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Dendritic cell vaccines in tumor immunotherapy: Immune activation strategies with ligands for the Toll-like receptors 7 and 9

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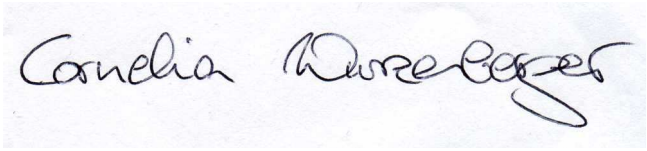
Erklärung

Diese Dissertation wurde im Sinne von § 13 Abs. 4 der Promotionsordnung vom 29. Januar 1998 von Herrn Professor Dr. Stefan Endres und Frau Dr. Dr. Carole Bourquin betreut und von Frau Professor Dr. Angelika M. Vollmar vor der Fakultät für Chemie und Pharmazie vertreten.

Ehrenwörtliche Versicherung

Diese Dissertation wurde selbständig, ohne unerlaubte Hilfe erarbeitet.

München, am 09.12.2008



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Dedicated to my family

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1 Summary

The main function of dendritic cells (DC) is to process antigenic material and present it to other cells of the immune system. As such, they act as mediators between the innate and the adaptive arm of the immune system. Due to their substantial ability to elicit effective memory responses and to break immunological tolerance to tumors, DC-based vaccines have emerged as a promising strategy for the immunotherapy of cancer. In these vaccines, the maturation state of the DC is of crucial importance for the success of vaccination, but the most effective mode of maturation is still a matter of debate.

In the first part of this work, short-term activation of DC with CpG oligonucleotides as Toll-like receptor 9 ligands was investigated as a novel strategy to achieve optimal DC maturation. The kinetics of DC maturation are a critical factor for the induction of efficient immune responses, as immature DC carry the risk of inducing tolerance whereas extensive stimulation may lead to DC unresponsiveness and exhaustion. Short activation of DC for as little as 4 hours (versus 24 to 48 hours) induced fully functional DC that rapidly secreted IL-12p70 and IFN-α, expressed high levels of costimulatory and MHC molecules and efficiently presented antigen to CD4 and CD8 T cells. Furthermore, short-term activated DC overcame immune suppression by regulatory T cells and acquired high migratory potential toward the chemokine CCL21 necessary for DC recruitment to lymph nodes. *In vivo*, vaccination with short-term activated DC induced a strong cytotoxic T-cell response and led to the eradication of tumors. Thus, short-term activation of DC generates fully functional DC for tumor immunotherapy. These results may guide the design of new protocols for DC generation in order to develop more efficient DC-based tumor vaccines.

The second part of this study centers on a novel finding characterizing Toll-like receptor 7 activation in a subpopulation of myeloid DC: in contrast to Toll-like receptor 9 ligands, Toll-like receptor 7 agonists fail to elicit secretion of bioactive IL-12p70 in these DC. This inhibition is highly selective, because other proinflammatory cytokines are efficiently induced by Toll-like receptor 7 stimulation. As IL-12 is an important cytokine for the induction of antitumoral immune responses, the regulation of IL-12p70 production upon Toll-like receptor 7 stimulation was examined by inhibition of signal transduction, use of gene-deficient mice, and a genome-wide expression analysis by microarray. Several genes were identified that modulate IL-12 production in myeloid DC. These results add to the understanding of Toll-like receptor 7 stimulation, which is a promising alternative for the maturation of human DC.

2 Introduction

2.1 Dendritic cells

2.1.1 Functions of dendritic cells

The immune system protects organisms from infection by pathogenic microorganisms. As a first line of defense, physical barriers prevent bacteria and viruses from entering the organism. If a pathogen breaches these barriers, the innate immune system constitutes a second layer of protection. It provides an immediate but non-specific response based on the recognition of conserved molecular patterns of microorganisms via pattern-recognition receptors. Innate immune responses are found in all plants and animals. Vertebrates have however acquired a third line of defense, the adaptive immune system, which is activated by the innate immune response. This immune response is “adapted” during an infection to improve the recognition of the pathogen. Adaptive immunity allows for a stronger immune response as well as the development of immunological memory that enables the rapid elimination of microorganisms upon reinfection. Both innate and adaptive immunity depend on the recognition of invading microorganisms as non-self. While the innate immune system recognizes conserved microbial structures, the adaptive immune response is antigen-specific, meaning that it detects specific features of each pathogen. Dendritic cells, macrophages, granulocytes and natural killer cells represent the most important cells of the innate immune system, whereas T and B cells are responsible for adaptive immune responses.

Antigen-presenting cells that process antigenic material and present it on their surface to other cells of the immune system act as mediator between the innate and the adaptive arm of the immune system. In peripheral lymphoid organs, there are three main types of antigen-presenting cells that can activate T cells – dendritic cells, macrophages, and B cells. The most potent of these are dendritic cells (DC), which are present in small numbers in tissues that are in contact with the external environment. Most DC reside in the skin, where a specialized DC type, now called Langerhans cell, was first described by Paul Langerhans in the late 19th century (Langerhans, 1868), and the mucosa of the respiratory and the intestinal tract. Langerhans cells can also be found in an immature state in the blood. At certain stages of development they grow branched projections, the dendrites, that gave the cell its name (Figure 2.1). Although first described by Paul Langerhans, it was not until 1973 that the term "dendritic cells" was coined by Ralph M. Steinman and Zanvil A. Cohn (Steinman and Cohn, 1973). In 2007, Steinman was awarded the Albert Lasker Medical Research Award for his discovery.

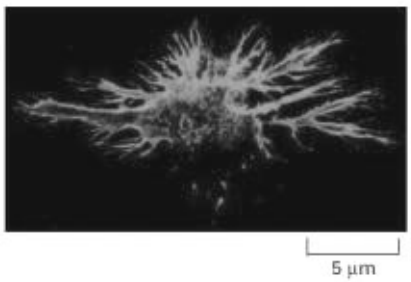

Figure 2.1: Immunofluorescence micrograph of a dendritic cell (from Alberts, 2008).
The distinct long processes called dendrites, which give the cell its name, are most pronounced in mature DC. This cell has been labeled with a monoclonal antibody that recognizes a surface antigen.

DC are derived from hematopoietic bone marrow progenitor cells. These progenitor cells initially transform into immature DC which constantly sample the surrounding environment for pathogens. DC use phagocytosis, endocytosis, pinocytosis, and specific receptors to capture microbial pathogens, dead or dying cells, immune complexes, and other antigens for immune presentation (Rossi and Young, 2005). Once they have come into contact with a presentable antigen, they mature and migrate to regional lymph nodes. Upon maturation, peptides derived from the captured antigens are presented by major histocompatibility complex (MHC) molecules at the cell surface. In the lymph nodes, the now-mature DC induce an immune response by activating T cells with receptors that recognize the foreign peptide – MHC complex on the DC surface. Antigen-presenting cells usually present antigens acquired from the extracellular environment on class II MHC complexes binding to T-cell receptors on $CD4^+$ T helper cells, whereas class I MHC molecules bear peptides synthesized in the cytosol and activate $CD8^+$ cytotoxic T cells. DC, however, are capable of cross-presenting exogenous antigens on class I MHC complexes, thus allowing the induction of a cytotoxic T-cell response to pathogens that do not infect DC (Groothuis and Neefjes, 2005).

Although effector T cells are triggered when their antigen-specific receptors bind to peptide - MHC complexes, ligation of the T-cell receptor does not, on its own, stimulate naïve T cells to proliferate and differentiate into effector T cells. The antigen-specific clonal expansion of naïve T cells requires a second costimulatory signal, which must be delivered by the same antigen-presenting cell on which the T cell recognizes its antigen. The best-characterized costimulatory molecules are the structurally related glycoproteins CD80 (B7.1) and CD86 (B7.2), whose expression on DC is upregulated upon maturation and which bind to CD28 on T cells. CD40 on the other hand provides a sustained or modifying co-stimulatory signal driving clonal expansion and differentiation of T cells by binding to CD40L on T cells. The effects of this receptor-ligand interaction are bidirectional, with both the T cell and the DC receiving activating signals (Janeway, 2005).

The requirement for the simultaneous delivery of antigen-specific and costimulatory signals in the activation of T cells means that only DC that migrate into lymphoid tissues after being activated by ingesting pathogens can initiate T-cell responses. This is important to prevent destructive immune responses to self tissues. Antigen binding in the absence of costimulation not only fails to activate T cells, it leads instead to the induction of anergic T cells, which are refractory to activation, or of regulatory T cells, which suppress immune responses (Hawiger et al., 2001; Steinman et al., 2003).

DC are constantly in communication with other cells in the body. This communication can take the form of direct cell-to-cell contact based on the interaction of cell-surface proteins or occur at a distance via cytokines. One of the most important cytokines secreted by DC is IL-12, which activates natural killer cells and polarizes T helper cells towards a T_h1 phenotype. However, depending on the type of DC and the maturation signal the DC receives, the cytokines produced may vary.

2.1.2 Dendritic cell subsets

Since the initial description of DC by Steinman and Cohn, it has become evident that DC are heterogeneous and consist of many distinct DC subtypes. Although all DC are capable of antigen uptake, processing and presentation to naïve T cells, the DC subtypes differ in location, migratory pathways, detailed immunological function and dependence on infections or inflammatory stimuli for their generation. DC subtypes in the steady state, in the absence of infection, include type I interferon-producing plasmacytoid DC (pDC), migratory DC located in peripheral tissues such as Langerhans cells, and lymphoid tissue-resident myeloid DC (mDC), also called conventional DC (Shortman and Liu, 2002). The latter group can further be subdivided into $CD8^+$ and $CD8^-$ mDC in mice, according to their expression of CD8α on the cell surface (Vremec et al., 2000). These two subsets differ in their immune functions, with only the $CD8^+$ mDC being able to cross-present exogenous antigens on MHC I complexes (den Haan et al., 2000). Lymphoid tissue-resident DC collect and present antigens in a single lymphoid organ without migrating. In contrast, migratory DC are the classical “text-book” DC that act as sentinels in peripheral tissues and migrate to the lymph nodes through the lymphatics, bearing antigens from the periphery. DC can also be divided into conventional DC which already have a dendritic form and exhibit DC function in steady state, and precursors of DC (pre-DC), which require further development to acquire a dendritic form and full DC function (Shortman and Naik, 2007). Different pre-DC populations produce different subtypes of DC, and further development of DC often requires a microbial or inflammatory stimulus. In this model, pDC are an example of pre-DC. DC, like other blood

cells, derive from hematopoietic stem cells, and both common myeloid and common lymphoid progenitor cells have the ability to form pDC, $CD8^+$ and $CD8^-$ mDC (Manz et al., 2001; Wu et al., 2001).

Plasmacytoid DC represent a rare but important cell type and are characterized by the production of high amounts of type I interferons in response to viruses. In 1958, Lennert and Remmele described the presence of cells with a plasma cell-like morphology located in what are now known to be the T-cell zones of human lymph nodes and spleen (Lennert and Remmele, 1958). More than 40 years later, in 1999, it was determined that natural interferon-producing cells were identical to the plasmacytoid subset of DC (Cella et al., 1999; Siegal et al., 1999) and the description of human pDC was followed by descriptions of pDC in mice (Asselin-Paturel et al., 2001; Nakano et al., 2001). Immature pDC are present in the blood, bone marrow and secondary lymphoid organs and can be recruited to the skin, the cerebrospinal fluid, the gut and the lung under stimulatory conditions. Moreover, pDC are known to infiltrate many tumors.

Interferon (IFN) was the earliest described cytokine and first discovered for its ability to "interfere" with viral replication (Isaacs and Lindenmann, 1957). Since then, we have learned that interferons are a large family of genes comprising at least three different major subtypes: the type I interferons which include the IFN-α family and IFN-β, -κ, and –ω, the single type II interferon, IFN-γ, and the IFN-λs, which are also known as IL-28 and IL-29 (Fitzgerald-Bocarsly and Feng, 2007). Type I interferons are known to have a number of effects on different cells of the immune system: they act as a survival factor for pDC and can enhance the maturation of mDC, while also upregulating the cytokine and chemokine response in DC in an autocrine or paracrine manner (Fitzgerald-Bocarsly and Feng, 2007). They lead to the activation of natural killer cells and are able to induce the production of IFN-γ by $CD4^+$ T cells, thus exerting a strong T_h1-bias on T-cell differentiation (Brinkmann et al., 1993). IFN-α was also demonstrated to enhance the antibody response to soluble antigen (Le Bon et al., 2001). Thus, type I interferons are not only important mediators of anti-viral immunity but also provide a link between the innate and adaptive immune system as do the dendritic cells.

2.1.3 Isolation or generation of dendritic cells

In the peripheral blood of healthy individuals, DC represent a minor fraction of mononuclear cells (less than 1%). The capacity of DC to efficiently initiate and regulate lymphocyte-mediated immunity has led to the study of DC as cellular vaccine adjuvants for

immunizations and for the immunotherapy of cancer (Schuler and Steinman, 1997). However, the feasibility of using DC in immunotherapy is limited by the small number of DC that can be isolated from peripheral blood. Therefore, the majority of applications currently rely on the *in vitro* generation of DC from precursor cells. In the field of DC biology, by far the most work has been carried out using mDC obtained by culturing peripheral blood monocytes or bone marrow cells in the presence of GM-CSF and IL-4. However, these cells do not show the same heterogeneity in phenotype and function as *ex vivo* DC, and the preferential expansion of only myeloid-related DC may not necessarily provide the appropriate signals that would result in the generation of optimal T-cell responses (Mortarini et al., 1997). In contrast, the hematopoietic growth factor fms-like tyrosine kinase 3 ligand (Flt3L) induces the proliferation and survival of hematopoietic progenitor and stem cells (Lyman and Jacobsen, 1998). The *in vivo* administration of Flt3L expands the number of both myeloid and plasmacytoid DC subsets in mice and men (Maraskovsky et al., 1996; Maraskovsky et al., 2000) and allows the isolation of large numbers of DC from blood that efficiently prime immune responses. This has also been shown in cancer patients, where Flt3L-expanded DC primed immune responses to cancer antigens (Davis et al., 2006). In a murine tumor model, the synergism of Flt3L-derived myeloid and plasmacytoid DC proved to be more effective in increasing the levels of antigen-specific cytotoxic T lymphocytes (CTL) and in inducing an enhanced antitumor response than immunizations with either DC subset alone (Lou et al., 2007). Culture of murine bone marrow cells with Flt3L was described to generate bona fide counterparts of splenic *ex vivo* DC subsets, those being plasmacytoid DC, $CD8^+$ and $CD8^-$ myeloid DC (Naik et al., 2005).

2.1.4 Dendritic cell vaccines

Vaccination against infectious diseases by injecting antigens mixed with adjuvants targeted and activated DC *in situ* long before the existence of these cells was known. Although this approach is highly effective against viral and bacterial pathogens in a prophylactic setting, therapeutic vaccines against cancer have yet to meet expectations (Zhong et al., 2007). Factors that contribute to the limited efficacy of cancer vaccines are the preexisting cancer load (i.e. the tumor), the need to stimulate the cellular arm of the immune response to eradicate established tumors, and the fact that immune responses are suppressed in cancer patients (Gilboa, 2007). DC have emerged as a promising tool due to their ability to present tumor antigens and to initiate an immune response, induce memory cells and break immunological tolerance against the tumor. The most popular approach in tumor immunology is to immunize cancer patients with autologous, patient-derived DC loaded with tumor antigens *ex vivo*. Figure 2.2 shows an overview of current vaccine protocols, where DC are

mainly derived from monocytes or $CD34^+$ precursor cells, cultured in the presence of various cytokine mixtures to produce immature DC, and loaded with antigen either before or following DC maturation. The clinical trials so far have provided valuable information on DC-based therapy: it is safe and well tolerated with side effects generally limited to induration at the injection site and a mild fever. Furthermore, the importance of the quality of the DC was emphasized, especially with regard to their migratory capacity and their ability to induce potent T-cell responses (Tacken et al., 2007). Although conclusions from the first clinical trials were fairly optimistic due to the fact that the majority of patients developed T cell-mediated antitumor immune responses, recent reviews, however, began to express skepticism regarding the clinical effectiveness of autologous tumor antigen-loaded DC (Zhong et al., 2007). Thus, all the steps depicted in Figure 2.2 are subject to optimizing strategies in order to influence the efficacy of DC-based tumor vaccines. Problems that remain to be solved despite extensive research on DC vaccination include the optimal DC subtype to be used, the most efficient antigen-loading and activation of DC, and the best route of DC administration. A list of published trials on DC-based cancer vaccines since the first clinical study in 1995 (Mukherji et al., 1995) has been made available on the Internet (http://www.mmri.mater.org.au/) and allows a comparison of strategies pursued so far.

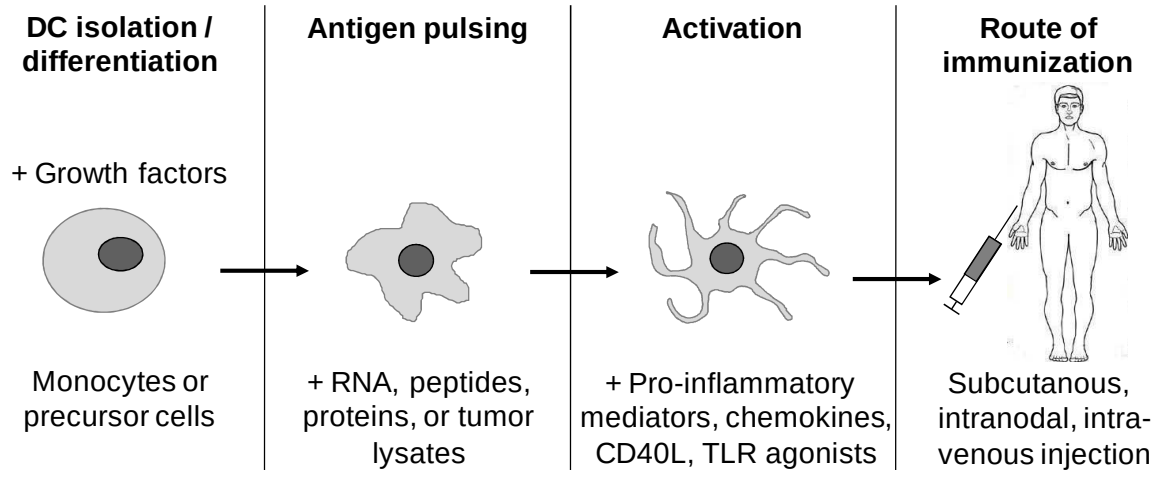

Figure 2.2: DC-based vaccines using *ex vivo* loaded DC to induce immunity.
Further research is needed to determine the optimal procedures for each step of the generation of DC vaccines, starting with the isolation or generation of DC, the optimal method to load DC with antigens, the most efficient maturationand activation stimulus, and the most efficient application of matured DC.

2.2 Toll-like receptors

2.2.1 The Toll-like receptors and their ligands

Toll-like receptors (TLRs) are of interest to immunologists because of their front-line role in the initiation of innate immunity against invading pathogens. They are evolutionarily conserved molecules that were originally described to stimulate the production of antimicrobial proteins in *Drosophila melanogaster* (Lemaitre et al., 1996). Mammalian TLRs are a family of membrane proteins that trigger innate immune responses through nuclear factor-κB (NF-κB)-dependent and IFN-regulatory factor (IRF)-dependent signaling pathways (Trinchieri and Sher, 2007). The TLR family members are pattern-recognition receptors that collectively recognize lipid, carbohydrate, peptide and nucleic-acid structures expressed by different groups of microorganisms. Some TLRs are found at the cell surface (TLR1, 2, 4, 5, and 6), while others are expressed on the membrane of endocytic vesicles (TLR3, 7, 8, and 9). Other groups of pattern-recognition receptors, such as the C-type lectin-like molecules on the cell surface and the cytosolic NLR (nucleotide-binding oligomerization domain-like receptors) and RIG-I-like helicases (retinoic-acid-inducible gene I), also participate in the binding and uptake of microbial components and can cooperate with the Toll-like receptors in the innate immune response to pathogens (Trinchieri and Sher, 2007).
Figure 2.3 shows a schematic representation of the different Toll-like receptors and their main target transcription factors.

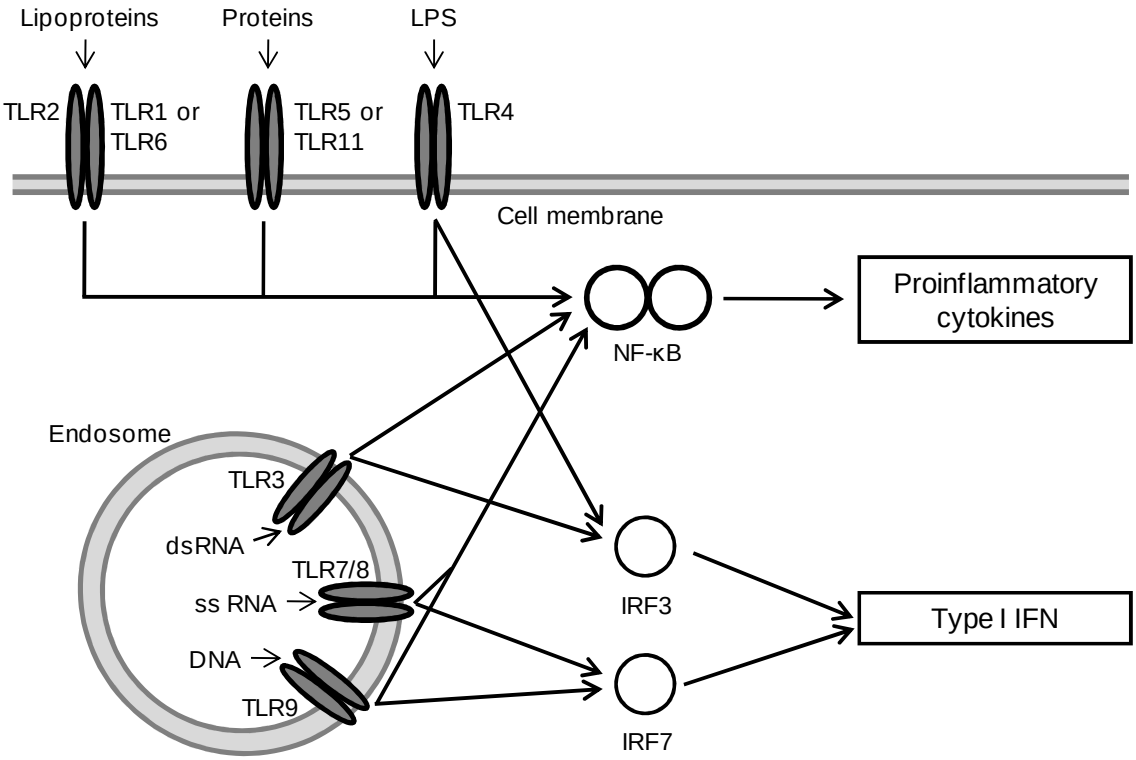

Figure 2.3: Overview of the Toll-like receptors and their main target transcription factors.
The family of Toll-like receptors includes cell-surface receptors (TLR1, 2, 4, 5, 6, and 11) that recognize microbial proteins and lipids, and endosomal receptors (TLR3, 7, 8, and 9) which bind to nucleic acids. Both types induce the activation of the NF-κB signaling pathway and the production of type I interferons. This schematic overview shows only receptors and main target transcription factors that differ between the different Toll-like receptors.
ds double-stranded, *IFN* interferon, *IRF* IFN-regulatory factor, *LPS* lipopolysaccharide, *NF-κB* nuclear factor-κB, *ss* single-stranded, *TLR* Toll-like receptor.

After the characterization of TLR4 as the first mammalian TLR, 12 other structurally related proteins were identified to date and named Toll-like receptors, although not all of them are conserved between species. Functionally, a critical role of TLR4 in the recognition of lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, was initially characterized (Poltorak et al., 1998). Further research subsequently identified ligands for most of the other TLRs as shown in Table 2.1. Accumulating evidence points to the possibility of endogenous molecules acting as TLR agonists, which is now thought to have an important role in the regulation of inflammation, but might also be responsible for certain autoimmune disorders. While TLR1, 2, and 6 recognize ligands by forming heterodimers, the other TLRs appear to function as homodimers, although the exact mechanisms of recognition have not yet been determined. Species-specific TLRs include TLR8, which is thought to be non- functional in murine immune cells, TLR10, which has only been identified in humans, and TLRs 11 to 13, that probably exist only in mice.

Table 2.1: Toll-like receptors and their ligands (adapted from Ishii et al., 2006)**.**

TLR	Exogenous ligands	Origin	Endogenous ligands
TLR1 + TLR2	Triacetylated lipopeptides	Bacteria	N.D.
TLR6 + TLR2	Diacetylated lipopeptides, Zymosan	Bacteria, fungi	N.D.
TLR3	Double-stranded RNA	Viruses	mRNA
TLR4	LPS, Taxol, fusion protein	Bacteria, plants, viruses	HSP, fibrinogen, fibronectin
TLR5	Flagellin	Bacteria	N.D.
TLR7 (TLR8)	Single-stranded RNA, Imidazoquinolines (Imiquimod, Resiquimod), Loxoribine (guanosine analog)	Viruses, synthetic	RNA
TLR9	CpG DNA Hemozoin	Bacteria, viruses, synthetic, Plasmodia	Chromatin immune complexes
TLR10	N.D.		N.D.
TLR11	N.D. Profilin-like protein	Uropathogenic bacteria, Toxoplasma	N.D.
TLR12	N.D.		N.D.
TLR13	N.D.		N.D.

HSP heat shock protein; *N.D.* not determined. Original references are listed in the review article (Ishii et al., 2006).

Toll-like receptors are expressed by a variety of immune and non-immune cells. Myeloid cells constitutively express TLR1 and TLR6, macrophages preferentially express TLR2, 3, 4 and 8 (in humans), and B cells express TLR7, 9 and 10. $CD4^+$ T cells and natural killer cells were reported to express TLR2, and some publications describe the expression of TLR2, 3, 5, and 9 on T cells (Ishii et al., 2006; Kabelitz, 2007). TLR expression on DC differs considerably between mice and men: human myeloid DC and monocyte-derived DC express all TLRs with the exception of TLR9 and therefore recognize a variety of pathogenic molecular patterns. The repertoire of TLRs expressed by plasmacytoid DC (TLR1, 6, 7, and 9) complements the TLRs on mDC, enabling the two subsets of DC to respond to distinct microorganisms. Murine DC subsets exhibit a broader distribution of TLR expression, with mouse pDC expressing all

TLRs but TLR3 and 4. In contrast to human DC, TLR9 and 7 are expressed by most subsets of DC, with the exception of $CD8^+$ DC, which lack TLR7. The expression pattern of TLRs on DC is reviewed in Mazzoni and Segal, 2004.

