Mechanism of dendritic cell-based vaccination against *Leishmania major*

Mechanismus der auf dendritischen Zellen beruhenden Impfung gegen *Leishmania major*

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submitted by

Johannes K. Schnitzer

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Submitted on:

Members of the *Promotionskomitee*:

Chairperson: Prof. Dr. rer. nat. Thomas Müller

Primary Supervisor: Prof. Dr. rer. nat. Dr. med. habil. Heidrun Moll

Supervisor (Second): Prof. Dr. rer. nat. Thomas Rudel

Supervisor (Third): Prof. Dr. med. Ulrich Vogel

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meiner Familie

1. Introduction

1.1. Leishmaniasis

1.1.1. Definition

In 1903 Leishman and Donovan described first the occurrence of later called Leishman-Donovan bodies in splenocytes of diseased patients in India (57, 119). The first described patient was affected by attacks of pyrexia, anaemia, and enlargement of the spleen wherein the amastigote form of the later called *Leishmania donovani* parasite was found (15). Nowadays several different *Leishmania* species are known, which are transmitted by sandflies of the genus *Phlebotomus* [Old World] and *Lutzomyia* [New World] and cause a variety of clinical symptoms collectively known as leishmaniasis.

1.1.2. The parasite

Protozoan parasites from the genus *Leishmania* are the causing agent of the disease leishmaniasis. These parasites belong to the Kingdom Protista, Subkingdom Protozoa, Phylum Sarcomastigophora, Subphylum Mastigophora, Class Zoomastigophora, Order Kinetoplastida, Family Trypanosomatidae, Section Salivaria. The Order Kinetoplastidae is determined by a characteristic structure called the kinetoplast that extends to a single large mitochondrion harboured by unicellular organisms, whereupon trypanosomes also belong to this group. Most forms of leishmaniasis are transmitted via sandflies from a rodent or mammal reservoir to the human host, whereas in Bihar, India, humans are the sole known reservoir for visceral leishmaniasis (200). In total, 20 out of 30 species can cause human infections.

The natural life cycle of *Leishmania* parasites comprises two forms which are morphologically distinguishable. In the female phlebotomine sandfly the promastigotes form can be found. These parasites possess a flagellum, have an elongated cell morphology and are motile. Seven to ten days after the uptake and transformation from the amastigote stage to the promastigote stage in the sandfly gut the parasites move to the proboscis and can be injected into the host subcutaneously during a blood-meal.

In contrast, the amastigote form of *Leishmania* parasites found intracellular has a roundish morphology, no flagellum and is not motile. As an obligate intracellular parasite, it infects neutrophils, macrophages [MΦ], dendritic cells [DC] and fibroblasts of the mammalian host (34, 211). These parasitized cells can be taken up during a blood meal.

Experimental evidence suggests a continuous progression in the developmental cycle between amastigote and promastigotes in nature (151).

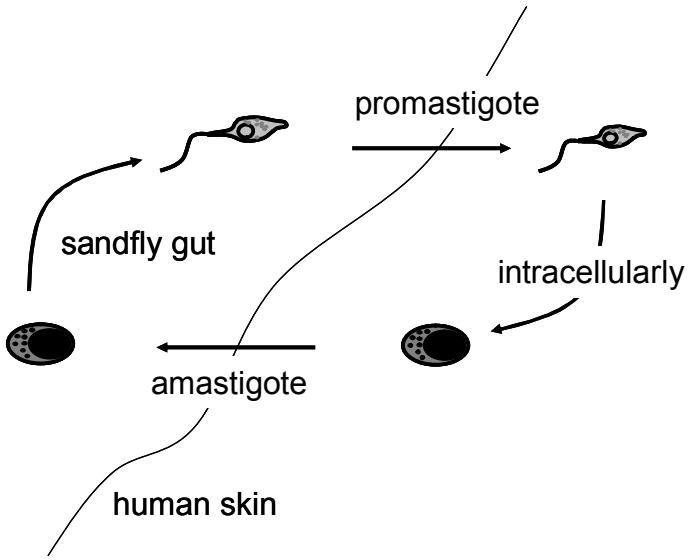

Fig. 1: *Leishmania* life cycle. Main characteristics of the *Leishmania* life cycle are the transformation from the amastigote stages into promastigote stages in the sandfly gut and vice versa intracellularly in the mammalian host.

1.1.3. The clinics

Cutaneous, mucocutaneous and visceral leishmaniasis are together with viscerotropic leishmaniasis the three major forms of the disease. Additionally, cutaneous manifestations can be further differentiated into diffuse leishmaniasis, leishmaniasis recidivans, localized leishmaniasis, and post-kala-azar dermal leishmaniasis.

The manifestation of localized cutaneous leishmaniasis [CL] is largely depending on the immune status of the host and varies depending on the stage of disease. The bite of a sandfly usually takes place at exposed areas of the skin, e.g. hands, face and legs. There, lesions develop beginning with a red papule which may have several centimetres in size. In later stages the lesion is crusting, shows granuloma formation and the development of an erythematous rim. The self-healing of this form can take two to twelve months and leaves scars with changes in pigmentation. In contrast, in anergic patients diffuse cutaneous leishmaniasis [DCL] can develop that is accompanied by formation of plaques, ulcers and nodules all over the body. This infection is chronic and may recur after treatment, which is most common in New World species but also for *L. aethiopica* in the Old World. The re-occurrence of lesions at the border or the centre of healed lesions [scars] is termed leishmaniasis recidivans. This can start years after the initial infection and is often resistant to treatment. Instead, post-kala-azar dermal leishmaniasis [PKDL] is the dermal re-occurrence of the disease after recovery from visceral leishmaniasis and had predominantly been described in Africa and India. Cases in India describe the formation of multiple, hypopigmented, erythematous macules years after the recovery from the initial affection. This form needs severe treatment whereas the less harmful African form spontaneously resolves within several months (84, 191).

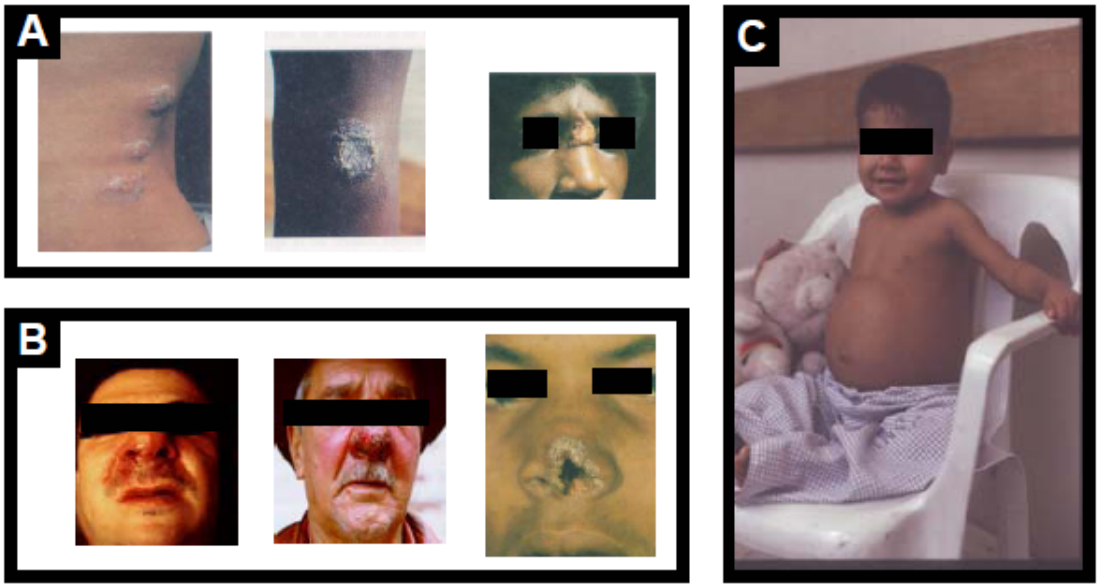

Fig. 2: Clinical presentations of leishmaniasis**.** A: cutaneous leishmaniasis, B: mucocutaneous leishmaniasis, C: visceral leishmaniasis.

The mucocutaneous form of leishmaniasis [ML] is commonly seen in the New World while cases of ML in the Old World are only reported for *L. aethiopica* infections. Initially, infections start with persistent cutaneous lesions which could be self-healing. Although, in 30% of the cases no infection with *Leishmania* parasites were reported prior to the outbreak of ML. Years later, oral and respiratory mucosal involvement occurs, causing inflammation and mutilation of the nose, mouth, oropharynx, and trachea with granula formation, ulceration and erosion of the palate, uvula, lips, pharynx, and larynx. Deformities occurring in time, especially of optical and genital mucosa in severe cases, need plastic surgery. Due to the fact that this form is difficult to treat, these disabilities can lead to death via malnutrition and secondary airway infections (84, 191).

Visceral leishmaniasis, also known as kala-azar or black fever, causes the most severe symptoms and leads to death if left untreated, due to immunosuppression and secondary infections. The disease results from both, New World as well as Old World species and

causes the pentad of fever, weight loss, hepatosplenomegaly, pancytopenia, and hypergammaglobulinemia due to the systemic infection of the liver, spleen and bone marrow (84, 191).

1.1.4. Epidemiology

According to reports of the world health organistion [WHO], 88 countries in intertropic and temperate regions of the world are endemic for leishmaniasis and in total 350 million people are at risk. Each year an estimate of 500,000 cases of visceral leishmaniasis occur mainly [90%] in Bangladesh, Brazil, India, Nepal and Sudan. Accordingly, an estimate of 1.5 million cases of cutaneous leishmaniasis occur yearly, mainly [90%] in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria. In the Old World - meaning the Middle East, Indian subcontinent, Asia, Mediterranean, East Africa, and republics of the former Soviet Union, these cases are caused by the species *L. tropica*, *L. major*, *L. aethiopica*, *L. donovani* and *L. infantum*. In the New World - throughout the Americas, with the exception of Canada, Chile, and Uruguay - species like *L. Leishmania mexicana*, *L. Leishmania amazonensis*, *L. Leishmania venezuelensis*, *L. viannia braziliensis*, *L. viannia guyanensis*, *L. viannia panamensis*, *L. viannia peruviana* and *L. donovani chagasi* cause the diverse types of leishmaniasis.

Primarily people of tropical and subtropical countries which are considered as developing or under-developed countries are affected. Although, a large number of cases in the European Mediterranean and, increasingly, also in Germany were reported (80, 144) the disease is found within the list of neglected tropical diseases.

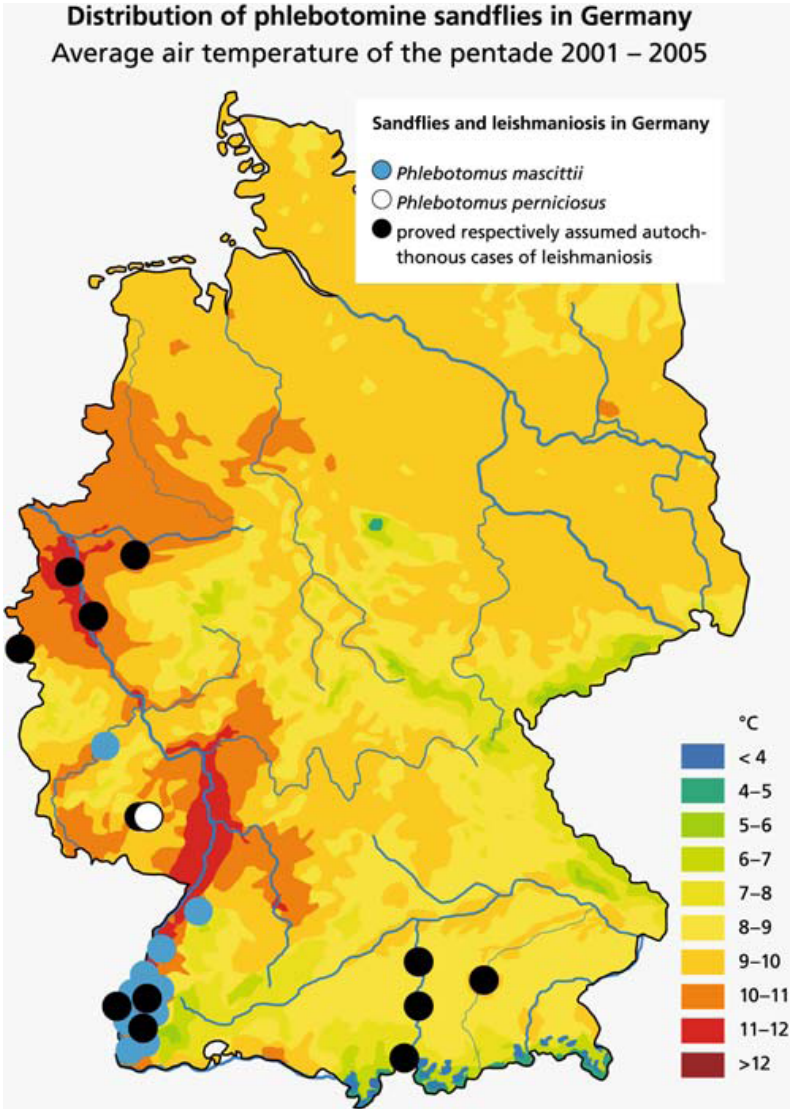

Fig. 3: Distribution of Phlebotomine sandflies and cases of autochthonous leishmaniasis **in Germany (144).**

Of major concern are actually co-infections of *Leishmania* with HIV. According to the WHO, respective cases have already been reported for more than 35 countries throughout southern Europe, the Mediterranean Basin, Central and South America, and India. As previously diseased people who recovered from leishmaniasis are not sterile and AIDS

leads to immunosuppression, re-occurrence and opportunistic infection with *Leishmania* is increasing in countries which are endemic for both (6).

Regarding the three major forms of leishmaniasis different numbers for mortality or morbidity have to be mentioned. CL mostly resolves within 3-6 months, whereas most cases of DCL, PKDL and leishmaniasis recidivans are associated with low mortality rates, chronic disease and resistance to treatment. ML is chronic as well as progressive and can lead to death because of secondary infection. VL has a mortality rate of 75-95% if left untreated. In this case, death usually occurs from malnutrition and secondary infection if the diseased people would have not been treated.

Normally, the age of a person itself is not decisive for a certain risk of getting infected, but changes of habits and behaviour which occur during life time may influence the specific risk. Exceptionally, a higher prevalence for VL is found for children in endemic areas with an animal reservoir. Regarding the gender, males have an increased incidence of infection [2:1] due to increased exposure to the habitat of the sandfly (191).

1.1.5. Experimental leishmaniasis

Leishmania spp. cause infections in a variety of animals. Naturally occurring infections of these animals are hard to recognize, as in these animals only minor to no symptoms can be detected. In contrast, infections of humans and dogs often have severe implications. But, in epidemiological terms, humans and dogs may be considered as secondary or accidental hosts in the *Leishmania*'s life-cycle (64). With regard to this situation the choice of an animal model is difficult and has to be applied to the individual goal (87).

Several experimental animal models of leishmaniasis have been established to study the pathogenesis and pathology of leishmaniasis, the impact of chemotherapy or the preventive effect of vaccines against the agents causing CL and VL (64, 81). Studies in mice, hamsters, dogs and non-human primates were carried out especially to determine the efficacy of chemotherapy and vaccination in consecutive steps. Whereas an early model using guinea pigs showed the relevance of cell mediated immunity [CMI] for the clearance of *Leishmania* parasites (36), the most prominent animal model for this disease is the mouse model due to the feasibility of genetic manipulation. Already since the 70's it was known that the genetic background of inbred mouse strains contributes to the outcome of leishmaniasis in the respective mice (79). In contrast to CBA mice or C57BL/6 mice BALB/c mice are highly susceptible to infections with different *Leishmania* spp. including *L. major* (219). Since then, a number of mouse strains have been used as experimental models of infection with different *Leishmania* species including *L. major*, *L. tropica*, *L. mexicana*, *L. amazonensis*, *L. donovani*, *L. infantum* and *L. braziliensis*. The availability of different mouse cell surface markers and the respective specific antibodies in combination with several dyes and technical improvements allow the assessment of immune pathways in wild type and modified animals. The impact of single cell populations in the progression of the disease can be monitored comparatively. Since different CD4+ T cells and effector cells like CD8+ T cells [cytotoxic T lymphocytes, CTL], NK cells and MΦ as well as cytokines like interleukin-4 [IL-4], IL-12, interferon gamma [IFNγ] and tumor necrosis factor alpha [TNFα] were known to play a major role in the progression of leishmaniasis their influence in shaping the immune response was under surveillance.

Here, BALB/c mice were used that are highly susceptible to *Leishmania* parasites. For the investigation of vaccine efficacy or to elucidate the mechanism of DC-based vaccination mice receive a single vaccination with antigen-loaded cells intravenously [i.v.] and a subsequent infection with 2×10^5 metacyclic *L. major* promastigotes subcutaneously [s.c.] into the right footpad. Generally, this is the high dose model for cutaneous leishmaniasis. Upon infection naïve BALB/c mice develop lesions that never heal (87, 158). Within the lesion the parasites replicate intracellularly in MΦ and in later stages disseminate to internal organs. This systemic infection leads to death if left untreated and resembles features of visceral leishmaniasis.

1.2. The immune system

The immune system consists of a very complex interplay between different cell types, molecules and specialized tissues. In front line the innate immune system responses to several pathogens where the complement system as well as cell-dependent clearance of cellular and bacterial material is involved. But, some infections need further mechanisms due to the pathogenicity or virulence of the causing agent. Therefore an adaptive immune response is needed to initiate the expansion and activation of specific effector cells against distinct pathogens.

Of course, this system fails when it is overwhelmed by a high number of pathogens or due to a weakened immune status because of the individual age, co-infections or immunosuppressive medication. To circumvent these situations we would like to strengthen and to teach the immune system ahead of the first encounter of a distinct pathogen. Therefore we have to exploit natural resources of the immune system. But, the

complexity of this system first has to be disclosed to understand how it can be manipulated.

In this study a focus was set on major players during the initial phases of immune instruction: DC as inducer of specific responses and T cells as messenger and effector cell types.

1.2.1. Dendritic cells

In 1973 Steinman and Cohn (193) first described a stellate cell population, which they called dendritic cells. But, cell types relating to these DC were already mentioned earlier by Paul Langerhans in 1886 (113). Later on, lots of studies were published, which demonstrated the diversity of these DC in terms of function, mode of action, lineage and cell-interactions (23, 106, 132, 174, 193). In the following chapters this diversity should be disclosed to give an overview about DC properties, which might be important for this study.

1.2.1.1. Dendritic cell lineages and subsets

Actually, there is a huge debate about DC lineages and subsets as not every individual DC prepared *ex vivo* resembles a specific subset and not every common or rare surface molecule is a linage marker. Additionally, the well-known plasticity of immune system related cell types also might play a role for DC development and differentiation (161). Lots of review articles do exist, which state commonly that DC linage specificity, differentiation and function is still an open field to be explored (161, 188).

To make the bunch of different DC subsets manageable Shalin H. Naik started to classify DC by some general properties (143). First, DC precursors were separated from their DC products. Furthermore migratory, resident and circulating DC were distinguished. Their presence during steady state or during inflammatory processes was also taken into account. Additional criteria have been the respective subtypes and their activation status.

According to the first discrimination argument, monocytes and plasmacytoid DC [pDC] are seen as non-DC antigen-presenting cells [APC] and DC precursors during inflammatory responses, which need certain stimuli for the differentiation towards the dendritic phenotype (188). pDC are of particular interest in this work here and are well known to produce interferon type I [IPC] upon stimulation via microbial products and need the same stimuli to differentiate into DC and to develop the abilities of antigen processing and presentation (75, 124, 149, 231).

The next discrimination argument leads to the differentiation between circulating, migratory and tissue resident DC. Resident DC are defined as immature DC $CD11c^{hi}$, $CD45RA^{lo}$ and major histocompatibility $complex^{int}$ [$MHCII^{int}$] DC, which can further be divided into $CD8^{+}$ and $CD8^{-}$ DC (143, 222). Associated to them are migratory DC which are characterized as DC residing in peripheral tissues until they receive certain signals. These signals induce the migration of DC into secondary lymphoid organs and additionally, these signals can induce the maturation of these respective DC towards a T cell activating phenotype (166). Quite contrary, other stimuli do not induce this kind of maturation, but still induce the migration of DC to lymph nodes in a transforming growth factor-beta 1 dependent manner (222, 223). Surprisingly, referring to the third class in this category, $CD11c^{hi}$ cells cannot be found in significant numbers in the circulation and among them there is no T cell activating capacity without further hematopoietic

development (150). This finding could argument against the idea of migratory DC in "clean" mice, whereas in "dirty" humans blood-DC (128) can be found, which gives rise to additional questions about the difference between mice and men.

Now the key players in DC immunity have to be classified. Among them there are monocytes, pDC, Langerhans cells, interstitial DC, resident $CD8^+$ and $CD8^-$ resident DC. As already mentioned, monocytes get recruited to several organs and into the blood stream under inflammatory conditions to act as one part of the innate immune defence against pathogens (185). But, it is also known that monocytes can differentiate into macrophages or $CD11c^+$ $MHCII^+$ DC to fulfil adaptive immune functions (188). Furthermore, as a second pre-DC cell type, pDC are found in many lymphoid organs under steady state (143). Here they act as sensors of certain toll-like receptor [TLR] agonists (27, 54, 96, 97, 189) and produce the well-known type I interferon burst and a wide set of chemokines and cytokines upon stimulation (159). The third subclass of DC is called Langerhans cells. These cells are high in numbers in the epidermis [2-4%] (113) but their role in adaptive immunity and tolerance is not quite clear and still under debate (5, 137, 183, 196, 221, 233). Langerhans cells and interstitial DC are the main subclasses of human conventional DC [cDC] (17, 187). Although these DC are present throughout the body and are thought to be involved in immune-regulation there is only little experimental evidence for their function during infection (216, 233). At last, $CD8^+$ and $CD8^-$ resident DC are mainly found in spleen and lymph nodes whereas they show differential spatial distribution (2, 51, 135). Their main role in the immune system is related to tolerance (24, 148), the ability of cross-presentation (52, 156) and T cell priming during viral and bacterial infections (5, 25, 52).

While applying vaccines i.v. all of these DC subtypes could be involved in anti *Leishmania* vaccination strategies. Therefore the outcome of such a complex immune stimulation depends on the immunostimulatory capacity of the respective adjuvant-antigen formulation.

