonous gastric tumors by adoptive T cell transfer in an SV40 T antigen mouse

model. J. Immunol. *185*, 2580–2588.

Briskin, M.J., McEvoy, L.M., and Butcher, E.C. (1993). MAdCAM-1 has homology to immunoglobulin and mucin-like adhesion receptors and to IgA1. Nature *363*, 461–464.

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

Campbell, J.J., Haraldsen, G., Pan, J., Rottman, J., Qin, S., Ponath, P., Andrew, D.P., Warnke, R., Ruffing, N., Kassam, N., et al. (1999). The chemokine receptor CCR4 in vascular recognition by cutaneous but not intestinal memory T cells. Nature *400*, 776–780.

Caswell, P., and Norman, J. (2008). Endocytic transport of integrins during cell migration and invasion. Trends Cell Biol. *18*, 257–263.

Caswell, P.T., and Norman, J.C. (2006). Integrin trafficking and the control of cell migration. Traffic *7*, 14–21.

Caswell, P.T., Vadrevu, S., and Norman, J.C. (2009). Integrins: masters and slaves of endocytic transport. Nature Reviews Molecular Cell Biology *10*, 843–853.

Cerottini, J.C., Von Fliedner, V., and Boon, T. (1992). Recognition of tumor-associated antigens by T lymphocytes: from basic concepts to new approaches. Ann. Oncol. *3*, 11–16.

Cho, Y., and De Bruyn, P.P. (1981). Transcellular migration of lymphocytes through the walls of the smooth-surfaced squamous endothelial venules in the lymph node: evidence for the direct entry of lymphocytes into the blood circulation of the lymph node. J. Ultrastruct. Res. *74*, 259–266.

Darnell, J.E., Jr, Kerr, I.M., and Stark, G.R. (1994). Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science *264*, 1415–1421.

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

Desmet, C.J., and Ishii, K.J. (2012). Nucleic acid sensing at the interface between innate and adaptive immunity in vaccination. Nature Reviews Immunology *12*, 479–491.

Dondi, E., Rogge, L., Lutfalla, G., Uzé, G., and Pellegrini, S. (2003). Down-modulation of responses to type I IFN upon T cell activation. J. Immunol. *170*, 749–756.

Duester, G. (2000). Families of retinoid dehydrogenases regulating vitamin A function: production of visual pigment and retinoic acid. Eur. J. Biochem. *267*, 4315–4324.

Dutt, S., Ermann, J., Tseng, D., Liu, Y.P., George, T.I., Fathman, C.G., and Strober, S. (2005). L-selectin and β7 integrin on donor CD4 T cells are required for the early migration to host mesenteric lymph nodes and acute colitis of graft-versus-host disease. Blood *106*, 4009–4015.

Ehl, S., Hombach, J., Aichele, P., Hengartner, H., and Zinkernagel, R.M. (1997). Bystander activation of cytotoxic T cells: studies on the mechanism and evaluation of in vivo significance in a transgenic mouse model. J. Exp. Med. *185*, 1241–1251.

Eksteen, B., Liaskou, E., and Adams, D.H. (2008). Lymphocyte homing and its role in the pathogenesis of IBD. Inflammatory Bowel Diseases *14*, 1298–1312.

Elangbam, C.S., Qualls, C.W., Jr, and Dahlgren, R.R. (1997). Cell adhesion molecules--update. Vet. Pathol. *34*, 61–73.

Enarsson, K., Johnsson, E., Lindholm, C., Lundgren, A., Pan-Hammarström, Q., Strömberg, E., Bergin, P., Baunge, E.-L., Svennerholm, A.-M., and Quiding-Järbrink, M. (2006). Differential mechanisms for T lymphocyte recruitment in normal and neoplastic human gastric mucosa. Clin. Immunol. *118*, 24–34.

Engelhardt, B., and Wolburg, H. (2004). Mini-review: Transendothelial migration of leukocytes: through the front door or around the side of the house? Eur. J. Immunol. *34*, 2955–2963.

Erle, D.J., Briskin, M.J., Butcher, E.C., Garcia-Pardo, A., Lazarovits, A.I., and Tidswell, M. (1994). Expression and function of the MAdCAM-1 receptor, integrin alpha 4 beta 7, on human leukocytes. J. Immunol. *153*, 517–528.

Evans, S.S., Collea, R.P., Appenheimer, M.M., and Gollnick, S.O. (1993). Interferon-alpha induces the expression of the L-selectin homing receptor in human B lymphoid cells. J. Cell Biol. *123*, 1889–1898.

Fasano, A., and Shea-Donohue, T. (2005). Mechanisms of disease: the role of intestinal barrier function in the pathogenesis of gastrointestinal autoimmune diseases. Nat Clin Pract Gastroenterol Hepatol *2*, 416–422.