While Toll-like receptors have been known only for the last decade, their ligands have been used as adjuvant components in vaccine formulations for a long time. Due to their efficacy in activating immune cells, TLR ligands are currently being used in clinical trials as adjuvants in protective vaccinations against bacterial and viral infections and in anti-tumor vaccines. However, recent studies suggest that tumor cells also bear TLRs and that TLR signaling may promote tumor growth and immune evasion (Huang et al., 2005; Kelly et al., 2006).

2.2.2 Signaling pathways of Toll-like receptors

TLRs belong to a superfamily called the Toll/IL-1 receptor (TIR) family. They are type I transmembrane receptors characterized by a highly variable extracellular region containing a leucine-rich repeat domain involved in ligand binding and an intracellular tail containing a highly conserved TIR homology domain which mediates homo-and heterophilic interactions between TLRs and TIR-containing adapters. Most TLRs signal through a common pathway, the MyD88 (myeloid differentiation primary response gene 88) -dependent pathway (Figure 2.4). Following TLR ligation, the adaptor protein MyD88 is recruited to the receptors by TIR-TIR interactions. The observation that MyD88-deficient mice still react to LPS suggested other adaptors involved in TLR4 signaling (Kawai et al., 2001). The discovery of the second TIR domain-containing adaptor, MyD88 adaptor-like (Mal), indicated a potential specificity in the signaling pathways of different TLRs, particularly when Mal was shown to have a role in TLR2 and TLR4 signaling but not in responses to other TLR ligands. Signaling via MyD88 as well as Mal eventually activates the IKK (inhibitor of κB kinase) complex. The IKK complex catalyzes IκB (inhibitor of κB) phosphorylation and degradation, thus allowing NF-κB to translocate into the nucleus. In addition, upstream kinases for p38 and JNK are activated, leading to the activation of AP-1 (activated protein 1). NF-κB and AP-1 control inflammatory responses by inducing proinflammatory cytokine expression.

TLR3 is the only TLR which signals only through the MyD88-independent pathway. A third adaptor, termed TIR-related adaptor protein inducing interferon (TRIF), is recruited by both TLR4 and TLR3, and is responsible for the activation of IRF3. The discovery of TRIF provided the first molecular basis for why TLR3 and TLR4 are able to induce the production of IFN-β. The fourth adaptor to be described was named TRIF-related adaptor molecule (TRAM), and acts only in TLR4 signaling, where it interacts with TRIF. These current articles

review Toll-like receptor signal transduction (Chen et al., 2008; Doyle and O'Neill, 2006; Krishnan et al., 2007; O'Neill, 2006). Figure 2.4 shows a schematic representation of the TLR adaptors and most important signaling molecules.

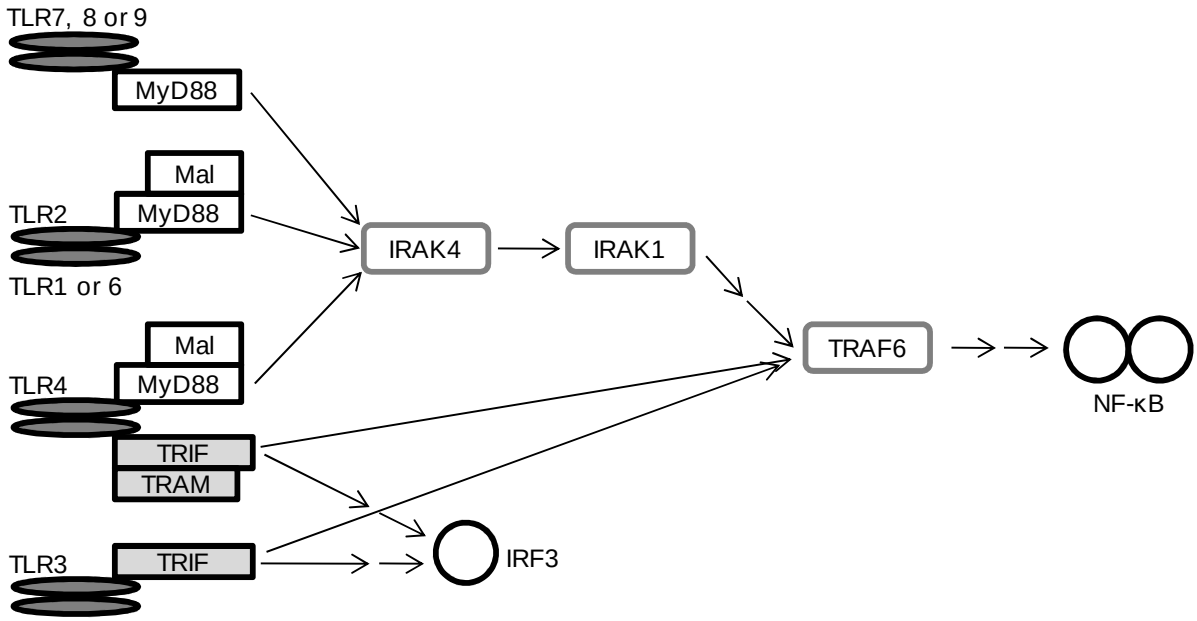

Figure 2.4: Overview of the TLR adaptors and main signaling molecules.
On TLR ligation, TLR4, 7, 8, and 9, and TLR2-TLR1 or TLR2-TLR6 heterodimers recruit MyD88 to their TIR domains. MyD88 binds IRAK4, which phosphorylates IRAK1, which in turn binds TRAF6 and other kinases. Subsequent ubiquitination and phosphorylation initiates the activation of the IKK complex and via IκB degradation the translocation of NF-κB into the nucleus. In the MyD88-independent pathway, TLR3 recruits TRIF directly to its TIR domain while TLR4 recruits TRAM which in turn recruits TRIF. TRIF binds TRAF6 directly in an IRAK1/4-independent manner. TRIF can also activate IRF3. *IRAK* IL-1R-associated kinase, *IRF* interferon regulatory factor, *MyD88* myeloid differentiation primary response gene 88.

Stimulation of cells through TLR-dependent pathways converges at the activation of the transcription factors NF-κB, IRF3, 7, and 5, and AP-1. These transcription factors collaborate in the production of a large number of cytokines, whose promoters contain multi-transcription factor binding sites leading to a highly specific activation. While NF-κB and AP-1 induce the expression of most proinflammatory cytokines (IL-1β, IL-6, IL-10, IL-12, IP-10, and TNF), the regulation of type I interferons is regulated by the IRF family of transcription factors. However, the IFN regulation seems to differ between cell types. In mDC, TLR signal transduction via TLR3 or 4 and TRIF leads to the expression of IFN-β, which in turn can induce the production of IRF7 and thus the secretion of IFN-α in an autocrine or paracrine manner (Au et al., 1998). Regulation of IFN production in pDC, however, is entirely different. Comparisons between IRF3$^{-/-}$ pDC and IRF7$^{-/-}$ pDC revealed that IFN production by TLR7, 8, and 9 was normal in IRF3$^{-/-}$ cells, but completely ablated in IRF7$^{-/-}$ cells, indicating that IRF7 is essential for type I interferon induction in pDC (Honda et al., 2005).

2.3 Objectives

In the field of cancer immunotherapy, dendritic cells have emerged as a powerful tool to initiate T-cell responses, induce immunological memory and break immunological tolerance to tumors. The ability of DC-based vaccines to stimulate cytotoxic T cells and to polarize T helper cells towards a T_h1 profile highlights their potential in the immunotherapy of cancer. However, despite rapid progress in the field of DC biology, clinical responses to DC vaccination have yet to meet expectations. Current research addresses the optimization of the different steps involved in the generation of DC vaccines. In the present study, DC were differentiated in the presence of the hematopoietic growth factor Flt3L (FL-DC) in order to generate both myeloid and plasmacytoid dendritic cell subsets. The first focus of this study was the development of an effective strategy for DC maturation. The kinetics of DC maturation are a critical factor, as immature DC carry the risk of inducing tolerance whereas extensive stimulation may lead to DC unresponsiveness and exhaustion. To optimize the DC maturation process, FL-DC were activated for a short versus a prolonged period by the TLR9 ligand CpG and analyzed for their capacity to elicit immune responses *in vitro* and *in vivo*.

In addition, DC maturation with TLR7 ligands was analyzed in more detail. This strategy is a more promising approach when dealing with human cells because of the restricted expression pattern of TLR9 on human DC. Although TLR7 and TLR9 stimulation are thought to activate the same intracellular signaling pathways, mDC generated by the Flt3L DC culture selectively fail to secrete detectable amounts of IL-12p70 after TLR7 stimulation. Due to the imperative of IL-12p70 secretion for the induction of efficient cell-mediated and T_h1-based immune responses, the mechanisms underlying the deficient IL-12p70 production upon TLR7 stimulation were examined by means of inhibition of signal transduction, knock-out mice, and a genome-wide expression analysis by microarray.

3 Materials and methods

3.1 Materials

3.1.1 Technical equipment

Agilent Bioanalyzer 2100	Agilent, Palo Alto, USA
Alpha Imager (gel documentation)	Alpha Innotech, San Leandro, USA
Balance (LP 6209)	Sartorius, Göttingen, Germany
BeadStation 500 (microarray reader)	Illumina, Essex, United Kingdom
Cell culture CO_2 incubator (BD 6220)	Heraeus, Hanau, Germany
Cell culture laminar flows	Heraeus, Hanau, Germany
Centrifuge 5424	Eppendorf, Hamburg, Germany
FACSCalibur	Becton Dickinson, San Jose, USA
Fluo-Link (UV irradiation)	Vilber Lourmat, Marne La Vallée, France
Gel electrophoresis systems	Bio-rad, Munich, Germany
MiniMACS, QuadroMACS	Miltenyi Biotec, Bergisch Gladbach, Germany
Mithras LB940 multilabel plate reader	Berthold Technologies, Bad Wildbad, Germany
Multifuge 3L-R	Heraeus, Hanau, Germany
Nanodrop ND-1000	NanoDrop, Wilmington, USA
Neubauer hemocytometer	Optik Labor Frischknecht, Balgach, Germany
pH meter	WTW, Weilheim, Germany
Power Supply 200/2.0	Bio-rad, Munich, Germany
Refrigerators (4℃, -20℃)	Bosch, Gerlingen-Schil lerhöhe, Germany
Refrigerators (-80℃)	Thermo Scientific, Waltham , USA
Shaker	NeoLab, Heidelberg, Germany
Thermocycler T3	Biometra, Göttingen, Germany
Thermomixer	Eppendorf, Hamburg, Germany
Vortex VF2	Janke & Kunkel, Staufen, Germany

3.1.2 Chemicals, reagents and buffers

Agarose LE	Biozym, Hess. Oldendorf, Germany
Aqua ad injectabilia	Braun Melsungen AG, Melsungen, Germany
Bovine serum albumine (BSA)	Sigma Aldrich, Steinheim, Germany
Brefeldin A	Sigma Aldrich, Steinheim, Germany
Chloroform	Sigma Aldrich, Steinheim, Germany
Dimethyl sulfoxide (DMSO)	Sigma Aldrich, Steinheim, Germany
Dulbecco's PBS (1x)	PAA, Pasching, Germany

Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich, Steinheim, Germany
FACSFlow, FACSSafe	Becton Dickinson, Heidelberg, Germany
Heparin-Natrium Braun 25000 I.E./5 ml	B. Braun Melsungen AG, Melsungen, Germany
Illumina TotalPrep RNA Amplification Kit	Applied Biosystems, Foster City, USA
Isoflurane (Forene®)	Abbott, Zug, Switzerland
Isopropanol (70 Vol%)	Apotheke Innenstadt, LMU Munich
Mouse-6 v1.1 Expression Bead Chips	Illumina, Essex, United Kingdom
Paraformaldehyde (PFA)	Sigma Aldrich, Steinheim, Germany
PharmLyse (10x) RBC lysis	Becton Dickinson, Heidelberg, Germany
Sigma fast OPD tablet sets	Sigma Aldrich, Steinheim, Germany
Sodium azide (NaN_3)	Sigma Aldrich, Steinheim, Germany
Sodium chloride (NaCl 0.9%)	Baxter S. A., Lessines, Belgium
Sulfuric acid (H_2SO_4, 2N)	Apotheke Innenstadt, LMU Munich
TMB Substrate Reagent Set	Becton Dickinson, Heidelberg, Germany
Trizol reagent	Invitrogen, Carlsbad, USA
Trypan blue	Sigma Aldrich, Steinheim, Germany
Trypsin (10x)	PAA, Pasching, Austria
Tween 20	Roth, Karlsruhe, Germany

MACS buffer

2 mM EDTA
10% FCS
in PBS

Fixation buffer for FACS samples

2% PFA
in PBS

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 20
in PBS

3.1.3 Kits

Cell proliferation ELISA, BrdU	Roche, Mannheim, Germany
Cell Trace CFSE Cell Proliferation Kit	Invitrogen/Molecular Probes, Eugene, USA

Cytokine ELISA sets

IFN-γ murine	BD Bioscences, San Diego, USA
IL-4 murine	BD Bioscences, San Diego, USA
IL-6 murine	BD Bioscences, San Diego, USA
IL-10 murine	BD Bioscences, San Diego, USA
IL-12p40 murine	BD Bioscences, San Diego, USA
IL-12p70 murine	BD Bioscences, San Diego, USA
IP-10 murine	R & D Systems, Minneapolis, 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
Detection of murine immunoglobulins:	
Anti-IgG (polyclonal, goat anti mouse)	Southern Biotech, Birmingham, USA
Anti-IgG1 (polyclonal, goat anti mouse)	Southern Biotech, Birmingham, USA
Anti-IgG2a (KLH/G2a-1-1)	Southern Biotech, Birmingham, USA

MACS cell separation

CD4 Micro Beads	Miltenyi Biotec, Bergisch Gladbach, Germany
CD11c MicroBeads	Miltenyi Biotec, Bergisch Gladbach, Germany
CD45R/B220 MicroBeads	Miltenyi Biotec, Bergisch Gladbach, Germany
Regulatory T Cell Isolation Kit	Miltenyi Biotec, Bergisch Gladbach, Germany
LS, MS and LD columns	Miltenyi Biotec, Bergisch Gladbach, Germany

3.1.4 Cell culture reagents and media

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
Hank's balanced salt solution (HBSS)	PAA, Pasching, Austria
L-glutamine 200mM	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
Ovalbumin from chicken egg	Sigma-Aldrich, Steinheim, Germany
$OVA_{257-264}$ peptide (SIINFEKL)	Metabion, Martinsried, Germany
Roswell Park Memorial Institute (RPMI) 1640 medium	PAA, Pasching, Austria
SB203580 (p38 inhibitor)	Invivogen, San Diego, USA
Sodium pyruvate	PAA, Pasching, Austria
Survivin protein	Generated in HEK293 cells, kindly provided by Jiwu Wei and Raffaela Tyroller
T1Db peptide (SAINNYAQKL)	Metabion, Martinsried, Germany
VLE RPMI 1640 medium (very low endotoxin)	Biochrom, Berlin, Germany

C26 medium

10% FCS
2 mM L-glutamine
100 IU/ml penicillin
100 µg/ml streptomycin
in DMEM

DC medium

10% FCS
2 mM L-glutamine
100 IU/ml penicillin
100 µg/ml streptomycin
in RPMI 1640

DC complete medium

10% FCS
2 mM L-glutamine
100 IU/ml penicillin
100 µg/ml streptomycin
1 mM sodium pyruvate
1% non-essential amino acids (MEM-NEAA)
3.75×10^{-4} % β-mercaptoethanol
in VLE RPMI 1640

Cytokines, growth factors, TLR ligands and stimuli

anti-CD3 (clone 500A2, syrian hamster IgG2,κ)	BD Biosciences, San Diego, USA
CCL21, mouse recombinant	tebu-bio, Offenbach, Germany
CpG 1826 (CpG) (5'-TCCATGA*CG*TTCCTGA*CG*TT-3')	Coley Pharmaceuticals, Langenfeld, Germany
Flt-3 ligand (Flt3L), human recombinant	tebu-bio, Offenbach, Germany
Granulocyte-macrophage colony-stimulating factor (GM-CSF), mouse recombinant	tebu-bio, Offenbach, Germany
Interleukin-4 (IL-4), mouse recombinant	tebu-bio, Offenbach, Germany
Resiquimod (R848)	Alexis Biochemicals, Lausen, Switzerland

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

3.1.5 FACS antibodies and MHC class I pentamers

Description	Isotype	Clone	Company
anti-CD4	Rat (DA) IgG2a,κ	RMA4-5	BD Biosciences
anti-CD8a	Rat (LOU/Ws1/M) IgG2a,κ	53-6.7	BD Biosciences
anti-CD11b	Rat (DA) IgG2b,κ	M1/70	BD Biosciences
anti-CD11c	Armenian Hamster IgG1a, λ2	HL3	BD Biosciences
anti-CD25	Rat (Lewis) IgM, κ	7D4	BD Biosciences
anti-CD40	Armenian Hamster IgM, κ	HM40-3	BD Biosciences
anti-CD45R/B220	Rat IgG2a,κ	RA3-6B2	BD Biosciences
anti-CD69	Armenian Hamster IgG1, λ3	H1.2F3	BD Biosciences
anti-CD80 (B7-1)	Armenian Hamster IgG2, κ	16-10A1	BD Biosciences
anti-CD86 (B7-2)	Rat (Louvain) IgG2a,κ	GL1	BD Biosciences
anti-CD197 (CCR7)	Rat IgG2a,κ	4B12	BioLegend
anti-H2D^{b} (MHC I)	Mouse (Balb/c) IgG2b,κ	KH95	BD Biosciences
anti-H2D^{d} (MHC I)	Mouse (C3H/HeJ) IgG2a,κ	34-2-12	BD Biosciences
anti-Ly6C	Rat IgM, κ	AL-21	BD Biosciences
anti-MHC II	Rat IgG2b	NIMR-4	Southern Biotech

isotype controls		BD Biosciences
Pro5-$OVA_{257-264}$-H2K^b	$OVA_{257-264}$-H-2K^b pentamers	Proimmune

3.1.6 Software

Adobe Creative Suite	Adobe Systems, San Jose, USA
CellQuest	BD Biosciences, San Diego, USA
FlowJo	Tree Star, Ashland, USA
Microsoft Office	Microsoft, Redmond, USA
NCBI Entrez Gene	www.ncbi.nlm.nih.gov/sites/entrez?db=gene
Pathway Studio	Ariadne Genomics, Rockville, USA
SPSS	SPSS, Chicago, USA

3.2 Cell culture

3.2.1 General culture conditions

All cell lines were cultured in tissue culture flasks at 37℃ in a humidified atmosphere and 5% CO_2. All manipulations were performed with sterile reagents under a laminar flow hood. Cell concentration and viability was determined by Trypan blue staining. Trypan blue is not absorbed in a viable cell; however, it traverses the membrane in a dead cell. Hence, dead cells are shown as a distinctive blue color under the microscope. Cell suspensions were mixed with 0,25% Trypan blue in PBS at appropriate dilutions and counted in a Neubauer hemocytometer under the microscope. Cell number was calculated as follows:

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

3.2.2 C26 colon carcinoma cell line

The murine colon carcinoma cell line C26 (Cell Lines Service, Heidelberg, Germany; first described in Griswold and Corbett, 1975) was grown in C26 medium and split in a 1:2 dilution three times a week. Cells were detached with cell scrapers, centrifuged (370 g, 7 min, 4℃), resuspended with a 20 G needle in fresh medium and transferred to culture flasks.

3.2.3 Dendritic cells

3.2.3.1 Generation of bone-marrow-derived dendritic cells

Immature murine DC were generated from bone marrow as described(Brasel et al., 2000; Brawand et al., 2002), with some modifications. Briefly, bone marrow cells were isolated as described in chapter 3.4.2.3, p. 35 and diluted to 1×10^6 cells/ml in DC medium supplemented with GM-CSF (20 ng/ml) and IL-4 (20 ng/ml) to generate myeloid DC (GM-DC). On day 4, fresh medium with GM-CSF and IL-4 was added (40% of the original volume). Two days later (d 6), differentiated DC were gathered by collecting the supernatant and rinsing the flasks with cold PBS to detach loosely adherent cells. DC ($CD11c^+$ cells) generally represented > 75% of the preparation.

To generate a mixture of plasmacytoid and myeloid DC, bone marrow cells were cultured at 2 to 3×10^6 cells/ml in DC complete medium containing 20 ng/ml recombinant Flt3L for 7 to 8 days and harvested as described above (FL-DC). DC ($CD11c^+$ cells) generally represented > 80% of the preparation with typically 40% being plasmacytoid DC ($B220^+$) and 60% being myeloid DC ($B220^-$, $CD11b^+$).

Figure 3.1 shows representative flow cytometry dot plots of GM-CSF / IL-4 DC in comparison to FL-DC. DC generated with GM-CSF and IL-4 are usually bigger (higher forward scatter, FSC) and more granular (higher side scatter, SSC) than FL-DC (Figure 3.1 A). They comprise only mDC, which do not express B220 (Figure 3.1 B) but high levels of CD11b (Figure 3.1 C). FL-DC on the other hand consist of mDC and pDC. pDC are characterized by a high expression of B220 and a low expression of CD11b. Gating of mDC and pDC for subsequent analysis of subsets was done according to their expression of B220, CD11b and CD11c.

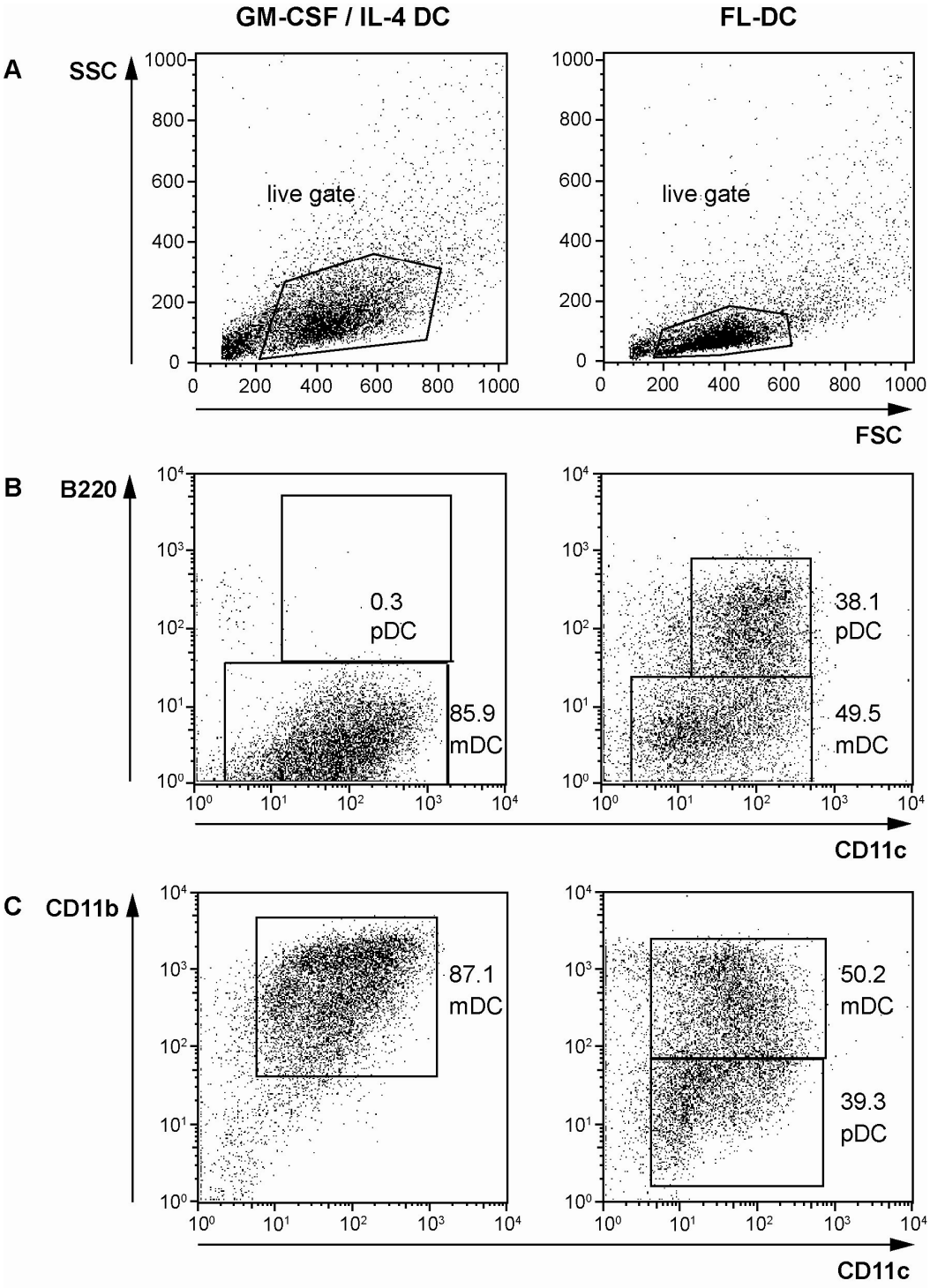

Figure 3.1: Comparison of GM-CSF / IL-4 DC and FL-DC.
Murine bone marrow cells were differentiated either with GM-CSF and IL-4 (left panel) or with Flt3L (right panel). Representative flow cytometry data is shown as dot plots. (A) Live gates were drawn in FSC-SSC plots to exclude dead cells or unneeded cell populations. (B) CD11c is expressed on all subsets of murine DC and the characteristic marker for DC. B220 (CD45R) is only expressed on pDC, which are not generated in GM-CSF / IL4 cultures. (C) mDC express high levels of CD11b, whereas pDC have low levels or no CD11b expression. Numbers show percentage of the indicated subset relative to live cells.