Different naturally occurring subsets of DC can now be pigeonholed. But the residual problem is to match these categorized DC with *in vivo*-generated DC. A first attempt to exploit the immunostimulatory capacities of DC was to load *ex vivo* prepared DC with antigen and to activate these DC in parallel to resemble naturally infected, fully activated DC. The most common method to generate these DC *in vivo* - as it is done here in this study - is the cultivation of bone marrow-derived cells with medium that is supplemented with granulocyte macrophage-colony stimulating factor [GM-CSF]. Here, monocytes and other precursors develop into adhering DC. As already mentioned, monocytes are recruited and differentiate towards a DC phenotype under inflammatory conditions. Therefore bone marrow-derived DC [BMDC] do not necessarily resemble steady state DC but inflammatory DC (227). But, here the common abilities of these cells - antigen uptake, antigen presentation via MHC class I and II molecules and T cell activation - are exploited to lead the immune system towards the favoured direction.

1.2.1.2. Properties: antigen uptake and degradation

The main mechanisms of antigen uptake by DC include macropinocytosis, receptor-mediated endocytosis and phagocytosis (205). These mechanisms are constitutively active at high levels already during an immature stage of the respective DC but can be further heightened via activation of DC with microbial stimuli (194, 220).

Macropinocytosis - from Greek “cell drinking” - is the constitutive engulfment of large entities of extracellular liquids via forming of cell membrane pockets with a diameter of 0.5-5 µm (60) that allows continuous capture of macrosolutes which are present in the liquid phase surrounding the respective cell (177). In addition, phagocytosis - from Greek “cell eating” - means the unspecific internalization of particles, including cell debris, which can be larger than 0.75 µm in diameter (129). Third, receptor-mediated endocytosis is more specific but is also restricted to a smaller volume. The uptake via clathrin-coated pits [or within caveolae] is restricted to a size up to 100 nm and is induced by as prominent receptors as the mannose receptor (164, 177) and the $F_c\gamma$ receptor (7) but also by a bunch of other membrane-bound receptors which are specific for self or pathogen-derived molecules.

Regarding degradation, every cell produces peptides that can be released into the cytoplasm or can be accumulated in vesicles. Therefore mechanisms are needed to degrade self-produced mis-folded, damaged or excess antigen in the cytoplasm as well as external antigen in vesicles for subsequent use in antigen presentation. These two different compartments use different mechanisms for degradation and additionally, these compartments are kept separate with the exception of antigen-exchange for cross-presentation (7). Cytoplasmic proteins get mainly degraded by the ubiquitin-proteasome-system (229). Here, a complex of 14 subunits is forming the 20S proteasome. The 20S proteasome in addition with the 19S regulators forms the 26S proteasome for the ATP-dependent degradation of ubiquitin-labelled proteins (230). Further more, for the formation of the immunoproteasome only three catalytically active subunits have to be exchanged for the production of a different spectrum of peptides which can be loaded onto MHC molecules. The immunoproteasome is active in almost every immune system-

related cell type and is inducible in almost all other cell type upon stimulation with IFNγ or TNFα (230). Differently, the degradation of external antigen accumulated in vesicles follows an alternative pathway. Here, peptides and proteins become degraded by different proteases, especially cathepsins, which degrade external proteins as well as MHC class II molecules for the proper presentation on the cell surface (44, 207). Therefore, peptides captured from the extracellular environment have to be directed from early endosomes via endosome carrier vesicles or multivesicular bodies to late endosomes. Then, depending on signals the respective vesicle carries itself, these vesicles either join the pathway for complete degradation and peptide re-cycling or the pathway for peptide presentation on the surface upon activation of the respective DC (155).

1.2.1.3. Properties: antigen presentation

Maturation of DC and antigen presentation by this potent cell type is closely associated. As mentioned above, the specific sequestration of cellular material is dependent on the activation status of the cell (31). Likewise, antigen presentation is modified upon DC maturation.

DC mature and get activated upon receptor activation by several ligands/signals which can be received on the surface of the respective DC as well as within endocytic vesicles and the cytoplasm. During steady state the activation status of DC is permanently under influence of several receptors which are able to sense host-derived as well as pathogen-derived molecules. These receptors are responsible for the determination of the surrounding milieu and the discrimination between self and foreign. First, pattern recognition receptors [PRR], which are able to sense pathogen associated molecular

patterns [PAMP] in forms of peptides, cell wall components, lipopolysaccharides [LPS] and nucleic acids like CpG-containing oligodeoxynucleotides [CpG ODN], play a major role in the activation of DC and other cell types (95, 98, 165, 208). Furthermore, TLR, a prominent family of PRR, can be exploited by some endogenous 'danger signals'. Thereby, the presence of non-self as well as differences between apoptotic and necrotic cell material or an overall inflammatory status of the surrounding milieu can be sensed (3, 20, 69, 182). This inflammatory milieu is generated by epithelial cells, non-hematopoietic and hematopoietic cells, which have gradual influence on DC maturation and activation (91, 117, 146, 170, 171). Here, pro-inflammatory cytokines like TNFα, type I interferons and interleukins like IL-18 play a major role. This enables DC to display the current status regarding immunogenic or non-immunogenic situations and to act on subsequent cell-types in the right manner. Additionally, it was shown that also activated T cells share the ability of activating DC at later stages. Therefore direct cell-cell-interaction - including cluster of differentiation 40 [CD40]–CD40L interactions - is needed to transmit a signal which enables DC to fully activate CTL.

In the case of pro-inflammatory signal-reception by DC, i.e. the reception of pathogen-derived material or alarmins, a lot of phenotypical changes regarding cell morphology, sequestration of cellular material, antigen-presentation and migration take place. As mentioned above, at the moment of receptor-mediated activation the overall capacity for antigen-uptake can be strengthened. Here and during steady state a clear discrimination between self non-inflammatory and self or non-self inflammatory is necessary to prevent excess presentation of self-antigen and subsequent failures of the immune system. Therefore, vesicles containing PAMP as well as PRR can be differently directed and processed than vesicles containing self non-inflammatory molecules. This mechanism is

well known for TLR. Here, the sequestration and the presentation of antigen is influenced on many levels like vesicle acidification, motor protein phosphorylation via an p38 scaffold, activation of distinct classes of proteases and invariant chain processing as reviewed by JM Blander in 2007 (29). In summary, antigen degradation in phagosomes and formation of antigen-MHC class II molecule-complexes, which are subsequently presented on the cell surface via fusion of the endosome and the cell membrane, is under control of vesicle activation.

The classical pathway of sequestration and presentation of exogenously acquired antigen as described above is supplemented by the mechanism of cross presentation. Here, exogenously acquired antigen finds its way into the MHC class I pathway (74) and is presented via MHC class I molecules on the surface of immunocompetent cells (77, 88). This is an important mechanism contributing to activation of specific CTL against host cells which are infected with viruses or intracellular parasites or bacteria. If this mechanism is just as well influenced by DC and/or vesicle activation like for the MHC class II pathway is still under investigation and debate (30).

1.2.1.4. Properties: migration

It is no surprise that DC migration shows comparable but counter-regulated dynamics as endocytosis and antigen-presentation. But, referring to the heterogeneity among DC, migration is not exclusively a property of activated and maturing DC (214). During steady state immature circulating DC permanently enter secondary lymphoid organs. In contrast, resident DC which show homing to peripheral tissues act as the ‘Langerhans paradigm’ predicts. Maturing DC start to migrate and get attracted by T cell-rich areas in secondary

lymphoid organs via up-regulation of chemokine receptor [CCR] 7 (56). Here, they find optimal circumstances for the interaction with and the activation of T cells. But first, upon stimulation they transiently arrest and show no migration (145, 218). This, in addition with the increased level of endocytosis, leads to enrichment of foreign antigen and may lead to full activation.

1.2.1.5. Properties: maturation and T cell priming

The term maturation describes the process in which DC change their morphology, regulate properties like antigen-uptake, -degradation and -presentation. Maturation takes place on a morphological as well as on a functional level. Further on, maturation is used as the term for the process that enables DC to activate T cells in the proper environment and therefore initiate protective immune responses.

Generally, the afore mentioned properties of DC account for the prominent ability of this cell type to specifically tailor immune responses in presence of various pathogens under several conditions. But these mechanisms were more or less prearrangements. The property which defines DC's ability for T cell priming is dependent on some more factors: First, higher levels of stable MHC class molecule-antigen-complexes (41, 92, 154, 207). Second, expression or up-regulation of co-stimulatory molecules which are needed for binding and activation of T cells (40, 94, 206). Third, cytokine expression stimulates proliferation and defines the route of differentiation of T cells (58, 112). These are the so-called 3 signals that are needed to generate differentiated, antigen-specific, fully activated T cells.

1.2.2. T cells

T cells develop beginning from stem cells of the bone marrow in the thymus towards naïve mature T cells. In contrast to DC, the term mature is used here for T cells that show full functionality but still are not activated. In this mature stage T cells are either $CD4^+$ T helper cells or $CD8^+$ which puts them into two distinct classes with different demands on activation stimuli. The fate of $CD8^+$ T cells is to become CTL which carry out their effector mechanisms upon specific interaction with MHC class I molecule-antigen-complexes. These CTL are able to selectively kill cells which show the specific antigen on the cell surface presented via MHC class I molecules but do not co-stimulate via CD80/86 molecules.

In contrast, the fate of naïve $CD4^+$ T helper [Th0] cells is more divers. Dependent on the quality and on the strength of the activation event, i.e. the cell-cell contact with DC, Th0 cells can develop into several subclasses with different obligations. For a long time already the concept of T cell differentiation towards Th1 and Th2 cells was known (59). Some more subclasses have been added since that. These subclasses need distinct differentiation factors and carry out different effector mechanisms ranging from acute inflammation [Th17] to immune silencing [Treg] which was nicely visualized on a poster of Chen Dong and Gustavo J. Martinez in Nature Reviews Immunology, 2010 [http://www.nature.com/nri/posters/tcellsubsets/index.html].

1.2.2.1. T cell instruction

As mentioned in 1.2.1.5 Th_0 cells need at least three signals for their full activation and proper differentiation. These signals provide mature, activated DC: Signal 1 - antigen

peptide presented via MHC class II - guaranties antigen specificity of the respective T cell. The duration of interaction between this complex and the T cell receptor [TCR] might also have influence on the stability and the quality of the differentiation (90). Second, signal 2 - co-stimulatory molecules of the B7 family - guaranties T cell activation. Perception of MHC-antigen-mediated signals without the perception of co-stimulatory signals would lead to anergy or apoptosis of the respective T cell (11). Third, signal 3 - cytokines in the surrounding milieu - drives proliferation and induction of differentiation of Th_0 towards the respective T helper cell subclass. Here, IL-2 is the prominent cytokine that induces proliferation whereas IL-6, transforming groth factor beta [TGF-β], IL-12, IL-10, IL-4 and IFNγ are the main cytokines which selectively induce differentiation (100).

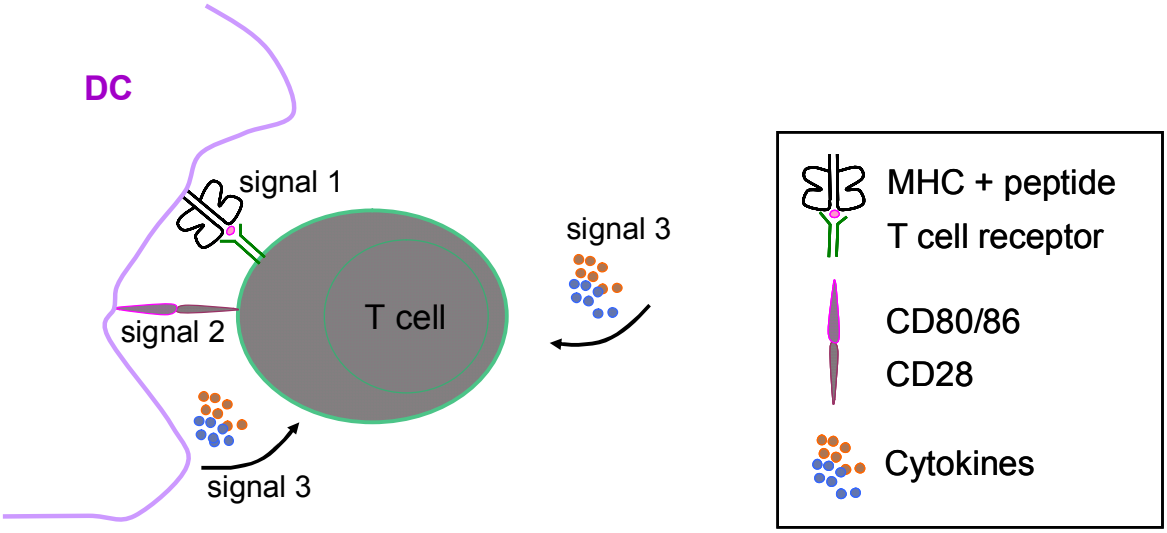

Fig. 4: Classical model of T cell instruction by activated DC. Naïve T cells need to receive 3 different signals to inititate differentiation, antigen-specific proliferation and activation. Interaction of the t cell receptor with peptide presented via MHC class molecules [signal 1], interaction of CD28 with co-stimulatory molecules like CD80/86 [signal 2] and perception of environmental cytokines via several receptors determines the fate of these T cells, respectively.

For induction of the Th_1 phenotype the cytokines IL-12 and IFNγ are greatly responsible. During activation of Th_0 cells the presence of IL-12 family members produced by appropriately activated DC and IFNγ produced by other cellular sources [e.g. NK cells] drive the expression of the transcription factor T-bet (142, 204). This leads to the expression of IFNγ by the respective T cell and the differentiation towards the Th_1 phenotype (102) as described in more details in the following chapter. In contrast, the Th_2 related phenotype is initiated by the presence of IL-4 produced by DC or e.g. mast cells. This cytokine initiates the transcription factor GATA-3 which in turn promotes IL-4 production (142). For both processes, i.e. Th_1 and Th_2 differentiation, other molecules, different interaction dynamics with membrane-bound ligands and receptors as well as timely coordination of these play a role (102). But, in this study a focus was set rather on the influence of the respective cytokines.

1.2.2.2. Th_1-related responses

Before carrying out their effector functions freshly activated Th_0 cells which differentiated towards the Th_1 phenotype in secondary lymphoid organs have to migrate to the site of microbial entry, i.e. the site of local inflammation. Therefore they express chemokine receptors [CXCR3 and CCR5] to migrate towards increasing levels of secreted chemokines which are related to local inflammation (104). Additionally, Th_1 cells express E- and P-selectin ligands for the adhesion to vesicle walls in close distance to inflammatory tissues. After transmigration through the vessel wall Th_1 cells can directly interact with APC. After recognition of the cognate antigen-MHC-complex they exert their effector mechanism.

The main effector mechanism is the production of IFNγ. This cytokine activates MΦ which in turn are able to kill intracellular pathogens (33). These MΦ are also important in later steps of the immune response when clearance of cellular material after silencing of immune responses becomes necessary. Furthermore, phagocytosis of pathogens by MΦ is greatly enhanced and supported by complement binding and opsonizing antibodies. The production of the respective antibodies is induced and enhanced by IFNγ production and direct interaction between Th_1 cells and B cells and therefore activation of the respective B cells. Furthermore, Th_1 cells activate neutrophils by secretion of TNF and lymphotoxin [LT]. This leads to enhanced microbial killing at the site of inflammation. In summary, Th_1 immune responses support cell-mediated clearance of intracellular and extracellular pathogens.

1.2.2.3. Th_2-related responses

In line with 1.2.2.2 Th_2 cells migrate towards increasing levels of chemokines. But, these chemokines are different from those which are involved in Th_1 cell migration. So are the involved chemokine receptors - CCR3, CCR4 and CCR8 (104). Ligands for these chemokine receptors are typically released at sites of helminth infection. Here, Th_2 cells release cytokines like IL-4, IL-13, IL-5 and IL-10. IL-4 stimulates activated B cells to produce IgE that in turn can bind to helminths and consequently serves as activating stimulus for mast cell degranulation. In combination with IgE-binding IL-5 serves as activator of eosinophils which also degranulate and therefore can attack helminths (39). In contrast to IFNγ IL-10 suppresses MΦ activation. Globally, IL-10 can be seen as an immune silencing cytokine. Nevertheless, in Th_2 related immune responses an alternative

pathway of MΦ activation can be triggered (71). In summary, the Th_2 immune response is a mainly antibody driven response against large extracellular pathogens that is not effective in *Leishmania* infections.

1.3. Immunobiology of leishmaniasis

1.3.1. Dependency on $CD4^+$ T cells

Mechanisms which confer resistance to *Leishmania* infections are greatly dependent on T cell help. But, the pathway leading to protective and long lasting immunity is not quite straight forward. It was shown that a clonal population of preformed Th_2-biased T helper cells respond early during infection (116). Although it was found that in some studies susceptible mice show stronger Th_2-biased immune responses, it is under discussion if this is crucial for the whole process of fighting the parasite (85). Nevertheless, the conversion of this preformed Th_2-related phenotype of the immune response towards the Th_1-related phenotype is absolutely necessary for the healing of lesions, the clearance of parasites and establishment of long-lasting immunity (175). As *Leishmania* parasites promote their own unrecognized infection in neutrophils and MΦ (115), MΦ are dependent on activation stimuli like IFNγ. These activation stimuli are released by Th_1 cells to a great extent.

1.3.2. Dependency on $CD8^+$ T cells

Considering the two experimental infection modes, for high dose infection models it was thought that $CD8^+$ T cells play only a role during re-infections (89, 203). This has been

challenged by experiments using the low dose infection model. Here $CD8^+$ T cells were necessary for the establishment of a Th_1-related immune response (22, 209). Hence, during beginning and ongoing *Leishmania* infections $CD8^+$ T cells exert their capacity for parasite elimination and support the Th_1 cell-driven immune response via production of IFNγ (83, 141).

1.3.3. Cytokines

A general feature of cytokines is to submit specific as well as generalized messages from one cell to the same [autocrine stimulation] or other cells [paracrine and endocrine stimulation]. IL-2 is initially released by DC and subsequently by T cells. Proliferation of the respective T cells is greatly dependent on this cytokine (73). IL-6 is a Janus-faced cytokine that serves as an inflammation marker but acts as a pro-inflammatory as well as an anti-inflammatory cytokine (18, 19). Cytokines which can be released by several cell types are known to promote either Th_1 or Th_2 cell differentiation or activate other cell types. It is worth to know that Th_1- and Th_2-related immune responses are not exclusive but inhibit each other by actions of their involved cytokines (175). In this study the balance between the respective cytokines was focused on to a certain extend. IL-4 and IL-10, the major cytokines during Th_2-related responses, inhibit differentiation of Th_0 cells towards Th_1 cells. Vice versa, IL-12 and IFNγ inhibit the establishment of Th_2-related immune responses. But, one has to mention that dependent on the point of time during the course of T cell activation and differentiation these cytokines might play different roles. Therefore, experimentally acquired levels of secreted cytokines have to be interpreted carefully.

1.3.4. Additional cell types and their effector mechanisms

Macrophages play the major role in neutralizing *Leishmania* parasites. Although they are one of the natural hosts of these pathogens, activation of MΦ by IFNγ initiates a cascade of effector mechanisms. Respectively, two molecules are indispensible for the effective clearance of *Leishmania* parasites during the *Leishmania*-specific response of the immune system. Nitric oxide (55, 122) and hydrogen peroxide are produced within endosomes of phagocytes and are potent and indispensible killers of intracellular parasites. Furthermore, Blos et al. demonstrated the organ-specific relevance of these two molecules (32). The inducible nitric oxide synthase has got a higher relevance for clearing the parasite within skin lesions and lymph nodes, whereas the NADPH oxidase, which converts molecular oxide to superoxide [O2-] is more important during the clearance of parasites in splenic cells.

Neutrophils do have a dual role. Besides the production of nitric oxide that contributes to the clearance of parasites they are exploited as “Trojan Horses” during the initial step of infection (115). These cells - with a high turnover - naturally ingest parasites and digest them in their vacuoles until they die of apoptosis. The apoptotic cell fragments are then consequently ingested by macrophages. But, *Leishmania* parasites prolong the life-span of and inhibit their own destruction within their neutrophilic hosts. Additionally, it is known that apoptotic vesicles have immune-silencing capabilities. Therefore it is not surprising that these parasite-filled vesicles are taken up by MΦ with no further activation of the MΦ. As a consequence, the “real” hosts of *Leishmania* parasites are infected without them triggering an immune response of these cells, respectively.

NK cells are not absolutely necessary for effective immune responses against *Leishmania* parasites (180). But, activation of NK cells by IL-12 during early phases of the infection

protects BALB/c mice from otherwise lethal infections (121). Naturally, NK cells are a source of IFNγ during infections with *Leishmania* in a TLR9, myeloid DC [mDC] and IL-12 dependent manner and therefore support the formation of protective Th_1 immune responses. More recently, it was shown that NK cells play a role in induction of protective immunity during vaccination with *Leishmania* antigen- and CpG-conditioned BMDC (168).

In this model of disease NKT cells act as bystander cells which support NK cell activity. After systemic infection the bystander help is needed to establish NK cell cytotoxicity and early IFNγ production in the spleen. Whereas, overall NK cell cytotoxicity, production of nitric oxide and IFNγ is not impaired in mice lacking NKT cells (131). However, absence of NKT cells during *Leishmania* infections worsens the clinical outcome.

1.3.5. Persistence of *L. major* in healed patients and mice

From different clinical and experimental studies it was known that viable *Leishmania* parasites can be found in dermis and draining lymph nodes (1, 195) of healthy individuals. Individual amastigotes may hide in so-called 'safe targets': immature myeloid precursor cells and monocytes (136) or fibroblasts (34) as shown for *L. major* For some *Leishmania* species also MΦ and hepatocytes have been determined as safe targets (49, 63, 118). But, if sterile cure is detrimental for the establishment of long-lasting immunity against *Leishmania* parasites or, vice versa, if containment of these parasites is necessary for preservation of T cell memory is under debate as reviewed by P. Scott in 2005 (184). There are studies emphasizing the necessity of parasite containment (21, 210) as well as a

study which showed long-lasting immunity against *Leishmania* infections in absence of *Leishmania* parasites (232).

Nevertheless, containment of these pathogens is dependent on different molecules which act against these parasites. Knock-out studies revealed that depletion of inducible nitric oxygen species [iNOS] (195) and phagocyte NADPH oxidase [Phox] (32) leads to effective emergence of the parasites and relapse of disease. More, production of IL-10 seems to play the major role for suppression of anti-*Leishmania*l activities in late phase of the immune response (176). Potent producers of this suppressive cytokine might be $CD4^+CD25^+CD45RB^{low}$ T cells which can be found in BALB/c mice (157) or regulatory $CD4^+CD25^+$ T cells (21).

Additionally, *Leishmania* parasites exhibit some mechanisms which allow immune evasion and might support containment in healed patients or mice. Partial resistance to reactive oxygen and nitrogen intermediates (68, 86, 140) as well as interference with antigen presentation of respective host cells (8, 43, 50, 105) and inhibition of oxidative burst account for these mechanisms.