Fausa, O., Schrumpf, E., and Elgjo, K. (1991). Relationship of inflammatory bowel disease and primary sclerosing cholangitis. Semin. Liver Dis. *11*, 31–39.

Ferrantini, M., Capone, I., and Belardelli, F. (2007). Interferon-alpha and cancer: mechanisms of action and new perspectives of clinical use. Biochimie *89*, 884–893.

Funk, J., Langeland, T., Schrumpf, E., and Hanssen, L.E. (1991). Psoriasis induced by interferon-

alpha. Br. J. Dermatol. *125*, 463–465.

Gagnon, J., Ramanathan, S., Leblanc, C., Cloutier, A., McDonald, P.P., and Ilangumaran, S. (2008). IL-6, in Synergy with IL-7 or IL-15, Stimulates TCR-Independent Proliferation and Functional Differentiation of CD8+ T Lymphocytes. J Immunol *180*, 7958–7968.

Galon, J., Costes, A., Sanchez-Cabo, F., Kirilovsky, A., Mlecnik, B., Lagorce-Pagès, C., Tosolini, M., Camus, M., Berger, A., Wind, P., et al. (2006). Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science *313*, 1960–1964.

Girard, J.P., and Springer, T.A. (1995). High endothelial venules (HEVs): specialized endothelium for lymphocyte migration. Immunol. Today *16*, 449–457.

González-Navajas, J.M., Lee, J., David, M., and Raz, E. (2012). Immunomodulatory functions of type I interferons. Nat. Rev. Immunol. *12*, 125–135.

Gorfu, G., Rivera-Nieves, J., and Ley, K. (2009). Role of beta7 integrins in intestinal lymphocyte homing and retention. Curr. Mol. Med. *9*, 836–850.

Graham, F.L., and Van der Eb, A.J. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology *52*, 456–467.

Grant, A.J., Lalor, P.F., Hübscher, S.G., Briskin, M., and Adams, D.H. (2001). MAdCAM-1 expressed in chronic inflammatory liver disease supports mucosal lymphocyte adhesion to hepatic endothelium (MAdCAM-1 in chronic inflammatory liver disease). Hepatology *33*, 1065–1072.

Haque, S.J., and Williams, B.R. (1994). Identification and characterization of an interferon (IFN)-stimulated response element-IFN-stimulated gene factor 3-independent signaling pathway for IFN-alpha. J. Biol. Chem. *269*, 19523–19529.

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

Hernández-Caselles, T., Martínez-Esparza, M., Lazarovits, A.I., and Aparicio, P. (1996). Specific regulation of VLA-4 and alpha 4 beta 7 integrin expression on human activated T lymphocytes. J. Immunol. *156*, 3668–3677.

Hesterberg, P.E., Winsor-Hines, D., Briskin, M.J., Soler-Ferran, D., Merrill, C., Mackay, C.R., Newman, W., and Ringler, D.J. (1996). Rapid resolution of chronic colitis in the cotton-top tamarin with an antibody to a gut-homing integrin alpha 4 beta 7. Gastroenterology *111*, 1373–1380.

Huovila, A.-P.J., Turner, A.J., Pelto-Huikko, M., Kärkkäinen, I., and Ortiz, R.M. (2005). Shedding light on ADAM metalloproteinases. Trends Biochem. Sci. *30*, 413–422.

Ihle, J.N., and Kerr, I.M. (1995). Jaks and Stats in signaling by the cytokine receptor superfamily. Trends Genet. *11*, 69–74.

Ishikawa, H., and Barber, G.N. (2008). STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. Nature *455*, 674–678.

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

Johansson-Lindbom, B., Svensson, M., Wurbel, M.-A., Malissen, B., Márquez, G., and Agace, W. (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*, 963–969.

Jones, M.C., Caswell, P.T., and Norman, J.C. (2006). Endocytic recycling pathways: emerging regulators of cell migration. Curr. Opin. Cell Biol. *18*, 549–557.

Jung, T.M., and Dailey, M.O. (1990). Rapid modulation of homing receptors (gp90MEL-14) induced by activators of protein kinase C. Receptor shedding due to accelerated proteolytic cleavage at the cell surface. J. Immunol. *144*, 3130–3136.

Kamphuis, E., Junt, T., Waibler, Z., Forster, R., and Kalinke, U. (2006). Type I interferons directly regulate lymphocyte recirculation and cause transient blood lymphopenia. Blood *108*, 3253–3261.

Kansas, G.S. (1992). Structure and function of L-selectin. APMIS *100*, 287–293.

Kastner, P., Mark, M., and Chambon, P. (1995). Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? Cell *83*, 859–869.

Kawai, T., and Akira, S. (2010). The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat. Immunol. *11*, 373–384.