3.2.3.2 Antigen loading of dendritic cells

Differentiated DC were harvested on day 6 (GM-CSF / IL-4 DC) or 7 (FL-DC) and pulsed with antigen to elicit specific immune responses in vaccinated animals. In this study, peptides, proteins and inactivated tumor cells were used as antigen sources. DC were pulsed with 100 nM peptide antigen ($OVA_{257-264}$ peptide, SIINFEKL) for one hour before using the cells for vaccination or *in vitro* assays. For *in vitro* assays, T1Db peptide (SAINNYAQKL) derived from the SV40 large T antigen was used as a negative control to test for antigen-specificity of T-cell responses. To test the ability of DC to cross-present protein antigens or to induce T helper cell responses, 100 µg/ml ovalbumin protein was added to immature DC 24 h before cells were matured with CpG. In *in vitro* assays, survivin protein was used as negative control. For the presentation of tumor antigens on DC, C26 tumor cells were first UV-irradiated at 0.7 J/cm^2 in a UV-crosslinker (Fluo-Link) and then added to immature DC in a ratio of 1:5 24 h before CpG stimulation.

3.2.3.3 In vitro *activation of dendritic cells*

DC were activated with the oligodeoxynucleotide CpG 1826 (CpG) as ligand for TLR9, with CL087 as TLR7 ligand, or with R848 (resiquimod) as ligand for TLR7 that also activates TLR8 in humans. DC that were used for *in vitro* analysis or RNA isolation were activated with 3 µg/ml CpG, 1 µg/ml CL087 or 0.5 µg/ml R848 for 2 to 48 h as indicated. For the inhibition of p38 MAPK, SB203580 (1.25 µM or 2.5 µM as indicated) was added to DC cultures 30 min before cells were stimulated with R848. Antigen-pulsed DC that were used to vaccinate mice were stimulated with 6 µg/ml CpG for 4 h or 20 h before injection, as indicated.

3.2.4 Isolation of DC and T-cell subtypes by magnetic cell separation (MACS)

MACS (magnetic cell separation or magnetic-activated cell sorting) is used for the isolation of viable and functionally active cells by magnetic labeling. The mixture of cells to be separated is incubated with magnetic beads (superparamagnetic particles of approximately 50 nm diameter) coated with antibodies against a particular surface antigen. This causes the cells expressing this antigen to attach to the magnetic beads. Afterwards, the cell solution is transferred on a column placed in a strong magnetic field. In this step, the cells attached to the beads (expressing the antigen) are retained on the column, while other cells (not expressing the antigen) flow through. The retained cells are eluted from the column after removal from the magnet. With this method, cells can be separated positively or negatively with respect to the particular antigen. In this study, reagents from Miltenyi Biotec were used according to the manufacturer's instructions. Plasmacytoid DC were sorted from FL-DC by positive selection using B220 microbeads, while myeloid DC differentiated by Flt3L were

obtained by depleting $B220^+$ plasmacytoid DC from FL-DC. For the analysis of DC migration *in vivo*, DC were separated from total spleen cells with CD11c beads. To examine T helper cell responses, $CD4^+$ T cells were isolated from splenocytes with CD4 beads. For the positive selection of labeled cells, LS and MS columns were used. Cells were labeled with the respective microbeads in MACS buffer for 15 min at 4℃ and washed with MACS buffer. LS columns were placed in the appropriate magnet and rinsed with 3 ml MACS buffer prior to loading the cells in a volume of 2 ml. Columns were washed three times with 3 ml MACS buffer. Effluent from cells and the first wash was gathered as negative fraction, flowthrough from the second and third wash was discarded. Positive cells were eluted by flushing the cells in 2 ml MACS buffer through the column dislodged from the magnet with the supplied plunger. To further enrich positively labeled cells, eluted cells were applied on a MS column which had been placed in a magnet and rinsed with 1 ml MACS buffer. Columns were washed twice with 1 ml MACS buffer and total effluent was discarded. Cells were flushed out in a volume of 1 ml after removing the column from the magnet. To increase the purity of negatively sorted mDC from FL-DC cultures, the negative flowthrough from LS columns was applied to LD columns which had been placed in a magnet and rinsed with 2.5 ml MACS buffer. Columns were washed with 2 ml MACS buffer and total effluent was gathered as myeloid DC (depleted from plasmacytoid DC). Regulatory T cells were isolated from spleen cells by first depleting non-$CD4^+$ cells and subsequent positive selection of $CD4^+$ $CD25^+$ regulatory T cells using the Regulatory T Cell Isolation Kit according to the manufacturer's instructions.

3.2.5 Assessment of T-cell responses *in vitro*

Induction of antigen-specific proliferation of T cells was measured by 5'-Bromo-2'-deoxy-uridine (BrdU) ELISA. This technique is based on the incorporation of the pyrimidine analogue BrdU instead of thymidine into the DNA of proliferating cells. After its incorporation into DNA, BrdU is detected by immunoassay. For this purpose, FL-DC were pulsed with different concentrations of ovalbumin (OVA) for 18 h before activation with CpG for 0, 4 or 20 hours. For the last hour of stimulation, unpulsed DC were cultured with $OVA_{257-264}$ peptide (SIINFEKL). Antigen-specific T-cell responses were analyzed by using transgenic mice expressing a T-cell receptor specific for OVA. OT-I mice express the specific T-cell receptor recognizing SIINFEKL bound to MHC class I complexes on $CD8^+$ T cells, while OT-II mice were used to analyze $CD4^+$ T-cell responses as they have OVA-specific T helper cells. To confirm antigen-specificity of T-cell responses, DC were pulsed with T1Db peptide (SAINNYAQKL) or survivin protein as irrelevant control antigen.

To analyze cytotoxic T-cell proliferation, 2 x 10^5 splenocytes from OT-I mice were cocultured with the indicated number of washed FL-DC for 48 h before 7.5 µM BrdU was added for the last 6 to 12 h of culture. Cell culture plates were centrifuged (300 g, 5 min) and medium was removed by flicking off before plates were dried with a hairdryer. Cell fixation, incubation with anti-BrdU antibody and substrate reaction were done according to the manufacturer's instructions. Chemiluminescence was measured in duplicate in relative light units (rlu) with a multilabel plate reader. IFN-γ secretion of CTL was measured in the supernatants of these cocultures by ELISA to check for functionality of CTL.

To analyze the induction of T_h1 versus T_h2 responses, splenocytes from OT-II mice were magnetically sorted using CD4 MicroBeads according to the manufacturer's instructions and 1 x 10^5 CD4 T cells were cocultured with the same number of FL-DC. After 48 h, supernatants were tested for IFN-γ (as an indicator for T_h1 responses) and IL-4 concentrations (as a marker for T_h2 responses) by ELISA.

3.2.6 Analysis of DC-induced suppression of regulatory T-cell function

For regulatory T-cell suppression assays, $CD4^+$ $CD25^-$ effector cells and $CD4^+$ $CD25^+$ regulatory T cells were sorted by MACS. The purity of T_{reg} cells was typically greater than 97%. $CD4^+$ $CD25^-$ effector T cells (7.5 x 10^4 cells per well) were cultured in the presence of 0.1 µg/ml soluble anti-CD3 antibody with 6 x 10^4 $CD4^+$ $CD25^+$ T_{reg} cells and 3.5 x 10^3 FL-DC. BrdU incorporation was measured in triplicate as described above and is shown in rlu. Suppression of T-cell proliferation by T_{reg} cells in coculture with unstimulated DC is defined as 100 % suppression. Suppression was calculated as follows:

Suppression (% of control) = [1-(rlu with T_{reg} / rlu without T_{reg})] / [1-(rlu of unstimulated DC with T_{reg} / rlu of unstimulated DC without T_{reg})].

3.2.7 Transwell migration assays

To assess the potential of DC to migrate towards CCL21, a cytokine strongly expressed in T-cell zones of spleen and lymph nodes, 2 x 10^4 FL-DC in 100 µl DC complete medium were put into 5 µm transwell inserts, placed into 24-well plates containing 600 µl DC complete medium with or without 100 ng/ml CCL21, and incubated for 2 hours at 37°C. The medium in the lower chambers was concentrated to 50 µl and cells were counted with a hemocytometer. Percentages of migrated DC are shown as means of triplicate samples.

3.3 Immunological methods

3.3.1 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay, or ELISA, is a technique used to detect the presence of an antibody or an antigen in a sample by enzyme-linked antibodies and the subsequent conversion of a colorless substrate to a colored reagent (first described by Engvall and Perlmann, 1971).

3.3.1.1 Cytokine ELISA

In this study, cytokines in cell culture supernatants were detected by sandwich ELISA: plates were coated with an antibody directed against the respective cytokine, and bound cytokines were revealed by a second, biotinylated antibody followed by streptavidin coupled to peroxydase. This enzyme catalyzes the oxidation of 3,3',5,5' – tetramethylbenzidine (TMB) with hydrogen peroxide to a blue chromogen. The detection of all cytokines except IFN-α was done with kits including all the necessary reagents according to the manufacturers' instructions. Samples were generally diluted 1:2. For IL-12p40 and IFN-γ ELISAs, samples were diluted 1:5 and 1:10, respectively.

Detection of IFN-α was done by a set of antibodies: plates were coated overnight at 4°C with 50 µl/well capture antibody in coating buffer (1 µg/ml). Unspecific binding sites on the plates were blocked with 150 µl/well assay diluent for 3 h at room temperature (RT). The plates were washed 3 times with wash buffer, before samples and standard protein were loaded on the plates and incubated overnight at 4°C. The highest standard was 10^5 IU/ml, samples were applied undiluted. Plates were washed again, and 50 µl/well detection antibody in assay diluent (625 ng/ml) was added for 3 h at RT. Horseradish peroxidase (HRP)-conjugated F(ab´)2 fragments (15 µg/ml) were used to detect bound detection antibody after washing the plates 10 times and incubated 3 h at RT. To develop a colored reaction, TMB and H_2O_2 were used as substrate for the HRP. 50 µl/well substrate solution were added to each well after washing the plates again 10 times, and the reaction was stopped by adding 25 µl/well of 2N H_2SO_4. All cytokine ELISAs were read at 450 nm with a wavelength correction subtraction at 590 nm.

3.3.1.2 Measurement of immunoglobulin concentrations in mouse sera

Serum antibody titers were determinded by coating 96-well ELISA plates with antigen (ovalbumin, 10 µg/ml in PBS) overnight at 4°C. Unspecific binding sites were blocked with

150µl/well assay diluent for 1 h at RT. Plates were washed 3 times with wash buffer before 50 µl/well of each sample were loaded in duplicate (mice sera, 1:100 diluted in PBS) and incubated for 1 h before plates were washed again. 50 µl/well anti-mouse antibodies coupled to HRP were added to detect the bound immunoglobulins and incubated for 1 h: anti-IgG (binds to all IgG subclasses) and anti-IgG1 were used in a 1:1000 dilution, whereas anti-IgG2a was diluted 1:4000 in PBS. After washing the plates 5 times, OPD substrate tablets (o-phenylenediamine dihydrochloride and urea hydrogen peroxide) dissolved in water were added to the wells and the reaction was developed for 5 to 20 min before it was stopped with 2N H_2SO_4. Plates were read at 490 nm with a wavelength correction subtraction at 690 nm.

3.3.2 Flow cytometry

Flow cytometry is a method that allows for the analysis of various properties of single cells or particles suspended in a fluid. The suspension of single cells emerges from the sample needle into a surrounding sheath fluid liquid that is moving with a greater velocity. The resulting acceleration at the orifice forces the particles to travel one by one in the central portion of the fluid jet that emerges from the flow chamber. This principle is called hydrodynamic focusing. The cells flow past the detector point and are illuminated by a focused laser beam. The illuminating light is scattered and simultaneously, if particles have been previously stained with a fluorescent dye capable of absorbing the illuminating light, fluorescence emission occurs. Scattered light and emitted fluorescence are collected and sent to different detectors by using optical filters. The relative size and granularity of a cell influence the way in which light is scattered as the cell passes through the laser beam. Low angle scattered light depends on cell size and is recorded as a parameter called forward scatter (FSC). Similarly, cell granularity and surface convolutions scatter light at higher angles. For convenience the signal is measured orthogonal to the stream and is referred to as side scatter (SSC). Fluorescence measurements are also made in the orthogonal direction with detectors appropriate to the specific emission spectrum of the fluorochrome. All measurements were performed on a FACSCalibur, where cells are illuminated by a 488 nm argon-ion laser to detect the following fluorochromes: fluorescein isocyanate (FITC, emission at 520 nm), phycoerythrin (PE, emission at 575 nm), and peridinin chlorophyll protein (PerCP, emission at 675 nm). A red diode laser was used to excite allophycocyanin (APC, emission at 660 nm).

3.3.2.1 Analysis of surface molecule expression

Expression of surface molecules was analyzed by staining 2×10^5 to 2×10^6 cells in 200 µl PBS or cell culture medium with fluorochrome-conjugated monoclonal antibodies against the

examined surface antigens for 25 min at 4°C (0.5 µl per antibody; see chapter 3.1.5, p. 25 for an overview of utilized antibodies). Cells were washed twice with PBS before data was acquired on a FACSCalibur. When cells were not analyzed immediately, they were resuspended in 200 µl fixation buffer and kept at 4°C until data was collected by flow cytometry. FlowJo software was used to analyze data.

3.3.2.2 MHC pentamer staining

Multimeric MHC-peptide complexes have a high avidity for T-cell receptors of a particular specificity as determined by the MHC allele and peptide combination, and their use as detection reagent allows the evaluation of specific T-cell immunity. To detect antigen-specific $CD8^+$ T-cell responses, 200 to 300 µl peripheral blood were incubated with 1 ml red blood cell lysis buffer for 8 min at RT. Cells were washed with PBS and resuspended in 50 µl PBS, before 4 µl Pro5-$OVA_{257-264}$-H-$2k^b$-PE pentamer were added and incubated for 15 min at RT. Cells were washed with PBS before surface staining was done with an anti-CD8 antibody as described above.

3.3.2.3 CFSE staining

Carboxyfluorescein diacetate succinimidyl ester (CFSE) is a dye often used to trace cells. The colorless molecule passively diffuses into cells, where the acetate groups are cleaved by esterases to yield highly fluorescent carboxyfluorescein succinimidyl ester. The succinimidyl ester groups then react with intracellular amines, forming fluorescent conjugates. The dye-protein adducts that form in labeled cells are retained by the cells throughout development and are inherited by daughter cells after cell division. Cells were resuspended in 1 ml HBSS and labeled with 15 µl 0.1 to 1 mM CFSE for 15 min at 37°C. Cells were washed and injected either subcutaneously to trace migration of DC or intravenously to analyze antigen-specific lysis of target cells. To detect labeled cells after injection, single cell suspensions were generated from appropriate organs and analyzed by flow cytometry. CFSE is detected in the FL1 channel.

3.4 Animal experimentation

3.4.1 Animals

Female balb/c and C57BL/6 mice were purchased from Harlan-Winkelmann (Borchen, Germany). Transgenic and knock-out mice mice were kindly provided by Prof. Th. Brocker (Institute of Immunology, Ludwig-Maximilian University Munich, Germany: TCR transgenic

OT-I and OT-II mice), Prof. J. Heesemann (Max von Pettenkofer Institute for Hygiene and Medical Microbiology, Ludwig-Maximilian University Munich, Germany: IL-10-deficient mice), Prof. S. Frantz (Medizinische Klinik und Poliklinik I, University Würzburg, Germany: p50-deficient mice), and Dr. A. Krug (Department of Internal Medicine II, Klinikum Rechts der Isar, Technical University Munich, Germany: IRF3/7-deficient mice). Mice were 6 to 10 weeks of age at the onset of experiments. Mice were anesthetized with isoflurane for all interventions but subcutaneous (s.c.) injections. All animal studies were approved by the local regulatory agency (Regierung von Oberbayern, Munich, Germany).

3.4.2 Organ and single cell preparation

3.4.2.1 Preparation of serum and isolation of blood cells

For the detection of specific antibodies in the serum and the analysis of specific T cells, blood was collected from the retro-orbital sinus with a glass capillary pipette. To collect serum, 2 drops of blood were collected in an Eppendorf tube and centrifuged at 3500 g for 10 min. Serum (the supernatant) was carefully taken and stored at -20℃ until further analysis. For the collection of blood cells, 200 to 300 µl blood were collected in an Eppendorf tube containing 50 µl heparin, mixed immediately, and kept on ice. 1 ml of PharmLyse ammonium chloride buffer was added to lyse erythrocytes, and samples were incubated for 10 min at RT before being centrifuged (350 g, 5 min) and washed with PBS to remove remaining lysed red blood cells. Cells were then analyzed by flow cytometry.

3.4.2.2 Spleen cell isolation

Mice were killed by cervical dislocation and spleens were harvested. Organs were passed through a 40 µm cell strainer to get single cell suspensions. Erythrocytes were lysed by resuspension of centrifuged cells in red blood cell lysis buffer. Splenocytes were kept in DC complete medium for *in vitro* experiments.

3.4.2.3 Preparation of bone marrow cells

Mice were euthanized by cervical dislocation and bone marrow was extracted from femura and tibiae by flushing DC medium through the bones and passing the cell suspension through a 40 µm cell strainer. Erythrocytes were lysed by resuspension of centrifuged cells in PharmLyse ammonium chloride buffer.

3.4.2.4 Isolation of lymph node cells

Mice were killed by cervical dislocation and brachial and axillary lymph nodes were harvested. Organs were passed through a 40 µm cell strainer. Single cell suspensions were centrifuged and resuspended in DC complete medium.

3.4.3 Immunization of mice

3.4.3.1 Dendritic cell immunizations

To assess the ability of differentially activated DC to elicit antigen-specific cytotoxic T-cell responses, naïve C57BL/6 mice were injected with antigen-pulsed DC. For this purpose, FL-DC were generated, pulsed with ovalbumin protein or peptide antigen and activated with 6 µg/ml CpG for 4 h or 20 h, as described in chapter 3.2.3 (p. 27). 4×10^5 antigen-pulsed activated DC were then injected s.c. together with 100 µg CpG two to three times at 7-day intervals. OVA-specific CTL responses were determined one week after the second immunization.

3.4.3.2 Detection of specific immune responses

Antigen-specific immune responses were detected by analyzing specific serum antibody titers, by staining $CD8^+$ T cells with MHC I pentamer complexes, and by examining cytotoxic lysis of antigen-loaded target cells. Mice were immunized with the model antigen ovalbumin to allow a detailed investigation of antigen-specific responses.

Serum antibody titers were detected by coating ELISA plates with ovalbumin protein. When the samples are loaded, specific antibodies bind to the attached protein and can be detected by secondary antibodies specific for the heavy chains of murine antibodies that determine the isotype of the antibody. By using different antibodies for IgG (all subclasses), IgG1 and IgG2a, the respective titers of these antibodies could be detected individually, with IgG2a titers being most important as they are indicative of a T_h1-type response. The IgG ELISA protocol is described in detail in chapter 3.3.1.2, p. 32.

For the detection of antigen-specific cytotoxic T-cells, two assays were used. On the one hand, T cells which express a T-cell receptor that recognizes ovalbumin presented in a MHC I complex were analyzed by fluorochrome-conjugated complexes of five synthetic MHC I molecules, each binding the immunodominant peptide $OVA_{257-264}$ (SIINFEKL). These complexes, also called MHC pentamers, bind to the specific T-cell receptor and allow the

assessment of the percentage of cytotoxic T cells specific for ovalbumin by flow cytometry. The staining procedure for MHC pentamers is described in chapter 3.3.2.2, p. 34.

An *in vivo* cytotoxicity assay was used to examine the cytotoxic function of antigen-specific $CD8^+$ T cells. In this assay, peptide-loaded labeled target cells are injected into immunized mice and lysis of the target cells is measured in comparison to unloaded cells. Target cells were prepared by isolating spleocytes and subsequent red blood cell lysis. The cells were divided into two populations and either left unpulsed or pulsed with $OVA_{257-264}$ peptide (10 µg / ml) for 1 h at 37°C. The cells were then washed twice with HBSS and each population was resuspended in 1 ml HBSS and labeled with a low concentration (1.5 µM) or a high concentration (15 µM) of carboxyfluorescein succinimidyl ester (CFSE), respectively. Peptide-pulsed $CFSE^{high}$ cells and unpulsed $CFSE^{low}$ cells were mixed 1:1 and a total of 10^7 target cells were injected i.v. into immunized mice. 3 days later, CFSE-labeled target cells from peripheral blood were analyzed by flow cytometry. Specific lysis was calculated as follows:

Specific lysis (%) = 100 x [1- ($CFSE^{high}$ cells / $CFSE^{low}$ cells) / ($CFSE^{high}$ cells in naïve mice / $CFSE^{low}$ cells in naïve mice)].

3.4.4 C26 tumor experiments

3.4.4.1 Tumor challenge

For tumor induction, 2.5 x 10^5 murine colon carcinoma C26 cells on a Balb/c background were injected s.c. into the right flank of Balb/c mice anesthetized with isoflurane. Palpable tumors developed from day 5 to 7 onwards. Tumor growth was monitored three times a week and expressed as the product of the perpendicular diameters of individual tumors. Animals were sacrificed by cervical dislocation when tumor size exceeded 225 mm^2. Mice were also monitored for signs of distress (see Table 3.1) and were killed when they reached values higher than 2 twice in 24 h. Untreated mice had to be killed or died between 20 and 30 days after tumor injection, as was already described in earlier studies (Brunner et al., 2000). Tumor growth and survival were the readouts of tumor experiments.

3.4.4.2 Dendritic cell therapy

For DC therapy, mice received 2 x 10^5 DC s.c. together with 100 µg CpG in the non tumor-bearing (contralateral) flank. Prior to injection, immature DC were loaded with UV-irradiated tumor cells (0.7 J / cm^2) in a ratio of 5:1 24 h before CpG stimulation for 4 or 20 h as indicated. In some experiments, mice were additionally injected with 100 µg CpG

peritumorally. Treatment was initiated at day 8 to 10 after tumor injection when tumor size was 10 to 25 mm^2. The interval between the first and second vaccination was 5 days, and two more vaccinations were performed at 7-day intervals. Mean tumor size curves of therapy groups were plotted until three mice of that group died or were killed.

3.4.4.3 Rechallenge of mice with tumor cells

To evaluate long-term protective immunity against tumor antigens, cured mice from previous tumor experiments were rechallenged s.c. with 2.5 x 10^5 C26 tumor cells 99 to 149 days after the initial tumor challenge, as indicated. Naïve mice were used as controls. Tumor growth was monitored as described above.

Table 3.1: Qualifying pain and distress in rodents (adapted from Morton and Griffiths, 1985)**.**

Score	Degree of distress	Physical appearance
0	None	Smooth coat, eyes clear and bright, normal behavior
1	Minor	Piloerection, reduced mobility, normal behavioral reactions to external stimuli
2	Intermediate	Rough coat, nasal/ocular discharge, abnormal posture (intermittent), depressed provoked behavior
3	Severe	Hunched posture (continuous), respiratory markedly increased or reduced, no reaction to external stimuli

3.4.5 Analysis of *in vivo* migration of dendritic cells

Activated DC migrate to draining lymph nodes in order to trigger an antigen-specific immune responses by lymphocytes. Migration of DC *in vivo* was assessed by tracing CFSE-labeled DC in the brachial and axillary lymph nodes which were injected in the ventral antebrachium. For this purpose, FL-DC were activated with 6 µg/ml CpG for 4 h or 20 h, washed with PBS and stained with 3 µM CFSE in PBS for 10 min at 37°C. Cells were washed once in cold PBS and once in DC medium, resuspended in PBS and counted. 2 to 4 x 10^6 cells were injected in a volume of 70 µl s.c. in the left forearm of anesthetized mice. 48 h later, mice were killed and brachial and axillary lymph nodes were removed from both sides. Lymph node cells were gathered and stained with an anti-CD11c antibody to allow the identification of DC before cells were analyzed by flow cytometry.

3.5 Statistical analysis

Statistical analyses were performed by unpaired, one-way analysis of variance (ANOVA) with the Newman-Keuls multiple comparison test. Significance was set at $P < 0.05$. Comparisons

in tumor size among groups were made using the Mann-Whitney test for various time points. Comparisons among groups regarding survival time were made using the log-rank test. Fisher's exact test was used for comparing tumor incidence in rechallenged mice. Statistical analyses were performed using SPSS software.

3.6 Molecular biology methods

3.6.1 RNA isolation

To examine which genes are expressed in unstimulated DC and DC after stimulation, total RNA was prepared by means of Trizol, a monophasic solution of phenol and guanidine isothiocyanate. FL-mDC were magnetically sorted by depletion of $B220^+$ pDC on day 8 of FL-DC culture as described in chapter 3.2.4, p. 29. 1.5 to 2 x 10^6 mDC were stimulated in 1 ml DC complete medium in Eppendorf tubes with 3 µg/ml CpG, 0.5 µg/ml R848, or were left untreated. 4 h later, cells were centrifuged for 5 min at 350 g, supernatants were taken and frozen for further analysis by ELISA, and cell pellets were resuspended and lysed in 1 ml Trizol. Insoluble material was removed by centrifugation at 12000 g for 10 min, and supernatants containing RNA were transferred to new tubes. Homogenized samples were incubated for 5 min at RT to permit the complete dissociation of nucleoprotein complexes, before 0.2 ml of chloroform were added. Tubes were shaken vigorously by hand for 15 seconds and then incubated for 3 min at RT before centrifugation at 12000 g for 15 min. The colorless upper aqueous phase containing the RNA was then transferred to a fresh tube and RNA was precipitated by mixing with 0.5 ml isopropanol and incubating for 10 min at RT prior to centrifugation at 12000 g for 10 min. Supernatants were removed and the RNA pellet was washed once with 75% ethanol by vortexing, centrifuged again at 7500 g for 5 min, and supernatants were removed again. Pellets were left to dry for 1 min at RT and dissolved in 40 µl H_2O. Dissolving was enhanced by heating samples to 55°C for 10 min. RNA concentration was measured by Nanodrop photometer. Samples were stored at -80°C for further analysis.

3.6.2 Analysis of gene expression profiles by microarray

To compare the genes transcribed after CpG and R848 stimulation, we analyzed mRNA levels by means of a microarray. Microarrays are two-dimensional arrays, typically on a glass or silicon wafer, upon which oligonucleotide sequences are synthesized. These oligonucleotides are used as probes to which only the complementary target sequence will hybridize. Microarrays are most commonly used for high throughput expression profiling. By

hybridizing cDNA or cRNA from specific tissues or cells onto the array, the expression levels of thousands of genes can be simultaneously monitored. In this study, cRNA from stimulated and unstimulated DC was hybridized to Illumina's Mouse-6 v1.1 Expression Bead Chips which contains probes for 46643 genes covering the whole mouse genome.