1.3.6. Interaction of DC and *Leishmania* parasites during the course of infection

Due to immunoevasive mechanisms of *Leishmania* parasites certain kinetics of cellular recruitment are necessary to establish Th_1 cell responses by DC (120). As *Leishmania* parasites are able to exploit specific cell types as Trojan horses (47) and thereby induce TGF-β expression by MΦ (76, 211) recruitment of and interaction with DC are necessary. With their capacity to mount strong IFNα/β-responses upon interaction with *Leishmania* parasites but without phagocytosis (179) pDC contribute to disease control (167). But, *in*

vivo studies revealed that the main effector cells for the control of leishmaniasis, i.e. activation of NK cell-cytotoxicity and IFNγ-production, are mDC (16, 179). Unfortunately, most of the studies aiming to investigate these DC-*Leishmania*-interactions did not consider the influence of sandfly bites for which it was shown that they do have impact on DC maturation and local inflammation (9, 48). Early *Leishmania*-specific antibody production contributes to enhanced uptake of opsonized parasites in mDC and LC (215, 224). As a negative result IL-10 can be found in *L. amazonensis* amastigote-infected DC (162). If this displays the situation with *L. major* and DC is not known so far. Nevertheless, IL-10 in infected DC seems not to be the trigger for down-modulation of DC activation. It is suggested that for this immunoevasive mechanism the ongoing expression of 154 peptidases and additional proteinases is the key element (26, 37, 162, 178, 225) which interfere with the JAK/STAT-, the NF-κB- and the IRF pathway. Furthermore, alteration of DC's migratory capacities (13, 192) influence the antigen transport to draining lymph nodes by dermal DC (172).

1.4. Immunomodulatory strategies

1.4.1. Vaccination strategies

During the last decades huge efforts have been made to develop specifically tailored vaccines to fight life threatening emerging diseases. But, it came out that some targets are not easily hit by 'simple' life attenuated or subunit vaccines. Therefore, the improvement of specific strategies was pushed forward. This includes improvements in determination of target epitopes of the respective pathogen (138), routes of vaccination, delivery systems to

specifically address APC (72), adjuvant formulations for specific instruction of effective immune responses (38) as well as usage of e.g. DNA (169), RNA (123), mimotopes (108) or peptides as carriers of information.

1.4.2. Use of dendritic cells and derivatives for immunotherapy

1.4.2.1. Dendritic cells as adjuvants for vaccination against infectious diseases and cancer

DC are of central importance for linking innate and adaptive immunity and subsequently inducing fine-tuned immune responses due to their ability to integrate several signals. As a logical consequence, experimental manipulation of DC for preventive and therapeutical approaches was used in *in vivo* as well as in *in vitro* studies using mouse model diseases and cancer in human. These studies made use of either *ex vivo* manipulated DC and injection in allogeneic individuals as well as of *in vivo* targeting of DC with recombinant or cellular material.

The '*ex vivo* approach' has the advantage of maturation and activation of the respective DC under theoretically total control. Hence, the balance between tolerance inducing and immunity inducing capacities could be influenced and monitored. But, due to the specific interaction of MHC class I and II molecules and TCR manipulated DC and the recipient individual have to have the same genetic background. However, a problem comes up. Experiments either are done in inbred strains, where one individual serves as donor of bone marrow and other individuals receive the expanded and differentiated cells, or e.g. in humans the donor and the recipient is the same individual. For studies in humans it is

absolutely necessary to generate these donor DC under GMP conditions and so this is a quite expensive effort. Consequently, studies in humans are almost exclusively done for therapeutical goals in cancer or AIDS.

For the induction of protective immune responses these DC have to be properly stimulated and antigen delivery has to be adequate for antigen presentation on MHC class I and II molecules. There are several studies using antigen delivery via co-incubation of DC with the respective antigen and maturation inducing adjuvants. These DC are able to induce protective Th1-related immune responses after injection into syngeneic mice as shown in our group for the model disease leishmaniasis (61, 163, 167). Stable or transient antigen expression by the DC themselves is a great advantage if strong CTL responses should be induced by TCR stimulation via antigen-MHC class I molecule-complexes. Therefore transfection of DC with bacterial plasmids which harbour CpG motifs was used (107, 111) which was able to brake regulatory T cell induced tolerance (228). Furthermore, lentiviral vectors were efficiently used for such an approach in tumor models (134, 139). Another technique, DC-tumor cell-fusion has the great advantage that hybrid-DC are generated which present all relevant antigens for the induction of tumor-interfering immune responses (14, 109) irrespective of the use of autologous (70) or allogeneic (199) DC.

1.4.2.2. Exosomes

On the way towards cell-free vaccines derivatives of DC - exosomes - were used for vaccination studies within models of infectious diseases and cancer (213). Exosomes are specifically formed vesicles which differ in their protein composition from whole cell lysates and are enriched with several molecules which are related to immune functions

(45). Here, very prominent proteins are the tetraspanin family with functions related to cell-fusion, stimulation and motility (82). Already in 1998 these exosomes efficiently induced $CD8^+$ T cell and MHC dependent growth retardation of tumors in different mouse models (234). Additionally, exosomes can serve as protective vaccines in infectious disease models as proven for *Toxoplasma gondii* in 2004 (4). While it was shown that exosomes are able to transfer MHC-peptide complexes to *ex vivo* generated DC or host DC and to indirectly activate T cells (10, 46, 201), Kovar et al. was able to demonstrate direct CD8+ T cell stimulation by exosomes *in vivo* (110). Hence, much potential is given towards exosomes-based stimulation of the immune system inducing cell-based immunity under controlled conditions.

1.4.2.3. DC as targets for immunostimulatory techniques

DC and exosome preparation *ex vivo* is a time and cost-expensive procedure. Hence, DC-targeted approaches were suggested to be promising. Nicely reviewed by Proudfoot, Apostolopoulos and Pietersz (160), many membrane-associated molecules serve as targets to deliver antigen more or less specifically to DC after administration via several routes. For example, lectins were targeted via mannan-coated vesicles or antibodies which were fused to the antigen of interest. Further more, TLR, the F4/80-like receptor FIRE, Fc receptors, chemokine receptors and others have been addressed so far. But, this technique still needs lots of further studies for each of the targets as the outcome of such approaches is not always predictable. Instead of inducing protective immunity against a pathogen or cure of the respective disease a misguided immune response towards the wrong type - Th1, Th2, tolerance or overshooting cytokine bursts - might be the result.

2. Aim of the study

In previous studies it was shown that like for cancer antigen-loaded DC are perfect vaccines or therapeutic agents. *Ex vivo* manipulated, they induce well controlled and observable immune responses. For the case of leishmaniasis it is known that antigen-loaded DC can induce Th1-based immune responses in mice, which lead to the induction of IFNγ production and, as a consequence, to the destruction of *Leishmania* parasites within MΦ. But, as therapeutic vaccination against leishmaniasis is only accessable via a complex combination therapy (67), establishment of preventive immunity would be the most reachable goal so far. To transfer this to worldwide vaccination of humans would have several drawbacks like DC preparation and manipulation under GMP conditions, individual autologous cell transfer in hospitals and
high costs.

Whereas leishmaniasis is one of the neglected diseases of which mainly poor people in underdeveloped countries suffer from, this type of vaccination would be affordable only for rich people in well developed countries. Therefore this type of vaccination has to be modified towards a simple and cheap formulation that brings the same properties like long lasting protective immunity and high reproducibility.
It is already known that host-derived cell types have to produce IL-12 upon vaccination with antigen-loaded DC (163). Therefore it seems to be a feasible goal to produce a leishmaniasis-tailored subunit vaccine that targets the respective cell population *in vivo*.

As a consequence the following questions were addressed:

Which cell population triggers the development of protective immunity after DC-based vaccination?

Which type of interaction is necessary between donor DC and recipient cell types?

What are the key factors which have to be monitored to ensure proper immune responses?

3. Materials and Methods

3.1. Device

Device	Brand
accu-jet®	Brand GmbH & Co. KG, Wertheim, Germany
analytical balance	Kern & Sohn GmbH, Balingen, Germany
AxioCam	Carl-Zeiss AG, Oberkochen, Germany
cell culture centrifuge, Megafuge 1.0R	Heraeus Sepatech GmbH, Osterode, Germany
electrophoresis power supply, EPS 500/400	Pharmacia, Piscataway, USA
electrophoresis power supply, model 200/2.0	BioRad, München, Germany
FACScalibur™	BD, Heidelberg, Germany
fluorescence microscope	Carl-Zeiss AG, Oberkochen, Germany
heater	Axon lab AG, Baden-Dättwil, Switzerland
incubator (for *Leishmania* promastigotes)	WTB Binder, Tuttlingen, Germany
incubator (primary cell culture)	Heraeus Instruments GmbH, Osterode, Germany
inverse microskope	Carl-Zeiss AG, Oberkochen, Germany
laser scanning confocal microscope (LSM)	Carl-Zeiss AG, Oberkochen, Germany
light microscope	Carl-Zeiss AG, Oberkochen, Germany
magnetic stirrer	Heidolph, Kehlheim, Germany
Mini Trans-Blot®	BioRad, Munich, germany
Mini-PROTEAN® II	BioRad, Munich, germany
Multiskan Ascent®	Thermo Fisher Scientific, Waltham, USA
pH-meter	Inolab/WTW, Weilheim, Germany
photometer	Eppendorf, Hamburg, Germany
sterile bench	Nuaire, Plymouth, MN, USA
table centrifuge, miniSpin	Eppendorf, Hamburg, Germany
Thermomixer 5436	Eppendorf, Hamburg, Germany
Sonoplus HD70	Bandelin, Berlin, Germany
vortex generator	Heidolph, Schwabach, Germany
water bath	GFL mbH, Burgwedel, Germany

Table 1: Alphabetic listing of technical equipment used in this study

3.2. Reagents for cell culture and plastic material

For the culture of primary cells and cell lines R-10 culture medium was used which contained RPMI 1640 [Invitrogen Karlsruhe, Germany] supplemented with 10% fetal calf serum [FCS, PAA Laboratories GmbH, Cölbe, Germany] 2 mM L-glutamine [Biochrom,

Berlin, Germany], 10 mM Hepes buffer [Biochrom], 60 µg/ml penicillin [Sigma, Taufkirchen, Germany], 20 µg/ml gentamycin [Sigma] and 0.05 mM 2-mercaptoethanol [Sigma]. For T cell proliferation assays cells were cultured in HL-1 medium [Lonza, Basel, Switzerland] supplemented similar to R-10 but without FCS. Sterile phosphate-buffered saline [PBS] was from Invitrogen. The counting of viable cells was done with 0.4% trypan Blue [Invitrogen] and the digestion of mouse tissue was performed with sterile Collagenase D [Roche Diagnostics, Mannheim, Germany]. Plastic 50 ml and 15 ml tubes were from Sarstedt [Nümbrecht, Germany]. 1.5 ml microfuge tubes were from Eppendorf [Hamburg, Gemany], ELISA plates and pipet tips were from Thermo Fisher Scientific [Dreieich, Germany]. Petri dishes [60 × 15 and 90 × 16 mm] and cell scrapers were from BD Falcon [Heidelberg, Germany].

Culture plates [6-, 12-, 24- and 96-well] and plastic pipets were from Greiner Bio-one [Frickenhausen, Germany]. 1 ml syringes were from Dispomed [Gelnhausen, Germany] and 2 ml and 5 ml syringes were from B. Braun Melsungen AG [Melsungen, Germany]. Cell strainers were purchased from BD Biosciences Pharmingen [Heidelberg, Germany].

3.3. Reagents and material for the treatment of mice

Recombinant murine granulocyte-macrophage colony-stimulating factor [GM-CSF] was from Invitrogen [London, UK]. Recombinant human FMS-like tyrosine kinase 3 ligand [Flt3-L] was from CellGenix Technologie Transfer GmbH [Freiburg, Germany] E. coli-derived LPS was from Sigma. The CpG-ODN 1668 [5′ TCCATGACGTTCCTGATGCT 3′] were manufactured by Eurofins MWG GmbH [Ebersberg, Germany].

3.4. Mice

Female BALB/c and C57BL/6 mice were purchased from Charles River Breeding Laboratories [Sulzfeld, Germany]. IL-12p40 and IL-12p35-deficient BALB/c mice were kindly provided by Dr. Gottfried Alber, University of Leipzig (130, 153) and bred in our institute's animal facility. Animals were 6 to 8 weeks old at the onset of experiments and were kept under conventional conditions.

For vaccination of BALB/c mice 5 × 105 viable BMDC or an equivalent amount of disrupted BMDC were injected i.v. into the tail vein of naive 6 - 8 week old mice. Control mice were treated with PBS. One week later, 2 × 105 stationary phase promastigotes were injected subcutaneously in the right hind footpad. The course of the infection was monitored weekly by measuring the increase in footpad size of the infected versus the non-infected footpad.

3.5. *L. major* parasites and preparation of antigen lysate

The *L. major* isolate MHOM/IL/81/FE/BNI used in this study has been described previously (190). Parasites were maintained by passage in BALB/c mice. Promastigotes were grown in conventional biphasic medium consisting of a solid base of rabbit-blood agar plus a liquid phase of R-10. Parasites used for *in vivo* and *in vivo* experiments were passaged 4 to 8 times *in vivo* to maintain maximal infectivity. For one experiment transgenic *Leishmania* promastigotes [luc2-*L. major*] constitutively expressing the firefly luciferase were kindly provided by Angela Bruder. Therefore, these *Leishmania* parasites had been stably transfected via recombination with the linearized pLEXSY-hyg2 vector [Jena Bioscience GmbH, Jena, Germany] where the firefly luc2 gene was introduced. *In vivo* detection of luciferase-expressing *Leishmania* was done via measuring the

luminescence emitted by parasites in the right footpad with a CCD camera 5 min. after an intraperitoneal [i.p.] injection of 3 mg D-Luciferin [Synchem OHG, Felsberg, Germany] in 200 µl per mouse together with peritoneal anaesthetic.

For the preparation of total *L. major* antigen, stationary-phase promastigotes were collected, washed three times with PBS, resuspended at 1×10^9/ml in PBS and subjected to six cycles of freezing and thawing at -80°C and 25°C, respectively. *Leishmania* antigen [Lmag] was stored at -80°C and aliquots were thawed not more than twice.

3.6. Cell culture methods

3.6.1. Generation of BMDC and pDC

BMDC were generated from bone marrow progenitors according to Lutz et al. with minor modifications (127). Mice were sacrificed at an age of six to ten weeks. After preparation, femurs and tibiae were flushed with a syringe containing culture medium. Cells were washed and resuspended in culture medium and cell number was determined. At day of preparation [day zero] two million bone marrow cells were seeded into a 9 cm bacteriological petri dish in a volume of 10 ml R-10 medium containing 200 U/ml GM-CSF [Pepro-Tech, London, UK]. On day three and six 5 ml of R-10 medium containing 200 U/ml GM-CSF were added to each culture-dish. On day ten non-adherent cells were harvested and counted for the use in further experiments.

Accordingly, pDC were generated from bone marrow progenitors as described by Brasel et al. (35) with minor modifications. Bone marrow cells were seed into cell culture flasks in a total volume of 5 ml with 2×10^6 cells/ml culture medium supplemented with 200

ng/ml FMS-like tyrosine kinase 3 [Flt3-L, CellGenix, Freiburg, Germany]. At day eight of culture non-adherent cells were harvested and $CD11b^+$ cells were depleted via magnetic cell sorting [MACS, Miltenyi, Bergisch Gladbach, Germany] to enrich for pDC.

3.6.2. Antigen loading and cell disruption

Cells were counted and one million cells per ml culture medium were seeded into petri dishes. Again 200 U/ml of GM-CSF were added. For antigen loading, in the presence or absence of 25 µg/ml CpG ODN 1668, a volume of 30µl per ml of Lmag were added, corresponding to 30 parasites per each cell. After 16 hours cells were harvested, washed three times and counted. Cell concentration was set to 5×10^6 cells per ml in ice cold PBS or warm R-10, depending on the further use.

For the co-culture or the injection of viable cells, the prepared solutions were used directly. Instead, suspensions of non-viable BMDC [from now on called fragments] were obtained by incubation of the cells with 4% para-formaldehyde [PFA] for 20 min, three times of rapid freezing in liquid nitrogen and thawing in a water bath [37°C], or disruption of the cells via sonication [30 sec, cycle 70%, power MS 72/D] with an ultrasonic homogenizer [Sonoplus HD70]. Sonication I included one cycle of sonication for 30 sec, sonication II included two cycles of sonication for 30 sec. The suspensions were used directly or stored at -80°C before usage.

3.6.3. Preparation of dendritic cell fractions

For the preparation of distinct cellular fractions of BMDC the cells were taken up in ISE buffer [3mM Imidazole pH 7.4, 250 mM sucrose 0.5 mM EDTA], counted and adjusted to 5×10^6 cells per ml. After three times of rapid freezing and thawing the cellular fragments were homogenized by flushing the cells for 5 times with high pressure through a 5 ml syringe with a 26G needle. With a first centrifugation step with 3000 g for 20 minutes at 4°C the larger cell debris could be removed. The aqueous fraction [supernatant] was taken to a second centrifugation step with 50,000 g [22,000 rpm, SW60 rotor] for 30 minutes at 4°C to separate membranous particles from cytoplasmic components. For a washing step both the aqueous fraction and the pellet, as it was resuspended in ISE buffer, were subsequently centrifuged again with 50,000 g for 60 minutes at 4°C. From the previous aqueous fraction again the supernatant was taken and from the previous pellet again the pellet was taken and resuspended in ISE buffer. Afterwards the solutions were stored at -80°C.

3.6.4. Cell labelling with a fluorescent dye

For the labelling of membranes with a red fluorescent dye PKH26 from Sigma was used. Cells were counted, centrifuged and resuspended in 50 µl per $1x10^6$ cells in Diluent C. The same amount of Diluent C supplemented with 4 µM PKH26 was added immediately and incubated for 5 minutes while inverting gently. The labelling was stopped via adding 100 µl per 1×10^6 cells of 1% BSA in PBS and incubating for one minute. After the addition of at least 2 ml of R-10 cells were washed one time with R-10 and one time with

PBS. At last, cells were counted and suspended in PBS or R-10 with the required concentration. The whole procedure was done at 25°C.

For the labelling of cells with a green fluorescent dye cells were labelled with CFDA from Invitrogen. Therefore a stock solution of 10 mM CFDA in DMSO was prepared and from this, a solution containing 5 µM CFDA in PBS was generated. Cells were washed and resuspended in PBS with a concentration of 1×10^7 cells per ml in a 15 ml Falcon tube. After the same amount of 5 µM CFDA in PBS was added the suspension was incubated for 10 minutes at room temperature [RT]. At next, the tube was filled with ice cold PBS and cells were washed twice with PBS at 4°C. Then, the cells were resuspended in R-10 or complete HL-1 medium according to the following experiment for 30 minutes. Then the cells were harvested, washed two times with PBS, counted and resuspended in PBS, complete HL-1 or R-10 with the required concentration.

3.6.5. Co-culture Assay

To determine the secretion of cytokines by splenocytes upon stimulation spleens of naïve or vaccinated mice were collected. After a digestive step of Collagenase D incubation for 30 minutes the spleen was mashed through a 70 µm cell strainer using the plunger end of a 5 ml syringe. Subsequently, cells were incubated in TAC buffer [0.83% NH_4Cl, 0.25 mM Tris, pH7.2] for 5 minutes at RT to lyse red blood cells. After one washing step the cell suspension was adjusted to the required concentration and seeded into 48 wells. Subsequently the cells were stimulated with either CpG [100 µg/ml], LPS [10 µg/ml], *Leishmania* parasites [1×10^7 per ml] or a solution of BMDC fragments. after 48 hours the

supernatants were collected and analysed via enzyme-linked immunosorbent assay [ELISA] for the amount of expressed cytokines.

3.6.6. Determination of cytokine release

Supernatants generated in co-culture experiments were used to evaluate the amount of expressed cytokines via sandwich enzyme-linked immuno sorbent assay [ELISA]. Plates were coated with 50 µl of purified rat antibodies against the respective cytokine in 0.1 M NaHCO3, pH 8.3. The concentration of capture and detection antibodies is given in Table 2.

Cytokine	capture mAb purified		detection mAb biotinylated		standard max. conc.		
IFNγ	0.30	µg/ml	2.00	µg/ml	25	ng/ml	all BD Biosiences Pharmingen
IL-2	1.67	µg/ml	1.00	µg/ml	2.5	ng/ml	all BD Biosiences Pharmingen
IL-4	2.00	µg/ml	1.00	µg/ml	2	ng/ml	all BD Biosiences Pharmingen
IL-6	1.67	µg/ml	1.00	µg/ml	10	ng/ml	all BD Biosiences Pharmingen
IL-10	5.00	µg/ml	1.00	µg/ml	2.5	ng/ml	all BD Biosiences Pharmingen
IL-12p70	1.50	µg/ml	2.50	µg/ml	2.5	ng/ml	all BD Biosiences Pharmingen
IL-12p40	3.00	µg/ml	2.50	µg/ml	10	ng/ml	all BD Biosiences Pharmingen
IL-17	1.67	µg/ml	0.17	µg/ml	1	ng/ml	all R&D Systems
TGFβ	1.67	µg/ml	1.67	µg/ml	2.5	ng/ml	all BD Biosiences Pharmingen

Table 2: Concentration of antibodies used for detection of cytokines via ELISA.

After washing with PBS-Tween [0.05% Tween 20, Sigma, in PBS] plates were blocked with 150 µl of blocking solution [10% FCS in PBS-Tween] for four hours at room temperature. Again, after washing 3 times with PBS-Tween 50µl of supernatants or one of eight 1:2-dilutions of the respective standards – with the maximum concentration as shown in Table 2 - were added and incubated at 4°C overnight. After three times washing

50 µl of detection antibody was added at RT for one hour and again, after three times washing 50 µl of streptavidin-alkaline phosphatase-complex [AKP Streptavidin, BD Pharmingen] was added and incubated at room temperature for one hour. At last, after washing three times 100 µl of 1 mg/ml p-nitrophylphosphate [phosphatase substrate, Sigma] in diethanolamine-buffer was added and the optical density [OD] was measured with a Multiskan Ascent ELISA-reader [Thermo Fisher Scientific] at a wavelength of 405 nm and a reference wavelength of 490 nm.

The concentrations of cytokines were calculated with the Ascent Software via extrapolating the OD values of the samples to the multiparametric regression of the standard curve. Cytokine detection thresholds were 200 pg/ml for IFNγ, 20 pg/mL for IL-2, 16 pg/mL for IL-4, 80 pg/mL for IL-6, 20 pg/mL for IL-10, 80 pg/mL for IL12-p40, 20 pg/mL for IL12-p70, 8 pg/mL for IL-17 and 20 pg/mL for TGFβ.