Kelly, K.A., Wiley, D., Wiesmeier, E., Briskin, M., Butch, A., and Darville, T. (2009). The combination of the gastrointestinal integrin (α4β7) and selectin ligand enhances T-Cell migration to the reproductive tract during infection with Chlamydia trachomatis. Am. J. Reprod. Immunol. *61*, 446–452.

Kim, B., Kaistha, S.D., and Rouse, B.T. (2006). Viruses and autoimmunity. Autoimmunity *39*, 71–77.

Kishimoto, T.K., Jutila, M.A., Berg, E.L., and Butcher, E.C. (1989). Neutrophil Mac-1 and MEL-14 adhesion proteins inversely regulated by chemotactic factors. Science *245*, 1238–1241.

Kita, Y., Takashi, T., Iigo, Y., Tamatani, T., Miyasaka, M., and Horiuchi, T. (1992). Sequence and expression of rat ICAM-1. Biochim. Biophys. Acta *1131*, 108–110.

Lamaze, C., Fujimoto, L.M., Yin, H.L., and Schmid, S.L. (1997). The Actin Cytoskeleton Is Required for Receptor-mediated Endocytosis in Mammalian Cells. J. Biol. Chem. *272*, 20332–20335.

Lampen, A., Meyer, S., Arnhold, T., and Nau, H. (2000). Metabolism of vitamin A and its active metabolite all-trans-retinoic acid in small intestinal enterocytes. J. Pharmacol. Exp. Ther. *295*, 979–985.

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

Liu, G., Xia, X.-P., Gong, S.-L., and Zhao, Y. (2006). The macrophage heterogeneity: difference between mouse peritoneal exudate and splenic F4/80+ macrophages. J. Cell. Physiol. *209*, 341–352.

López, C.B., García-Sastre, A., Williams, B.R.G., and Moran, T.M. (2003). Type I interferon induction pathway, but not released interferon, participates in the maturation of dendritic cells induced by negative-strand RNA viruses. J. Infect. Dis. *187*, 1126–1136.

Luster, A.D., Alon, R., and Andrian, U.H. von (2005). Immune cell migration in inflammation: present and future therapeutic targets. Nature Immunology *6*, 1182–1190.

Luthman, H., and Magnusson, G. (1983). High efficiency polyoma DNA transfection of chloroquine treated cells. Nucleic Acids Res. *11*, 1295–1308.

Mangelsdorf, D.J., and Evans, R.M. (1995). The RXR heterodimers and orphan receptors. Cell *83*, 841–850.

Marelli-Berg, F., Cannella, L., Dazzi, F., and Mirenda, V. (2008). The highway code of T cell trafficking. The Journal of Pathology *214*, 179–189.

Marlin, S.D., and Springer, T.A. (1987). Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). Cell *51*, 813–819.

Marshall, H.D., Urban, S.L., and Welsh, R.M. (2011). Virus-induced transient immune suppression and the inhibition of T cell proliferation by type I interferon. J. Virol. *85*, 5929–5939.

McGettrick, A.F., and O'Neill, L.A.J. (2010). Localisation and trafficking of Toll-like receptors: an important mode of regulation. Curr. Opin. Immunol. *22*, 20–27.

Medzhitov, R., Preston-Hurlburt, P., Kopp, E., Stadlen, A., Chen, C., Ghosh, S., and Janeway, C.A., Jr (1998). MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. Mol. Cell *2*, 253–258.

Merigan, T.C., Oldstone, M.B., and Welsh, R.M. (1977). Interferon production during lymphocytic choriomeningitis virus infection of nude and normal mice. Nature *268*, 67–68.

Monroe, K.M., McWhirter, S.M., and Vance, R.E. (2010). Induction of type I interferons by bacteria. Cell. Microbiol. *12*, 881–890.

Monteleone, G., Pender, S.L., Alstead, E., Hauer, A.C., Lionetti, P., McKenzie, C., and MacDonald, T.T. (2001). Role of interferon alpha in promoting T helper cell type 1 responses in the small intestine in coeliac disease. Gut *48*, 425–429.

Mora, J.R., and Von Andrian, U.H. (2006). T-cell homing specificity and plasticity: new concepts and future challenges. Trends Immunol. *27*, 235–243.

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

Mora, J.R., Iwata, M., and Von Andrian, U.H. (2008). Vitamin effects on the immune system: vitamins A and D take centre stage. Nat. Rev. Immunol. *8*, 685–698.

Morales, J., Homey, B., Vicari, A.P., Hudak, S., Oldham, E., Hedrick, J., Orozco, R., Copeland, N.G., Jenkins, N.A., McEvoy, L.M., et al. (1999). CTACK, a skin-associated chemokine that preferentially attracts skin-homing memory T cells. Proc. Natl. Acad. Sci. U.S.A. *96*, 14470–14475.