3.6.2.1 Generation and hybridization of cRNA

Total RNA was isolated from R848- and CpG-stimulated and untreated cells after 4 h stimulation as described above. Each condition was done in quadruplicates. RNA was reversely transcribed into first strand cDNA with a subsequent synthesis of the second strand to obtain a double-stranded template for *in vitro* transcription with T7 polymerase. During this *in vitro* transcription, biotinylated nucleotides are incorporated to generate labeled cRNA. cRNA was prepared using the Illumina TotalPrep RNA Amplification Kit according to the manufacturer's instructions. The purity and quality of total RNA and cRNA was tested by capillary electrophoresis in the Agilent Bioanalyzer. Samples were hybridized to two Mouse-6 v1.1 Expression Bead Chips (Illumina), each containing 6 arrays. To prevent imprecise values due to a bias of the different arrays, duplicates of each condition were bound to both chips. cRNA synthesis, quality control, and hybridization were done by Peter Weber at the Max Planck Institute of Psychiatry in Munich, Germany.

3.6.2.2 Data readout and analysis

Microarrays were analyzed using the Beadstation 500. Array scanning and statistical evaluation of data was done by Peter Weber and Benno Pütz at the Max Planck Institute of Psychiatry, Munich. Quality control of array data was done with Illumina's scanning software, normalization of data was performed by variance stabilization (Huber et al., 2002), and differential expression was analyzed by means of the LIMMA package (linear models for microarray data, described in Smyth, 2005). Functional analysis of data was perfomed using Pathway Studio Software and NCBI's Entrez Gene database.

4 Results

4.1 Kinetics of dendritic cell activation by TLR9 in tumor therapy

The maturation state of DC is of critical importance for the success of DC tumor vaccines, but the most effective mode of maturation is still a matter of debate. In the present study, the kinetics of DC activation were analyzed in detail by examining the influence of short-term versus long-term activation on DC phenotype and function. DC were differentiated in the presence of Flt3L to generate both myeloid and plasmacytoid DC subsets and activated by the TLR9 ligand CpG.

4.1.1 Cytokine production

As a first step, the kinetics of cytokine production by Flt3L-derived DC (FL-DC) after stimulation for increasing times with the TLR9 ligand CpG 1826 (CpG) were investigated. FL-DC comprise both plasmacytoid and myeloid DC in a relation of approximately 40% pDC and 60% mDC. To examine potential differences between pDC and mDC, both populations were also separated by magnetic cell sorting and analyzed individually.

DC were stimulated with CpG for 2 h (short-term) up to 24 h (long-term). After the stimulation period, cytokine levels were measured in the supernatant (Figure 4.1, grey bars). Cells were washed to remove free CpG and culture was continued for a total of 48 h. Cytokine secretion after this additional incubation without stimulus indicates the therapeutically relevant fraction of cytokines that can be produced *in vivo* following DC transfer in therapeutic applications (Figure 4.1, black bars).

Cytokine production by total FL-DC (Figure 4.1, left column) showed that the bioactive heterodimer of IL-12, IL-12p70, was detected as early as 2 h after stimulation with CpG. Secretion persisted after removal of the stimulus, so that a 2 h activation period was sufficient to induce subsequent IL-12 production, but production ceased after 8 h. IFN-α was also produced early, with most of the secretion taking place in the first 8 h after activation. The time course of IL-6 secretion showed a sustained production for over 24 h even with short activation times. In contrast, IL-10 secretion was initiated late between 12 and 24 h after the onset of stimulation, and levels were highest after a stimulation of 24 h. As IL-10 suppresses the differentiation of T_h1 cells, a short stimulation may help to support a T_h1-inducing cytokine milieu. T_h1 cells drive cell-mediated immunity in contrast to the humoral immunity triggered by T_h2 cells.

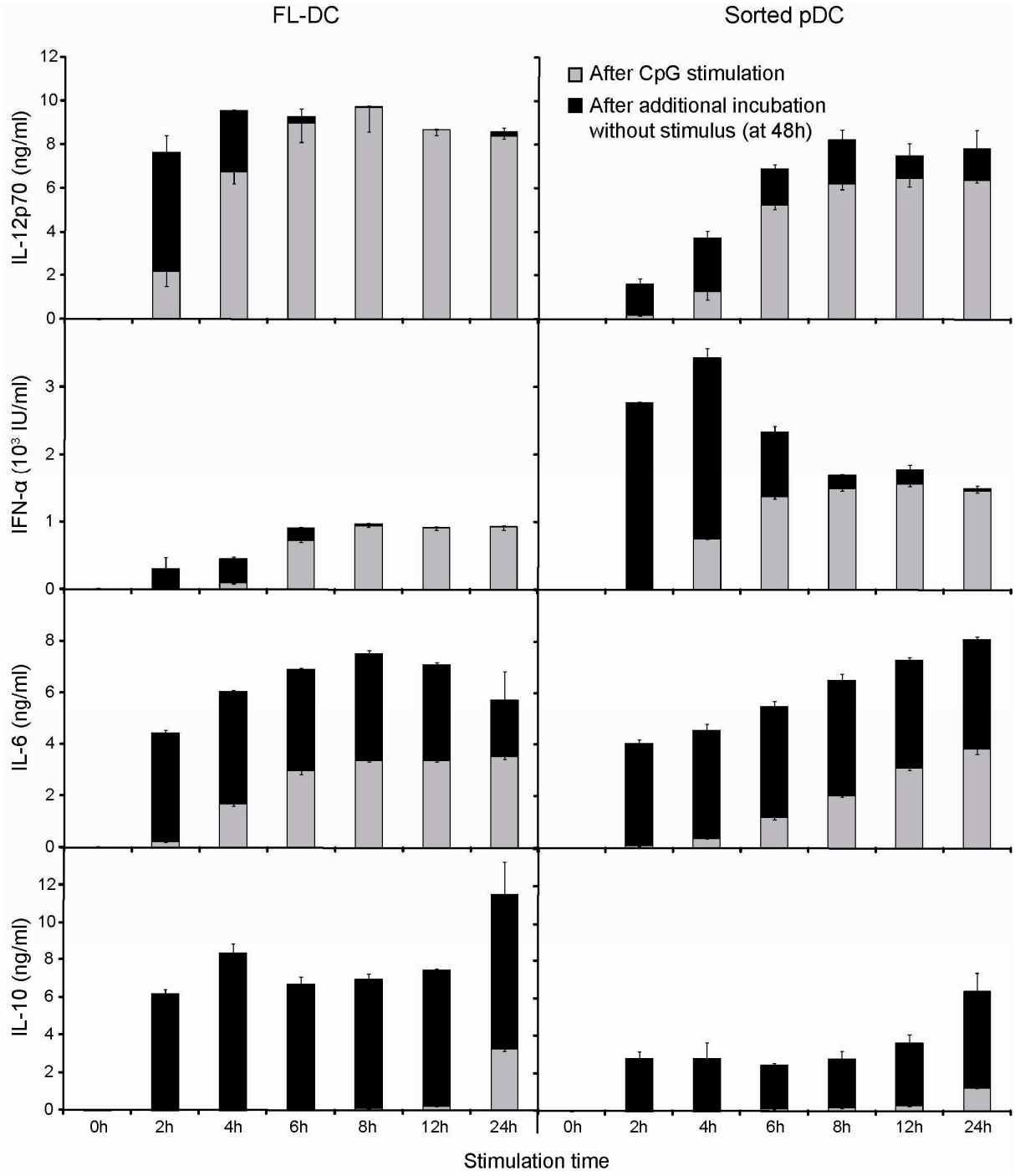

Figure 4.1: Kinetics of cytokine production of FL-DC and sorted pDC.
Total FL-DC (a mixture of approx. 40% pDC and 60% mDC; left column) and sorted pDC (right column) were stimulated with CpG for 2 to 24 h, extensively washed to remove excess CpG and cultured without further stimulation for a total of 48 h. Cytokine concentration in the supernatant was measured directly after the 2 to 24 h stimulation period (grey bars) and at 48 h, after additional incubation without stimulus (black bars). Results show mean and SEM of one representative experiment of four.

When analyzing differential cytokine production by the pDC and mDC subpopulations isolated from FL-DC, secretion patterns of pDC were similar to that of total FL-DC. pDC are the main producers of IFN-α upon TLR stimulation and levels were accordingly high upon

activation (Figure 4.1, right column). Cytokine secretion by mDC was also consistent with that of total FL-DC, except for IFN-α, which is not produced by mDC upon CpG stimulation (Kadowaki et al., 2001 and data not shown).

Thus, activation of total FL-DC for as little as 4 h induces efficient production of all cytokines examined. Importantly, the T_h1-type cytokines IL-12p70 and IFN-α are produced nearly exclusively during the first 8 h after initiation of activation, independently of the duration of stimulation. This proposes a higher benefit of remaining cytokine production *in vivo* when using short-term stimulated DC instead of extensively activated, exhausted DC that have ceased secretion of IL-12 and IFN-α.

4.1.2 Expression of costimulatory molecules

To further characterize the potential of short-term CpG-activated DC to initiate T-cell responses, we examined the expression of costimulatory and MHC molecules on CpG-activated DC by flow cytometry. The antigen-specific clonal expansion of naïve T cells always requires two signals: on the one hand, the T-cell receptor binds to the appropriate peptide - MHC complex on the antigen-specific cell, on the other hand costimulatory molecules on the same antigen-presenting cell are needed to avoid the development of anergic T cells, that are refractory to activation by specific antigen. The expression of MHC and costimulatory molecules therefore reflects the potential of the DC to activate T-cell responses.

We analyzed the expression of the costimulatory molecules CD40, CD80, and CD86, and of both antigen-presenting complexes, MHC I and MHC II, on FL-DC that were stimulated either transiently for 4 h (4h-DC) or long-term for 20 h (20h-DC). For total FL-DC, 4h-DC showed a 2.5 to 3-fold increase in expression of the costimulatory molecules CD40, CD80 and CD86 relative to unstimulated DC (Figure 4.2 A and B, first row). Expression of MHC class I and MHC class II molecules was also increased. In 20h-DC, costimulatory and MHC molecules were further upregulated. In contrast, 48 h after the initiation of stimulation, both 4h-DC and 20h-DC expressed similar levels of costimulatory and MHC molecules. Expression at 48 h was increased compared to unstimulated DC, although to a lesser extent than at earlier time points.

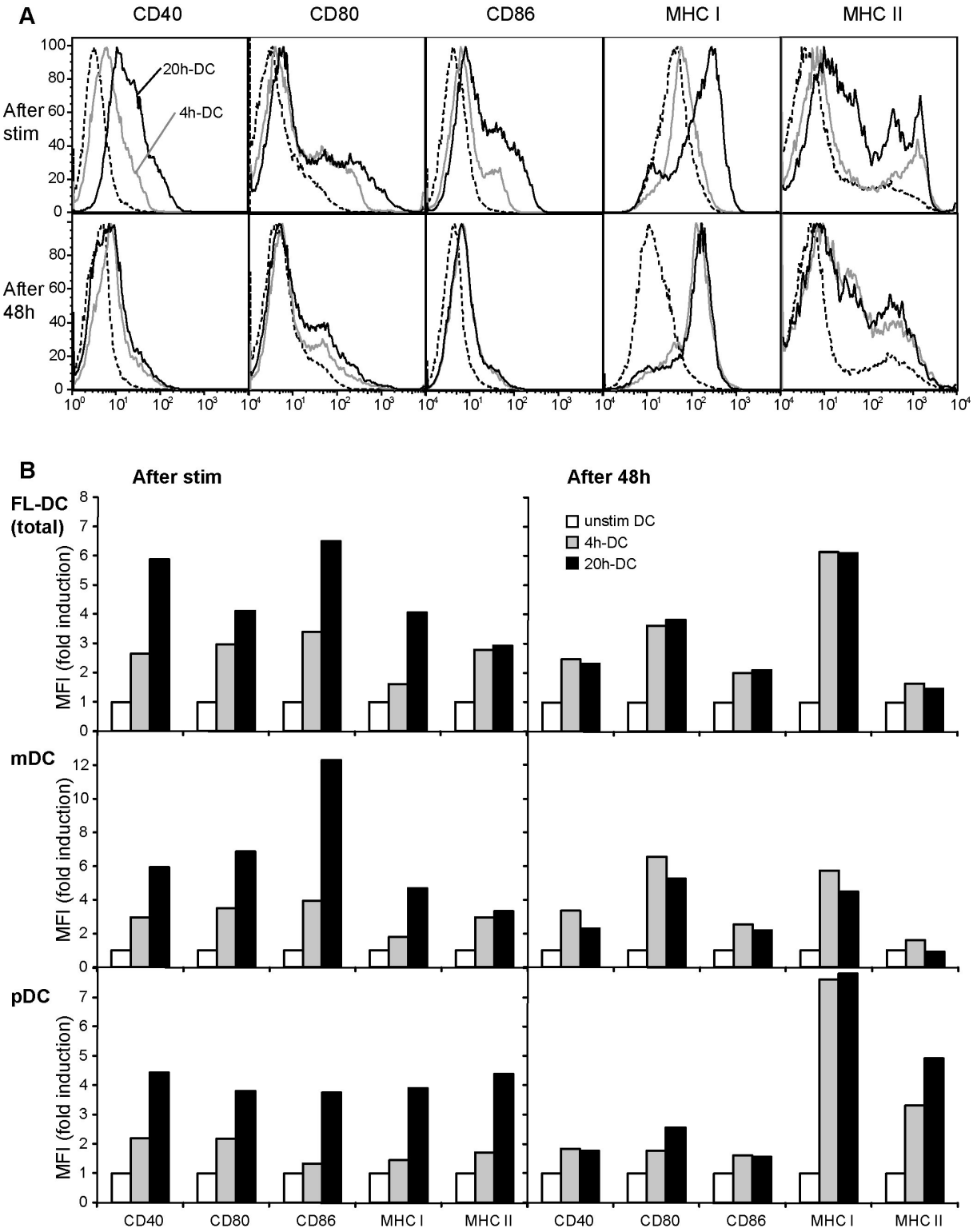

Figure 4.2: Expression of costimulatory and MHC molecules on short- and long-term activated DC subsets.
FL-DC were stimulated with CpG for 4 h (4h-DC) or 20 h (20h-DC), washed to remove excess CpG and cultured without further stimulus for a total of 48 h. Surface expression of the indicated markers was measured by flow cytometry directly after stimulation and at 48 h. Histograms of total FL-DC (A) illustrate the expression of depicted markers on unstimulated cells (dashed line), 4h-DC (grey line) and 20h-DC (black line). Graphs (B) show mean fluorescence intensity (MFI) of total FL-DC, mDC and pDC (differentiated by FACS analysis) depicted as fold increase relative to unstimulated cells. One representative experiment of four is shown.

Gating for mDC ($CD11c^+$, $CD11b^{high}$) and pDC ($CD11c^+$, $CD11b^{low}$) during FACS analysis allowed the differentiation of expression levels of costimulatory molecules on mDC and pDC, respectively (Figure 4.2 B). Both subsets showed a similar pattern of expression of costimulatory and MHC molecules, although the increase of expression levels was more pronounced in mDC.

We thus show that short-term stimulation is as efficient as a long-term stimulation to induce an activated DC phenotype at 48 h. Furthermore, expression of costimulatory molecules decreases after an initial peak, suggesting that the immunostimulatory potential of DC may be higher at early time points after activation.

4.1.3 Induction of T helper cell responses

The early production of the T_h1-inducing cytokines IL-12p70 and IFN-α by FL-DC suggested that short-term activated DC could be more efficient in the induction of T_h1 responses than long-term activated DC. T_h1 responses are characterized by $CD4^+$ T helper cells that produce high amounts of IFN-γ after activation by antigen-presenting cells and that promote cell-mediated immunity. Ovalbumin (OVA), the chicken egg albumin, is often used as model antigen, as many reagents are available for the detection of OVA-specific immune responses. To examine the capacity of FL-DC to generate T_h1 responses, OVA protein-pulsed DC were cocultured for 48 h with OVA-specific CD4 T cells from OT-II mice. T cells from these mice bear a transgenic T-cell receptor which recognizes the $OVA_{323-339}$ peptide in the context of a MHC II molecule. After 48 h, the hallmark cytokines for T_h1- and T_h2-type responses, IFN-γ and IL-4, were measured in the supernatants. As predicted, 4h-DC induced high levels of IFN-γ, whereas T cells cocultured with 20h-DC produced no more IFN-γ than T cells cultured with unstimulated DC (Figure 4.3). No IFN-γ was produced when DC were pulsed with a control protein. FL-DC did not promote T_h2 responses, as IL-4 was not detected in any supernatants (data not shown).

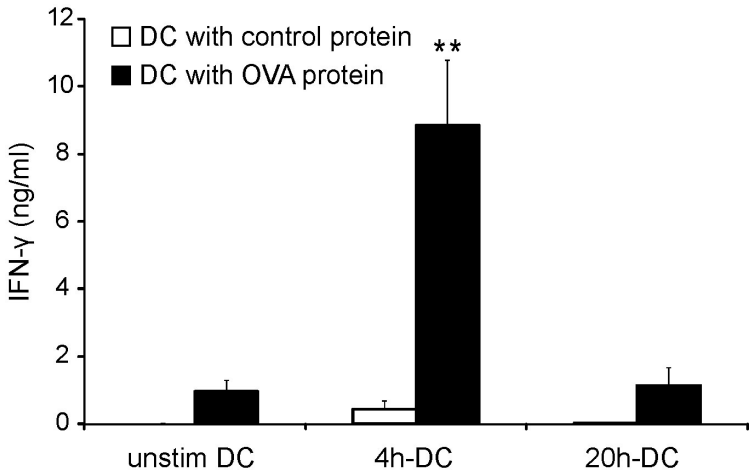

Figure 4.3: Induction of T_h1 responses by short-term activated FL-DC.
DC were pulsed with OVA (black bars) or survivin protein as control antigen (white bars), stimulated for 4 h or 20 h with CpG and cocultured with sorted OT-II CD4 T cells. IFN-γ production was measured in culture supernatants after 48 h. Results show means + SEM of quadruplicates of 2 independent experiments (** $P < 0.01$).

4.1.4 Initiation of cytotoxic T-cell responses

One of the main goals of DC vaccination is the induction of an efficient cytotoxic T-cell response against tumor-associated antigens. To assess the efficiency of short-term activated DC to present antigen to CTL, 4h-DC and 20h-DC were pulsed with the MHC I-restricted peptide $OVA_{257-264}$ derived from the OVA protein. Unstimulated DC or DC pulsed with the control peptide T1Db derived from the SV40 large T antigen were used as control. DC were then cocultured with splenocytes from OT-I mice. T cells from these mice bear a transgenic T-cell receptor which recognizes the $OVA_{257-264}$ peptide in the context of a MHC I molecule. In this assay, T-cell proliferation is used as an indicator of DC ability to induce efficient cytotoxic T-cell responses. Proliferation of T cells was assessed by a quantitative measurement of incorporated BrdU after a 48 h coculture of DC and splenocytes.

Both short- and long-term CpG stimulation enhanced the ability of DC to activate OVA-specific $CD8^+$ T cell proliferation to a similar extent compared to unstimulated DC (Figure 4.4 A), when DC were pulsed with OVA peptide. We could not detect a difference between short- and long-term activation, showing that a 4 h activation is as effective as an extensive stimulation of DC for 20 h.

To initiate a protective CTL response *in vivo*, extracellular antigens must be taken up, then processed and presented in the context of MHC class I molecules by mechanisms termed cross-presentation and cross-priming, respectively (Groothuis and Neefjes, 2005). To

determine the ability of 4h-DC and 20h-DC to cross-prime antigen-specific T cells, FL-DC were pulsed with complete OVA protein before activation with CpG. Antigen-pulsed, activated DC were then cocultured with OT-I splenocytes and proliferation was measured after 48 h. While unstimulated DC induced little CTL proliferation, activation of DC with CpG increased their efficiency to induce CTL proliferation independently of the duration of CpG stimulation (Figure 4.4 B).

The effector function of stimulated CTL was analyzed by measuring IFN-γ concentrations in the supernatant of DC – OT-I splenocyte cocultures. 4h-DC as well as 20h-DC promoted a strong IFN-γ secretion by OT-I CTL, while IFN-γ levels in cocultures with unstimulated DC were low. When DC were pulsed with a control peptide, no IFN-γ was induced (Figure 4.4 C), confirming the antigen-specificity of the DC-induced T-cell priming.

Thus, 4h-DC induce as efficient a response as 20h-DC when analyzing antigen-specific T-cell proliferation *in vitro*. Short-term activated DC were also highly efficient in cross-priming CTL, implying a potent induction of cytotoxic T-cell responses.

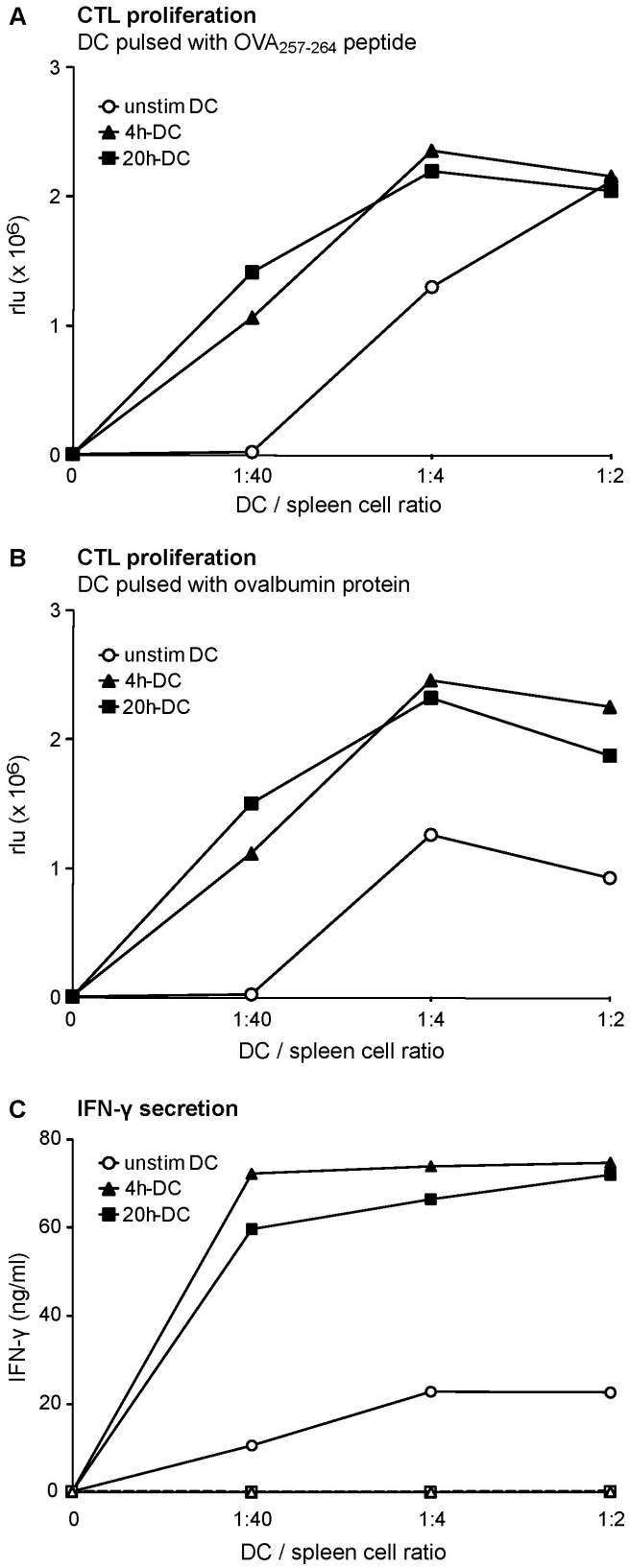

Figure 4.4: Induction of CTL responses by 4h-DC and 20h-DC.
(A, B) FL-DC were pulsed with $OVA_{257-264}$ peptide (A) or ovalbumin protein (B), stimulated for 4 h or 20 h with CpG and cocultured with splenocytes from OT-I mice. DC-induced T-cell proliferation was assessed after 48 h by measuring BrdU incorporation. (C) OT-I splenocytes were cocultured with peptide-pulsed DC to induce IFN-γ secretion by CTL. DC were loaded with $OVA_{257-264}$ peptide (black lines) or the irrelevant T1Db peptide as control antigen (dashed lines). One representative experiment of three is shown.

4.1.5 Inhibition of regulatory T-cell function

In addition to effector lymphocytes, which are capable of attacking invading microbes, the immune system also harbors an inhibitory population of T cells called regulatory T (T_{reg}) cells. These cells are specialized in suppressing excessive or misguided immune responses that can be harmful to the host, such as responses directed against normal self-constituents (Sakaguchi, 2000). On the other hand, overzealous T_{reg} cell responses can impede host protective immunity, which plays an important role in the immune evasion of tumors. High numbers of T_{reg} cells have been found in lung, pancreas, breast, liver, and skin cancer patients, either in peripheral blood or around and within the tumor (Cools et al., 2007). However, activation of DC by TLR ligands was shown to overcome the suppression mediated by T_{reg} cells (Pasare and Medzhitov, 2003). Due to the importance of T_{reg} cell-mediated immune suppression in cancer, inhibition of T_{reg} cell function is an essential step for the success of any DC vaccination. Therefore we investigated whether short-term activated DC can suppress T_{reg} cell function, or if DC need to be activated extensively in order to mediate the TLR ligand-induced effects on T_{reg} cells. For these experiments, proliferation of $CD4^+$ effector T helper cells was assessed by using an *in vitro* suppression assay. In this assay, $CD4^+$ T helper cell proliferation is induced by cross-linking T-cell receptors with an anti-CD3 antibody. When T_{reg} cells are cocultured with anti-CD3-stimulated T helper cells, they potently suppress T helper cell proliferation. TLR ligand-stimulated DC can inhibit T_{reg} cell-induced suppression and restore T helper cell proliferation, mainly by soluble factors such as cytokines (Pasare and Medzhitov, 2003).

To examine the effects of short- and long-term activated DC on T_{reg} cell suppression, 4h-DC and 20-DC were added to cocultures of $CD4^+$ T helper cells and T_{reg} cells in the presence of anti-CD3 antibody. Proliferation was measured by BrdU incorporation for 12 h after an incubation of DC and T cells for 48 h. Cocultures of T helper cells and DC without T_{reg} cells were used as control.

While T_{reg} cells suppressed $CD4^+$ T-cell proliferation down to 53 % of initial levels when cocultured with unstimulated DC, 4h-DC and 20h-DC restored CD4 T cell proliferation to 74% and 78% of the levels reached without regulatory T cells, respectively (Figure 4.5 A). In Figure 4.5 B, suppression was normalized to levels of cocultures with unstimulated DC. These results imply that FL-DC are able to overcome T_{reg}-mediated suppression of T-cell proliferation independently of the duration of CpG-activation of the DC.