3.6.7. Proliferation assay

To determine the capability of BMDC fragments to induce T cell proliferation a suspension of three different cell populations was set up. The population containing the *Leishmania* antigen specific T cells was obtained from mice which were vaccinated with sonicated, Lmag-loaded and CpG-activated BMDC [BMDC L/C, son.] and infected with 2×10^5 *L. major* parasites into the right footpad one week later. One week post infection spleens were collected, digested and single cell suspension was prepared. As a source for DC, spleens of naïve mice were collected. After preparation of single cell suspension DC were enriched via MACS using CD11c [N418] MicroBeads [Miltenyi] according to manufacturer's guidelines. The third population consisted of a suspension of sonicated

Lmag-loaded, CpG-activated BMDC. All viable cells were labelled with CFSE as described in 3.6.4. All cells were resuspended in HL-1 medium and co-cultured at a ratio of 2×10^6 splenocytes to 2×10^5 DC to 2×10^5 BMDC. After 3 to 5 days the cell suspensions were centrifuged, supernatants were collected and stored at -20°C for cytokine analysis. After a washing step with PBS the cells were labelled with antibodies against CD4 and CD8 [BD Pharmingen]. Via FACS analysis the CFSE-dilution of CD4+ and CD8+ cells was measured.

3.6.8. Mouse perfusion and cryosectioning of spleens

For the tracking of i.v.-injected DC, 5×10^5 CFSE-labelled BMDC were injected into the left tail vein. Three hours later mice were anesthetized with a lethal dose of up to 2 mg Ketanest S [Pfizer Pharma GmbH, Berlin, Germany] and 0.2 mg Rompun [Bayer Animal Health GmbH, Leverkusen, Germany] per mouse. Then the mice were fixed and the breast was opened so that the left ventricle could be penetrated. After a few seconds of PBS pumping the vein on the right side of the heart was cut to let the blood flow out. PBS pumping was followed until the efflux was cleared of blood. Then PBS was replaced with a solution of 4% PFA in 0.1 M sodium phosphate buffer with a pH of 7.2 and the mouse was perfused for 25 minutes. After this, the spleen was collected from the mouse and was post-fixed with the same solution for 24 h. Before the spleen was frozen, it had been cryo-protected via incubation in 20% sucrose solution for about 48 h. Afterwards the spleen sample was arranged in plastic Cryomolds and embedded in Tissue Tek O.C.T. [both Sakura Finetek Europe B.V., Zoeterwoude, Netherlands] and frozen in a cup that had been

filled with isopentane and cooled by a bath of liquid nitrogen. For short time storage the samples were then kept at -80°C.

Spleen samples were then cut into 10 - 20 µm thick sections using Microm blades [Thermo Fisher Scientific] in a microtome cryostat HM 500 CM [Microm International GmbH, Walldorf, Germany] and placed onto SuperFrost Plus glas slides [Menzel GmbH & Co KG, Braunschweig, Germany].

3.6.9. Immunofluorescence staining

For immunofluorescence staining of spleen sections samples were treated as follows: Prior to staining glas slides were incubated in acetone at -20°C for 10 minutes. For some antibodies, as shown in

Table **3**, some slides were blocked with antibodies against Fc receptors [CD16/32] in a solution consisting of 20% rat serum in PBS-Tween. Afterwards slides were incubated with the respective antibodies. After the staining procedure glas slides were prepared for microscopical analysis via the LSM.

antibody	source	isotype	prestaining procedure		incubation	conc. of antibody
mabαCD4-PE	rat	IgG2a	10min acetone	30min mabαCD16/32 in 20% rat serum	30min in 20% rat serum	1µg/ml
mabαCD11c-PE	armenian hamster	IgG1	10min acetone	w/o	60min in 5%FCS	1µg/ml
mabαCD45R-PE	rat	IgG2a	10min acetone	30min mabαCD16/32 in 20% rat serum	30min in 20% rat serum	0.2µg/ml

Table 3: Staining procedure of cryo-sections of spleen samples for different markers. Sections were pre-treated as indicated. Briefly, sections were post-fixed with acetone and blocked with monoclonal antibody against [mabα] CD16/32 in the respective serum. The same serum was used to incubate with the staining antibody with the indicated concentrations, PE: phycoerythrin.

3.6.10. Limiting dilution assay

The number of infected cells in the lymph nodes draining the infected footpads was determined by limiting dilution analysis (202). Six to seven weeks post infection, mice were sacrificed and single cell suspensions were obtained from pooled draining lymph nodes. Serial dilutions ranging from 2×10^6 to 1.2×10^2 cells per ml [protected mice] or 2×10^5 to 12 cells per ml [susceptible mice] were seeded into microculture plates [100 µl/well] containing 50 µl of a blood-agar slant to support the growth of parasites. For each dilution, 16 replicates were set up and incubated for 10 days at 28°C and 5% CO_2 in a humidified atmosphere. *Leishmania*-negative wells were identyfied using an inverted microscope and the estimation of infected cells in the draining lymph nodes was calculated.

3.6.11. Analysis of cytokine production of lymph node cells and splenocytes

To elucidate the cytokine expression of lymph node cells and splenocytes in the course of disease lymph nodes and spleens were harvested from sacrificed mice 6 – 7 weeks post infection. After preparing a single cell suspension as mentioned above splenocytes were incubated for 5 minutes in TAC buffer at RT and both cell suspensions were washed subsequently, resuspended in R-10, counted and adjusted to 1×10^6 cells per ml. Per vaccination group and treatment – re-stimulation with 10 µl L.major lysate or 10 µl PBS - 1 ml of each cell suspension was seeded in 48wells as duplicates. 48 hours later supernatants were collected and the amount of produced cytokine was measured via ELISA.

3.6.12. Statistical analysis.

Results of all experiments were analyzed using the GraphPad Prism 4.03 software. For determining statistical significance of the vaccination experiments and each of the co-culture experiments, multiple group comparisons were performed by one-way ANOVA with Dunnett's post test to compare all samples against the control sample. For comparison of all co-culture experiments a two-way ANOVA was performed. Additionally Bonferroni's post test was used to compare all groups. To determine the statistical significance between two distinct samples, the two-tailed unpaired t-test was used.

4. Results

4.1. *In vitro* generation of pDC

4.1.1. Growth kinetics

Ambitious scientific approaches regarding pDC-based vaccination against *Leishmania* parasites are exacerbated due to the large numbers of mice needed to isolate appropriate amounts of pDC as pDC are low in cell numbers and not easy to handle during the process of preparation, antigen-loading and re-injection into the recipient mice. Therefore we started to raise pDC *in vivo*. For this purpose, we cultivated bone marrow stem cells in the presence of the growth factor Flt3-L for eight days. Fig. 5A shows the growth kinetics of bone marrow stem cells cultured in Flt3-L-containing medium. Cells were harvested at day eight for further use in experiments. Therefore $CD11b^+$ cells, i.e. conventional or myeloid dendritic cells, were depleted via MACS. Fig. 5B shows an average rate of yield between three to four million pDC per culture flask.

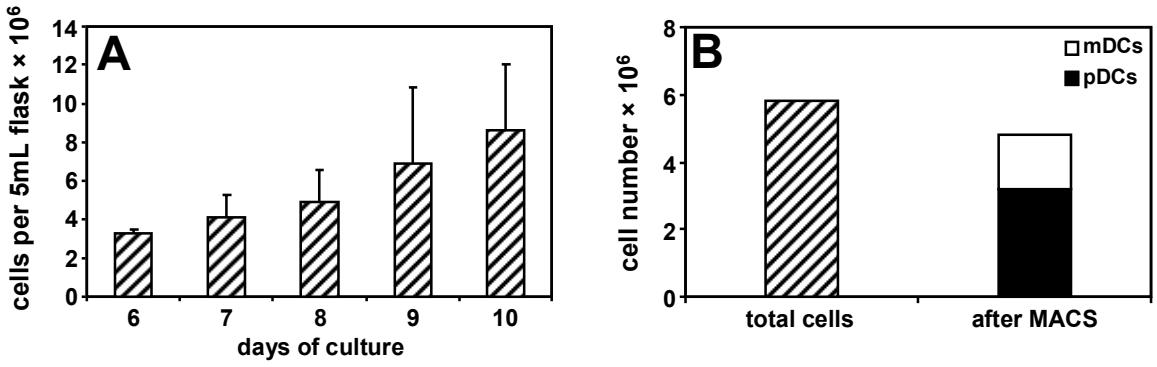

Fig. 5: Analysis of cells derived from bone marrow and grown in culture medium supplemented with Flt3-L. A: Growth kinetics of total cells per 5 ml culture medium supplemented with 200 ng/ml Flt3-L. **B**: Representative yield of bone marrow cells after eight days of culture in Flt3-L-containing medium before and after MACS for CD11b. mDCs: conventional myeloid dendritic cells; pDCs: plasmacytoid dendritic

The high yield of pDC generated from precursor cells *in vitro* emerged to be an attractive method to be used in our model of pDC-based vaccination. From this moment on sufficient numbers of cells were available to conduct substantial studies in this field.

4.1.2. *In vitro* grown pDC do not need pre-activation via CpG ODN

It was established in our laboratory that *Leishmania* lysate serves as a sufficient whole-parasite antigen for the presentation of relevant epitopes to induce protective immune responses. But likewise, the low stimulatory activity of *Leishmania* lysate in terms of DC activation is known. Using different DC subsets for our DC-based vaccination strategy revealed that *ex vivo* antigen-loaded pDC do not need additional activation via CpG ODN to serve as a potential vaccine carrier against infections with *L. major* (167).

To evidence, whether this holds true for *in vitro*-cultured pDC, we injected antigen-loaded pDC i.v., co-incubated with lysate either in the presence or absence of CpG ODN. Control mice were treated with PBS or naïve pDC.

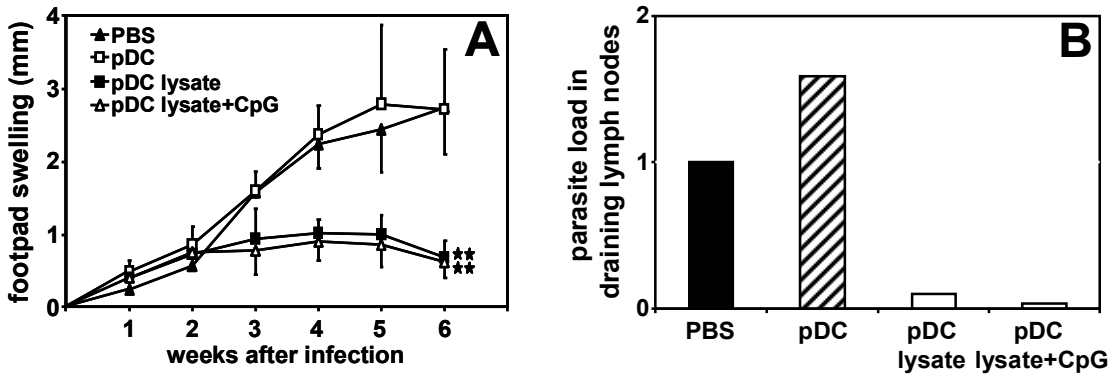

Fig. 6: Monitoring of disease parameters after vaccination and subsequent infection with *L. major*. pDC were pretreated as indicated **A**: Development of footpad swelling after infection with *L. major* into the right footpad of vaccinated mice. ANOVA p value < 0.0001; Dunnett's post test: **, $p \leq 0.01$. **B**: Determination of parasite load in pooled draining lymph nodes of the respective vaccination groups six weeks post infection. pDC lysate: *Leishmania* lysate-loaded pDC; pDC lysate+CpG: *Leishmania* lysate-loaded and CpG-activated pDC. The data shown is representative for three independent experiments with at least 4 mice per group.

Fig. 6 clearly demonstrates the successful immunization, comparable to *ex vivo*-prepared pDC (167), using *in vitro*-grown pDC as vaccine carrier, independent of additional activation with CpG ODN,. In contrast to both control groups, mice of both groups that were vaccinated with antigen-loaded pDC perform equally in terms of controlled footpad swelling and reduced parasite load in draining lymph nodes. Differences were only seen for cytokine production upon re-stimulation with *L.m.* lysate of splenocytes and lymph node cells six weeks post infection (Fig. 7).

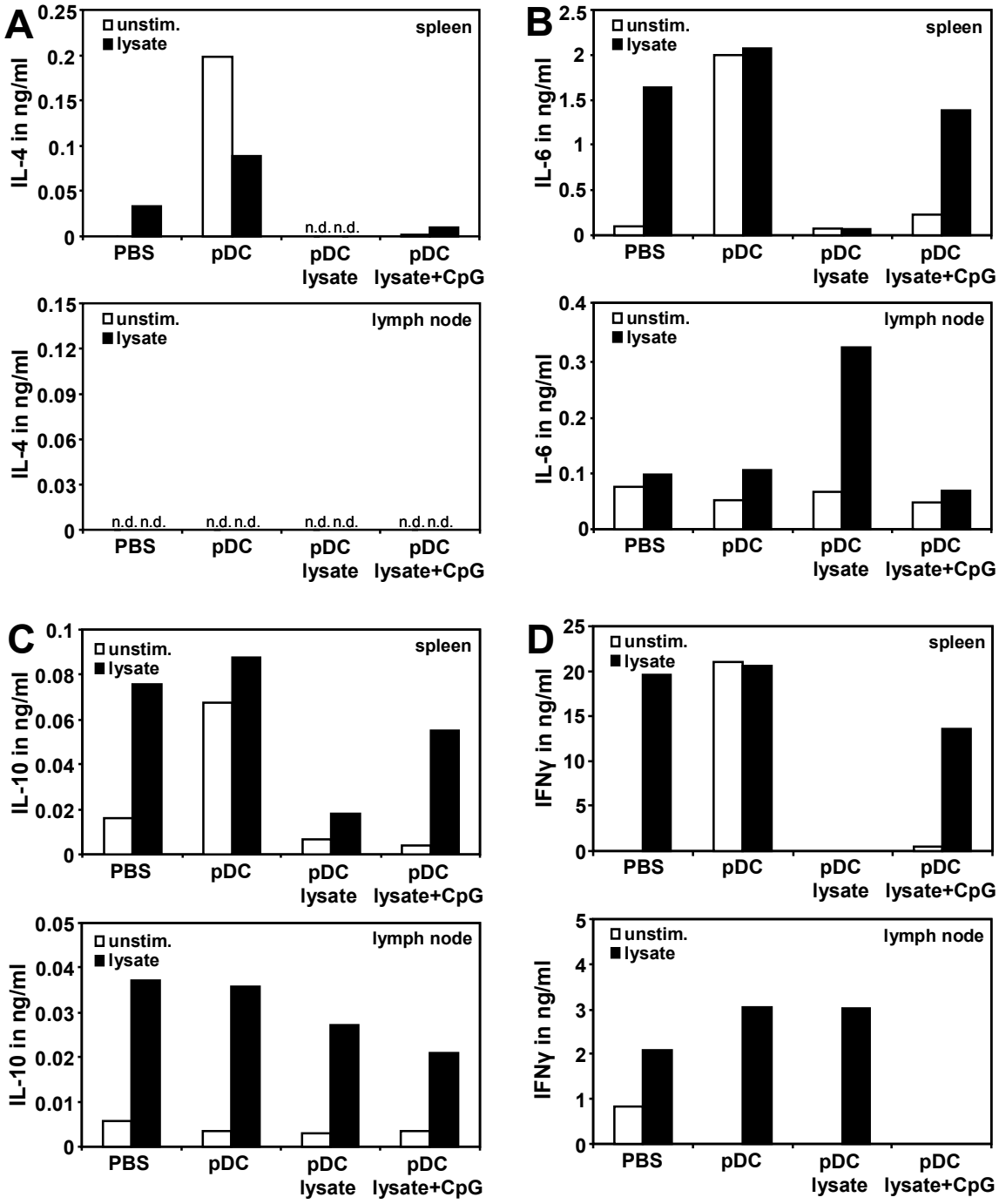

Fig. 7: Cytokine profiles of spleen and LN cells after challenge with *L. major antigen*. Six weeks post infection splenocytes and dLN cells of vaccinated mice were re-stimulated with *Leishmania* lysate. Supernatants were collected and cytokine levels of IL-4 [**A**], IL-6 [**B**], IL-10 [**C**] and IFNγ [**D**] were determined via ELISA. unstim.: without stimulation, open bars; lysate: re-stimulation with *Leishmania* lysate, black bars; pDC lysate: *Leishmania* lysate-loaded pDC; pDC lysate+CpG: *Leishmania* lysate-loaded and CpG-activated pDC. The data are representative of three independent experiments.

Here, the strong immune-stimulatory capacity of antigen-loaded pDC was indicated by elevated levels of IL-6, IFNγ and IL-10, whereas these cytokines were differentially expressed by these two groups. Vaccination with antigen-loaded and CpG-activated pDC results in high levels of IL-6, IL-10 and IFNγ within the spleen but not within the lymph node. Compared to this, antigen-loaded pDC caused high levels of IL-6- and IFNγ by lymph node cells. These differences become more evident by calculating the ratio of expressed IFNγ vs. IL-10 [Fig. 8].

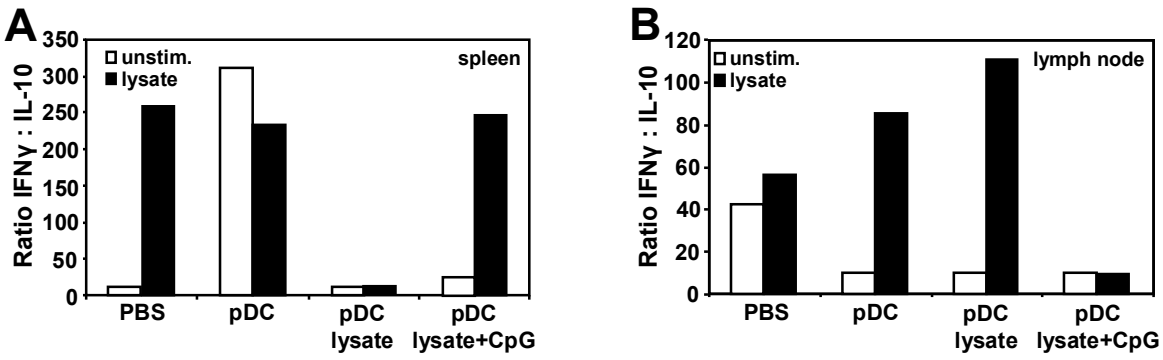

Fig. 8: Ratio of IFNγ to IL-10 in the supernatants of spleen and LN cells 6 wk after footpad infection with *L. major*. Ratios of IFNγ to IL-10 in the supernatants were calculated from the cytokine levels detected by ELISA for spleens [**A**] and lymph nodes [**B**]. Where no cytokine was detected, the value of the detection threshold of the ELISA was used for calculating the ratio. pDC lysate: *Leishmania* lysate-loaded pDC; pDC lysate/CpG: *Leishmania* lysate-loaded and CpG-activated pDC The data are representative of three independent experiments.

4.2. Antigen presentation in the spleen after i.v. injection of antigen-loaded BMDC

Commonly, DC are well known for their ability to catch antigens, to present the relevant epitopes via MHC molecules and to mediate coordinated immune responses. These

abilities are taken in advantage when injecting DC-based vaccines into animals and men. To gain closer insights into the mode of action of our well established DC-based vaccination with viable antigen-loaded BMDC against *L. major*, we started to monitor the fate of i.v. injected BMDC after administration. For that reason 5×10^5 CFSE-labelled BMDC were injected into the tail vein.

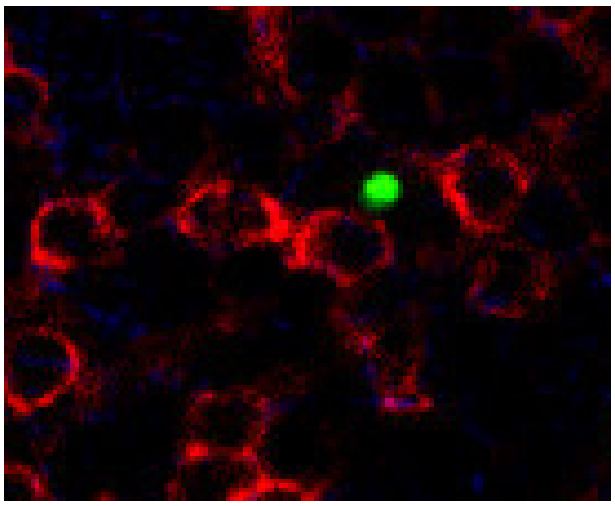

Fig. 9: Immunohistochemistry of spleen sections. The picture shows a green fluorescent particle in contact with a $CD4^+$ [red] T cell. 5×10^5 CFSE-labeled antigen-loaded BMDC were injected i.v. into the tail vein of a BALB/c mouse. 3 hours later the spleen was harvested after mouse-perfusion and cryo-sections of the spleens were prepared. The picture is representative for three independent experiments.

At different time points, mice were PFA-perfused and cryo-section of spleens were prepared to localize these CFSE-labelled BMDC in the spleen. Surprisingly, already three hours post administration the BMDC did not appear as intact cells, but small CFSE-labelled particles were detected in the spleen, unlike our expectations. This unexpected finding gave rise to the question whether protective T cell immunity is initiated before fragmentation of antigen-loaded BMDC or whether fragments of antigen-loaded DC on

their own, posses the ability to serve as potential adjuvant for sufficient antigen-presentation. 20 – 25% of all CFSE-labelled particles were taken up by cells which directly interact with $CD4^+$ T cells, but only 3.5 – 4.5% interact with $CD45R^+$ B cells. This correlates with the fact that for efficient vaccination against *Leishmania* parasites and the efficient destruction of replication sites a cell-based immune response is necessary.

4.3. Minimal requirements for BMDC-based vaccination

4.3.1. Viability and integrity of dendritic cells as a cell-based vaccine is not an prerequisite to mediate immunity

It was believed that DC-based vaccination is dependent on active and direct antigen-specific interaction between the vaccinating DC and T cells in secondary lymphoid organs (61, 197). The results of 4.2 together with preliminary tests using PFA-fixed or UV-irradiated antigen-loaded CpG-activated BMDC showed that viability of BMDC is not required for the efficiency of cell-based vaccination strategies (181). To test whether the initiation of a protective T cell immunity *in vivo* depends on intact cell membranes of the vaccine carrier, we disrupted antigen-loaded CpG-activated BMDC prior to administration to BALB/c mice. These BMDC were either disrupted via rapid freezing and thawing or via sonication. This leads to fragments of BMDC ranging from the size of large cell ghosts to small fragments which are hardly visible with conventional light microscopy.