Morita, S., Kojima, T., and Kitamura, T. (2000). Plat-E: an efficient and stable system for transient packaging of retroviruses. Gene Ther. *7*, 1063–1066.

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

Mosmann, T.R., and Coffman, R.L. (1989). TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu. Rev. Immunol. *7*, 145–173.

Mrass, P., Takano, H., Ng, L.G., Daxini, S., Lasaro, M.O., Iparraguirre, A., Cavanagh, L.L., Von Andrian, U.H., Ertl, H.C.J., Haydon, P.G., et al. (2006). Random migration precedes stable target cell interactions of tumor-infiltrating T cells. J. Exp. Med. *203*, 2749–2761.

Muraro, P.A., Leist, T., Bielekova, B., and McFarland, H.F. (2000). VLA-4/CD49d downregulated on primed T lymphocytes during interferon-beta therapy in multiple sclerosis. J. Neuroimmunol. *111*, 186–194.

Musch, E., Andus, T., Malek, M., Chrissafidou, A., and Schulz, M. (2007). Successful treatment of steroid refractory active ulcerative colitis with natural interferon-beta--an open long-term trial. Z Gastroenterol *45*, 1235–1240.

Nakache, M., Berg, E.L., Streeter, P.R., and Butcher, E.C. (1989). The mucosal vascular addressin is a tissue-specific endothelial cell adhesion molecule for circulating lymphocytes. Nature *337*, 179–181.

Neumann, K., Kruse, N., Szilagyi, B., Erben, U., Rudolph, C., Flach, A., Zeitz, M., Hamann, A., and Klugewitz, K. (2012). Connecting liver and gut: Murine liver sinusoidal endothelium induces gut tropism of CD4(+) T cells via retinoic acid. Hepatology *55*, 1976–1984.

Ngo, M.C., Rooney, C.M., Howard, J.M., and Heslop, H.E. (2011). Ex vivo gene transfer for improved adoptive immunotherapy of cancer. Hum. Mol. Genet. *20*, R93–99.

Ngoi, S.M., St Rose, M.-C., Menoret, A.M., Smith, D.E., Tovey, M.G., Adler, A.J., and Vella, A.T. (2012). Presensitizing with a Toll-like receptor 3 ligand impairs CD8 T-cell effector differentiation and IL-33 responsiveness. Proc. Natl. Acad. Sci. U.S.A. *109*, 10486–10491.

Nishijima, K., Ando, M., Sano, S., Hayashi-Ozawa, A., Kinoshita, Y., and Iijima, S. (2005). Costimulation of T-cell proliferation by anti-l-selectin antibody is associated with the reduction of a cdk inhibitor p27. Immunology *116*, 347–353.

Nogai, A., Siffrin, V., Bonhagen, K., Pfueller, C.F., Hohnstein, T., Volkmer-Engert, R., Brück, W., Stadelmann, C., and Kamradt, T. (2005). Lipopolysaccharide injection induces relapses of experimental autoimmune encephalomyelitis in nontransgenic mice via bystander activation of autoreactive CD4+ cells. J. Immunol. *175*, 959–966.

Novick, D., Cohen, B., and Rubinstein, M. (1994). The human interferon alpha/beta receptor: characterization and molecular cloning. Cell *77*, 391–400.

Olsson, R., Danielsson, A., Järnerot, G., Lindström, E., Lööf, L., Rolny, P., Rydén, B.O., Tysk, C., and Wallerstedt, S. (1991). Prevalence of primary sclerosing cholangitis in patients with ulcerative colitis. Gastroenterology *100*, 1319–1323.

Olsson, S., and Sundler, R. (2006). The role of lipid rafts in LPS-induced signaling in a macrophage cell line. Mol. Immunol. *43*, 607–612.

Osborn, L., Hession, C., Tizard, R., Vassallo, C., Luhowskyj, S., Chi-Rosso, G., and Lobb, R. (1989). Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. Cell *59*, 1203–1211.

Park, T.S., Rosenberg, S.A., and Morgan, R.A. (2011). Treating cancer with genetically engineered T cells. Trends Biotechnol. *29*, 550–557.

Pellinen, T., and Ivaska, J. (2006). Integrin traffic. J. Cell. Sci. *119*, 3723–3731.

Picarella, D., Hurlbut, P., Rottman, J., Shi, X., Butcher, E., and Ringler, D.J. (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*, 2099–2106.

Picker, L.J., Kishimoto, T.K., Smith, C.W., Warnock, R.A., and Butcher, E.C. (1991). ELAM-1 is an adhesion molecule for skin-homing T cells. Nature *349*, 796–799.