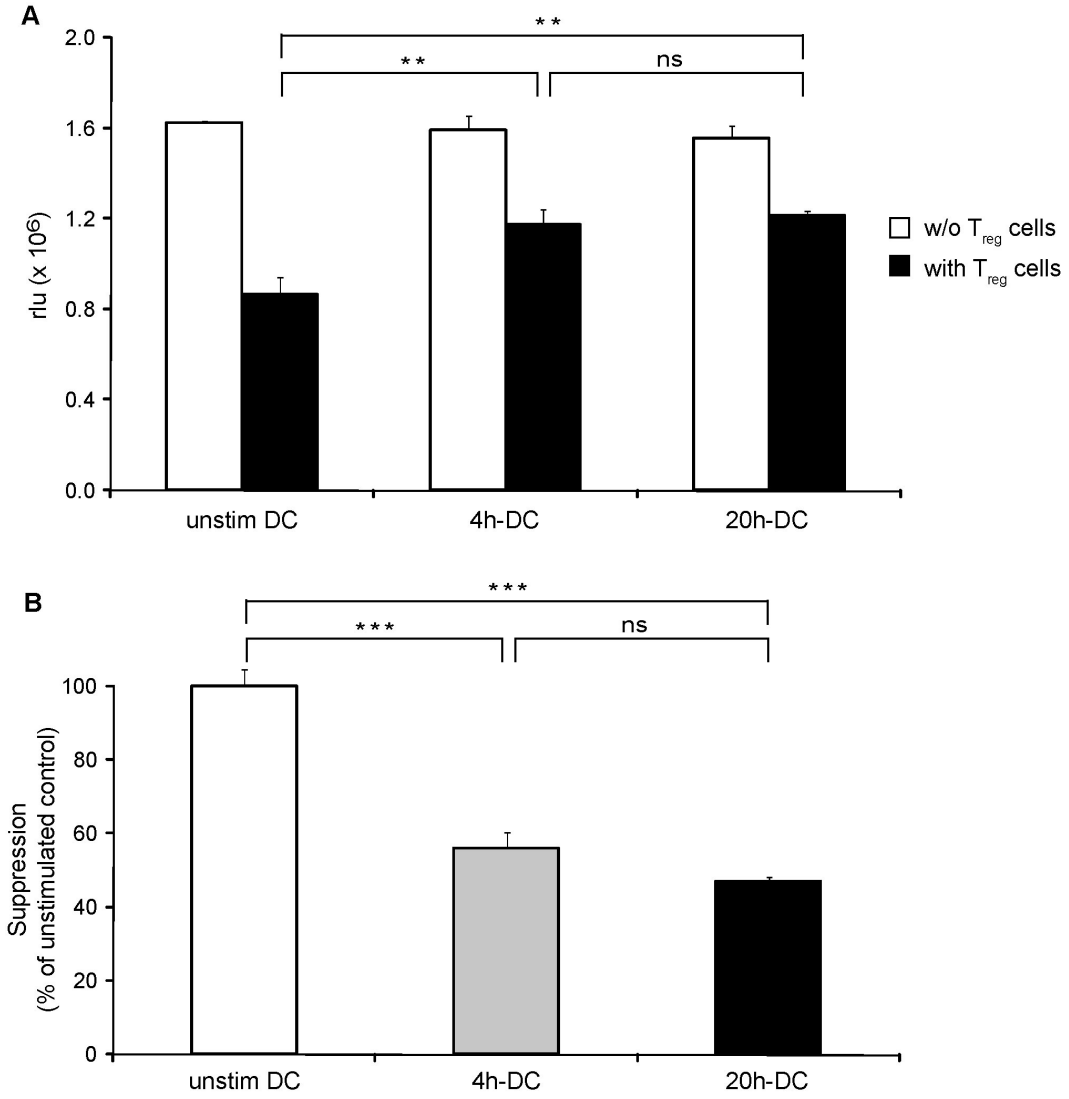

Figure 4.5: Inhibition of regulatory T-cell function.
4h-DC or 20h-DC were added to a coculture of $CD4^+$ T cells and T_{reg} cells in the presence of anti-CD3 antibody. Proliferation was measured by chemiluminescent detection of incorporated BrdU. (A) White bars show proliferation of T helper cells in the absence of T_{reg} cells, black bars show proliferation of T helper cells in coculture with T_{reg} cells. (B) The suppressive effect of T_{reg} cells on T helper cell proliferation was calculated by defining T_{reg} cell-mediated suppression in the presence of unstimulated control DC as 100% (for exact formula see Materials and Methods, p. 30). Results show means and SEM of one representative experiment of three (** $P < 0.01$, *** $P < 0.001$, *ns* not significant).

4.1.6 Migratory capacity of short-term activated dendritic cells

Upon activation, DC upregulate MHC molecules, CD40, CD80, and CD86, that are required for effective interaction with T cells. They also upregulate CCR7, a chemotactic receptor that guides DC to lymph nodes, where the DC - T cell interactions take place.

To assess the migratory potential of short- and long-term activated FL-DC, DC were analyzed for their expression of CCR7 by flow cytometry. CCR7 expression was rapidly

upregulated by CpG activation after 4 h of stimulation and did not further increase when stimulation was extended to 20 h (Figure 4.6).

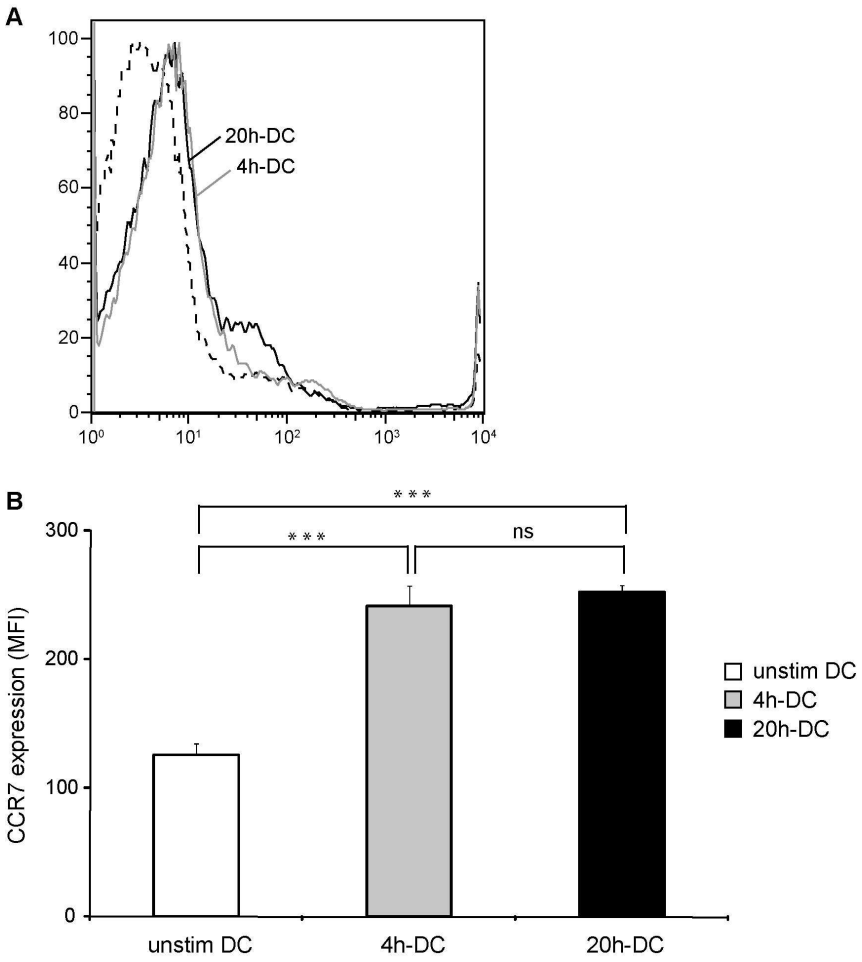

Figure 4.6: CCR7 expression of short- and long-term activated DC.
DC were activated for 4 h or 20 h with CpG and surface expression of CCR7 was measured by flow cytometry directly after stimulation. (A) Histograms illustrate the expression of CCR7 on unstimulated cells (dashed line), 4h-DC (grey line) and 20h-DC (black line). Graphs (B) show mean fluorescence intensity (MFI) of CCR7 expression as mean and SEM of MFI. One representative experiment of three is shown (***$P < 0.001$, *ns* not significant).

The migratory function of DC was examined in a transwell chemotaxis assay. In these assays, migration across a membrane towards the gradient of a chemokine is measured by counting the transmigrated cells.

Here, short- and long-term activated DC were analyzed for their capacity to migrate towards the CCR7 ligand CCL21 (6Ckine), which is important for DC homing to the lymph node. 4h-DC as well as 20h-DC migrated very efficiently towards the chemokine gradient compared to unstimulated DC, which showed only a poor migration towards the lower chamber containing CCL21 (Figure 4.7). Conditions without CCL21 were used as control.

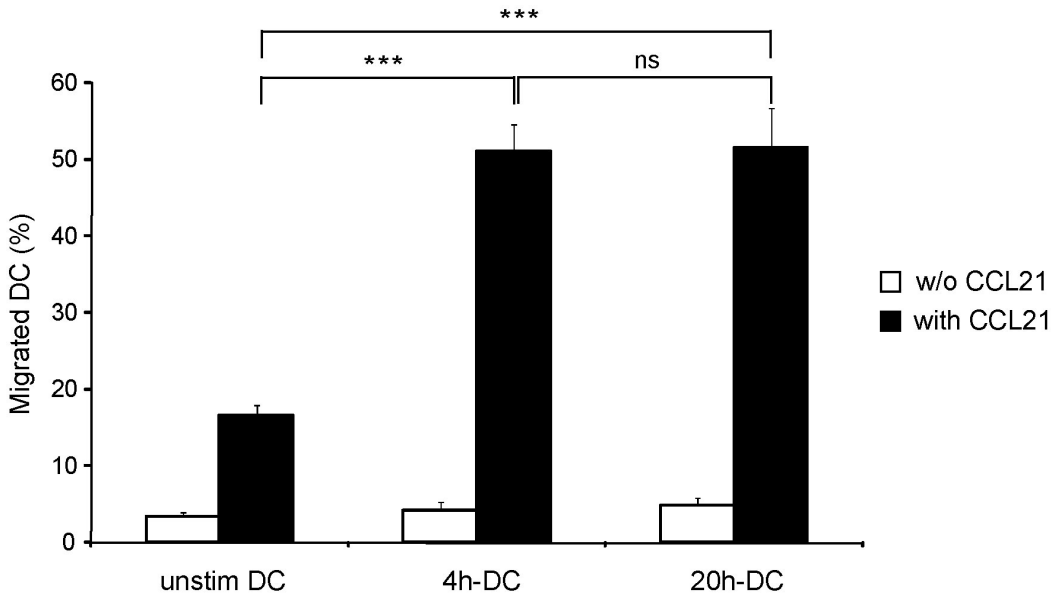

Figure 4.7: Migration of 4h-DC and 20h-DC towards the CCR7 ligand CCL21.
FL-DC were activated for 4 h or 20 h with CpG or left untreated before migration of differentially stimulated DC toward CCL21 was assessed in a transwell chemotaxis assay. White bars show percentage of migrated cells in conditions without CCL21, black bars illustrate migration of DC when CCL21 was added to the lower chamber. Mean and SEM from two experiments are shown (****P* < 0.001, *ns* not significant).

4.1.7 *In vivo* migration to draining lymph nodes

As a next step, the migratory potential of FL-DC was examined *in vivo*. For this purpose, DC were activated for 4 h or 20 h with CpG or left unstimulated, labeled with CFSE, and injected s.c. into the forelimb of naïve mice. 48 h later, the draining (brachial and axillary) lymph nodes were harvested and analyzed by flow cytometry. Contralateral lymph nodes were excised as control.

Migration of CpG-activated DC to the draining lymph node was increased compared to unstimulated DC, although the difference was not significant ($P > 0.1$). There was no difference in the migratory capacity of 4h-DC and 20h-DC (Figure 4.8). Migration only took place via the lymphatic vessels as there were no CFSE-positive cells in the contralateral lymph nodes or spleen (Figure 4.8 B and data not shown).

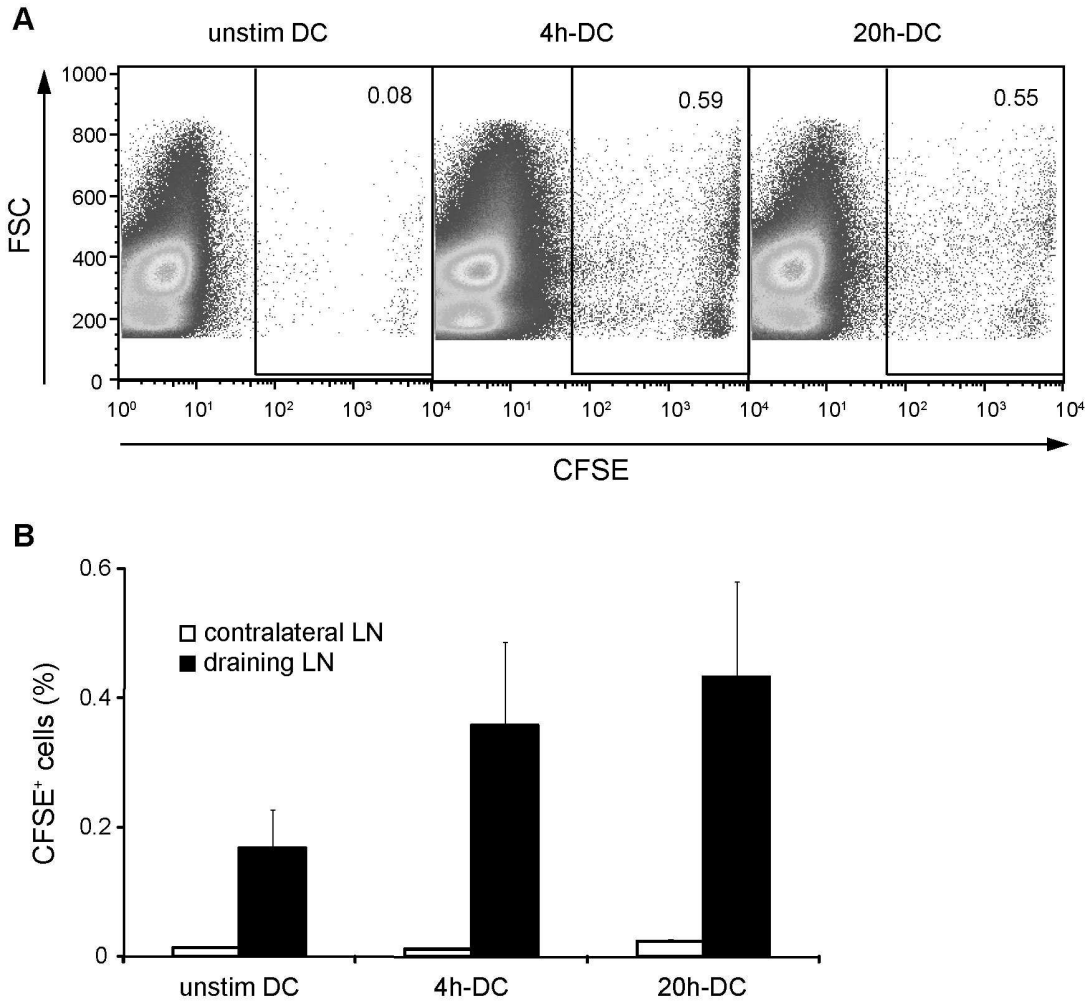

Figure 4.8: Migration of FL-DC *in vivo*.
DC were activated for 4 h or 20 h with CpG or left untreated, labeled with CFSE, and 3 x 10^6 cells were injected s.c. into the left antebrachium of naïve mice. 48 h later, draining (black bars) and contralateral (white bars) brachial and axillary lymph nodes were harvested and the percentage of $CFSE^+$ cells was analyzed by flow cytometry. (A) Dot plots show representative data of one mouse per group. Numbers indicate the percentage of $CFSE^+$ cells in the draining lymph node. (B) Results depicted are mean percentages of $CFSE^+$ cells + SEM of 3 mice per group. One representative experiment of three is shown.

4.1.8 Immunization with short-term activated dendritic cells

We demonstrated that short-term activated FL-DC induce antigen-specific IFN-γ secretion by T_h1 cells and CD8 T-cell proliferation. They can also overcome T_{reg} cell-mediated suppression *in vitro*. To examine the induction of antigen-specific immune responses *in vivo*, naïve C57BL/6 wild-type mice were immunized s.c. with OVA-pulsed FL-DC stimulated with CpG for 4 h or 20 h twice at a weekly interval. Serum antibody titers, frequency and cytolytic function of $CD8^+$ T cells were examined one week after the last immunization.

Serum levels of OVA-specific antibodies were measured to evaluate the effect of vaccination with short-term stimulated DC on B-cell responses. Compared to naïve mice, OVA-specific total IgG were increased significantly in mice immunized with both OVA protein-pulsed 4h-DC and 20h-DC (Figure 4.9).

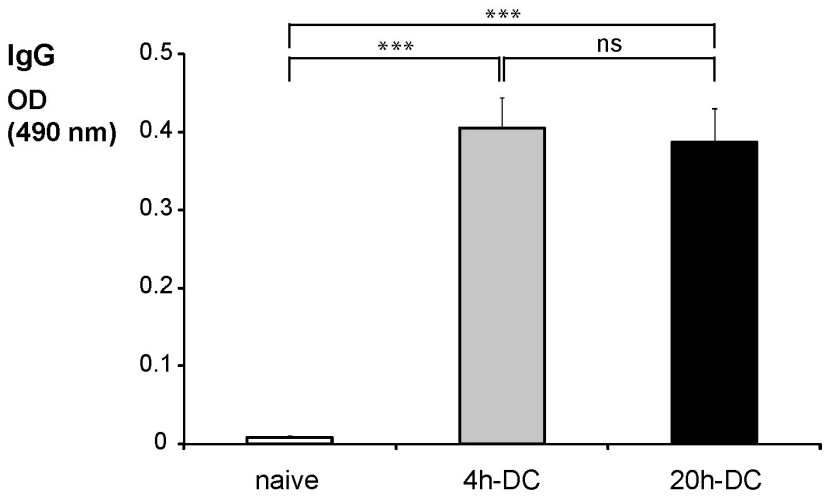

Figure 4.9: OVA-specific antibodies after immunization with FL-DC.
C57BL/6 mice were immunized twice s.c. with 2 x 10^5 FL-DC pulsed with OVA protein and stimulated for 4 h or 20 h with CpG. Concentration of OVA-specific total IgG in serum was measured by ELISA. Data show mean values + SEM of three mice per group. Results are representative of two independent experiments (***$P < 0.001$, *ns* not significant).

In addition to antibody responses, T-cell responses were analyzed after immunization with peptide-loaded FL-DC. The focus here was on cytotoxic T-cell responses, as they are crucial for the eradication of tumor cells. Synthetic MHC class I pentamers were used to identify cytotoxic T cells bearing a T-cell receptor that recognizes the $OVA_{257-264}$ peptide. Pentamer staining of peripheral blood cells showed that immunization with short-term activated FL-DC was sufficient to induce a high percentage of OVA-specific CTL, and thus a strong OVA-specific CTL response (Figure 4.10).

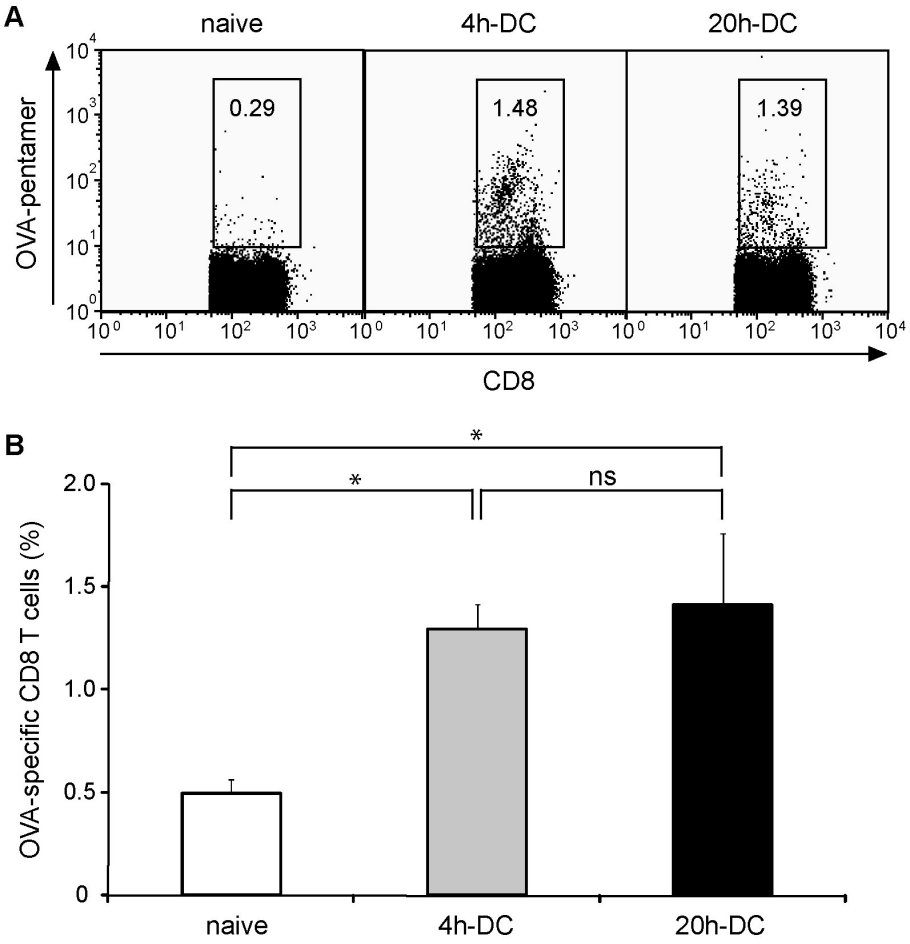

Figure 4.10: MHC pentamer staining of OVA-specific CTL.
C57BL/6 mice were immunized twice s.c. with 2 x 10^5 FL-DC stimulated for 4 h or 20 h with CpG and pulsed with $OVA_{257-264}$ peptide. Antigen-specific CD8 T cells in peripheral blood were measured by flow cytometry with H2-K^b $OVA_{257-264}$ pentamers one week after the last immunization. (A) Dot plots show representative data gated on CD8 T cells. Numbers indicate the percentage of CTL that are OVA-pentamer-positive. (B) Graph shows mean and SEM of eight mice from two independent experiments (*P < 0.05, *ns* not significant).

In vivo cytotoxicity assays were done to examine the cytotoxic T-cell response induced by vaccination with short- and long-term activated FL-DC. In these assays, peptide-loaded labeled target cells were injected into immunized mice and lysis of the target cells was measured in comparison to the lysis of unloaded cells, thus allowing an evaluation of the cytotoxic function of the antigen-specific CTL. Longer activation of DC for 20 h did not result in an increase in the frequency or cytotoxic activity of specific CTL in mice immunized with peptide-loaded DC (Figure 4.11 A). Similarly, no increase of cytotoxic activity was seen in mice immunized with 20h-DC compared to 4h-DC when DC were pulsed with whole OVA protein (Figure 4.11 B). Hence, 4h-activated FL-DC induced an efficient antibody and CTL response *in vivo* that was not increased by longer CpG activation.

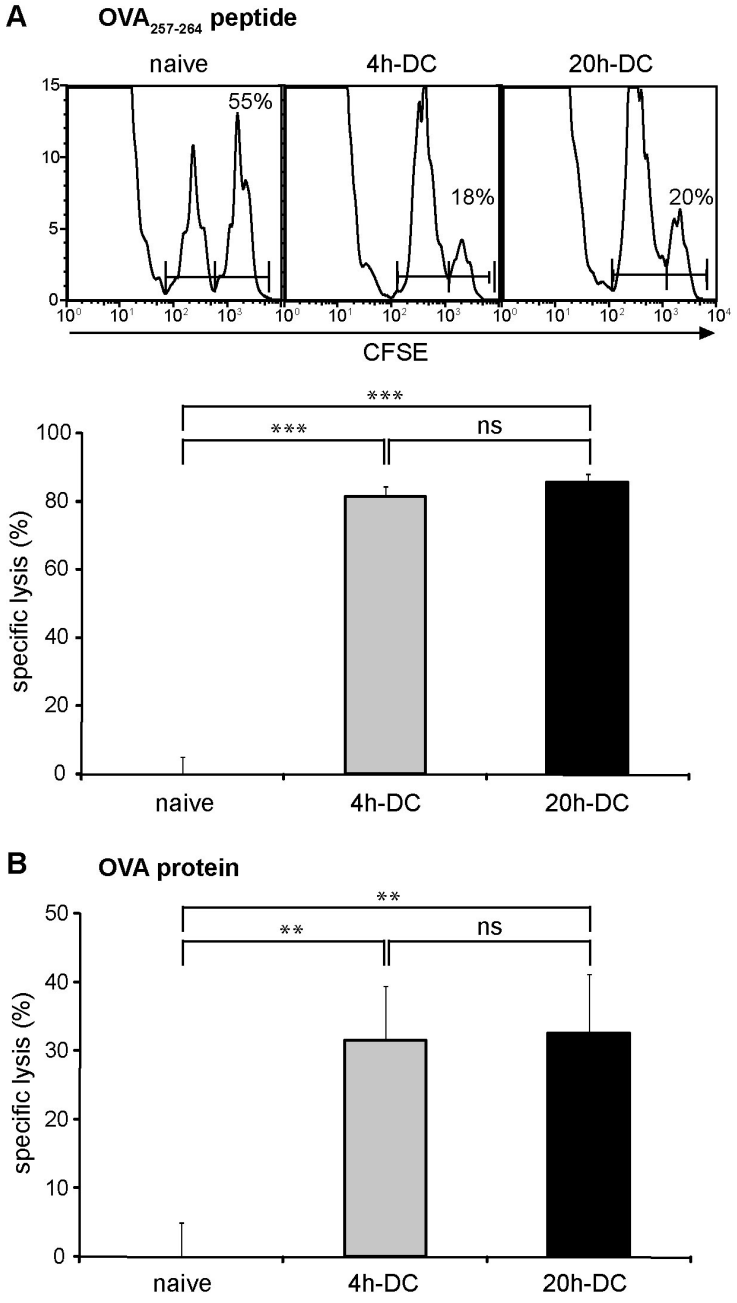

Figure 4.11: *In vivo* cytotoxicity of OVA-specific CTL.
To assess function of CTL, an *in vivo* cytotoxicity assay was performed by transferring CFSE-labeled unpulsed ($CFSE^{low}$) and peptide-pulsed ($CFSE^{high}$) target cells into immunized mice. Labeled cells were detected by flow cytometry in peripheral blood on day 3 after injection. Mice were immunized with peptide-pulsed DC (A) or protein-pulsed DC (B). Mean specific lysis and SEM of 5 mice per group is shown. Results are representative of two independent experiments (***P* < 0.01, ****P* < 0.001, *ns* not significant).