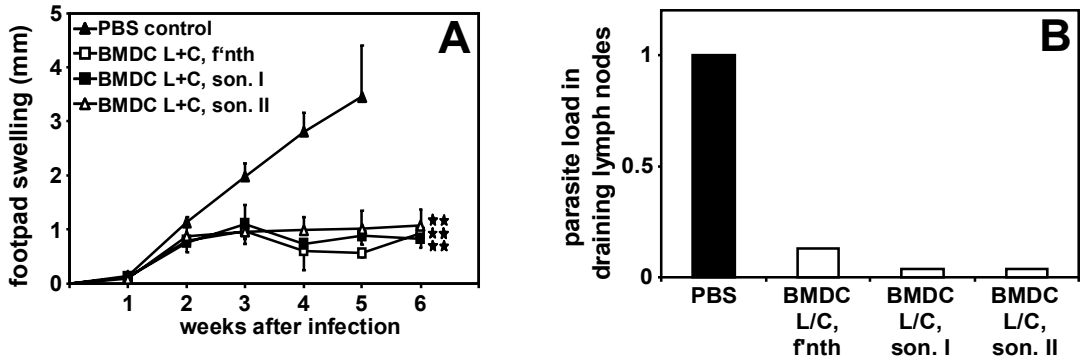

Fig. 10: Monitoring of disease parameters after vaccination and subsequent infection with *L. major*. **A**: Development of footpad swelling after infection with *L. major* into the right footpad of mice which have been vaccinated as indicated. ANOVA p value = 0.0002; Dunnett's post test: **, $p \leq 0.01$. **B**: Determination of parasite load in pooled draining lymph nodes of the respective vaccination groups six [five for PBS] weeks post infection. BMDC L+C: *Leishmania* lysate-loaded CpG-activated BMDC; f'nth: freeze and thaw; son. I: one cycle of sonication; son. II: two cycles of sonication. The data shown are representative for three independent experiments with at least 4 mice per group.

BMDC-vaccinated mice were protected against a subsequent infection with *L. major* promastigotes in terms of reduced footpad swelling compared to the PBS treated mice. In addition, a limiting dilution assay of draining lymph nodes revealed reduced numbers of *Leishmania*-infected cells. This demonstrates that effective vaccination against experimental leishmaniasis is independent of the size of disrupted BMDC.

4.3.2. Pre-activation of BMDC prior to vaccination is not necesarry

The results of 4.2 and 4.3.1 suggest a possible indirect mechanism of T cell-activation by vaccination with antigen-loaded CpG-activated DC. I.e., antigen-loaded CpG-activated DC do not interact directly with T cells but might serve as antigen-carrier with immunogenic activity. Nonetheless, host DC might be the major player in this setting. But, presentation of co-stimulatory molecules by the immunogenic cell or compound, like

expression of e.g. CD80 and CD86, prior to disruption of transferred DC might play a minor role for proper activation of host DC. To test whether pre-activation of BMDC is still necessary in this setting of BMDC fragments-based vaccination mice were vaccinated with fragments either derived from antigen-loaded BMDC, prepared in the presence or absence of CpG or PBS as control. The results clearly demonstrate that activation of antigen-loaded BMDC prior to cell-disruption is not a prerequisite for vaccine efficiency [Fig. 11]. Mice vaccinated with sonicated antigen-loaded BMDC performed at least equal to mice which were vaccinated with pre-activated antigen-loaded BMDC in terms of footpad swelling and parasite load in the draining lymph nodes seven weeks post infection with *L. major*.

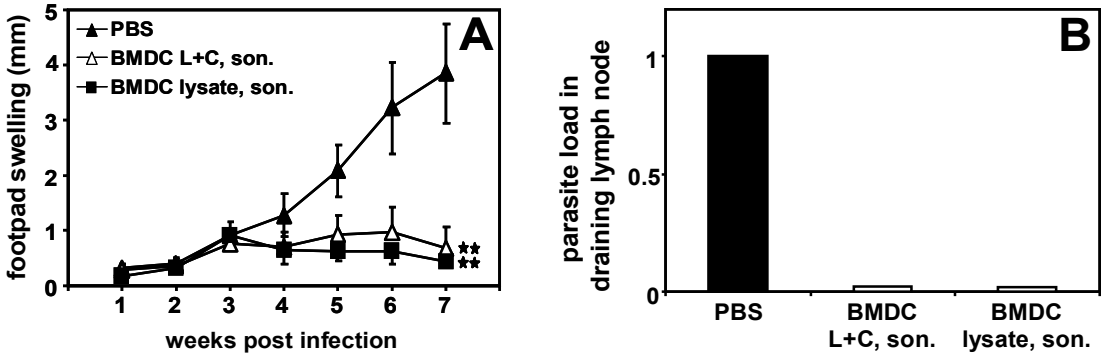

Fig. 11: Monitoring of disease parameters after vaccination and subsequent infection with *L. major*. **A**: Development of footpad swelling after infection with *L. major* into the right footpad of mice which have been vaccinated as indicated. ANOVA p value < 0.0001; Dunnett's post test: **, $p \leq 0.01$. **B**: Determination of parasite load in pooled draining lymph nodes of the respective vaccination groups seven weeks post infection. BMDC L+C, son.: sonicated *Leishmania* lysate-loaded CpG-activated BMDC; BMDC lysate, son.: sonicated *Leishmania* lysate-loaded BMDC. The data shown are representative for three independent experiments with at least 4 mice per group.

4.3.3. Disrupted antigen-loaded pDC can serve as a vaccine carrier

Obviously, we were interested in assessing a common ability of disrupted antigen-loaded BMDC and pDC, as disrupted antigen-loaded BMDC showed good efficiency in mediating protective immunity,. To investigate DC-based vaccination strategies further, it was necessary to discover if vaccination with disrupted cells is unique for antigen-loaded BMDC. Therefore, mice were vaccinated either with sonicated antigen-loaded pDC and pure pDC or PBS as controls. In comparison to the results shown in 4.3.2 sonicated antigen-loaded pDC show the same ability of providing immunity in the same way as sonicated antigen-loaded BMDC.

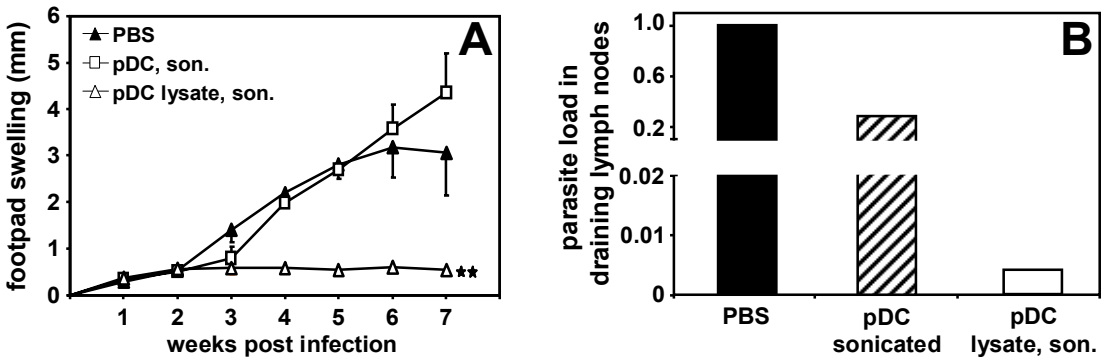

Fig. 12: Monitoring of disease parameters after vaccination and subsequent infection with *L. major*. **A**: Development of footpad swelling after infection with *L. major* into the right footpad of mice which have been vaccinated as indicated. ANOVA p value = 0.0002; Dunnett's post test: **, $p \leq 0.01$. **B**: Determination of parasite load in pooled draining lymph nodes of the respective vaccination groups seven weeks post infection. pDC son.: sonicated pDC; pDC lysate, son.: sonicated *Leishmania* lysate-loaded pDC. The data shown are representative for two independent experiments with at least 4 mice per group.

A tendency of progressive footpad swelling of mice upon immunization with naïve pDC in comparison to PBS-treated mice was observed in these and other experiments. This

might be associated with a misled immune response as an reaction to a stimulus without foreign peptides. In contrast, the decrease of footpad swelling in the PBS control group during the last week of experiment seems to be related to dissemination of parasites as these mice appeared to be sick and less vital as the other mice. This is backed up by the very low number of infected cells in draining lymph nodes of clinically protected mice. According to Fig. 7a similar cytokine pattern was observed after re-stimulation of splenocytes and lymph node cells seven weeks post infection [Fig. 13].

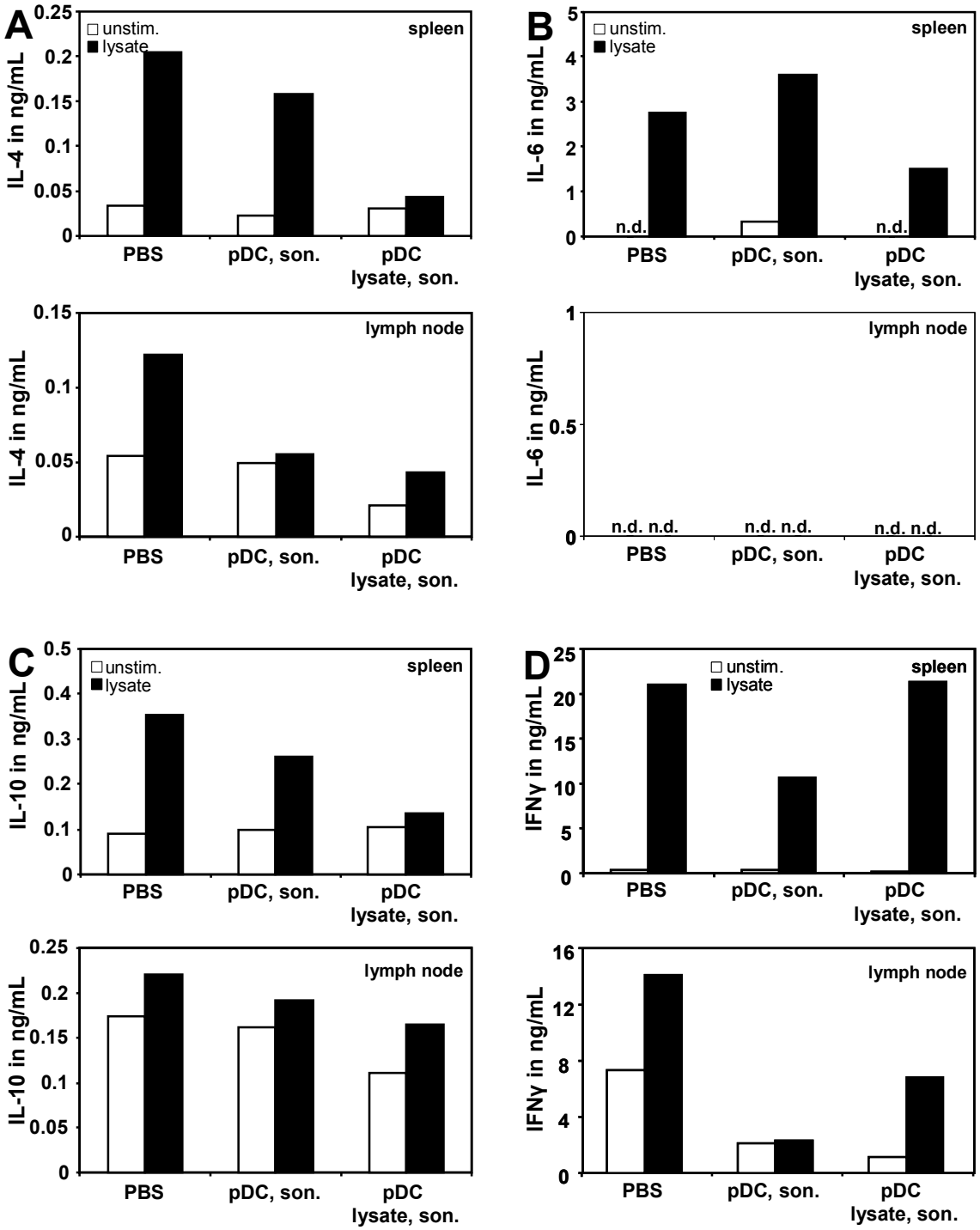

Fig. 13: Cytokine profiles of spleen and LN cells after challenge infection with *L. major*. Seven weeks post infection splenocytes and dLN cells of vaccinated mice were re-stimulated with *Leishmania* lysate. Supernatants were collected and cytokine levels of IL-4 [**A**], IL-6 [**B**], IL-10 [**C**] and IFNγ [**D**] were determined via ELISA. unstim.: without stimulation, open bars; lysate: re-stimulation with *Leishmania* lysate, black bars. pDC son.: sonicated pDC; pDC lysate, son.: sonicated *Leishmania* lysate-loaded pDC. The data are representative of two independent experiments.

Again, ratios of IFNγ vs. IL-10 were calculated to emphasise the difference between protected and non-protected vaccination groups.

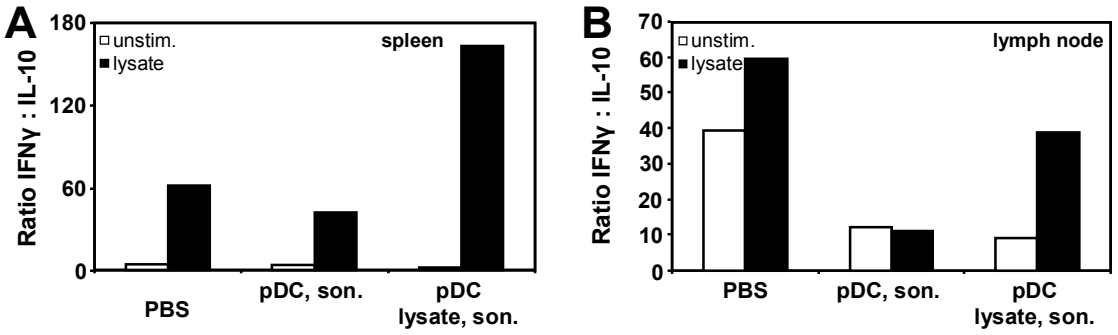

Fig. 14: Ratio of IFNγ to IL-10 in the supernatants of spleen and LN cells 6 wk after footpad infection with *L. major*. Ratios of IFNγ to IL-10 in the supernatants were calculated from the cytokine levels detected by ELISA for spleens [**A**] and lymph nodes [**B**]. Where no cytokine was detected, the value of the detection threshold of the ELISA was used for calculating the ratio. pDC son.: sonicated pDC; pDC lysate, son.: sonicated *Leishmania* lysate-loaded pDC. The data are representative of two independent experiments.

4.3.4. The protective effect of disrupted cell-based vaccination is independent of the type of APC and the H-2 haplotype

It became clear that neither cell viability or cell integrity [4.3.1] nor types of DC [4.3.3] were important for the protective effect of DC-based vaccination against *L.major* In consequence, the question arose which mechanisms cause the success of disrupted DC-based vaccination. To answer this question, we examined the necessity of appropriate antigen presentation via syngenic MHC molecules to provide protection in BALB/c mice. Accordingly, solutions of sonicated antigen-loaded cells derived from *in vitro* grown bone marrow-derived BALB/c MΦ and a BALB/c derived MΦ cell line [J-774A.1] were tested in vaccination experiments. MΦ do have the ability of antigen uptake, processing and

presentation but lack some DC-specific features. Additionally, sonicated antigen-loaded C57BL/6 BMDC [H-2k^b] were tested in this experiment as they exhibit a different MHC haplotype in comparison to BALB/c mice [H-2k^d].

The vaccination experiment revealed that antigen-loaded CpG-activated MΦ and C57BL/6 BMDC have the ability to provide protective immunity against *L. major* infections in BALB/c mice with regard to controled footpad swelling and reduced parasite load in the draining lymph nodes [Fig. 15]. Therefore, neither a DC-specific ability nor antigen-presentation via MHC class molecules by the donor DC are necessary for the induction of protective immunity against a subsequent infection with *Leishmania major* in the recipient mice.

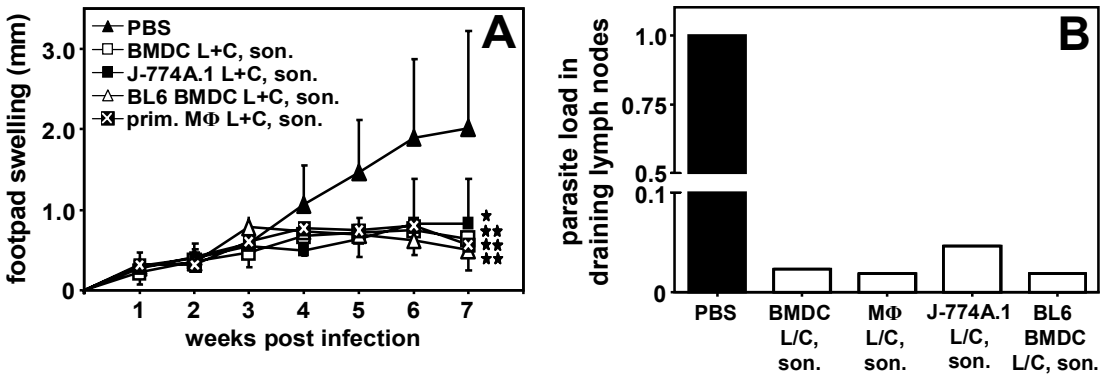

Fig. 15: Monitoring of disease parameters after vaccination and subsequent infection with *L. major*. **A**: Development of footpad swelling after infection with *L. major* into the right footpad of mice which have been vaccinated as indicated. ANOVA p value = 0.0063; Dunnett's post test: *, $p \leq 0.05$; **, $p \leq 0.01$. **B**: Determination of parasite load in pooled draining lymph nodes of the respective vaccination groups seven weeks post infection. BMDC L+C, son.: sonicated *Leishmania* lysate-loaded CpG-activated BMDC; BL6 BMDC: BMDC derived from C57BL6 mice; prim MΦ: BALB/c bone marrow-derived MΦ. The data shown are from a single experiment with five mice per vaccination group.

The elevated footpad swelling of the MΦ cell line-vaccine together with a tendency to a higher level of parasitized cells in draining lymph nodes might be due to normal variation during experiments. Beyond this, all mice which received disrupted antigen-loaded cells performed almost equally in terms of footpad swelling and parasite load in draining lymph nodes.

Looking at cytokines released by splenocytes or cells of lymph nodes, it becomes clear that after six weeks post infection LN are of more importance than the spleen. Only in lymph nodes IFNγ was expressed to a higher extend by cells of protected mice than by cells of PBS control-treated mice**.** Again, looking for the IFNγ : IL-10 ratio the difference between spleen and LN becomes apparent. Further more, no difference was seen between the cytokine patttern of BALB/c or BL6 DC.

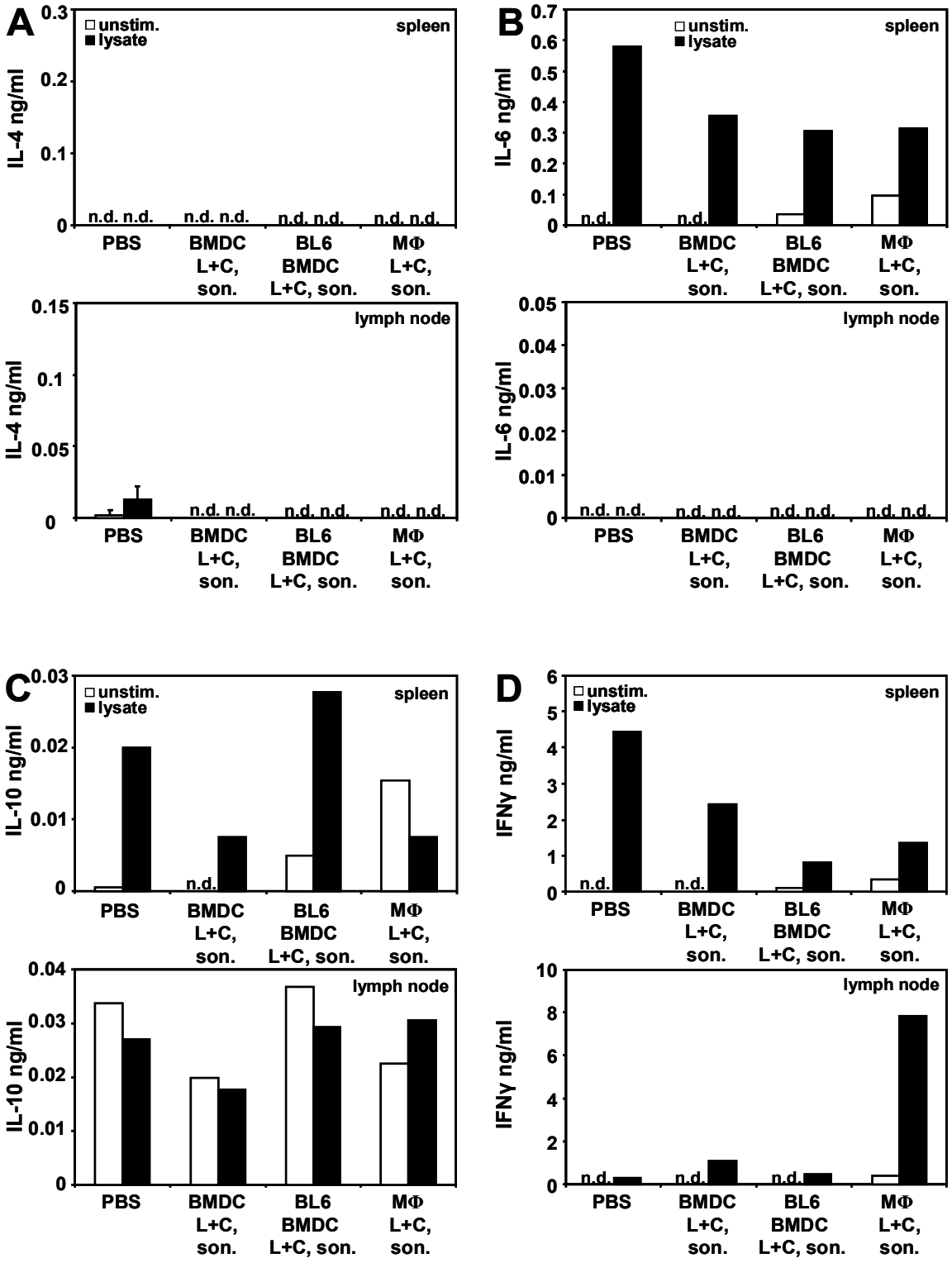

Fig. 16: Cytokine profiles of spleen and LN cells after challenge infection with *L. major*. Seven weeks post infection splenocytes and dLN cells of vaccinated mice were re-stimulated with *Leishmania* lysate. Supernatants were collected and cytokine levels of IL-4 [**A**], IL-6 [**B**], IL-10 [**C**] and IFNγ [**D**] were determined via ELISA. unstim.: without stimulation, open bars; lysate: re-stimulation with *Leishmania* lysate, black bars; n.d.: not detected. The data shown are from a single experiment with five mice per vaccination group.