Podolsky, D.K., Lobb, R., King, N., Benjamin, C.D., Pepinsky, B., Sehgal, P., and deBeaumont, M. (1993). Attenuation of colitis in the cotton-top tamarin by anti-alpha 4 integrin monoclonal antibody. J. Clin. Invest. *92*, 372–380.

Ponsioen, C.Y. (2012). Recent insights in primary sclerosing cholangitis. J Dig Dis *13*, 337–341.

Postigo, A.A., Sánchez-Mateos, P., Lazarovits, A.I., Sánchez-Madrid, F., and De Landázuri, M.O. (1993). Alpha 4 beta 7 integrin mediates B cell binding to fibronectin and vascular cell adhesion molecule-1. Expression and function of alpha 4 integrins on human B lymphocytes. J. Immunol. *151*, 2471–2483.

Prager, E., Staffler, G., Majdic, O., Säemann, M., Godár, S., Zlabinger, G., and Stockinger, H. (2001). Induction of hyporesponsiveness and impaired T lymphocyte activation by the CD31 receptor:ligand pathway in T cells. J. Immunol. *166*, 2364–2371.

Qureshi, S.A., Salditt-Georgieff, M., and Darnell, J.E., Jr (1995). Tyrosine-phosphorylated Stat1 and Stat2 plus a 48-kDa protein all contact DNA in forming interferon-stimulated-gene factor 3. Proc. Natl. Acad. Sci. U.S.A. *92*, 3829–3833.

Randolph, G.J., Angeli, V., and Swartz, M.A. (2005). Dendritic-cell trafficking to lymph nodes through

lymphatic vessels. Nature Reviews Immunology *5*, 617–628.

Reiss, Y., Proudfoot, A.E., Power, C.A., Campbell, J.J., and Butcher, E.C. (2001). CC chemokine receptor (CCR)4 and the CCR10 ligand cutaneous T cell-attracting chemokine (CTACK) in lymphocyte trafficking to inflamed skin. J. Exp. Med. *194*, 1541–1547.

Restifo, N.P., Dudley, M.E., and Rosenberg, S.A. (2012). Adoptive immunotherapy for cancer: harnessing the T cell response. Nat. Rev. Immunol. *12*, 269–281.

Reynolds, J.M., Martinez, G.J., Chung, Y., and Dong, C. (2012). Toll-like receptor 4 signaling in T cells promotes autoimmune inflammation. PNAS *109*, 13064–13069.

Rivera-Nieves, J., Olson, T., Bamias, G., Bruce, A., Solga, M., Knight, R.F., Hoang, S., Cominelli, F., and Ley, K. (2005). L-selectin, alpha 4 beta 1, and alpha 4 beta 7 integrins participate in CD4+ T cell recruitment to chronically inflamed small intestine. J. Immunol. *174*, 2343–2352.

Rönnblom, L. (2011). The type I interferon system in the etiopathogenesis of autoimmune diseases. Ups. J. Med. Sci. *116*, 227–237.

Rot, A., and Von Andrian, U.H. (2004). Chemokines in innate and adaptive host defense: basic chemokinese grammar for immune cells. Annu. Rev. Immunol. *22*, 891–928.

Schindler, C., and Darnell, J.E., Jr (1995). Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. Annu. Rev. Biochem. *64*, 621–651.

Schmid, P., Itin, P., Cox, D., McMaster, G.K., and Horisberger, M.A. (1994). The type I interferon system is locally activated in psoriatic lesions. J. Interferon Res. *14*, 229–234.

Schoefl, G.I. (1972). The migration of lymphocytes across the vascular endothelium in lymphoid tissue. A reexamination. J. Exp. Med. *136*, 568–588.

Schweighoffer, T., Tanaka, Y., Tidswell, M., Erle, D.J., Horgan, K.J., Luce, G.E., Lazarovits, A.I., Buck, D., and Shaw, S. (1993). Selective expression of integrin alpha 4 beta 7 on a subset of human CD4+ memory T cells with Hallmarks of gut-trophism. J. Immunol. *151*, 717–729.

Seidelin, J.B., Rogler, G., and Nielsen, O.H. (2011). A role for interleukin-33 in T(H)2-polarized intestinal inflammation? Mucosal Immunol *4*, 496–502.

Shortman, K., and Wu, L. (2004). Are dendritic cells end cells? Nat. Immunol. *5*, 1105–1106.

Springer, T.A. (1994). Traffic signals for lymphocyte recirculation and leukocyte emigration: the

multistep paradigm. Cell *76*, 301–314.

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

Stark, G.R., Kerr, I.M., Williams, B.R., Silverman, R.H., and Schreiber, R.D. (1998). How cells respond to interferons. Annu. Rev. Biochem. *67*, 227–264.

Staunton, D.E., Dustin, M.L., and Springer, T.A. (1989). Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1. Nature *339*, 61–64.