4.1.9 Tumor therapy with short-term activated dendritic cells

To examine whether the CTL response generated by short-term activated FL-DC is able to control tumor growth, 4h-DC and 20h-DC were used for DC vaccination in a murine model of colon cancer. An immunotherapy protocol combining CpG-activated GM-CSF / IL-4 DC with

peritumoral CpG injections to treat established C26 tumors has been established previously in our group (Bourquin et al., 2006; Heckelsmiller et al., 2002a). Here, immature FL-DC were pulsed with UV-irradiated tumor cells, acti-vated *in vitro* with CpG, and injected together with 100 µg CpG on the flank opposite the tumor. In some treatment groups, an additional dose of 100 µg CpG was injected in the peritumoral area to enhance the immunostimulatory effect.

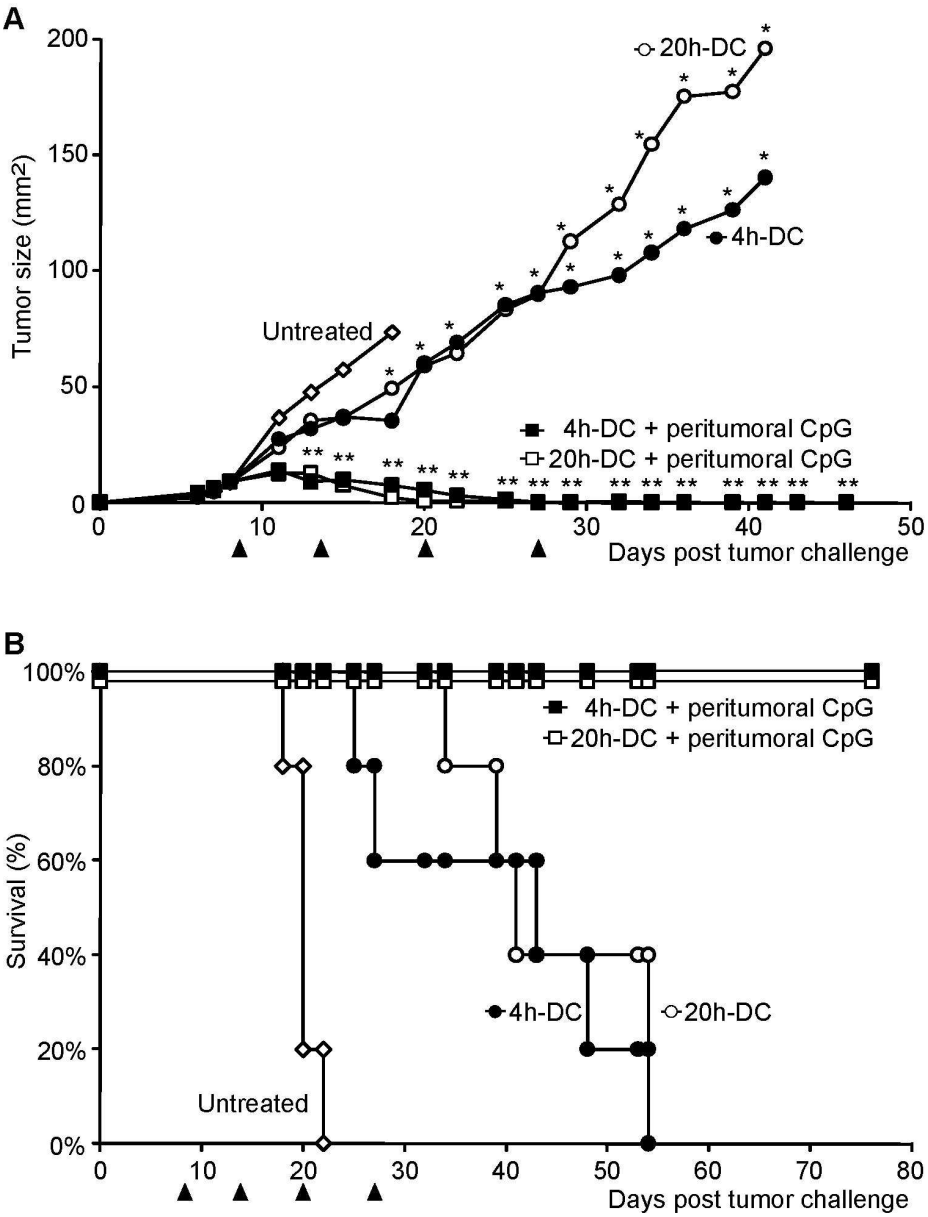

Figure 4.12: Tumor therapy with short-term activated FL-DC.
Balb/c mice bearing palpable C26 tumors were injected s.c. with 100 µg CpG and CpG-activated FL-DC pulsed with irradiated tumor cells in the flank opposite the tumor. Two groups received an additional 100 µg CpG peritumorally. The treatment was administered four times at 5- to 7-day intervals (arrows). (A) 4h-DC and 20h-DC significantly reduced tumor growth compared to untreated mice (**P* < 0.05 from day 18 onwards). The addition of peritumoral CpG led to complete tumor regression in all treated mice (***P* < 0.01 compared to untreated mice at all time points from day 13). Mean tumor size of treatment groups (n=5) is plotted until two mice per group remain. (B) Treatment with either 4h-DC or 20h-DC significantly increased survival (*P* < 0.01). Similar results were obtained in four independent experiments.

Antigen-pulsed, activated FL-DC injected together with 100 µg CpG on the contralateral flank reduced tumor growth and led to a significant increase in survival compared to untreated mice (Figure 4.12). This therapeutic effect was independent of the duration of the *in vitro* activation of the DC. When the therapy was supported with an additional injection of CpG in the peritumoral area, all mice rejected their tumors and remained tumor-free for four months after the last DC vaccination. It was previously shown that using this protocol, peritumoral CpG injection alone does not result in a significant reduction of tumor growth (Heckelsmiller et al., 2002a). In addition, immunization with irradiated tumor cells and CpG in the absence of DC did not protect against a subsequent tumor challenge and could not cure established C26 tumors (Heckelsmiller et al., 2002b).

To evaluate long-term protection, the cured mice were rechallenged s.c. with C26 tumor cells four months after the last treatment. Naïve mice were used as controls. None of the cured mice developed a tumor. In contrast, all naïve control mice developed tumors (Table 4.1, $P = 0.008$). There was no difference between mice treated with 4h-DC or 20h-DC. Thus, short-term activated DC effectively cured established tumors and at the same time induced a potent memory response that provided protection until after several months after the last treatment.

Table 4.1: Tumor formation in mice cured from C26 tumors and rechallenged with C26 cells.

	naïve	**4h-DC + CpG**	**20h-DC + CpG**
Proportion of mice with tumor	5 of 5	0 of 5	0 of 5

4.2 Distinctive features of TLR7 stimulation

CpG as a TLR9 ligand enhances immune responses in many murine tumor models and has shown substantial evidence of antitumor activity in human clinical trials (Krieg, 2007). However, the distribution of TLR9 differs substantially between murine and human DC populations. In mice, TLR9 is expressed by pDC and mDC, whereas in humans TLR9 expression is mainly restricted to pDC (Krug et al., 2001). Similarly to TLR9, DC can also be stimulated via TLR7 and TLR8, which are expressed on both human and murine mDC and pDC (Hornung et al., 2002) and ligands of TLR7/8 have already been successfully used as vaccine adjuvants in clinical trials (Shackleton et al., 2004). TLR7/8 and TLR9 are described to activate the same intracellular pathways via MyD88. In view of the therapeutic potential of TLR7 ligands, their influence on DC maturation was analyzed in closer detail.

4.2.1 Kinetics of TLR7-induced cytokine production

As analyzed in chapter 4.1.1 for TLR9 stimulation with CpG, the kinetics of FL-DC activation were examined for ligands of TLR7 and 8. The experimental setup was identical to the stimulation with CpG: Total FL-DC were stimulated with R848 as ligand for murine TLR7 (and TLR8 in humans), or CL087 as a pure TLR7 ligand, for 2 h up to 24 h. After the stimulation period, cytokine levels were measured in the supernatant (Figure 4.13, grey bars). Cells were washed and culture was continued for a total of 48 h, when again cytokine secretion was determined (Figure 4.13, black bars).

For both ligands, IL-12p70 secretion through TLR7 was induced even more rapidly than with the TLR9 ligand CpG and persisted after removal of the stimulus (Figure 4.13). As with CpG stimulation, production ceased after 6 h. IL-6 showed a sustained secretion for over 24 h. IL-10 was again secreted late, and levels were highest after long stimulation times. Thus, as for TLR9, TLR7/8 activation induces rapid and efficient production of T_h1 cytokines by DC that is restricted to the first 6 to 8 hours after initiation of stimulation.

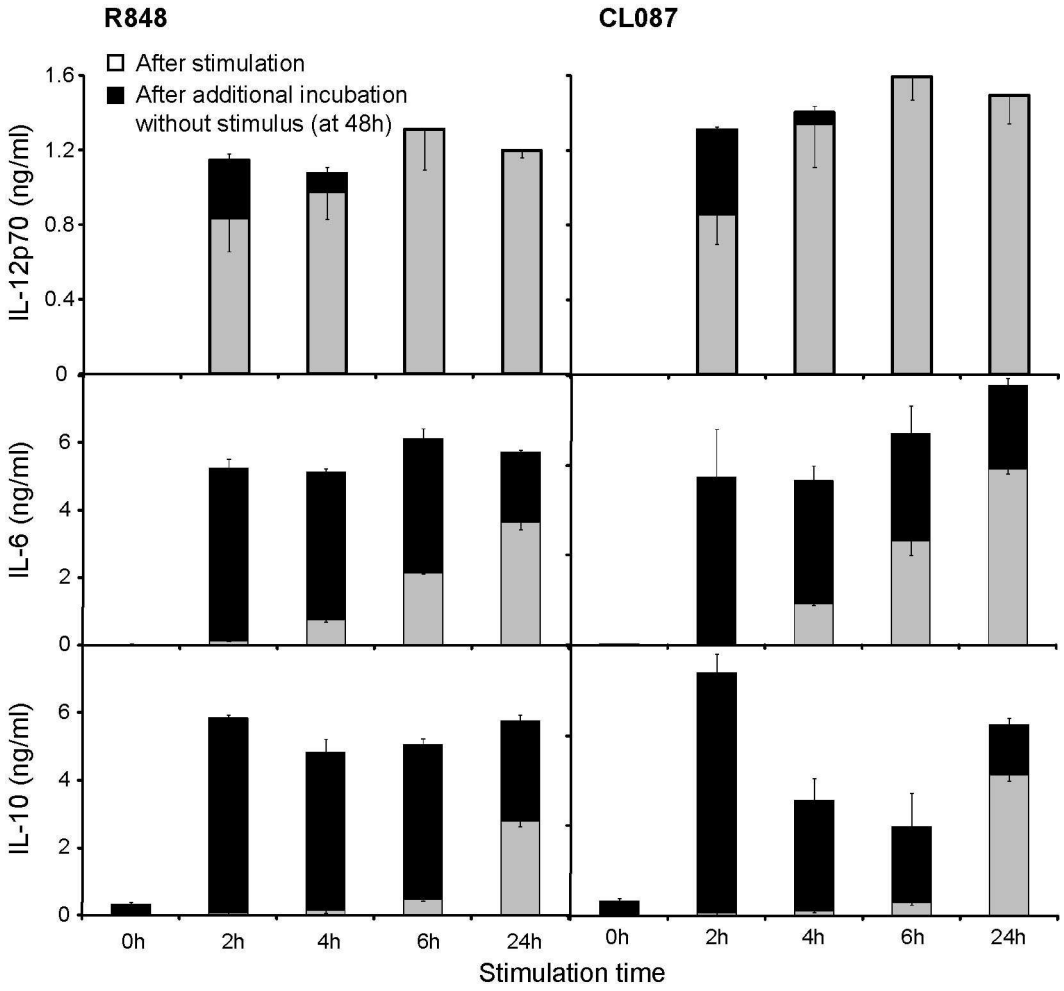

Figure 4.13: Kinetics of cytokine production of FL-DC after TLR7 stimulation.
Total FL-DC were stimulated with the TLR7/8 ligand R848 or CL087 as TLR7 ligand for 2 to 24 h, extensively washed to remove excess ligands and cultured without further stimulation for a total of 48 h. Cytokine concentration in the supernatant was measured directly after the 2 to 24 h stimulation period (grey bars) and at 48 h, after additional incubation without stimulus (black bars). Results show mean and SEM of one representative experiment of three.

4.2.2 Lack of IL-12p70 production in TLR7-activated dendritic cells

Interestingly, cytokine secretion differed substantially between DC subsets after stimulation with the TLR7/8 ligand R848. When FL-DC were magnetically sorted into $B220^+$ pDC and $B220^-$ mDC, mDC produced IL-12p70 only upon TLR9 but not TLR7 activation. Yet, pDC as well as mDC generated by conventional GM-CSF / IL-4 culture responded to both CpG and R848 with a similar production of IL-12p70 (Figure 4.14 A). FL-mDC did secrete other cytokines including the IL-12p40 subunit upon R848 stimulation, indicating the expression of functional TLR7 receptors on these cells (Figure 4.14 B). Stimulation by other TLR7 and TLR7/8 ligands, such as Imiquimod, Gardiquimod, CL079, CL087, and CL097 showed the same pattern of cytokine production (data not shown). This discrepancy between TLR7- and TLR9-induced IL-12 secretion is likely to be a general feature FL-mDC, as it was seen in

cells from mice with different genetic backgrounds (Balb/c and C57BL/6, data not shown). As TLR7 and TLR9 are thought to activate the same intracellular signaling pathways, we decided to further analyze this difference.

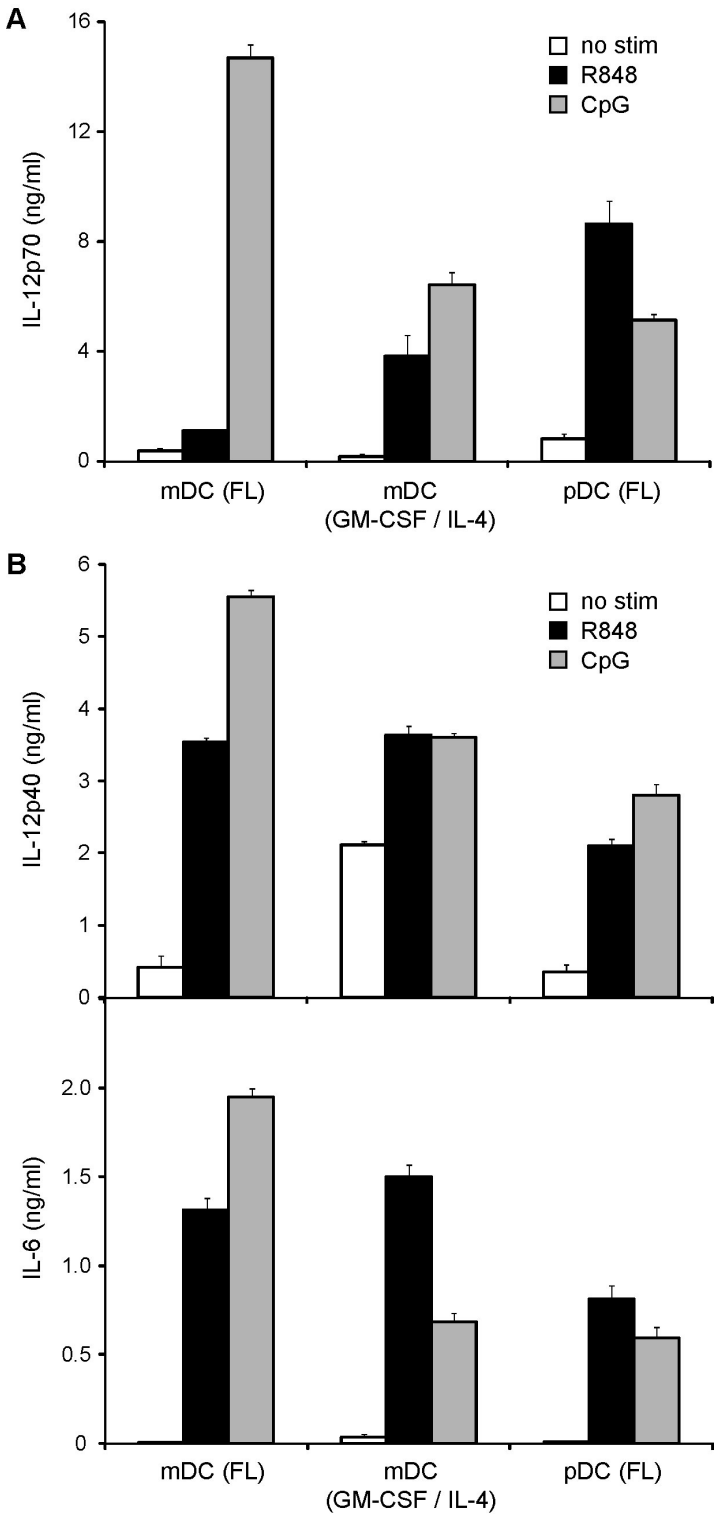

Figure 4.14: Cytokine production by DC subsets upon R848 stimulation.
Total FL-DC and sorted pDC and mDC of the FL-DC culture as well as mDC generated by culture with GM-CSF and IL-4 were stimulated with R848 (black bars), CpG (grey bars), or left untreated (white bars) for 20 h and cytokine concentration in the supernatant was measured by ELISA. Results show mean and SEM of one representative experiment of five.

4.2.3 The role of p38 MAPK for IL-12p70 inhibition

The molecular mechanisms underlying the IL-12 production by DC are not fully understood, although different molecules have been identified that play a role in its regulation. The family of mitogen-activated protein kinases (MAPK) has been implicated in the regulation of IL-12 by different authors, and especially the p38 MAPK was shown to control transcription of both the p40 and the p35 subunit of the bioactive IL-12p70 heterodimer (Lu et al., 1999). Further, inhibition of p38 MAPK by the pyridinyl imidazole inhibitor SB203580 reduced the amounts of IL-12p70 secreted upon LPS stimulation or CD40 ligation in bone-marrow derived DC generated with GM-CSF and an immature murine splenic DC cell line (Fukao et al., 2002; Kikuchi et al., 2003; Yanagawa and Onoe, 2006). To examine whether TLR7-induced IL-12 production was influenced by p38 MAPK, IL-12p70 secretion of R848-stimulated FL-DC subsets was examined in the absence and presence of the p38 MAPK inhibitor SB203580.

FL-mDC, which do not secrete IL-12p70 production upon TRL7 stimulation without p38 MAPK inhibition, showed a marked increase of IL-12p70 secretion when p38 MAPK was blocked by SB203580. In contrast, IL-12p70 production in pDC was inhibited (Figure 4.15). Thus, pDC react to p38 MAPK inhibition similarly as described for LPS-stimulated GM-CSF DC, whereas FL-mDC seem to rely on a different regulation of IL-12 production that is inhibited by p38 MAPK.

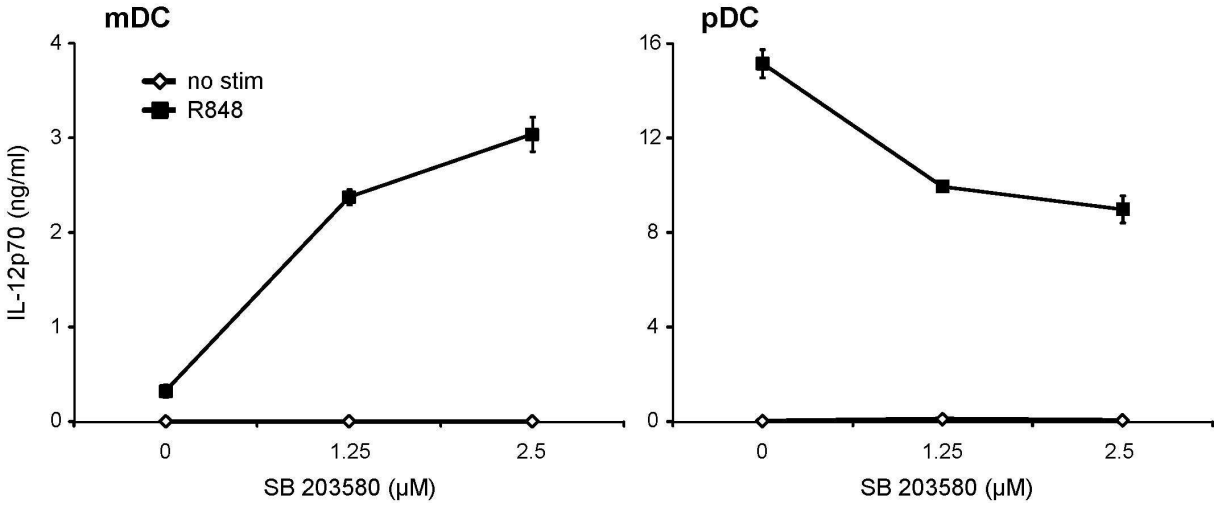

Figure 4.15: IL-12p70 secretion after inhibition of p38.
mDC and pDC sorted from FL-DC were incubated with different concentrations of the p38 inhibitor SB203580 for 30 min before cells were stimulated with R848 or left unstimulated for 20 h. IL-12p70 concentration in the supernatant was measured by ELISA. Results show mean and SEM of triplicate wells.

4.2.4 Influence of various cytokines on IL-12p70 secretion

To further characterize the regulation of IL-12p70 production in FL-mDC, cells from mice deficient for different signaling molecules were analyzed. IL-10 has been known to negatively regulate the secretion of IL-12 for more than 15 years (D'Andrea et al., 1993). The NF-κB family member p50 was proposed to induce the transcription of IL-10 (Cao et al., 2006), and heterodimers of p50 and other NF-κB transcription factors like p65 and c-Rel were described to contribute to the transcription of many inflammatory cytokines, including IL-12 (Doyle and O'Neill, 2006). Transcription factors of the IRF family on the other hand are responsible for the induction of interferons. As especially type I interferons have been shown to promote IL-12 production via an autocrine-paracrine feedback loop (Gautier et al., 2005), knock-out mice that lack both IRF3 and IRF7 were also examined.

FL-DC were generated from bone marrow cells from wild-type C57BL/6 mice and mice deficient for either IL-10, p50, or both IRF3 and IRF7. Sorted mDC were stimulated with R848 or CpG as positive control, and supernatants were analyzed for IL-12p70 concentrations.

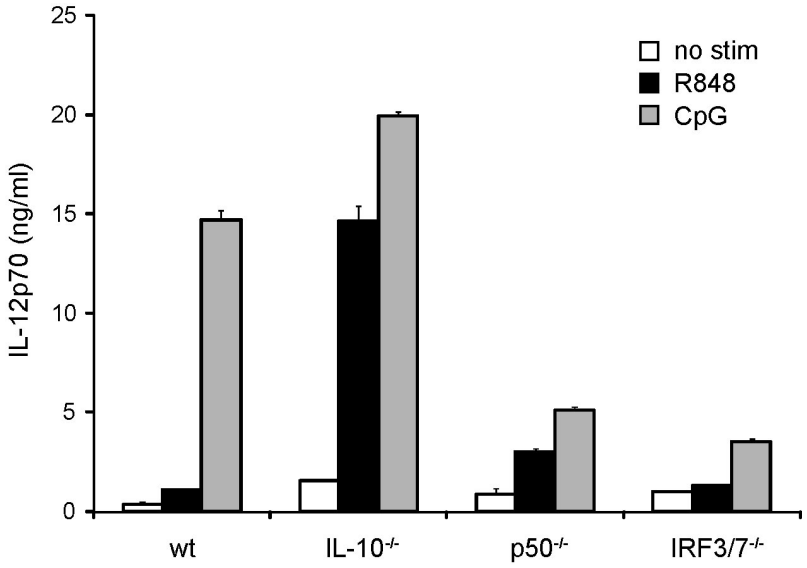

Figure 4.16: IL-12p70 production in different knock-out mice.
mDC from different mouse strains were sorted by B220 depletion from FL-DC and stimulated with R848 (black bars) or CpG (grey bars) for 20 h. IL-12p70 concentration in the supernatant was measured by ELISA. Results show mean and SEM of one representative experiment of two. *wt* wild-type, $^{-/-}$ homozygous knock-out.

As expected, IL-12p70 secretion was higher in cells from IL-10 knock-out mice than in wild-type cells, confirming the negative regulation of IL-12 by IL-10 (Figure 4.16). Yet, while IL-12 production after CpG was increased only by about 30%, IL-12 concentration in R848-stimulated cells was induced much more potently than in wild-type cells with levels that were

almost 15-fold increased compared to wild-type cells. IL-12p70 secretion was reduced but still existent in both p50- and IRF3/7-deficient cells (Figure 4.16), indicating an important but not exclusive role for these transcription factors in the induction of IL-12. While IRF3/7 knock-out cells showed the same pattern as wild-type cells with no IL-12p70 secretion upon R848 stimulation, p50-deficient cells produced low amounts of IL-12 after TLR7 stimulation. This is probably due to the reduced IL-10 levels in these cultures (Cao et al., 2006) and therefore mimics the results of the IL-10 knock-out cells.

Thus, IL-10 seems to be important for the inhibition of IL-12p70 production upon R848 stimulation. Yet, CpG induces even higher levels of IL-10 secretion in wild-type FL-mDC than TLR7 activation by R848 (Figure 4.17), indicating an additional or different regulatory mechanism underlying the lack of IL-12p70 production after TLR7 stimulation. The delayed kinetics of IL-10 secretion compared to IL-12p70 production (see Figure 4.13, p. 62) further support the necessity of another regulatory factor controlling IL-12 secretion in FL-mDC.