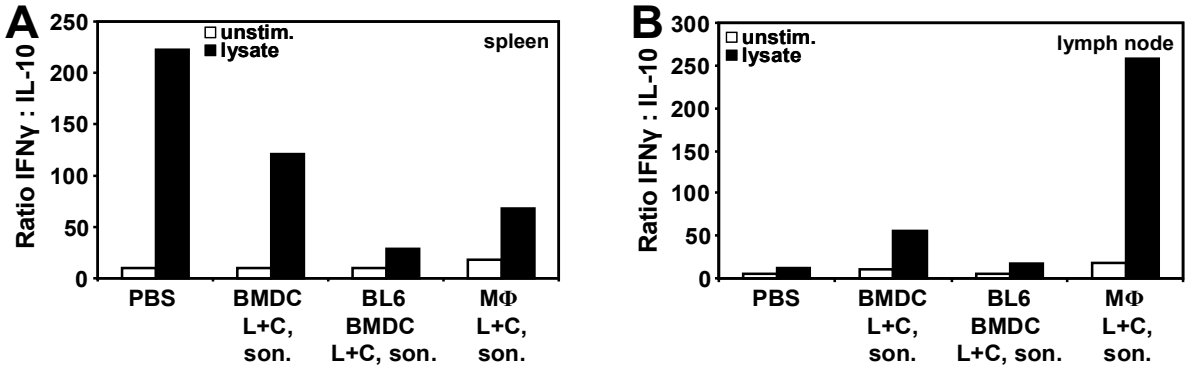

Fig. 17: Ratio of IFNγ to IL-10 in the supernatants of spleen and LN cells 7 wk after footpad infection with *L. major*. Ratios of IFNγ to IL-10 in the supernatants were calculated from the cytokine levels detected by ELISA for spleens [**A**] and lymph nodes [**B**]. Where no cytokine was detected, the value of the detection threshold of the ELISA was used for calculating the ratio.

4.4. *In vitro* studies of the stimulatory effect of DC fragments on murine splenocytes

4.4.1. Fragments of antigen-loaded DC induce antigen-specific T cell proliferation

While it has been described that viable antigen-loaded DC trigger the activation, maturation and proliferation of naïve and primed T cells, the effect of disrupted antigen-loaded DC used as vaccine against *Leishmania* parasites on T cells was not known so far. The above shown results demonstrate the terminal outcome of disrupted cell-based vaccination against *L. major* infections. Reduced footpad swelling and reduced parasite load in draining lymph nodes has been demonstrated several times. But, early events during activation of protection-transferring immune responses have not been elucidated so far. Consequently, we started to investigate the capacity of sonicated antigen-loaded CpG-activated BMDC to activate T cells in *in vitro* co-cultures of disrupted DC together with

the respective cell populations. However, neither co-cultures of $CD4^+$ T cells nor co-cultures of $CD8^+$ T cells together with fragments of antigen-loaded BMDC showed any T cell proliferation in comparison to controls [data not shown], even if the T cells were isolated from primed mice. Additionally, co-cultures of splenic DC from naïve mice together with T cells and the respective solutions of sonicated antigen-loaded BMDC were ineffective in inducing T cell proliferation [data not shown]. Effective $CD4^+$ and $CD8^+$ T cell proliferation only took part in co-cultures of complete splenocytes of primed mice together with splenic DC of naïve mice which were stimulated with fragments derived from antigen-loaded CpG-activated BMDC or viable antigen-loaded BMDC as control [Fig. 18].

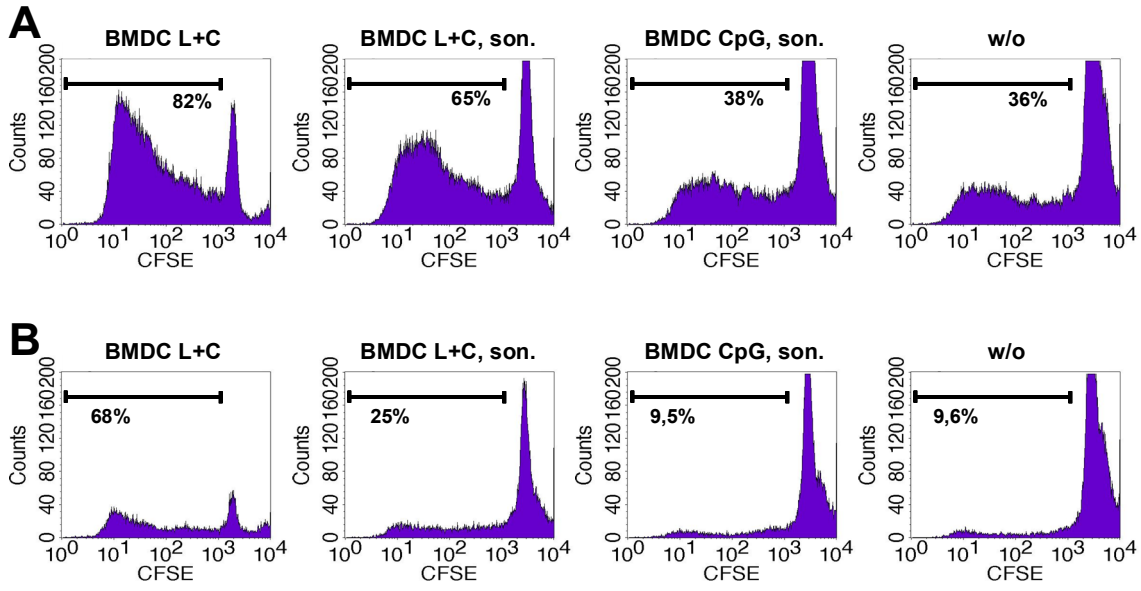

Fig. 18: CFSE-proliferation assay of splenocytes stimulated with antigen-loaded BMDC. Antigen-specific T cell proliferation upon stimulation with disrupted antigen-loaded BMDC. Therefore splenocytes of primed mice, splenic DC of naïve mice and BMDC, treated as indicated, were CFSE-stained and cultured together for five days. After staining for CD4 [**A**] or CD8 [**B**] using PE-labelled antibodies CFSE-dilution was determined via FACS analysis. The data are representative of three independent experiments. Cells were prepared in each case from two identically pre-treated mice. The data shown are representative of three independent experiments

Despite the antigen-unspecific T cell proliferation observed in co-cultures where no *Leishmania* antigen has been introduced [right columns, Fig. 18], marked antigen-specific T cell proliferation could be observed for splenocyte co-cultures which were stimulated with either viable or disrupted antigen-loaded CpG-activated BMDC. Both populations of T cells, i.e. $CD4^+$ and $CD8^+$ T cells show high proliferation rates on day 5. This clearly demonstrates that signal 1 and signal 2 which are needed for sufficient T cell activation and proliferation are provided in this setting of lymphocyte co-cultures.

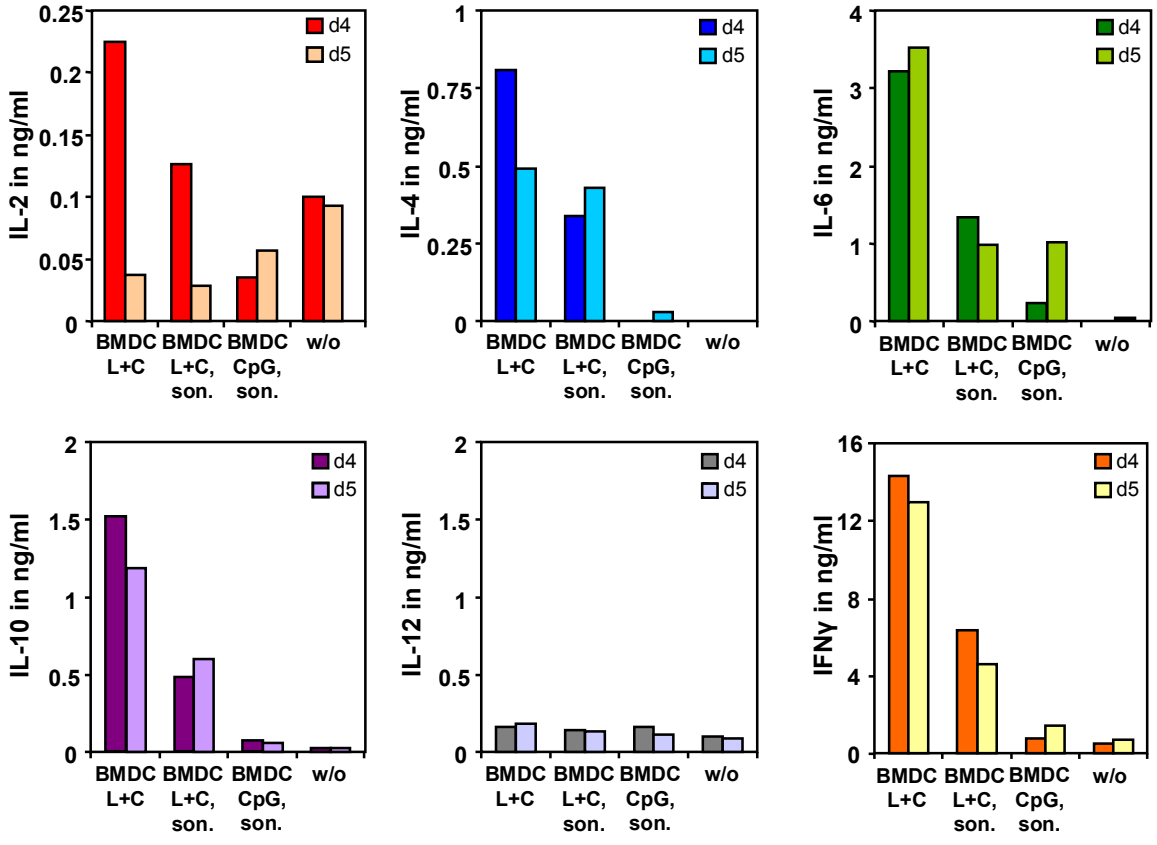

Fig. 19: Cytokine release of splenocytes during antigen-specific T cell proliferation. According to Fig. 18, splenocytes and BMDC were CFSE-labelled and co-cultured. After four and five days supernatants were taken for subsequent Cytokine analysis via ELISA. Data are representative for three independent experiments. Cells were prepared in each case from two identically pre-treated mice.

Although, the specific clearance of pathogens depends on both, the activation of T cells via signal one and two and directing T helper cell fate which are necessary during initial and continuous T helper cell priming. Therefore, the release of cytokines which are related to a Th_1-related or Th_2-related immune response was monitored. Accordingly, we determined the amount of IL-2, IL-4, IL-6, IL-10, IL-12p70 and IFNγ for 5 days, beginning at day 1 after co-incubation [data shown for days 4 and 5]. In Fig. 19 it is shown that a high amount of IFNγ is produced upon stimulation with antigen-loaded BMDC - either viable or disrupted - but not by splenocytes which were stimulated in the absence of *Leishmania* antigen. IL-2 was produced concomitantly or rather initially to the T cell proliferation on days 1 to 3. Because of that reason, already decreasing levels of IL-2 have been detectable on day 4 and 5. Antigen-specific production of IL-4 and IL-10 which act as counterparts to the Th1-related cytokines was also observed. At last, IL-6, a marker for inflammatory immune responses was also up-regulated upon stimulation with BMDC. A control sample containing splenocytes of primed mice and fragments of antigen-loaded CpG-activated BMDC but no splenic DC of naïve mice did not show antigen-specific proliferation but the same amount of IL-4 compared to the equivalent sample with naïve splenic DC. These results showed that IL-4 production in this setting might be rather a side effect of splenocyte stimulation than a specific signal 3 to T cells.

4.4.2. Fragments of antigen-loaded BMDC induce Th1-biased cytokine production

The importance of a cytokine shift from a Th_2-biased towards a Th_1-biased immune response in non-self-healing leishmaniasis is well known (66, 125). Additionally, it is known that BALB/c mice tend to promote a Th_2-related immune response that can be

reverted via DC-based vaccination towards a self-healing state of immunity. For the use of disrupted BMDC so far it was not known in which way the type of immune response is modulated. As the spleen is mainly targeted via the i.v. injection route we started extensive *in vitro* studies as a first step to determine the impact of fragments derived from antigen-loaded BMDC on the cytokine release of splenocytes of naïve mice. For that reason we stimulated splenocytes of naïve mice either with PBS, LPS or CpG as controls or with disrupted antigen-loaded BMDC to simulate the act of vaccination.

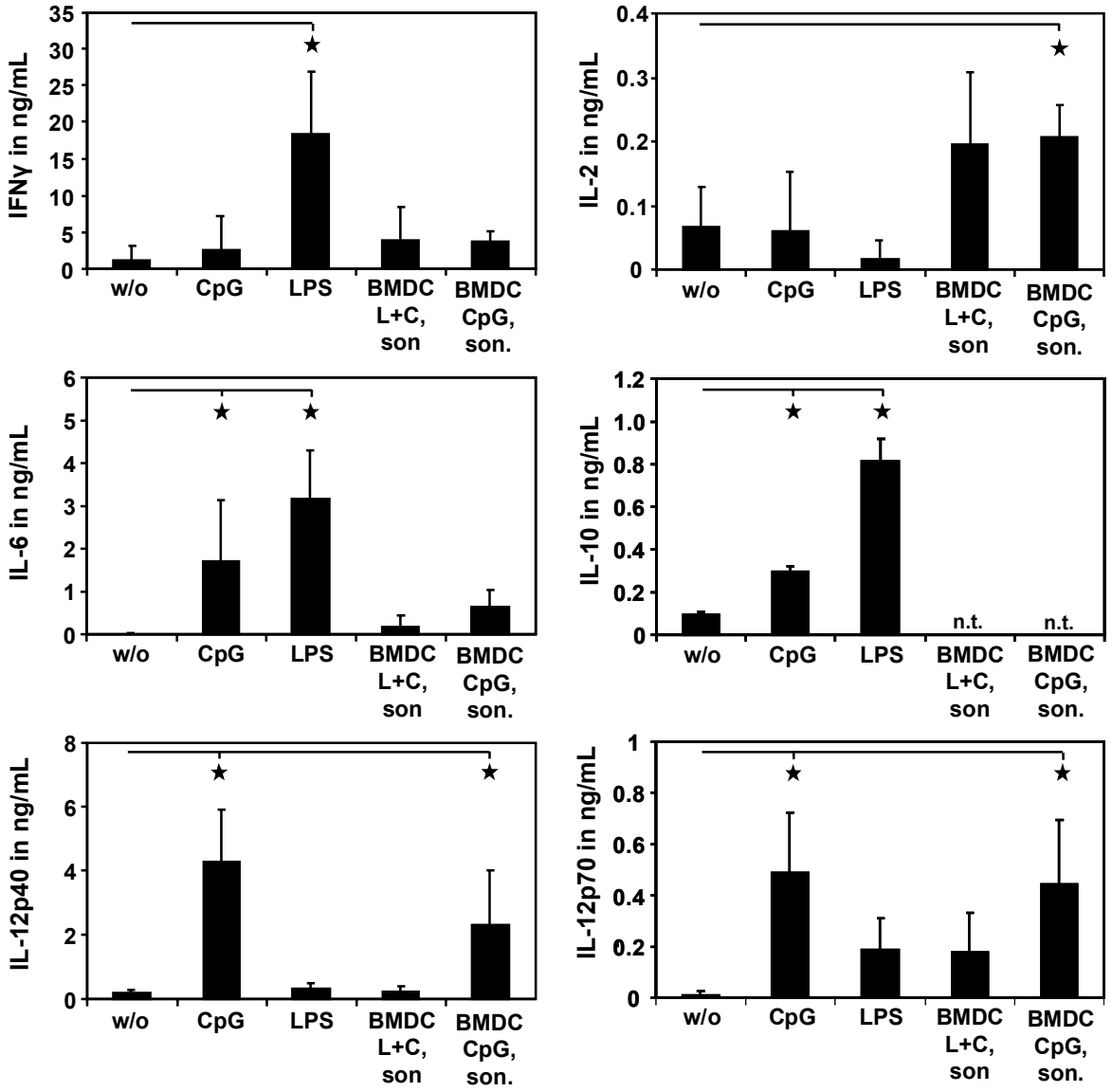

Fig. 20: Cytokine production of naïve splenocytes after stimulation with disrupted BMDC. Single-cell suspensions of spleens were prepared from naïve BALB/c mice. Cells were cultured for 48 hours with the respective stimuli. Afterwards supernatants were collected and cytokine levels were determined via ELISA. ANOVA *p* values: $p < 0.0001$ for IFNγ; $p = 0.0088$ for IL-2; $p < 0.0001$ for IL-6; $p < 0.0001$ for IL-12p40; $p < 0.0001$ for IL-12p70. Dunnett's post test: *, $p < 0.05$. Data represent the mean ± SD of at least four independent experiments. BMDC L+C, son.: sonicated *Leishmania* lysate-loaded CpG-activated BMDC; n.t.: not tested.

The results in Fig. 20 show enhanced production of IL-2 and IL-12p40/p70 upon stimulation of naïve splenocytes with disrupted BMDC. IL-4 was never detected in the

samples with disrupted BMDC [data not shown]. Therefore a Th1-favoring and T cell proliferation-initialising cytokine milieu was found in these culture samples.

Many *in vitro* assays showed that *Leishmania* lysate has some suppressive impact on the cytokine production of lymphocytes [data not shown]. Hence, slight differences in cytokine production between naïve splenocytes upon stimulation with either disrupted antigen-loaded BMDC or disrupted BMDC occur randomly. This effect is not a *Leishmania* antigen-specific feature of disrupted cells but rather the suppressive effect of *Leishmania* antigen lysate itself. This is supported by the *in vivo* data and the results of *in vitro* proliferation assay. These results demonstrated that disrupted antigen-loaded BMDC serve as a sufficient stimulus for protective immune responses as well as a source for antigen-specific T cell proliferation.

4.4.3. Vaccination with disrupted antigen-loaded BMDC alters the cytokine response of splenocytes upon stimulation with *Leishmania* parasites

The next step in our conventional animal experiments is the infection of the mice with 2×10^5 *Leishmania* parasites into the right footpad one week post vaccination. Beginning from this point of time or shortly after, the immune response of the murine host should be transformed from a IL-4 and IL-10-driven Th_2- response to an IFNγ and IL-12p70-driven Th_1- response. To determine their cytokine response, splenocytes of mice vaccinated with the protective vaccine, i.e. sonicated antigen-loaded CpG-activated BMDC, were isolated one week post vaccination and stimulated with either PBS, CpG or LPS as controls or *Leishmania* either viable [*L. major*] or dead [Lmag].

As expected, splenocytes of BALB/c mice expressed dramatically elevated amounts of IFNγ one week after vaccination [first pair of bars, Fig. 21, IFNγ] compared to naïve splenocytes. But, after stimulation of these differently pre-treated splenocytes with lysed or viable *Leishmania* parasites enhanced IFNγ production has been observed [third and fourth pair of bars, Fig. 21, IFNγ]. This shows that these splenocytes are already pre-stimulated towards a Th_1-related phenotype by the vaccination with disrupted antigen-loaded splenocytes.

In addition, due to the enhanced production of IL-2 by vaccinated splenocytes and the evidence for antigen-specific T cell proliferation shown in Fig. 18, we assumed a Th_1-related T cell proliferation initiated by the vaccination with disrupted antigen-loaded BMDC. In addition, this effect is dependent on the antigen-specific stimulus of disrupted BMDC used for vaccination as naïve and pre-stimulated splenocytes do not respond on CpG or LPS with expression of IL-2. Nevertheless, elevated amounts of IL-6 and IL-10 were observed.

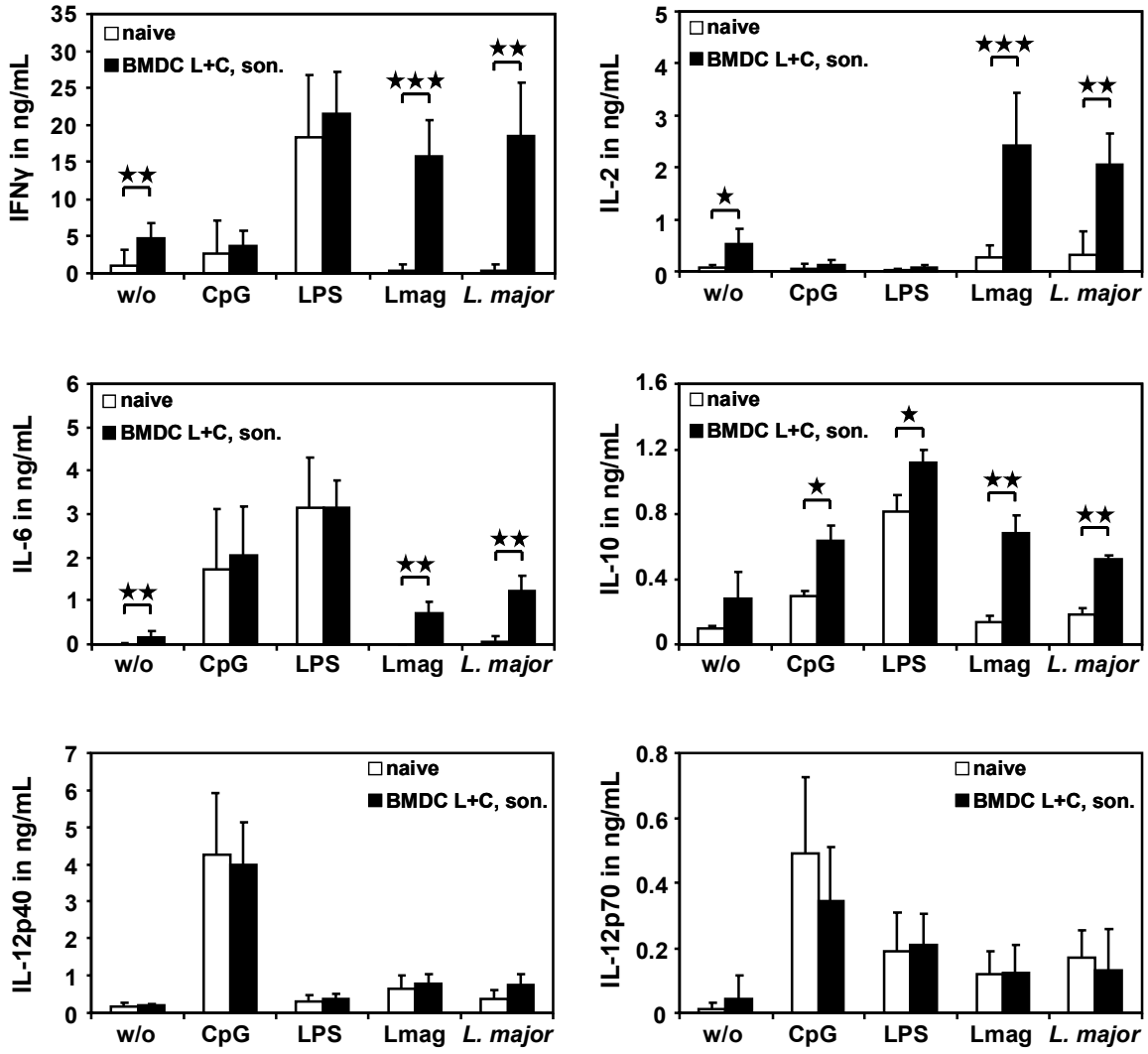

Fig. 21: Cytokine production of naïve and vaccinated splenocytes after stimulation with *Leishmania* parasites. Single-cell suspensions of spleens were prepared from naïve BALB/c mice or mice which were vaccinated with sonicated *Leishmania* lysate-loaded CpG-activated BMDC. Cells were cultured and stimulated as indicated for 48 hours. Afterwards supernatants were collected and cytokine levels were determined via ELISA. T test p values: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Data represent the mean ± SD of at least four independent experiments, except for IL-10. BMDC L+C, son.: sonicated *Leishmania* lysate-loaded CpG-activated BMDC; Lmag: *Leishmania* lysate; *L. major*: viable *Leishmania* promastigotes.