Stenstad, H., Svensson, M., Cucak, H., Kotarsky, K., and Agace, W.W. (2007). Differential homing mechanisms regulate regionalized effector CD8alphabeta+ T cell accumulation within the small intestine. Proc. Natl. Acad. Sci. U.S.A. *104*, 10122–10127.

Stockinger, H., Schreiber, W., Majdic, O., Holter, W., Maurer, D., and Knapp, W. (1992). Phenotype of human T cells expressing CD31, a molecule of the immunoglobulin supergene family. Immunology *75*, 53–58.

Stout, R.D., and Suttles, J. (2005). Immunosenescence and macrophage functional plasticity: dysregulation of macrophage function by age-associated microenvironmental changes. Immunol. Rev. *205*, 60–71.

Streeter, P.R., Berg, E.L., Rouse, B.T., Bargatze, R.F., and Butcher, E.C. (1988). A tissue-specific endothelial cell molecule involved in lymphocyte homing. Nature *331*, 41–46.

Svensson, H., Olofsson, V., Lundin, S., Yakkala, C., Björck, S., Börjesson, L., Gustavsson, B., and Quiding-Järbrink, M. (2012). Accumulation of $CCR4^{+}$CTLA-4 $FOXP3^{+}$CD25(hi) regulatory T cells in colon adenocarcinomas correlate to reduced activation of conventional T cells. PLoS ONE *7*, e30695.

Sydora, B.C., Wagner, N., Lohler, J., Yakoub, G., Kronenberg, M., Muller, W., and Aranda, R. (2002). beta7 Integrin expression is not required for the localization of T cells to the intestine and colitis pathogenesis. Clin. Exp. Immunol. *129*, 35–42.

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

Takaoka, A., and Yanai, H. (2006). Interferon signalling network in innate defence. Cell. Microbiol. *8*, 907–922.

Takeda, K., Kaisho, T., and Akira, S. (2003). Toll-like receptors. Annu. Rev. Immunol. *21*, 335–376.

Uzé, G., Schreiber, G., Piehler, J., and Pellegrini, S. (2007). The receptor of the type I interferon family. Curr. Top. Microbiol. Immunol. *316*, 71–95.

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

Wagner, N., Löhler, J., Tedder, T.F., Rajewsky, K., Müller, W., and Steeber, D.A. (1998). L-selectin and beta7 integrin synergistically mediate lymphocyte migration to mesenteric lymph nodes. Eur. J. Immunol. *28*, 3832–3839.

Waldman, E., Lu, S.X., Hubbard, V.M., Kochman, A.A., Eng, J.M., Terwey, T.H., Muriglan, S.J., Kim, T.D., Heller, G., Murphy, G.F., et al. (2006). Absence of beta7 integrin results in less graft-versus-host disease because of decreased homing of alloreactive T cells to intestine. Blood *107*, 1703–1711.

Wang, B.X., and Fish, E.N. (2012). The yin and yang of viruses and interferons. Trends Immunol. *33*, 190–197.

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

Welzel, T., Radtke, I., Meyer-Zaika, W., Heumann, R., and Epple, M. (2004). Transfection of cells with custom-made calcium phosphate nanoparticles coated with DNA. Journal of Materials Chemistry *14*, 2213.

Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K., et al. (2003). Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science *301*, 640–643.

Yoneyama, M., and Fujita, T. (2007). Function of RIG-I-like receptors in antiviral innate immunity. J. Biol. Chem. *282*, 15315–15318.

Yoneyama, M., and Fujita, T. (2010). Recognition of viral nucleic acids in innate immunity. Rev. Med. Virol. *20*, 4–22.

Yoshida, R., Imai, T., Hieshima, K., Kusuda, J., Baba, M., Kitaura, M., Nishimura, M., Kakizaki, M., Nomiyama, H., and Yoshie, O. (1997). Molecular cloning of a novel human CC chemokine EBI1-ligand chemokine that is a specific functional ligand for EBI1, CCR7. J. Biol. Chem. *272*, 13803–13809.

Yoshida, R., Nagira, M., Kitaura, M., Imagawa, N., Imai, T., and Yoshie, O. (1998). Secondary lymphoid-tissue chemokine is a functional ligand for the CC chemokine receptor CCR7. J. Biol. Chem. *273*, 7118–7122.

Ytterberg, S.R., and Schnitzer, T.J. (1982). Serum interferon levels in patients with systemic lupus erythematosus. Arthritis Rheum. *25*, 401–406.

Zhang, Y., and Wang, H. (2012). Integrin signalling and function in immune cells. Immunology *135*, 268–275.

Zhou, S., Cerny, A.M., Zacharia, A., Fitzgerald, K.A., Kurt-Jones, E.A., and Finberg, R.W. (2010). Induction and Inhibition of Type I Interferon Responses by Distinct Components of Lymphocytic Choriomeningitis Virus. J. Virol. *84*, 9452–9462.