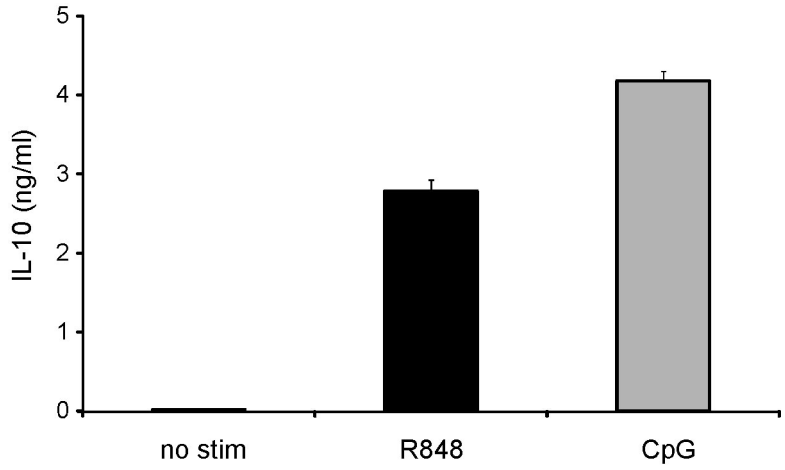

Figure 4.17: IL-10 production by FL-mDC.
Magnetically sorted FL-mDC from wild-type mice were stimulated with R848, CpG, or left untreated for 20 h and IL-12p70 concentration in the supernatant was measured by ELISA. Results show mean and SEM of one representative experiment of three.

4.2.5 Gene expression profiles of TLR7- versus TLR9-stimulated FL-mDC

To further examine the regulation of IL-12 production in FL-mDC, we analyzed genome-wide expression profiles of FL-mDC stimulated with R848 and CpG by means of a microarray. FL-mDC were magnetically sorted by depletion of $B220^+$ pDC from total FL-DC from wild-type C57BL/6 mice and examined by FACS analysis for their purity. Cells were stimulated with R848 or CpG for 4 h or left untreated before RNA was isolated. A short 4 h stimulation period was chosen because of the rapid induction of IL-12p70 and because of possible

indirect effects of other newly induced genes that would bias the results. cRNA was prepared and hybridized to two Illumina Mouse-6 v1.1 Expression Bead Chips as described in chapter 3.6.2.1, p. 40. Hybridization, scanning and statistical analysis of the microarrays were done by Peter Weber and Benno Pütz at the Max Planck Institute of Psychiatry in Munich, Germany. Cytokine concentration in the supernatants was analyzed by ELISA to test for a successful stimulation and showed the induction of IL-6 in all R848- and CpG-stimulated samples and the production of IL-12p70 by CpG-stimulated samples, as expected (data not shown).

Microarray data was normalized and further submitted to a cluster analysis to visualize similarities between the expression profiles of the different samples. As shown in the dendrogram in Figure 4.18, the quadruplicates of each condition are clearly separated into distinct branches, implying a good reproducibility of results despite the distribution of the samples on two different arrays. It also shows that the expression profiles of R848- and CpG-stimulated cells are more similar to one another than to those of unstimulated cells, indicating the induction of similar gene transcription networks in both TLR7- and TLR9-stimulated cells.

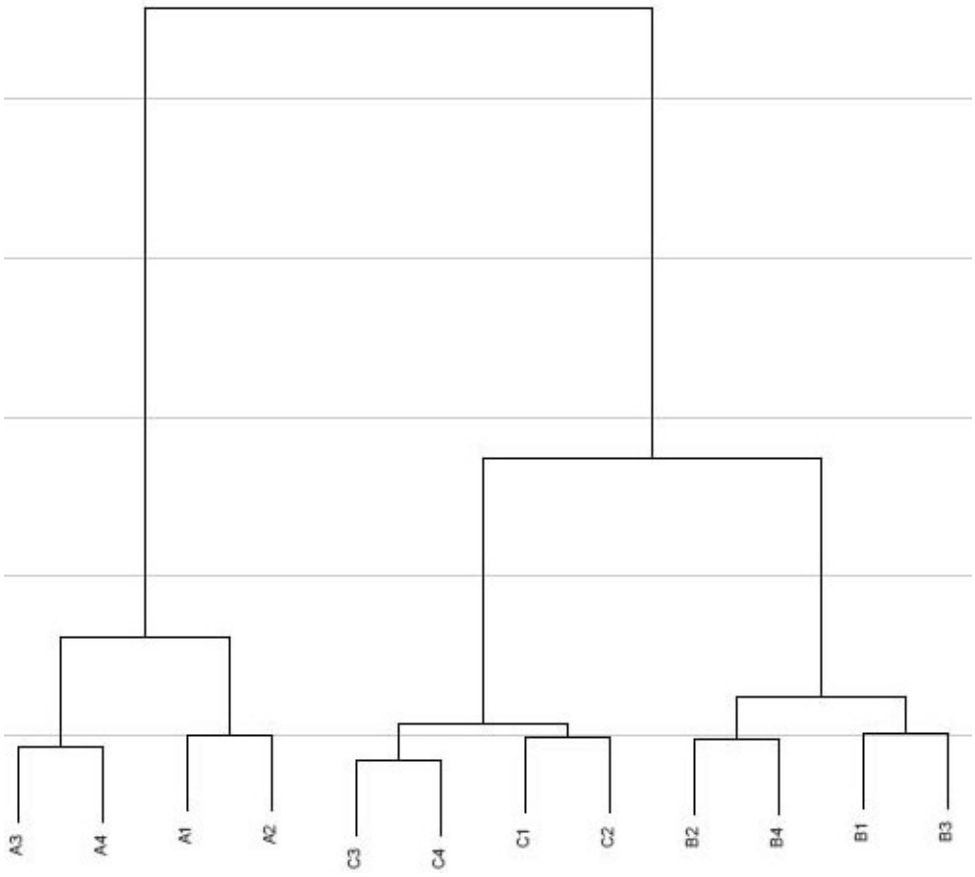

Figure 4.18: Clustering of normalized data.
Cluster analysis of normalized microarray data visualizes similarities between the expression profiles of different samples. *A* unstimulated cells, *B* R848 stimulated cells, *C* CpG stimulated cells, *1-4* number of quadruplicates.

For further analysis, differentially expressed genes were identified by comparing gene expression levels between the different samples. The evaluation of all conditions as a combined contrast was done by ANOVA; for single contrast analysis, Student's t-test was used. 4603 genes showed no expression signals in any sample and were excluded from subsequent analysis. Due to the high number of differentially expressed genes, the level of significance was set at $P < 0.001$. Table 4.2 gives an overview of the number of regulated genes determined by t-test.

Table 4.2: Differentially expressed genes in microarray experiment ($P < 0.001$).

	Total	Down	Up
R848 vs unstim	3367	1721	1646
CpG vs unstim	5749	3313	2436
R848 vs CpG	1375	681	694

Down downregulated , *Up* upregulated.

To examine the distinctive mechanism of TLR7 and TLR9 stimulation, pathways or regulatory genes were sought that are only expressed with either TLR7 or TLR9 stimulation. For this purpose, genes that were differentially expressed in R848-stimulated cells compared to unstimulated cells were matched with genes that were either up- or downregulated in CpG-stimulated cells compared to unstimulated cells. Due to the high number of differentially expressed genes in both conditions, the cutoff for further analysis was set more stringently: Differentially expressed genes were sorted by their respective fold change in expression. For R848 versus unstimulated, genes with a fold change of more than 2, for CpG versus unstimulated, genes with a fold change of more than 2.5 were chosen for further analysis, yielding approximately 1000 genes in each condition. Differentially expressed genes in CpG-stimulated cells were computationally subtracted from those genes that were regulated after R848 stimulation and vice versa. These genes were manually matched up reciprocally. For this purpose, fold changes in expression levels of stimulated cells versus unstimulated cells were compared between CpG and R848 stimulation. For R848-stimulated samples, only genes with a fold change differing by more than 1.5 to the fold change induced by CpG stimulation were selected, yielding 15 genes that were stimulated after R848 but not CpG stimulation. 4 of these genes play a role in inflammatory responses, and 3 are described to act in signal transduction (Table 4.3). For CpG-stimulated samples, the required difference in fold changes was set to 2.5, resulting in 46 genes. Here, 8 and 13 genes are responsible for inflammatory responses and signaling, respectively (Table 4.4). Expression levels of these genes will be verified by quantitative real-time PCR.

Table 4.3: Genes differentially expressed after R848 but not CpG stimulation.

Gene	Biological process	Fold change R848 vs unstim	Fold change CpG vs unstim	Difference R848 vs CpG (ranking value)
CSF3	Inflammatory response	6.2	n.s.	6.2
IL21R	Inflammatory response	2.1	-1.5	3.6
ORM1	Inflammatory response	2.9	n.s.	2.9
FCGR1A	Inflammatory response	3.1	1.3	1.8
GPR35	Signaling	2.1	-1.7	3.8
SDCBP2	Signaling	2.0	n.s.	2.0
JAK3	Signaling	2.0	n.s.	2.0
PIWIL2	Other	3.2	-1.5	4.7
KIAA0062	Other	2.1	-1.3	3.4
IER3	Other	2.4	n.s.	2.4
CCND1	Other	2.2	n.s.	2.2
SIAT9	Other	-2.1	n.s.	2.1
DNMT3L	Other	2.0	n.s.	2.0
retnlg	Unknown	-4.3	-1.7	2.6
CRA	Unknown	2.1	n.s.	2.1

Table 4.4: Genes differentially expressed after CpG but not R848 stimulation.

Gene	Biological process	Fold change CpG vs unstim	Fold change R848 vs unstim	Difference CpG vs R848 (ranking value)
igh-1a	Inflammatory response	8.9	n.s.	8.9
LTA	Inflammatory response	10.2	1.7	8.5
FCGRT	Inflammatory response	-7.1	-1.6	5.5
TZFP	Inflammatory response	7.0	1.7	5.2
IL6ST	Inflammatory response	-4.2	n.s.	4.2
TNFSF15	Inflammatory response	3.6	n.s.	3.6
IFNB1	Inflammatory response	3.2	n.s.	3.2
CD8B1	Inflammatory response	4.9	2.0	2.9
PLEKHG2	Signaling	-2.7	1.6	4.3
MYLK	Signaling	4.1	n.s.	4.1
Tim2	Signaling	4.0	n.s.	4.0
EDG7	Signaling	-2.5	1.4	3.9
CREM	Signaling	-3.9	n.s.	3.9
GPR146	Signaling	-3.6	n.s.	3.6
CD19	Signaling	4.8	1.8	3.0
DLL4	Signaling	2.9	n.s.	2.9
NR4A2	Signaling	-2.9	n.s.	2.9
ARHE	Signaling	2.8	n.s.	2.8
PLXNB2	Signaling	-2.7	n.s.	2.7
WNT11	Signaling	-2.7	n.s.	2.7
TCF7L2	Signaling	3.9	1.3	2.6

Gene	**Biological process**	**Fold change CpG vs unstim**	**Fold change R848 vs unstim**	**Difference CpG vs R848** (ranking value)
HIP1	Other	-3.8	1.5	5.3
ITGA3	Other	-6.6	-1.8	4.9
HS3ST1	Other	4.5	n.s.	4.5
CACNA1S	Other	4.6	1.3	3.3
TPBG	Other	3.3	n.s.	3.3
OLFM1	Other	-4.5	-1.4	3.1
GFI1B	Other	4.9	1.8	3.1
FLJ20559	Other	4.6	1.6	3.1
ITGB8	Other	4.6	1.8	2.9
CKIP-1	Other	-4.4	-1.6	2.8
MAP1B	Other	-2.7	n.s.	2.7
TOSO	Other	2.7	n.s.	2.7
BMF	Other	-2.6	n.s.	2.6
RBPMS	Other	4.4	1.9	2.6
ST14	Other	2.5	n.s.	2.5
dhrs8	Other	4.1	1.7	2.5
au044919	Unknown	5.7	1.3	4.4
C10orf81	Unknown	4.0	n.s.	4.0
Phxr4	Unknown	5.4	1.8	3.6
RHBDL3	Unknown	3.6	n.s.	3.6
C1orf161	Unknown	3.0	n.s.	3.0
WDR59	Unknown	2.7	n.s.	2.7
KRT222P	Unknown	2.5	n.s.	2.5
TLCD1	Unknown	4.0	1.5	2.5
C18orf17	Unknown	4.2	1.8	2.5

Protein names in italics show genes that are represented on the array with more than one probe but showed similar results with all probes.

Expression data confirmed cytokine production from previous experiments, with high levels of IL-12p35 transcripts only after CpG but not R848 stimulation (Figure 4.19). The increase of RNA levels of the p40 subunit was also higher in CpG- than in R848- stimulated cells, reflecting p40 concentrations in supernatants. Expression of IL-10 was upregulated with TLR7 as well as TLR9 stimulation and confirmed previous data. Expression of MAPK14 (p38α), the only isoform of p38 MAPK which showed transcriptional regulation in this assay, was downregulated with both stimulations (Figure 4.19).

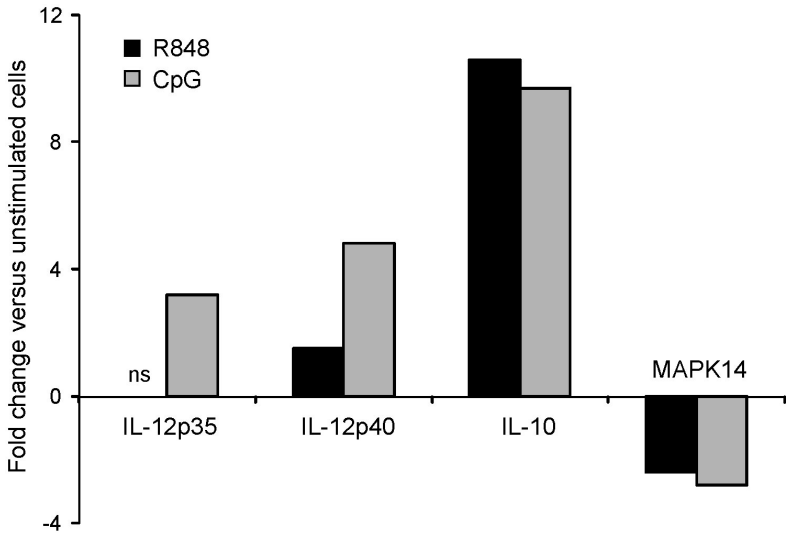

Figure 4.19: Expression of candidate genes in microarray.
Expression levels of IL-12p35, IL-12p40, IL-10 and MAPK14 (p38α) were compared between unstimulated cells and R848- and CpG-stimulated cells, respectively. Different RNA levels are shown as fold change in stimulated cells versus unstimulated cells. *ns* not significant.

4.2.6 Integrating data by pathway analysis

To further evaluate the biological pathways responsible for IL-12p35 transcription, differentially expressed genes were analyzed by pathway analysis using the Pathway Studio Software. When the genes that were only regulated with either TLR7 or TLR9 stimulation were examined for connective pathways by literature information embedded in the Pathway Studio software, no signaling network was identified.

By integrating gene expression levels from the present study with described regulators of IL-12 transcription, only IL-10, IFN-γ, and IRF1 showed higher expression levels after stimulation. Since these genes were all upregulated with TLR7 as well as TLR9 stimulation, they are probably not responsible for the differences in IL-12p35 transcription described above.

As a next step, common regulators for IL-10 and IL-12 were analyzed for their expression levels in the microarray experiment. Figure 4.20 shows genes described to regulate both IL-10 and IL-12 synthesis that were differentially expressed in stimulated compared to unstimulated cells. Expression levels after R848- and CpG stimulation were similar for all genes but IFN-β1, which was found to be regulated only with CpG but not R848 stimulation. Further analysis is necessary to examine the importance of IFN-β production for IL-12p70 secretion and additional mechanisms regulating IL-12 production after TLR7 and TLR9 stimulation.

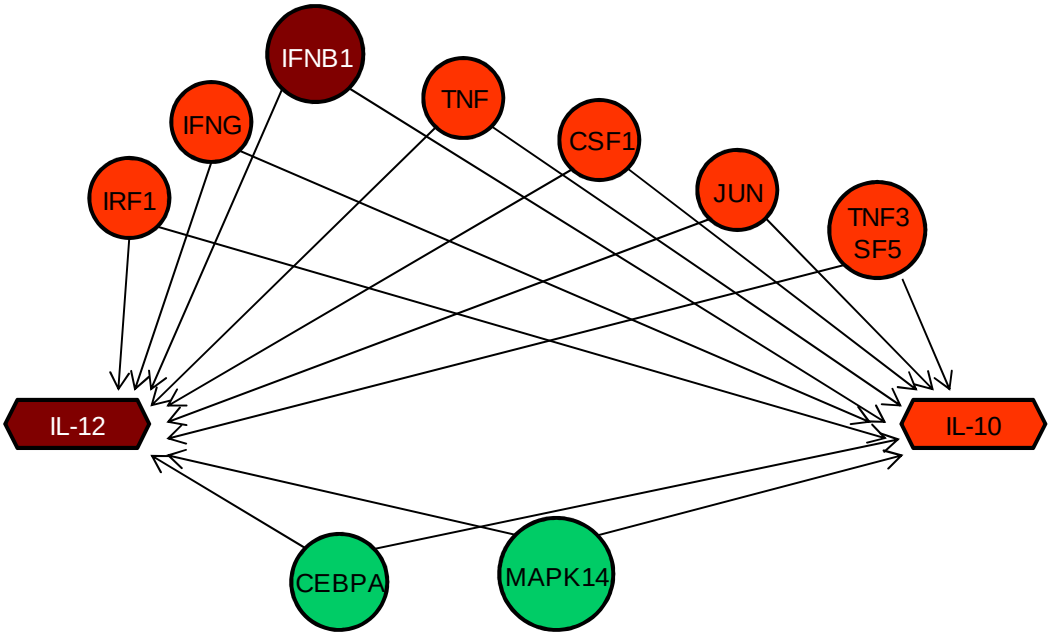

Figure 4.20: Expression pattern of common regulators of IL-12 and IL-10 transcription after TLR7 and TLR9 stimulation.
Genes described to regulate production of IL-12 and IL-10 were analyzed for their expression in the microarray experiment. Only genes that were differentially expressed after TLR stimulation are shown. Red symbols indicate genes that were upregulated, green symbols indicate genes that were downregulated by both R848 and CpG stimulation (compared to unstimulated cells). Expression of IFN-β1 (IFNB1) and IL-12p35 was only upregulated after CpG but not R848 stimulation (shown as dark red).

5 Discussion

5.1 Kinetics of dendritic cell activation in tumor therapy

Dendritic cells are able to initiate T-cell responses, induce immunological memory and break immunological tolerance to tumors. Their ability to stimulate cytotoxic T cells and to polarize T helper cells towards a T_h1 profile makes them promising elements of cancer immunotherapy (Tuyaerts et al., 2007). Nevertheless, DC-based vaccines have so far failed to meet expectations in clinical settings (Zhong et al., 2007). Several questions remain to be resolved in order to improve the efficacy of DC vaccines, such as the development of effective strategies for DC maturation and the appropriate usage of DC subtypes.

5.1.1 Advantages of Flt3L-generated dendritic cells for immunotherapy

DC subsets represent a complex immunological system distributed in different microenvironments within the body. They also sense different types of pathogens and modulate different classes of immune responses (Pulendran et al., 2008). Nevertheless, recent work suggests several mechanisms of crosstalk between the different DC subsets. Langerhans or dermal DC have been reported to interact with $CD8^+$ lymph node DC in the cross-presentation of s.c. injected antigens (Allan et al., 2003; Lund et al., 2003), whereas pDC cooperate with lymph-node mDC to generate antiviral CTL in herpes simplex virus infection (Yoneyama et al., 2005). In murine *L. monocytogenes* infections, IL-15-dependent crosstalk between mDC and pDC was necessary for CpG-induced immune activation (Kuwajima et al., 2006). A better understanding of the functional heterogeneity of DC subsets and their interactions is required to optimize the use of different subsets in immunotherapy. Currently, most clinical trials use the single subset of monocyte-derived mDC (Proudfoot et al., 2007). However, the most successful immunotherapeutic strategies will likely be those that maintain the diversity of DC subsets. The ability of the hematopoietic growth factor Flt3L to expand the number of DC *in vitro* and *in vivo* offers an alternative strategy that allows the generation of a diverse repertoire of DC subsets (Maraskovsky et al., 1996; Maraskovsky et al., 2000). Flt3L also regulates DC development *in vivo* as it was shown that Flt3L-deficient mice have reduced DC numbers (McKenna et al., 2000), injection of mice with Flt3L greatly induces DC numbers (O'Keeffe et al., 2002), and that only bone-marrow progenitor cells expressing Flt3 are effective precursors of DC (D'Amico and Wu, 2003). In the present study, we have differentiated DC in the presence of Flt3L in order to generate both myeloid and plasmacytoid DC (Brasel et al., 2000). pDC represent key effector cells in both innate and adaptive immunity due to their efficient production of type I interferons upon stimulation (Ito et al., 2004). Lou et al. could show that pDC alone are able to stimulate antigen-specific immune responses in a murine tumor model, but that the combination of pDC and mDC

promotes the strongest CTL responses (Lou et al., 2007). We show that the combination of short-term activated myeloid and plasmacytoid DC is effective for the immunotherapy of established tumors. Several experiments in our laboratory compared the effectivity of GM-CSF- plus IL-4-generated mDC with FL-DC. FL-DC were always superior in eliciting antitumor responses, which also underlines the importance of a crosstalk between the different DC subsets (data not shown). Due to these promising results in murine models, DC-based cancer vaccines should be optimized by using the interplay between multiple cell types which can be achieved by generating DC with Flt3L.

5.1.2 The difficult choice of the right stimulus

Maturation and activation of DC are essential in order to elicit the protective T-cell responses required for tumor immunotherapy. Activated DC produce proinflammatory cytokines, upregulate surface expression of MHC and costimulatory molecules and migrate from peripheral tissues to lymph nodes. There, DC present antigen to T cells in order to initiate an antigen-specific immune response. In contrast, immature splenic DC are tolerogenic in the steady-state, taking up antigen from the environment, and presenting it to T cells in the absence of a costimulatory signal, thereby inducing a tolerogenic outcome (Hubert et al., 2007). A growing body of evidence now indicates that immature DC can also actively maintain peripheral T-cell tolerance by the induction of regulatory T cells (Belz et al., 2002; Jonuleit et al., 2000). This induction of suppressive T cells has also been shown *in vivo* in humans, where antigen-bearing immature DC induced peptide-specific $CD8^+$ regulatory T cells (Dhodapkar and Steinman, 2002). Two clinical studies compared the effects of immature and mature DC on tumor immune responses directly: In melanoma patients, mature DC were superior in eliciting T-cell responses, which resulted in objective clinical responses in two patients (de Vries et al., 2003; Jonuleit et al., 2001).

Many signals have been shown to induce at least some aspects of DC activation. In the majority of published clinical trials to date, DC are matured with a cytokine cocktail consisting of TNF, IL-1β, IL-6 and prostaglandin E_2 (PGE_2), first described by Jonuleit et al., 1997. This protocol induces a mature DC phenotype with upregulation of costimulatory molecules that supports clonal expansion of $CD4^+$ T cells. However, recent findings show that DC that have been activated by inflammatory mediators are poor producers of IL-12p70 and thus do not promote T-cell differentiation towards a T_h1 phenotype *in vivo* (Lee et al., 2002; Sporri and Reis e Sousa, 2005). Furthermore, cytokine-activated DC, which are sometimes

characterized as “semi-mature”, have been shown to induce immunosuppressive regulatory T cells (Lutz and Schuler, 2002; Menges et al., 2002).

In contrast, TLR ligands induce the complete activation of DC: On the one hand, they control the expression of many innate immune response genes and can directly promote DC activation. On the other hand, signaling from these receptors leads to the synthesis of inflammatory cytokines such as IFN-α or IFN-β, TNF, and IL-1, all of which can also trigger DC activation indirectly (Sporri and Reis e Sousa, 2005; Winzler et al., 1997). In addition, TLR signaling promotes the synthesis of other inflammatory mediators such as chemokines, prostaglandins and leukotrienes, which can also lead to DC activation (Reis e Sousa, 2004). DC activated by agonists of TLRs induce efficient T_h1 responses both *in vitro* and *in vivo* and lead to the efficient generation of cytotoxic T cells (Iwasaki and Medzhitov, 2004; Krieg, 2007). In the present study, we show that antigen-pulsed CpG-activated DC were able to induce strong T_h1 and CTL responses that led to the eradication of established tumors. DC activated by TLR ligands may therefore provide more effective antitumoral immunity in clinical trials. Indeed, maturation of human DC with a combination of the TLR3 ligand poly I:C and inflammatory cytokines induced more potent CTL responses *in vitro* than the classical cytokine cocktail (Mailliard et al., 2004).

To date, the TLR9 ligand CpG has been identified as the most potent immune enhancer in mouse tumor models (Krieg, 2007). However, TLR9 expression in humans is mainly restricted to plasmacytoid DC (Krug et al., 2001). Nevertheless, CpG has to date shown substantial evidence of antitumor activity in human clinical trials (Krieg, 2007). DC can also be stimulated via TLR7 and 8 and ligands of TLR7 and 8 have already been successfully used as vaccine adjuvants in clinical trials (Shackleton et al., 2004). In the future, specific RNA oligonucleotides may also prove suitable for tumor therapy, as immunostimulatory RNA oligonucleotides induce potent T-cell responses in mice and activate both myeloid and plasmacytoid human DC through TLR7 (Bourquin et al., 2007; Heil et al., 2004). Furthermore, the combination of ligands for different TLRs can lead to potent synergy for the induction of IL-12 and T_h1-polarization by DC in both mice and men (Napolitani et al., 2005; Warger et al., 2006).

5.1.3 The danger of “exhausting” dendritic cells

Although the importance of DC maturation for tumor immunotherapy is now widely accepted, the timing of DC activation remains to be investigated in detail. In most clinical trials,

maturation protocols require extensive *in vitro* activation of DC for 48 h. However, it has been shown that this long activation time leads to a cutback of cytokine production, in particular for the key T_h1-polarizing cytokine IL-12p70. This phenomenon, termed DC exhaustion, occurs independently of the stimulus used for maturation and is associated with the absence of DC response to further stimulation both *in vitro* and *in vivo* (Kalinski et al., 1999; Langenkamp et al., 2000; Reis e Sousa et al., 1999). Since excess IL-12 can lead to lethal tissue damage, its production must be tightly regulated. IL-10, which is usually synthesized together with IL-12, plays a major role in protecting the host from permanent damage by downregulating IL-12 expression (Berg et al., 1995). Although DC exhaustion is beneficial in regulating immune responses in inflammation and infection, it may prove to be deleterious when exhausted DC are injected as a tumor vaccine, where IL-12 secretion is needed for the induction of T_h1 and CTL responses. Short activation times could possibly prevent DC exhaustion, but little is known about the type of immune response induced *in vivo* by DC activated through short TLR stimulation (Macagno et al., 2007).