4.5. The protective principle of disrupted antigen-loaded BMDC is membrane associated

4.5.1. Preparations of membranous particles derived from antigen-loaded BMDC serve as a protective vaccine against infections with *L. major*

Solutions of disrupted antigen-loaded BMDC contain all molecules of viable antigen-loaded BMDC. But, the integrity of plasma membranes is disrupted and cytosolic molecules loose their compartmentalisation by the surrounding plasma membrane and therefore become solubilised in the respective medium, either PBS or R-10. But still these solutions can serve as protective vaccines in animal studies as shown above. While taking a closer look at the mechanism of this DC-fragment-based vaccination strategy the question arose whether the immunity mediating effect of these solutions is associated with any distinct component of the disrupted cells. In consequence, we started to separate the aqueous phase containing all molecules that were solubilised during disruption and homogenisation from membranous particles via ultracentrifugation. After a washing step the resulting fractions, the membranous particles-containing pellet and the soluble molecules-containing aqueous phase, were resuspended used as vaccines in an animal experiment. Vaccination of mice with the membrane fraction of antigen-loaded BMDC mediated immunity against subsequent infections with *L. major* promastigotes in each individual mouse.

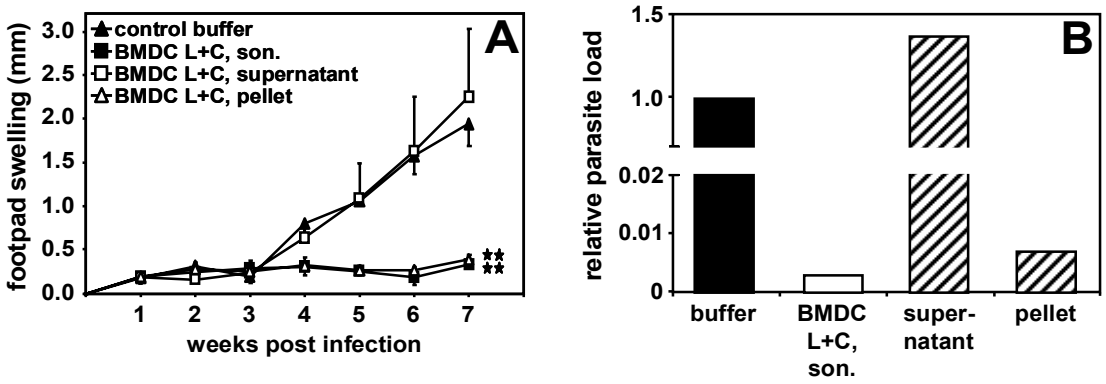

Fig. 22: Monitoring of disease parameters after vaccination and subsequent infection with *L. major*. **A**: Development of footpad swelling after infection with *L. major* into the right footpad of mice which have been vaccinated as indicated. ANOVA p value < 0.0001; Dunnett's post test: **, $p \leq 0.01$. **B**: Determination of relative parasite load of the respective vaccination groups seven weeks post infection via measuring the luminescence emitted by luciferase expressing *Leishmania*. BMDC L+C, son.: sonicated *Leishmania* lysate-loaded CpG-activated BMDC; supernatant or pellet respectively: supernatant or pellet after ultra-centrifugal processing of disrupted *Leishmania* lysate-loaded CpG-activated BMDC. The data shown are from a single experiment with five mice per vaccination group.

Additionally, Fig. 22 clearly demonstrates that the membrane fraction of antigen-loaded BMDC shows the same ability for providing protection against *L .major* as complete solutions of disrupted antigen-loaded BMDC, whereas the cytoplasmic fraction fails to confer protective immunity to these mice.

In contrast to animal experiments described above, luciferase-expressing *L. major* promastigotes were used [*luc2-L.major*] in this set up. After administration of the respective substrate into mice the luminescence of viable parasites was detected with a CCD camera. Angela Bruder clearly demonstrated that parasite load in infected footpads and in draining lymph node show a good correlation with detected luminescence from infected footpads [A. Bruder, MD thesis, in preparation]. In consequence, comparison between relative light units emitted from infected footpads of protected vs. non-protected mice is expressed again as relative parasite load and therefore we were able to detect a

reduction of overall parasite load. Expression of cytokines by splenocytes or LN cells does not give a significant pattern that is assignable to protected or non-protected mice, except for IL-12 that is expressed in higher amounts by splenocytes and LN cells of protected mice [see Fig. 23]. But, there is a tendency of LN cells of non-protected mice to express higher ratios of IFNγ : IL-10 without stimulation than LN cells of protected mice. In contrast, after stimulation LN cells of protected mice express higher ratios of IFNγ : IL-10 than non-protected ones [see Fig. 24].

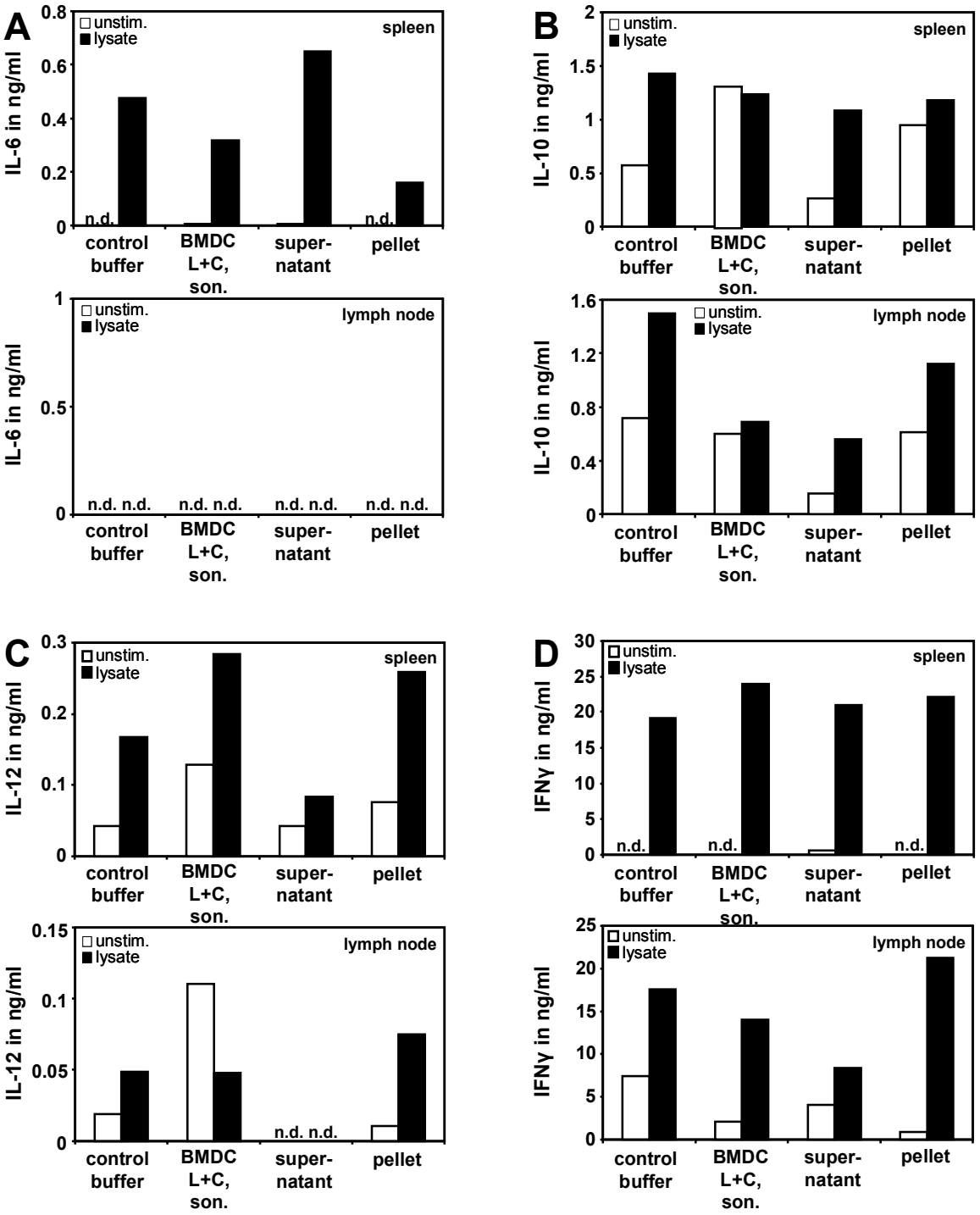

Fig. 23: Cytokine profiles of spleen and LN cells after challenge infection with *L. major*. Seven weeks post infection splenocytes and dLN cells of vaccinated mice were re-stimulated with *Leishmania* lysate. Supernatants were collected and cytokine levels of IL-4 [**A**], IL-6 [**B**], IL-10 [**C**] and IFNγ [**D**] were determined via ELISA. unstim.: without stimulation, open bars; lysate: re-stimulation with *Leishmania* lysate, black bars; BMDC L+C, son.: sonicated *Leishmania* lysate-loaded CpG-activated BMDC; supernatant or pellet respectively: supernatant or pellet after ultra-centrifugal processing of disrupted *Leishmania* lysate-loaded CpG-activated BMDC; n.d.: not detected. The data shown are from a single experiment with five mice per vaccination group.

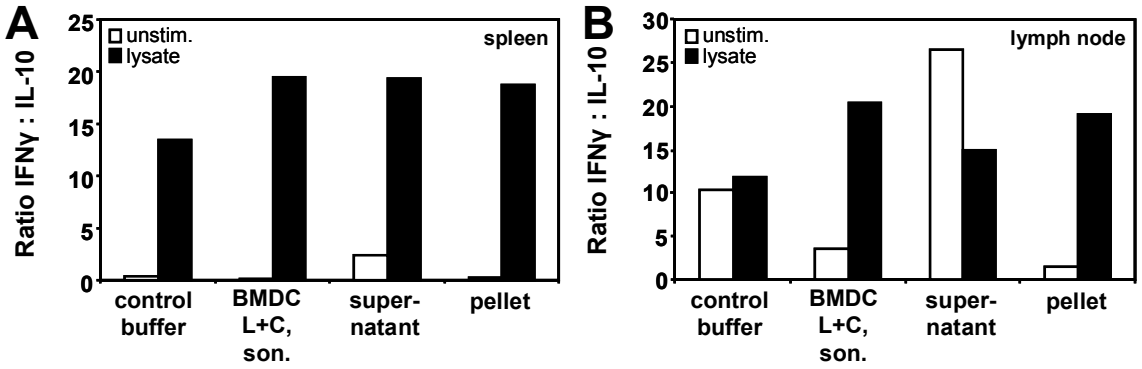

Fig. 24: Ratio of IFNγ to IL-10 in the supernatants of spleen and LN cells 7 wk after footpad infection with *L. major*. Ratios of IFNγ to IL-10 in the supernatants were calculated from the cytokine levels detected by ELISA for spleens [**A**] and lymph nodes [**B**]. Where no cytokine was detected, the value of the detection threshold of the ELISA was used for calculating the ratio.

4.5.2. *In vitro* analysis of cell fractions derived from antigen-loaded BMDC

Fractions of antigen-loaded BMDC were tested *in vitro* to compare the immunogenic capacity of soluble and membrane fractions of antigen-loaded BMDC. Therefore, an analogous setting to the culture and the stimulation of splenocytes, as described above, was used. First, splenocytes of naïve mice were taken and stimulated with the respective cell suspensions or with PBS, CpG or LPS as controls.

Regarding IFNγ, IL-2 and IL-6 the purified cytosolic and the purified membranous fraction showed almost equal immunostimulatory capacity like disrupted antigen-loaded BMDC. Contrary to our expectations raised as a consequence of the successful membrane fraction vaccine (see Fig. 22) and by previous experiments (see Fig. 20), no IL-12p40 and no IL-12p70 was detectable. Regarding effective vaccination, we have been able to detect clear differences between mice vaccinated with the aqueous fraction or membrane fraction (see Fig. 22). Here, only mice vaccinated with the pellet fraction were protected against *L. major* infections. In contrast, Fig. 25 does not show any differences in cytokine production

of splenocytes upon stimulation with either one of the two fractions. Low amounts of IL-2 indicate a T cell proliferation-promoting milieu but again, there was no difference in IL-2 production after the respective stimulus either. Therefore, both fractions share the same adjuvant activity whereas the sufficient combination or the sufficient amount of antigen and adjuvant lies within the membranous fraction.

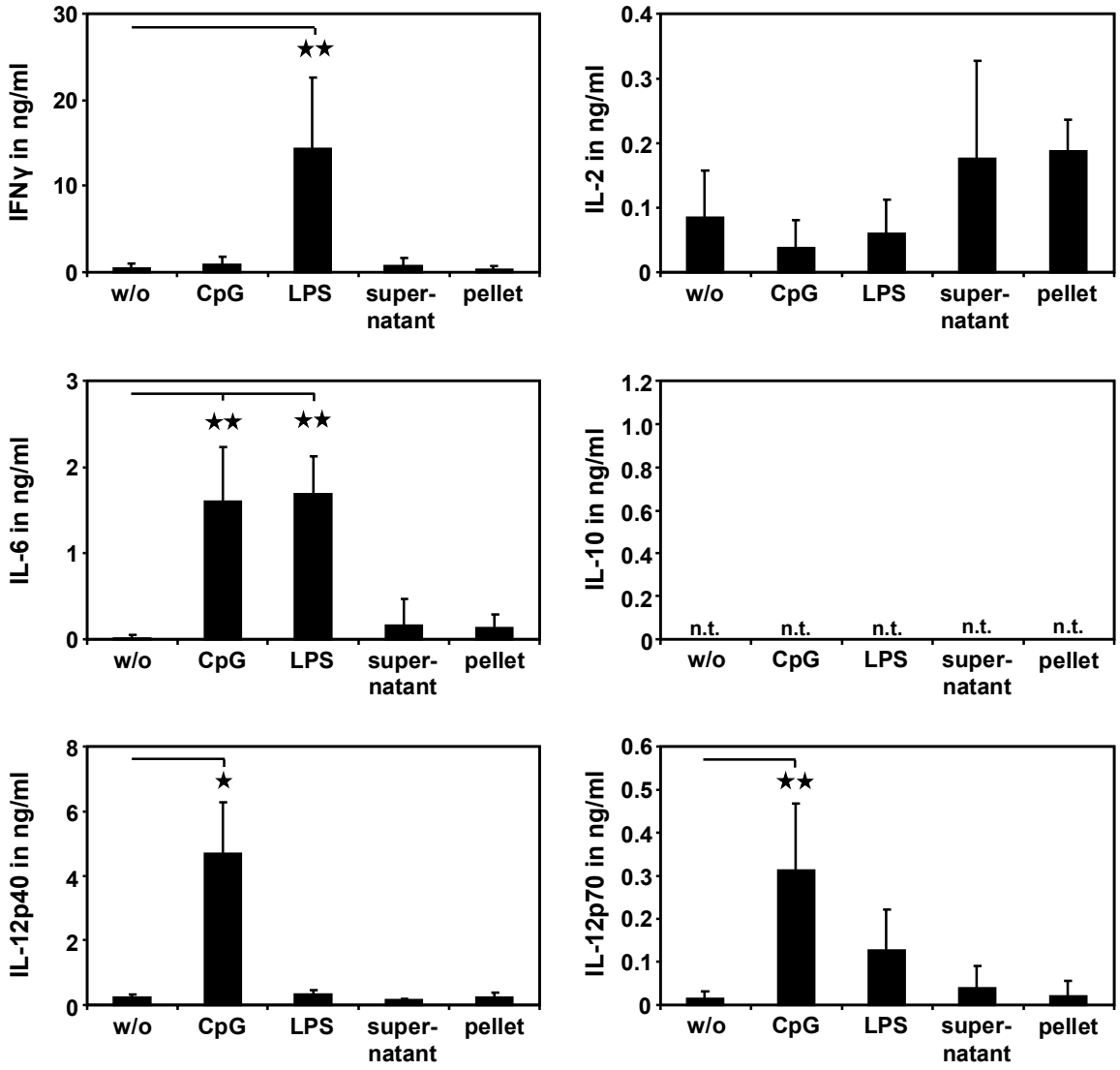

Fig. 25: Cytokine production of naïve splenocytes after stimulation with disrupted BMDC. Single-cell suspensions of spleens were prepared from naïve BALB/c mice. Cells were cultured for 48 hours with the respective stimuli. Afterwards supernatants were collected and cytokine levels were determined via ELISA. ANOVA p values: $p < 0.0001$ for IFNγ; $p = 0.3193$ for IL-2; $p = 0.0005$ for IL-6; $p < 0.0001$ for IL-12p40; $p < 0.0001$ for IL-12p70. Dunnett's post test: *, $p < 0.05$; **, $p < 0.01$. Data represent the means ± SD of four independent experiments. supernatant or pellet respectively: supernatant or pellet after ultra-centrifugal processing of disrupted *Leishmania* lysate-loaded CpG-activated BMDC; n.t.: not tested.

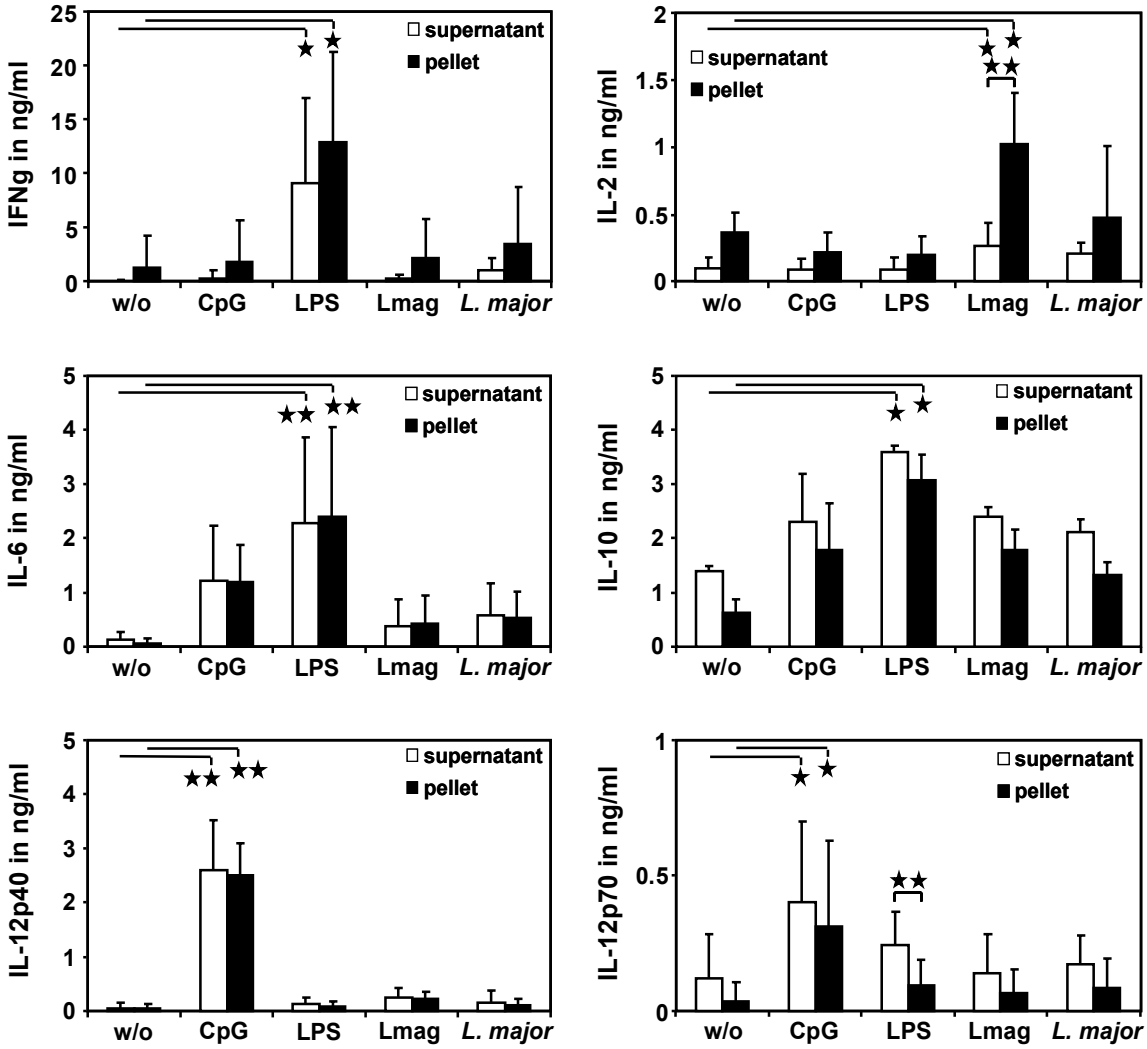

Fig. 26: Cytokine production of naïve and vaccinated splenocytes after stimulation with *Leishmania* parasites. Single-cell suspensions of spleens were prepared from mice which were vaccinated with the supernatant or the pellet-fraction of ultra-centrifugally processed *Leishmania* lysate-loaded CpG-activated BMDC. Cells were cultured and stimulated as indicated for 48 hours. Afterwards supernatants were collected and cytokine levels were determined via ELISA. *p* values: *, $p < 0.05$; **, $p < 0.01$. Data represent the mean ± SD of four independent experiments, except for IL-10 [two independent experiments]. Lmag: *Leishmania* lysate; *L. major*: viable *Leishmania* promastigotes.