Zhu, J., and Paul, W.E. (2008). CD4 T cells: fates, functions, and faults. Blood *112*, 1557–1569.

7 Appendices

7.1 Abbreviations

A	
APC	Antigen-presenting cell
AP1	Activator protein 1
B	
BSA	Bovine serum albumin
C	
CCL	Chemokine ligands
CCR	Chemokine receptors
CD (e.g. CD3)	Cluster of differentiation
cDNA	complementary DNA
CFSE	Carboxyfluorescein succinimidyl ester
CLA	Cutaneous lymphocyte antigen
CTL	Cytotoxic T cell
CpG	Oligonucleotide with cytosine-(phosphate)-guanine motifs
D	
DAI	DNA-dependent activator of IFN-regulatory factors
DC	Dendritic cell
ddH_2O	Double-distilled water
DMSO	Dimethyl sulfoxide
dsDNA	Double-stranded DNA
E	
EAE	Experimental autoimmune encephalitis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMCV	Encephalomyocarditis virus
F	
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FSC	Forward scatter
G	
GALT	Gut-associated lymphoid tissue
H	
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HEV	High endothelial venules
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase

I	
ICAM-1/2	Intercellular Adhesion Molecule 1/2
IFN	Interferon
IFNAR	Interferon-α/β receptor
Ig	Immunoglobulin
IL (e.g. IL-2)	Interleukin
IRF	Interferon regulatory factor
ISGF3	Interferon-stimulated gene factor 3
ISRE	Interferon-sensitive response element
J	
Jak1	Janus kinase 1
L	
LB medium	Lysogeny broth medium
LFA-1	Lymphocyte function-associated antigen 1
LMCV	Lymphocytic choriomeningitis virus
LPAM-1/$\alpha_4\beta_7$	Lymphocyte Peyer's patch adhesion molecule 1
LPS	Lipopolysaccharides
M	
MAdCAM-1	Mucosal addressin cell adhesion molecule 1
MAVS	Mitochondrial antiviral-signaling protein
MDA-5	Melanoma Differentiation-Associated protein 5
MEM-NEAA	Minimum Essential Medium Non-Essential Amino Acids
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
MOI	Multiplicity of infection
M-protein	Matrix protein
MyD88	Myeloid differentiation primary response gene 88
N	
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cells	Natural killer cells
ns	Not significant
O	
OT-I	Transgenic mice with ovalbumin specific $CD8^+$ T cells
OVA	Ovalbumin
P	
PCR	Polymerase chain reaction
PBS	Phophate-buffered saline
PAMPs	Pathogen-associated molecular patterns
PLN	Peripheral lymph node
PP	Peyer’s patch
PTK	Protein tyrosine kinase

R

RA	Retinoic acid
RALDH	Retinaldehyde dehydrogenase
RAR/RXR	Retinoic acid receptor/retinoid X receptor
RARE	Retinoic acid response element
RIG-I	Retinoic acid inducible gene-I
RPMI medium	Roswell Park Memorial Institute medium
RT	Room temperature

S

SSC	Side scatter
ssRNA	Single-stranded RNA
STAT	Signal Transducer and Activator of Transcription
STING	Stimulator of IFN-genes

T

TCR	T cell receptor
TECK/CCL25	Thymus-expressed Chemokine
T_h	T helper
TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TRIF	TIR-domain-containing adapter-inducing interferon-β
Tyk2	Protein-tyrosine kinase 2

U

UV	Ultraviolet

V

VCAM-1	Vascular cell adhesion protein 1
VLE	Very low endotoxin
VSV	Vesicular stomatitis virus

7.2 Publications

7.2.1 Original publications

1. Efficient eradication of subcutaneous but not of autochthonous gastric tumors by adoptive T cell transfer in a SV40 T antigen mouse model (Journal of Immunology, 2010)
   Bourquin C, von der Borch P, Zoglmeier C, Anz D, Sandholzer N, **Suhartha N**, Wurzenberger C, Denzel A, Kammerer R, Zimmermann W, Endres S.
   Journal of Immunology, 2010; 185(4):2580-8. (Journal Impact Factor, JIF, 5.74)

2. Cellular Immunostimulation by CpG-Sequence-Coated DNA Origami Structures.
   Schüller VJ, Heidegger S, Sandholzer N, Nickels PC, **Suhartha N**, Endres S, Bourquin C, Liedl T.
   ACS Nano. 2011 Dec 27;5(12):9696-702. (JIF 9.86)

3. TLR activation excludes circulating naïve $CD8^+$ T cells from gut-associated lymphoid organs in mice.
   Heidegger S, Kirchner SK, Bohn B, **Suhartha N**, Hotz C, Anz D, Sandholzer N, Rüssmann H, Endres S, Bourquin C.
   Journal of Immunology, in revision