In the present study, short activation of DC for 4 hours by the TLR9 ligand CpG triggered a complete maturation program including the rapid production of T_h1-type cytokines, upregulation of costimulatory molecules, migration towards a CCR7 ligand and efficient antigen presentation to CTL. Importantly, the T_h1-polarizing cytokines IL-12p70 and IFN-α were produced only in the early phase following DC stimulation, leading to a more efficient induction of IFN-γ-producing T_h1 cells by short-term activated DC. Taken together, these results suggest that vaccination with short-term activated DC may ensure the benefit of fully mature migratory DC that still produce high levels of T_h1 cytokines and may thus emerge superior to extensively matured DC in tumor vaccines.

5.1.4 Duration and combination: signal integration in dendritic cells

In a physiological context, DC are exposed sequentially to different stimuli: microbial products and inflammatory cytokines are encountered initially in peripheral tissues, whereas T cell-derived signals such as CD40 – CD40L interaction and binding of the T-cell receptor to the peptide-MHC complex are delivered when DC have reached the T-cell areas of secondary lymphoid organs. The importance of sustained receptor stimulation in the context of T-cell activation is widely acknowledged. The signaling process is described to be sustained by sequential engagements of T-cell receptors, which are serially triggered by low affinity peptide-MHC complexes. Blocking of the T-cell receptor engagement results in the immediate termination of the signaling process, with hierarchical thresholds for the induction

of T-cell proliferation and differentiation (Lanzavecchia and Sallusto, 2002). For the duration of TLR stimulation and the activation of DC, investigations did not always lead to consistent results. For TLR4 stimulation with LPS, upregulation of MHC and costimulatory molecules and CCR7 was induced very rapidly, while secretion of IL-12p70, IL-6, and IL-10 needed a sustained stimulation of more than 6 hours in one report (Macagno et al., 2006). Warger et al. showed that DC needed to be activated for 20 h with R848 or polyI:C, a synthetic TLR3 ligand, to induce specific immune responses in mice (Warger et al., 2006). In our hands, short-term activation of DC with CpG was sufficient to induce complete maturation of DC. Extensive stimulation of DC could not enhance the production of the T_h1 cytokines IL-12p70 and IFN-α, which were secreted for only 8 hours after stimulation. Other studies support our results and show a potent activation of DC with short TLR stimulations: Camporeale et al. investigated the immune responses in mice elicited by DC activated for 8 h or 48 h with LPS and saw that 8h-DC were more effective in inducing antitumoral immunity in the B16 model of melanoma (Camporeale et al., 2003). When human DC were stimulated with a defined bacterial extract, which probably activates different TLRs simultaneously, the best induction of antigen-specific T cells was reached with DC activated for 6 h, while 20h-DC did not trigger CTL activity (Kaiser et al., 2003). Furthermore, DC activated for 48 h with LPS were shown to promote T_h2 responses in contrast to 8h-DC, which induced T_h1 responses (Langenkamp et al., 2000).

DC can be exposed to a variety of stimuli including microbial products, inflammatory cytokines and signals provided by T cells. Several studies have documented how different stimuli can cooperate or synergize in the induction of particular aspects of DC maturation. In particular IL-12p70 secretion is boosted by a combination of different stimuli including IFN-γ, TLR agonists, and CD40L (Macagno et al., 2007). Still, IL-12 production is not only regulated by the combination of stimuli, but also by the order in which the stimuli are delivered over a defined period of time. IFN-γ enhances IL-12 secretion only if given before TLR agonists, and CD40 stimulation is maximally effective when given after a TLR stimulus (Schulz et al., 2000). Unsurprisingly, this is the temporal order in which these stimuli can be encountered by the maturing DC under physiological conditions. The exposure of DC to different stimuli in an appropriate time frame represents an effective way to sustain signaling. However, there is a defined temporal window in which various signals can accumulate and integrate. This integration is optimal during cytokine gene expression, because DC become exhausted at later time points after stimulation, stop producing cytokines, and become refractory to further stimulation, as described above (Kalinski et al., 1999; Macagno et al., 2007). Thus, a short TLR stimulation may provide the necessary signals for DC maturation while offering a

temporal window for a potent CD40 activation by T cells once the injected DC have reached the draining lymph node.

5.1.5 The need to generate multifunctional dendritic cells

Apart from cytokine secretion and upregulation of the expression of MHC and costimulatory molecules, the upregulation of the chemokine receptor CCR7 is one functional consequence of DC maturation. Migration of DC towards gradients of the lymph node-directing chemokines CCL21 and CCL19 is key for enabling antigen-loaded DC to efficiently interact with T cells in draining lymph nodes (Forster et al., 1999). However, it has been demonstrated that chemokine receptor expression is not predictive of the migratory capacity of some DC types (Luft et al., 2002). Exposure of DC to PGE_2 induces a migratory phenotype due to its participation in podosome dissolution and high-speed migration (van Helden et al., 2006). On the other hand, the presence of PGE_2 during maturation of DC inhibits IL-12p70 production, thus impairing T_h1-directed immunity (Kalinski et al., 2001; Luft et al., 2002). Together, these data suggest that highly migratory DC would be unable to produce IL-12, raising the question of the source of intranodally produced IL-12. Yet, DC used for clinical vaccination studies require both migratory and cytokine production capacities. Protocols that describe the generation of multipotent DC using TLR ligands or combinations of the proinflammatory cytokine cocktail with TLR ligands for DC maturation have not been examined with DC meeting clinical grade standard yet (Boullart et al., 2008; Dauer et al., 2008; Lehner et al., 2007). Thus, there is currently no clinical grade maturation protocol available that yields DC combining migratory capacity and IL-12 production.

In this study, short-term activation of DC by CpG enhanced the expression of CCR7 on DC as well as the migratory potential of DC to the lymph-node directing chemokine CCL21. In contrast, extensive activation of DC for 24 h through CD40 stimulation inhibited DC migration in another study (Watanabe et al., 2003). Thus, short-term activation of DC with a TLR9 ligand has the advantage of inducing multifunctional DC capable of both lymph node migration and IL-12p70 production, that fit the desired physiological role to induce tumor specific IFN-γ-producing T_h1 cells and CTL.

5.2 Pathways to IL-12 production: TLR7 versus TLR9 signaling

The endosomal Toll-like receptors TLR7, 8, and 9, which all recognize specific patterns within nucleic acids, are thought to activate the same signaling pathway upon ligand binding. Strikingly, we observed a pronounced difference in IL-12p70 production after stimulation with TLR7 ligands compared to the TLR9 ligand CpG: Whereas CpG induced the secretion of large amounts of IL-12, the TLR7 ligand R848 did not trigger IL-12p70 production in mDC derived from FL-DC. We examined the underlying pathways to analyze this unexpected difference.

5.2.1 IL-12 as a crucial regulator of immune responses

The first response of an organism to pathogens is an inflammatory reaction, which constitutes the effector phase of innate resistance. Complete control of an infection however generally requires the induction of adaptive immunity. Yet, innate and adaptive immunity are not simply sequential and complementary mechanisms of resistance to pathogens, but regulate each other through cellular contacts and the secretion of soluble mediators, one of which is IL-12. The cytokine IL-12 is a heterodimer formed by a 35-kDa light chain known as p35 or IL-12A, and a 40-kDa heavy chain known as p40 or IL-12B. IL-12p40 associates not only with IL-12p35, but also with the molecule p19, thereby forming IL-23 (Oppmann et al., 2000). IL-12p40 is produced in large excess over the p35 and p19 subunits and can also be secreted as homodimer, at least in the murine system (Heinzel et al., 1997). However, p40 mRNA seems to be restricted to cells that produce the biologically active heterodimer, while mRNA encoding IL-12p35 is present in many cell types (D'Andrea et al., 1992). Transcription of both genes is induced after cell activation; the expression of p35 is considered to be rate-limiting for heterodimer production. IL-12p35 gene transcription upon TLR engagement was found to depend on p65 and c-Rel members of the NF-κB family as well as on recruitment of Sp1. Furthermore, IL-12p35 gene expression is enhanced by IFN-γ via the recruitment of IRF-1 and ICSBP (interferon consensus sequence-binding protein, or IRF-8; Trinchieri, 2003). The initial production of IL-12 occurs rapidly and has been shown to be independent of IFN-γ and CD40L-signals from T cells, although both stimuli enhance its production (Gazzinelli et al., 1994).

The IL-12 receptor is expressed mainly by activated T cells and natural killer cells, but has also been detected on DC and B cell lines (Trinchieri, 2003). IL-12 has direct proliferative effects on pre-activated T cells and natural killer cells and induces their secretion of several cytokines, particularly IFN-γ, thus promoting T_h1 and cell-mediated immunity. The importance

of IL-12 as an IFN-γ inducer lies not only in its high efficiency at low concentrations, but also in its synergy with many other activating stimuli, for example IL-2 and CD3 as well as CD28 receptor signals (Trinchieri, 2003). The ability of IL-12 to induce and maintain antigen-specific T_h1 responses is essential to control infections with many microbial pathogens (Trinchieri, 1998). In addition, IL-12 has also been shown to have a marked anti-tumor effect in mouse tumor models by using effector mechanisms of both innate and adaptive immunity (Brunda et al., 1993). In the present study, DC activation with the TLR9 ligand CpG induced the secretion of high amounts of bioactive IL-12p70. However, when DC were activated with TLR7 ligands, mDC derived from FL-DC cultures did not produce significant amounts of IL-12. The promising data from pre-clinical models of anti-tumor immunotherapy indicates that IL-12 might be a powerful therapeutic agent against cancer, although excessive toxicity and a modest response in clinical trials have dampened the enthusiasm (Colombo and Trinchieri, 2002). Nevertheless, the ability of DC-based tumor vaccines to elicit IL-12 production may to some extent determine their success. As TLR7 ligands are promising alternatives to CpG in clinical studies, the induction of IL-12 production upon TLR7 stimulation needs to be examined in closer detail. Comparably to the divergence between murine Flt3L-derived mDC and GM-CSF plus IL-4 – generated mDC, IL-12p70 secretion may be differentially regulated in different human DC subpopulations. This should also be analyzed in order to establish successful DC vaccination protocols.

5.2.2 IL-10 – an anti-inflammatory cytokine exerting regulatory feedback

Although IL-12 is essential to elicit potent cellular immune responses, excessive inflammatory cytokine production results in tissue damage and toxicity that is harmful to the host. Thus, strong inducers of inflammatory cytokines also activate homeostatic mechanisms that serve to limit cell activation, cytokine production, and tissue damage (Hu et al., 2006). The induction of IL-10 is one key regulatory feedback loop that limits inflammatory cytokine production by inhibiting multiple effector functions of DC and macrophages. The importance of the regulatory role of IL-10 is shown by the uncontrolled lethal inflammatory response to various pathogens in IL-10-deficient mice (Gazzinelli et al., 1996).

IL-10 was first described as cytokine synthesis inhibitory factor (CSIF) produced by T_h2 cells that inhibits activation and cytokine production of T_h1 cells. The functional IL-10 receptor complex is a tetramer consisting of two α-chains and two β-chains. Activation of the IL-10 receptor leads to the activation of the JAK-STAT signaling pathway and the transcription factors STAT1, STAT3 and STAT5 (Wehinger et al., 1996). SOCS3 (suppressor of cytokine

signaling 3) is one of the IL-10-responsive genes inhibiting the expression of various inflammatory genes. The ability of IL-10 to inhibit cytokine production by T cells and natural killer cells was found to be indirect via inhibition of antigen-presenting cells, where it suppresses the production of a number of pro-inflammatory cytokines including IL-12, TNF, IL-1β, and IL-6, at the same time as inhibiting the expression of MHC class II and costimulatory molecules. In monocytes and macrophages, IL-10 also inhibits the synthesis of superoxide anions and NO (Moore et al., 2001). Considering that IL-10 as well as IL-12 play pivotal roles in immune regulation, the elucidation of the molecular mechanism underlying their expression would shed new light on therapeutic approaches toward cancer on the one hand, but also in the context of autoimmune diseases on the other. In this study, we identified IL-10 as a key inhibitor of IL-12 production in FL-mDC after TLR7 stimulation, as IL-10-deficient cells secreted high amounts of IL-12 upon R848 stimulation. Nevertheless, other mechanisms are obviously enforcing the suppression of IL-12, as the IL-10 levels after CpG stimulation are at least as high as after R848 stimulation but do not inhibit CpG-induced IL-12 production.

5.2.3 IFN-β: the pivotal molecule?

In murine as well as human DC, IFN-γ priming is usually required in order to observe significant IL-12p70 secretion in response to TLR stimulation (Hayes et al., 1995). As IFN-γ and type I IFN receptors activate a common signaling pathway, the role of endogenous type I IFNs in IL-12p70 production has been examined previously. An autocrine feedback loop of type I IFNs, especially IFN-β, which induces STAT1 phosphorylation was found to be required for the secretion of bioactive IL-12 in myeloid DC after TLR7/8 stimulation (Gautier et al., 2005), while another group reported the inhibition of IL-12p70 production by IFN-β in DC infected with respiratory syncytial virus (Rudd et al., 2007). Data from our microarray experiment did indeed show an upregulation of IFN-β expression after CpG but not after R848 stimulation. Expression of IFN-α was also upregulated slightly but significantly after TLR9 activation only. In addition, FL-mDC from IRF3/7 knock-out mice deficient in type I IFN production showed markedly reduced levels of IL-12p70 secretion after CpG stimulation, indicating the importance of type I IFN priming for IL-12p70 production in our experiments. Recent publications have identified IL-27, another member of the IL-12 family of cytokines, as yet another modulator of cytokine responses after TLR stimulation: It was shown to be specifically induced by IFN-β and to enhance STAT1-dependent inflammatory gene transcription at the same time as altering IL-10 signaling (Kalliolias and Ivashkiv, 2008; Remoli et al., 2007). Interestingly, expression of IL-27 subunits was also increased more

potently after CpG than after R848 stimulation, further supporting a key role of this pathway in the IL-12 regulation in our settings. Further experiments are necessary to specify the influence of IFN-β, IL-27, and STAT1 phosphorylation in the induction of IL-12p35 expression upon TLR9 but not TLR7 stimulation.

5.3 Conclusion and perspectives

DC play a pivotal role in the initiation, programming, and regulation of tumor-specific immune responses. DC vaccines using the immunostimulatory power of activated DC have emerged as one of the most promising strategies for the immunotherapy of cancer (Melief, 2008; Steinman and Banchereau, 2007). In the present study, we show that a short-term activation of DC by the TLR 9 ligand CpG for as little as 4 hours irreversibly programs DC to produce T_h1-associated cytokines and is highly effective in inducing an antitumoral T-cell response. Short-term activated DC also acquired migratory function towards a CCR7 ligand and were able to overcome the immune suppression mediated by T_{reg} cells. Thus, short-term activation of DC by CpG as a novel strategy of DC maturation generates fully functional DC for tumor immunotherapy. These results may help to develop new concepts for DC activation in DC-based tumor vaccines.

Many of the molecular changes that allow a tumor to grow also create an immunosuppressive milieu that counteracts tumor rejection by the immune system and may thus restrict the effects of DC-based immunotherapies. Until now, DC-based vaccines have mostly been used to treat late-stage tumors in patients, where the presence of large tumor masses impedes the access of tumor-specific lymphocytes to the tumor. Hence, immunotherapeutic approaches may confer a more pronounced benefit when applied earlier during the course of disease. Combining DC-based therapies with other strategies such as chemotherapy may also prove an effective strategy: Interestingly, the simultaneous administration of chemotherapy does not reduce the effects of immunotherapy despite the immunosuppression often associated with chemotherapy (Bourquin et al., 2006). It may even enhance antitumoral effects by “resetting” the relationship between the tumor and the immune system: chemotherapy can, by simply reducing the tumor mass, reduce its immunosuppressive properties. In addition, the chemotherapy-induced lymphopenia reduces the number of regulatory cells and may promote the proliferation of immune effectors that are particularly active in the anticancer response (Zitvogel et al., 2008). Thus, a combination of chemotherapy and optimized DC-based immunotherapies might help to further improve the clinical outcome of tumor therapy.

In the second part of this study, the deficient IL-12p70 production upon Toll-like receptor 7 stimulation in Flt3L-derived mDC was examined. By analyzing inhibitors of signal transduction, knock-out mice, and genome-wide expression levels of R848- versus CpG-stimulated cells, p38 MAPK, IL-10, and IFN-β were identified as regulators of IL-12. Further experiments will elucidate the respective importance of these molecules and identify putative additional regulators of IL-12 expression.

IL-12 production in DC is clearly regulated by several different pathways. The phospho-inositide 3-kinase (PI3K) pathway has recently been shown to play an additional role in IL-12 and IL-10 expression: PI3K negatively regulates IL-12 production while inducing IL-10 transcription in DC stimulated with TLR ligands. Certain signaling molecules involved in the PI3K pathway, namely Akt, mTOR (mammalian target of rapamycin), and GSK3 (glycogen synthase kinase 3), have been shown to play a role in this process (Fukao et al., 2002; Ohtani et al., 2008). However, these regulatory pathways have so far only been examined in human monocytes and/or murine GM-CSF DC stimulated with LPS. The influence of these pathways on the signaling of other TLRs and in other cell types deserve further analysis and might help to elucidate the complex regulation of IL-12 production.

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7 Appendices

7.1 Abbreviations

A

Ab	Antibody
ANOVA	Analysis of variance
AP-1	Activated protein 1
APC	Allophycocyanin

B

BrdU	5’-Bromo-2’-deoxyuridine
BSA	Bovine serum albumin

C

CCL	Chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
cRNA	Complementary RNA
CFSE	Carboxyfluorescein succinimidyl ester
CpG	Oligonucleotide with cytosine-(phosphate)-guanine motifs
CTL	Cytotoxic T lymphocyte

D

d	Day
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

E

EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay

F

FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FL-DC	Flt3L-derived dendritic cells
Flt3L	Fms-like tyrosine kinase 3 ligand

FSC	Forward scatter

G

GM-CSF	Granulocyte-macrophage colony stimulating factor
GM-DC	Dendritic cells differentiated with GM-CSF and IL-4
GSK3	Glycogen synthase kinase 3

H

HBSS	Hank's balanced salt solution
HRP	Horseradish peroxidase

I

ICSBP	Interferon consensus sequence-binding protein
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IκB	Inhibitor of κB
IKK	Inhibitor of κB kinase
IL	Interleukin
IP-10	Interferon-induced protein 10
IRAK1/4	IL-1R-associated kinase 1/4
IRF	Interferon-regulatory factor

J

JAK	Janus kinase

L

LPS	Lipopolysaccharide

M

MACS	Magnetic cell separation or magnetic-activated cell sorting
Mal	MyD88 adaptor-like
MAPK	Mitogen-activated protein kinase
mDC	Myeloid dendritic cell
MHC	Major histocompatibility complex
MyD88	Myeloid differentiation primary response gene 88
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin

N

NF-κB	Nuclear factor-κB
NK cells	Natural killer cells
ns	Not significant

O

OPD	O-phenylenediamine dihydorochloride
OT-I	Transgenic mice with ovalbumin-specific $CD8^+$ T cells
OT-II	Transgenic mice with ovalbumin-specific $CD4^+$ T cells
OVA	Chicken egg ovalbumin

P

PBS	Phosphate-buffered saline
pDC	Plasmacytoid dendritic cell
PE	R-phycoerythrin
PerCP	Peridinin chlorophyll protein
PI3K	Phosphoinositide 3-kinase
PFA	Paraformaldehyde
PGE_2	Prostaglandin E_2
pre-DC	Precursor of DC

R

RBC	Red blood cells (erythrocytes)
RNA	Ribonucleic acid
RT	Room temperature

S

SOCS3	Suppressor of cytokine signaling 3
SSC	Side scatter
STAT	Signal transducer and activator of transcription

T

TAB1/2	TAK1-binding protein 1/2
TAK1	TGF-β-activated protein kinase 1
TBK1	Traf family member-associated NF-κB activator binding protein
TCR	T-cell receptor
T_h	T helper
TIR	Toll/IL-1 receptor

TLR	Toll-like receptor
TMB	Tetramethyl benzidine
TNF	Tumor necrosis factor
TRAF6	TNF receptor-associated factor 6
TRAM	TRIF-related adaptor molecule
TRIF	TIR-related adaptor protein inducing interferon
T_{reg} cells	Regulatory T cells

7.2 Publications

7.2.1 Original publications

1. Bourquin C, Schmidt L, Hornung V, **Wurzenberger C**, Anz D, Sandholzer N, Schreiber S, Voelkl A, Hartmann G, Endres S.
   Immunostimulatory RNA oligonucleotides trigger an antigen-specific cytotoxic T-cell and IgG2a response.
   Blood 2007 Apr 1;109(7):2953-60 (JIF: 10.9)

2. Bourquin C, Anz D, Zwiorek K, Lanz AL, Fuchs S, Weigel S, **Wurzenberger C**, von der Borch P, Golic M, Moder S, Winter G, Coester C, Endres S.
   Targeting CpG oligonucleotides to the lymph node by nanoparticles elicits efficient antitumoral immunity.
   Journal of Immunology 2008 Sep 1;181(5):2990-8 (JIF: 6.1)

3. **Wurzenberger C**, Koelzer VH, Schreiber S, Anz D, Vollmar AM, Schnurr M, Endres S, Bourquin C.
   Short-term activation induces multifunctional dendritic cells that generate potent antitumor T-cell responses *in vivo*.
   Cancer Immunology, Immunotherapy 2008 Oct 25; Epub ahead of print (JIF: 3.7)

4. Bourquin C, Schmidt L, Lanz AL, Storch B, **Wurzenberger C**, Anz D, Berger M, Poeck H, Hartmann G, Hornung V, Endres S.
   Immunostimulatory RNA oligonucleotides induce an effective antitumoral NK-cell response through the Toll-like receptor 7
   Cancer Research, in revision 2008 (JIF: 7.7)

Submitted and in preparation:

5. Anz D, Thaler R, Koelzer VH, Golic M, **Wurzenberger C**, Lahl K, Rothenfusser S, Sparwasser T, Noessner E, Endres S, Bourquin C.
   Toll-like receptor agonists control trafficking of regulatory T cells.
   Submitted 2008

6. **Wurzenberger C**, Weber P, Schlee M, Bscheider M, Schreiber S, Anz D, Vollmar AM, Endres S, Bourquin C.
   IL-12 production in murine dendritic cells is differentially regulated after TLR7 versus TLR9 stimulation.
   In preparation 2008

7.2.2 Oral presentations

1. The duration of dendritic cell activation with CpG oligonucleotides is critical for the outcome of immunotherapy in a mouse model of colon cancer
   9th International Conference on Dendritic Cells, Edinburgh, UK, 2006

2. Immunotherapy of tumors
   1st Annual Retreat, Graduiertenkolleg 1202, Frauenchiemsee, Germany, 2006

3. Kinetics of dendritic cell activation in tumor therapy
   ICE School (Immunology Course Engadina), Bos-Cha, Switzerland, 2007

4. Dendritic cell activation in tumor therapy
   2nd Annual Retreat, Graduiertenkolleg 1202, Tutzing, Germany, 2007

5. DC vaccination for tumor therapy
   Junior Science Faculty, SFB-Transregio 36, Tutzing, Germany, 2007

7.2.3 Poster presentations

1. Bourquin C, **Wurzenberger C**, Schreiber S, Hartmann G, Endres S.
   Flt3L-generated dendritic cells are superior to GM-CSF- plus IL-4 – derived dendritic cells for immunotherapy with CpG oligonucleotides in a mouse model of colon cancer.
   Tegernsee Conference on Immunotherapy of Solid Cancer, Tegernsee, Germany, 2005

2. **Wurzenberger C**, Schreiber S, Endres S, Bourquin C.
   The duration of DC activation with CpG is critical for the outcome of immunotherapy in a mouse model of colon cancer.
   9th International Conference on Dendritic Cells, Edinburgh, UK, 2006

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

4. **Wurzenberger C**, Schreiber S, Koelzer VH, Weigel S, Endres S, Bourquin C. Kinetics of dendritic cell activation by CpG oligonucleotides for the induction of CD8 T cell responses.
   3rd Annual Meeting of the Oligonucleotide Therapeutics Society, Berlin, Germany, 2007

5. **Wurzenberger C**, Koelzer VH, Schreiber S, Anz D, Endres S, Bourquin C. Short-term activation induces multifunctional dendritic cells that generate potent antitumor T-cell responses *in vivo*.
   10th International Symposium on Dendritic Cells, Kobe, Japan, 2008

7.3 Curriculum vitae

Persönliche Daten

Name:	Cornelia Wurzenberger
Geburtsdatum:	11. September 1979
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Schulbildung

1986 bis 1990	Grundschule an der Forellenstraße, München
1990 bis 1999	Michaeli-Gymnasium München
Schulabschluss	Abitur, Juni 1999, Endnote 1,0

Studium

1999 bis 2004	Studium der Molekularen Medizin an der Friedrich-Alexander-Universität Erlangen-Nürnberg:
September 2001	Vordiplom, Gesamtnote 1,4
Nov. 2003 bis Mai 2004	Diplomarbeit: „Positionsklonierung eines Gens für die Osteopathia striata“, Institut für Humangenetik, FAU Erlangen
Studienabschluss	Diplom-Molekularmedizinerin, Mai 2004, Endnote 1,1

Promotion

Seit Juli 2004	Promotion in der Arbeitsgruppe Tumorimmunologie (Dr. Dr. Bourquin) in der Abteilung für Klinische Pharmakologie der Medizinischen Klinik Innenstadt der LMU München unter Leitung von Prof. Dr. Endres; Fachvertretung der Fakultät für Chemie und Pharmazie: Prof. Dr. Vollmar

Stipendien

1999 bis 2004	Hochbegabtenförderung des Bayerischen Staatsministeriums für Unterricht, Kultus, Wissenschaft und Kunst
2002 bis 2004	Stipendium der e-fellows.net GmbH & Co. KG, München
2005 bis 2008	Stipendiatin des Graduiertenkollegs “Oligonukleotide in Zellbiologie und Forschung“, gefördert durch die DFG

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