4. Virus-associated activation of innate immunity induces rapid degeneration of Peyer's patches
   Heidegger Simon, Anz David, Bohn Bernadette, Stephan Nicolas, **Suhartha Nina**, Sandholzer Nadja, Kobold Sebastian, Hotz Christian, Radtke-Schuller Susanne, Krug Anne, Endres Stefan, Bourquin Carole
   Manuscript in preparation

5. Viral infection modulates expression of the gut homing molecule $\alpha_4\beta_7$ on naïve T cells.

   Suhartha Nina, Heidegger Simon, Sandholzer Nadja, Kirchner Sophie-Kathrin, Bohn Bernadette, Stephan Nicolas, Fertig Marina, Endres Stefan, Bourquin Carole.

   Manuscript in preparation

7.2.2 Oral presentations

1. Lymphocyte migration into gastrointestinal tract: immunotherapy of gastric cancer

   2nd Autumn School Current Concepts in Immunology, Bad Schandau, Germany, 2010

2. *Lymphocyte homing to the gut: IFN-α*
   5th Grako Annual Retreats, Sylvensteinspeicher, 2010

3. *Lymphocyte homing to the gut: LPAM-1 and IFN-α*
   6th Grako Annual Retreats, Schloss Fürstenried, München, 2011

4. IFN-α modulates the expression of the gut-homing molecule receptor $\alpha_4\beta_7$

   24th Meeting of the Swiss Immunology PhD students, Wolfsberg, Switzerland, 2012

7.2.3 Poster

Following TLR activation naive CD8+ T cells are excluded from gut-associated lymphoid tissue in an IL-6 dependent manner

9th Joint Meeting of ICS-ISICR: Cytokines and Interferons: from the bench to the bedside, Florence, Italy, 2011

7.3 Curriculum vitae

Name : Nina Aryani Suhartha

Date of Birth : March 2, 1983

Place of Birth: Aachen

Nationalities : German and Indonesian

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Education

2009 – 2012 **Work towards PhD in Tumor Immunology**, Division of Clinical Pharmacology, University of Munich, Germany

Graduate School 1202 “Therapeutic Oligonucleotides” from the German Research Foundation (DFG)

Topic: Lymphocyte migration into the gut

Supervisor: Prof. Carole Bourquin

11/2011 – 10/2012: research stay funded by the Graduate School 1202 at the University of Fribourg, Switzerland (Prof. Carole Bourquin, Chair of Pharmacology)

2007 – 2009 **Master in Biochemistry**, University of Munich, Germany

Major subjects: Biochemistry, Molecular and Tumor Cell Biology.

Master thesis title: Gene expression analysis of thymocytes lacking Rho GTPases (performed at Biotech and Research Innovation Center, Copenhagen, 08/2008 – 03/2009)

Supervisor: Prof. Cord Brakebusch

2002- 2006	**Bachelor in Biochemistry & Chemistry**, University of Munich, Germany
	Bachelor thesis title: Characterization of She2p, Puf1p-Puf5p, Slf1p, Tis11p in *Saccharomyces cerevisiae* (performed at the Gene Center of the University of Munich)
	Supervisor: Dr. Tung-Gia Du
2001 – 2002	**Preparatory school for the qualification for entrance university in Germany**, Studienkolleg, Munich, Germany
1998 - 2001	**High school diploma**, Sekolah Menengah Negeri 3, Denpasar, Indonesia

7.4 Acknowledgments

On the first place, I would like to thank Prof. Endres for the opportunity to work in his division.

I would especially like to thank Prof. Carole Bourquin for her excellent continuous support and feedbacks during the last 3.5 years.

Many thanks to Nadja Sandholzer for teaching me the adoptive transfer method and for helping me in organ isolations during big experiments. Also thanks to Markus Reinholz for explaining me the surgery method and immunhistochemistry. I also would like to thank Georg Wedekind for taking time to teach me how to use the FACS machine. Also many thanks to Melanie Merk for her tipps in primer design and Moritz Rapp for the PCR and cloning tipps.

I would also like to thank Sebastian Kobold, Yi Zeng, Simon Grassmann for teaching and providing me tipps in transfection and transduction methods.

Thanks to Simon Rothenfußer's group for kindly providing me the viruses.

I would also like to thank my colleagues in Fribourg Marina Fertig, Tina Herbst, Isabelle Wey, Jérôme Widmer, Thibaud Spinetti, Christian Hotz and Esther Mauron for all the feedbacks and fun during my stay in Switzerland.

My special thank to Ralf Löwa for his patience and support during my doctoral thesis.

Last but not least, I would like to thank my family their support.