Second, to investigate these observations further splenocytes of mice vaccinated either with the supernatant fraction or the pellet fraction were prepared and stimulated either with viable *L. major* promastigotes or *Leishmania* lysate. Again, controls were stimulated

with PBS, CpG and LPS. Unfortunately, no substantial differences in the cytokine milieu expressed by the differentially stimulated populations have been observed (Fig. 26), but splenocytes of mice vaccinated with the pellet fraction showed statistically significant differences in their level of IL-2 production. This indicates the capability to induce T cell proliferation upon infection with *L. major*.

4.5.3. Different immunostimulatory capability of purified fractions and complete fragments derived from antigen-loaded BMDC

For the demonstration of vaccination efficacy or for the discovery of cell interactions modulating the immune response and control of disease development, the vaccination strategy using viable antigen-loaded DC is a very useful tool. For a widespread usage of an anti-leishmanial vaccine, a cheap and simple vaccination method with no need of cooling would be favored. Therefore, an effective vaccine of low complexity, like stabilized protein together with an adjuvant has great advantages over autologous cell therapy. To transfer the knowledge from bench towards bedside, essential components of antigen-adjuvant combinations have to be studied. The knowledge of their mode of action will lead the way towards the development of the desired vaccine.

It was demonstrated that vaccination with *Leishmania* lysate alone did not induce protective immunity against *Leishmania* infections (61). This failure was due to inadequate immunostimulatory capacity of *Leishmania* lysate. In further experiments different cell types were used as shuttles for antigen. Using viable DC, disrupted DC and cellular fractions of these cells respectively, it was shown *in vivo* that elements of DC can serve as adjuvant for protective vaccination. In contrast, the model micro milieu did not

give identical images of what happens during vaccination or during pathogen encounter so far.

To determine the relative cytokine production of splenocytes demonstrating the mode of immune stimulation of different vaccine formulations, comparative studies of splenocyte samples treated with Lmag or viable *L. major* were performed. For visualization, all data were transformed to a relative amount of secreted cytokines in comparison to the levels expressed by splenocytes of naïve mice [Fig. 27]. Slight differences in cytokine release between splenocytes of mice which were treated either with the aqueous fraction or with the membranous fraction have been observed, whereas only for IL-2 this difference is statistically significant. Pellet-pretreated splenocytes have the same bias for the release of IL-2 and IFNγ like splenocytes pretreated with disrupted antigen-loaded DC. However, it is clearly demonstrated that disrupted cells and purified cell fractions do have different immunostimulatory capacities.

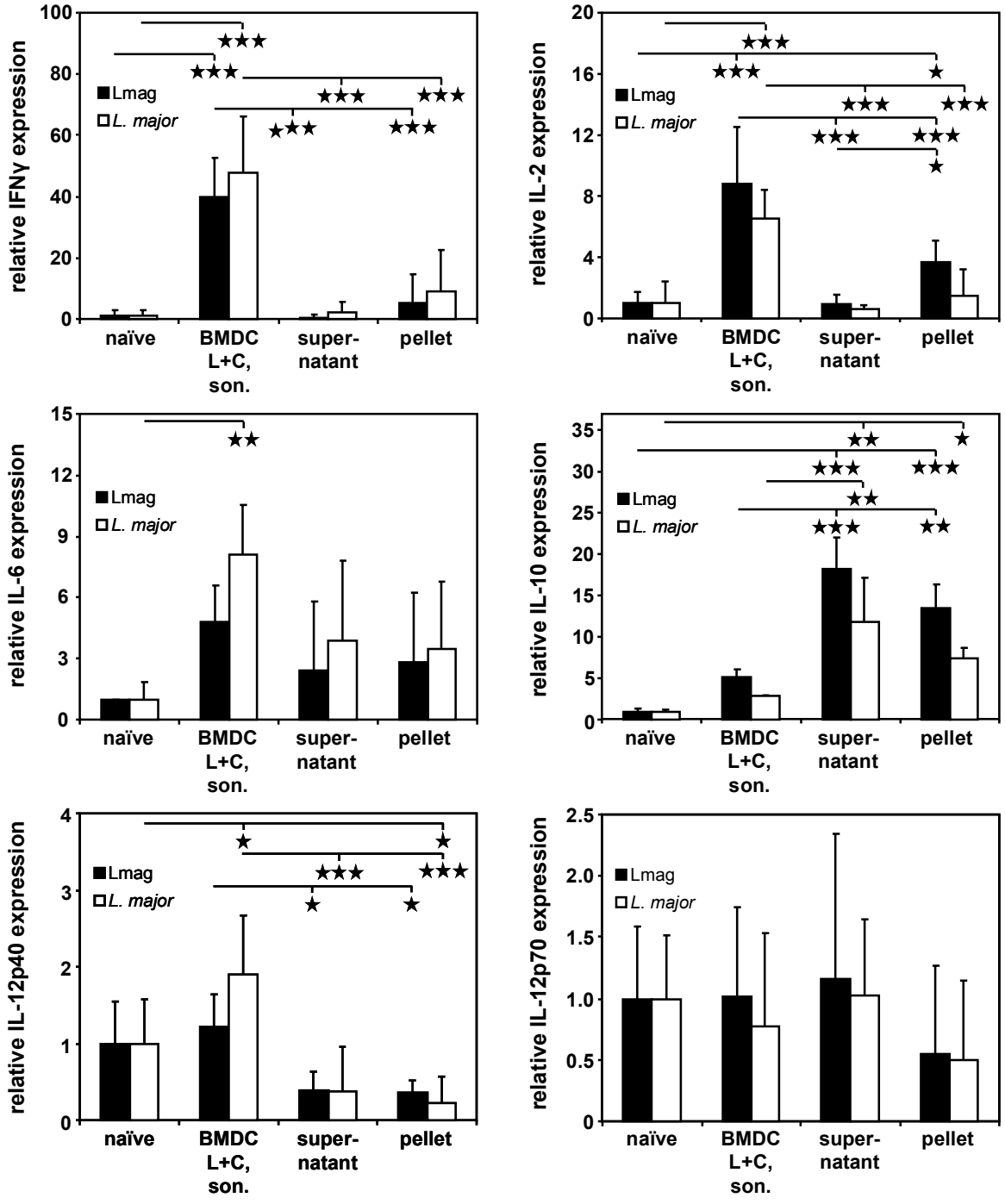

Fig. 27: Relative cytokine secretion of splenocytes upon stimulation with *L. major* parasites. Relative cytokine expression was calculated from the previously shown data. Amount of cytokine release was set to one for naïve mice of both stimuli. Accordingly, the amount of cytokine secretion by splencytes of the other groups was calculated as a factor of one. Data represent means ± SD. Two-way ANOVA p values for pre-treatments [column factor]: $p < 0.0001$ for IFNγ; $p = 0.3193$ for IL-2; $p = 0.0034$ for IL-6; $p < 0.0001$ for IL-10; $p < 0.0001$ for IL-12p40; $p = 0.4577$ for IL-12p70. Bonferroni's post test: *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$. Lmag: *Leishmania* lysate; *L. major*: viable *Leishmania* promastigotes; BMDC L+C, son.: sonicated *Leishmania* lysate-loaded CpG-activated BMDC; supernatant or pellet respectively: supernatant or pellet after ultra-centrifugal processing of disrupted *Leishmania* lysate-loaded CpG-activated BMDC.

5. Discussion

The present study demonstrates that intracellularly processed *Leishmania* parasites in combination with cell-derived fragments are able to induce protective immunity against *L. major* infections in otherwise susceptible BALB/c mice. Here, *Leishmania* antigen-loaded DC serve as the antigen processing machinery and later on - after disruption - as source of immune stimulatory molecules in combination with antigenic determinants. After i.v. injection, this formulation is taken up by host-derived DC which in turn induce antigen-specific T cell proliferation, differentiation and activation and later on MΦ activation via initiation of IFNγ production. First, DC get activated during reception of the vaccine components and, second, produce co-stimulatory molecules and Th_1-related cytokines for the proper stimulation of a protective immune response. Therefore, the protective effect of this vaccination is dependent neither on IL-12 production via the transferred DC nor by their type of MHC [H2-k^b or H2-k^d] or their activation status. These findings extend the knowledge of how to use DC for vaccination against intracellular pathogens and of what is necessary on a molecular level to prevent leishmaniasis in susceptible mice.

First, earlier studies using viable antigen-loaded CpG-activated DC showed that there is no need of IL-12 production by the transferred cells (163). IL-12 is a potent inducer of Th_1-based immune responses which are essential for the effective elimination of *Leishmania* parasites. In conclusion, other host-derived cells support the establishment of protective immunity [Fig. 28]. Second, DC-based vaccination is a time consuming and expensive procedure when curing or preventing human diseases. Preparation, expansion, antigen-loading and i.v. injection of human DC under GMP conditions is a huge effort that has to be made if treatment of lethal diseases like cancer is feasible. But, for worldwide preventive vaccination against infectious diseases this might be not practicable. Because

of these two reasons, it is necessary to focus on downstream events during the process of induction of preventive immunity for later simplification of the primary vaccine.

To investigate the fate of injected viable DC these DC were stained with CFSE prior to injection. Consequently, it was able to find these cells or fragments of these cells in cryo-sections of host spleens [see chapter 4.2]. Additionally, it was determined that DC fragments appear more often in the spleen than viable DC and these fragments are taken up by cells which locate in T cell rich areas. These finding were looked at more closely in parallel studies which are already published in Schnitzer et al., 2010 (181).

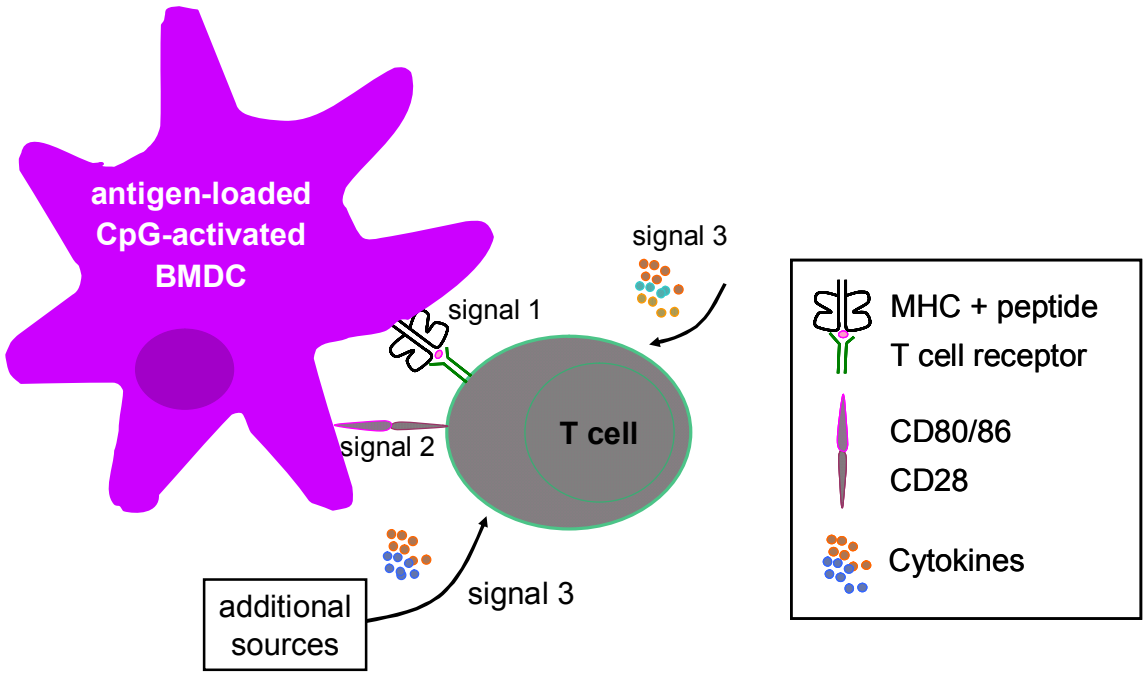

Fig. 28: Current paradigm of T cell activation after BMDC-based vaccination against leishmaniasis. I.v. injected BMDC provide antigenic peptides presented via MHC molecules together with co-stimulatory molecules for proper activation of T cells. Cytokines which are needed for directing the immune system towards a Th1-based immune response are provided from cellular sources of the recipient host.

Here it was shown that these fragments are taken up within the LAMP-1^+ endosomal pathway of $CD11c^+$ cells [DC]. The consequence of uptake of cellular fragments within the lysoendosomal pathway already was shown by Inaba et al. in 1998 (93). There, uptake and processing of cellular fragments led to the presentation of these antigens via MHC class II molecules. Furthermore, efficient targeting of antigen towards the LAMP-1^+ endosomal pathway was shown to be an effective tool to enforce antigen presentation via MHC class I and II molecules (101). Therefore it was concluded that cellular material derived from i.v. injected DC is taken up by host DC in the spleen or is subsequently transported to the spleen by these DC. Afterwards it is presented via MHC molecules to T cells [and B cells with smaller degree]. Thus, DC still are the key players during protection of BALB/c mice from otherwise lethal infections with *L. major* via initiating and guiding the adaptive immune response after vaccination with either viable or disrupted antigen-loaded DC. As a consequence for vaccine design, the target cell type stays the same. But now, *in vivo* host DC are the target for DC-based vaccination.

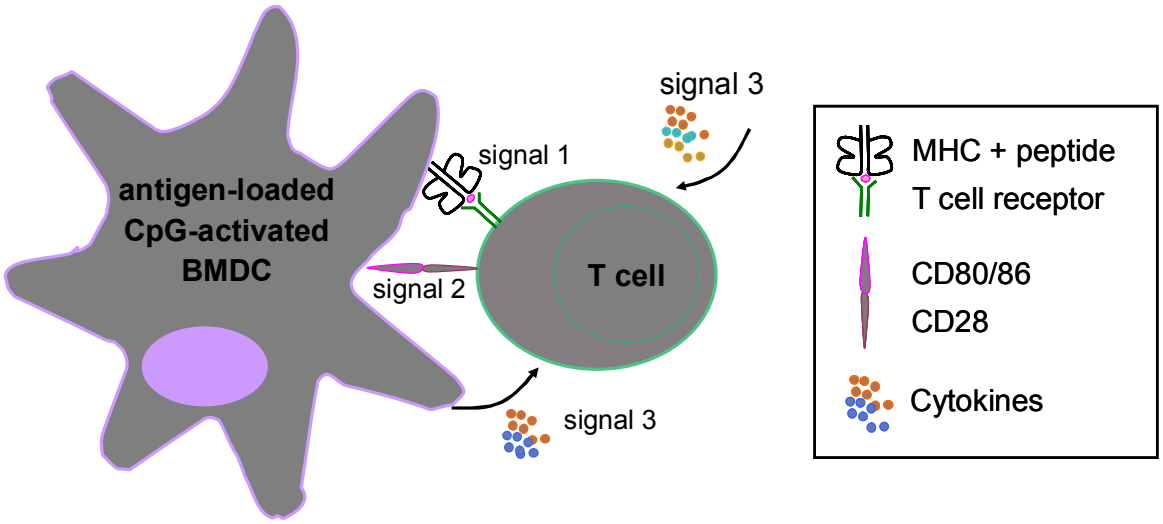

Fig. 29: Classical paradigm of DC-based vaccination. The classical paradigm of DC-based vaccination proposes direct interaction of i.v. injected antigen-loaded and activated DC. All signals which are necessary for complete T cell activation, differentiation and proliferation are provided by these DC.

Nevertheless, direct interaction between transferred antigen-loaded DC and T cells in the spleen can not be ruled out in a setting where viable DC were transferred. This direct interaction still can be responsible for sufficient T cell activation and therefore uptake of transferred DC might be an artefact. The question which has to be addressed now is: Is direct contact between viable antigen-loaded DC and T cells needed for the induction of a Th_1-based immune response? First answers to that question are given by experiments where PFA-fixed antigen-loaded DC were injected into BALB/c mice. Here, these cells still transfer protective immunity (181). More, it was revealed in this study that disrupted antigen-loaded DC show equal properties regarding establishment of protective immunity against subsequent infections with *L. major*, independent of the type of DC. Beyond this, the size of these disrupted cell fragments does not influence the efficacy of the vaccination which was shown by using different disrupting techniques. But, phospholipids of disrupted cell membranes tend to form vesicular structures (114). Kovar et al. clearly

demonstrated that membranous vesicles which were derived from disrupted antigen-loaded activated DC2.4 cells show the property of $CD8^+$ T cell activation *in vivo* (110). Additionally, *in vivo* assays revealed that these vesicles interact directly with $CD8^+$ T cells. *In vivo* these vesicles were able to induce anti-tumor immunity which was accompanied by $CD8^+$ T cell memory.

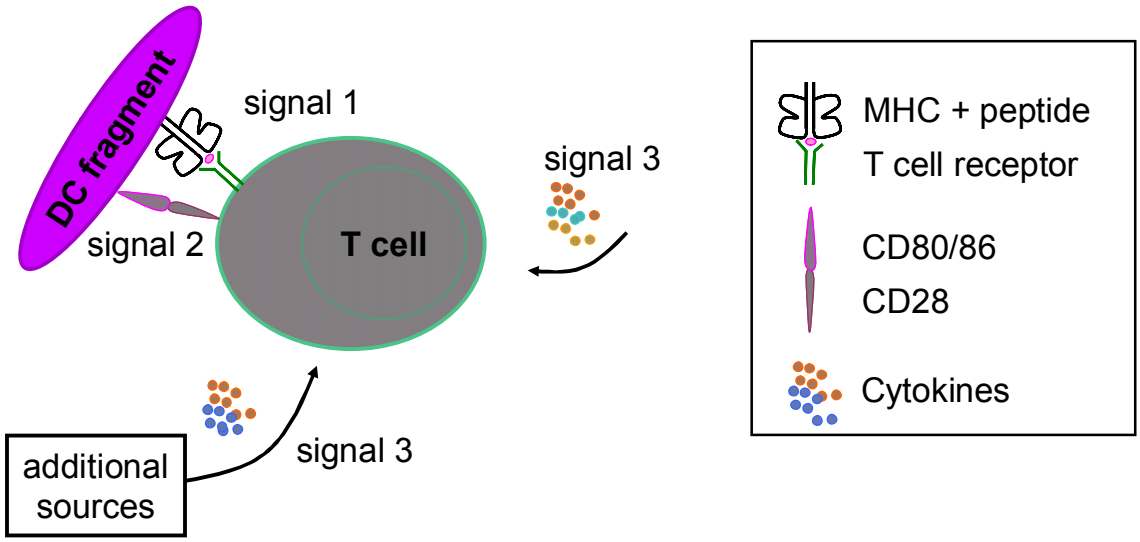

Fig. 30: Possible mechanism of T cell activation via fragments of antigen-loaded CpG-activated BMDC. I.v. injected disrupted antigen-loaded CpG-activated BMDC provide antigenic peptides presented via MHC molecules together with co-stimulatory molecules for proper activation of T cells. Cytokines which are needed for directing the immune system towards a Th1-based immune response are provided from cellular sources of the recipient host.

The efficacy of disrupted DC as vaccines against *Leishmania* parasites together with the findings of Kovar et al. points towards a direct interaction of antigen-loaded CpG-activated BMDC-derived fragments with the respective T cell population in naïve BALB/c mice that is able to induce protective immunity in these mice [Fig. 30]. Nevertheless, these findings contradict with the current DC-based vaccination paradigm which demands direct

interaction of viably transferred DC with T cells *in vivo* (61, 163). But in this experimental setup, it was clearly shown that no viable cells with intact metabolism or cellular integrity are needed for the transfer of activation stimuli together with antigen-specific determinants for the induction of protective immunity against subsequent infections with *L. major*.

But, proper T cell activation - one of the principal mechanisms in immunology - demands both co-stimulatory molecules and antigen which has to be presented via MHC molecules to the respective T cell. Whether both are needed to be present in the respective formulations used here for vaccination against *L. major* was the next question that had to be addressed. For vaccines using viable BMDC it was known that activation via stimulation with CpG ODN is a prerequisite for vaccine efficacy (163). In contrast, this holds not true for *ex vivo* prepared pDC and LC (61, 167). Furthermore, using disruption as pre-treatment prior to transfer activation of DC by CpG is neither a prerequisite for BMDC nor for *in vivo* grown pDC. In terms of reduced footpad swelling as well as parasite load in draining lymph nodes, mice which were vaccinated with one of these formulations showed full protection against subsequent infection with *L. major* parasites. If pre-existing co-stimulatory molecules like CD80/86 on the surface of transferred DC were necessary for effective T cell priming, there would have been the need of pre-activation of BMDC. Reverse, stimulation of BMDC via CpG ODN, as done in earlier studies, could have led to enhanced uptake of transferred viable cells by host DC, whereas LC and pDC already showed good performance, respectively. As this is just a presumption without experimental evidence, it is still possible that dual mechanisms of T cell activation after vaccination with viable antigen-loaded CpG-activated BMDC account for the establishment of protective immunity against *Leishmania* parasites. Nevertheless, no co-

stimulatory signal expressed by transferred DC is needed for the induction of protective immunity after transfer of disrupted antigen-loaded DC as a vaccine.

On the one hand, the so far proposed mechanism of T cell activation via fragments of antigen-loaded DC utilizes direct interaction between T cells and these fragments. On the other hand, stimulation of T cells via co-stimulatory molecules [signal 2] and antigen presented by MHC class molecules [signal 1] is necessary for the direct activation of T cells. But, antigen-loaded BMDC do not have efficient amounts of co-stimulatory molecules unless they were treated with CpG ODN (163). Therefore, bystander help from surrounding cells would be necessary to complete the obligatory signals for T cell activation. Only signal 1 could efficiently be presented to T cells by disrupted antigen-loaded DC. Hence, the next question that had to be addressed was whether direct interaction is needed between disrupted antigen-loaded DC and T cells via antigen-MHC molecule-complexes and the respective T cell receptors.

A simple way to do so is to bring MHC molecules on APC together with T cell receptors which were selected on a different MHC background. T cells exclusively can be activated by MHC class I or II molecules, respectively, which were present in the thymus during T cell development (28). In consequence, if there is direct interaction needed between these fragments and the T cells then there will be only activation of T cells if DC were used which share the same MHC background. In this study BALB/c mice were almost exclusively used for all of the experiments. These syngenic mice share the H2-k^d type of MHC. In experimental setups, so-called Balb/b mice [data not shown] and C57BL/6 mice were used as donors for bone marrow and subsequently for BMDC. These mice share the H2-k^b type of MHC. Although these both types - H2-k^d and H2-k^b - are different, fragments of antigen-loaded C57BL/6 BMDC were able to induce protective immunity in

BALB/c mice after i.v. injection. These mice showed a statistically significant reduction of footpad swelling together with a reduced parasite load in draining lymph nodes. This finding demonstrates that there is no direct interaction needed between the injected cellular fragment and the respective T cells. Or vice versa, host cells of the recipient mice have to fulfil this duty [Fig. 31].

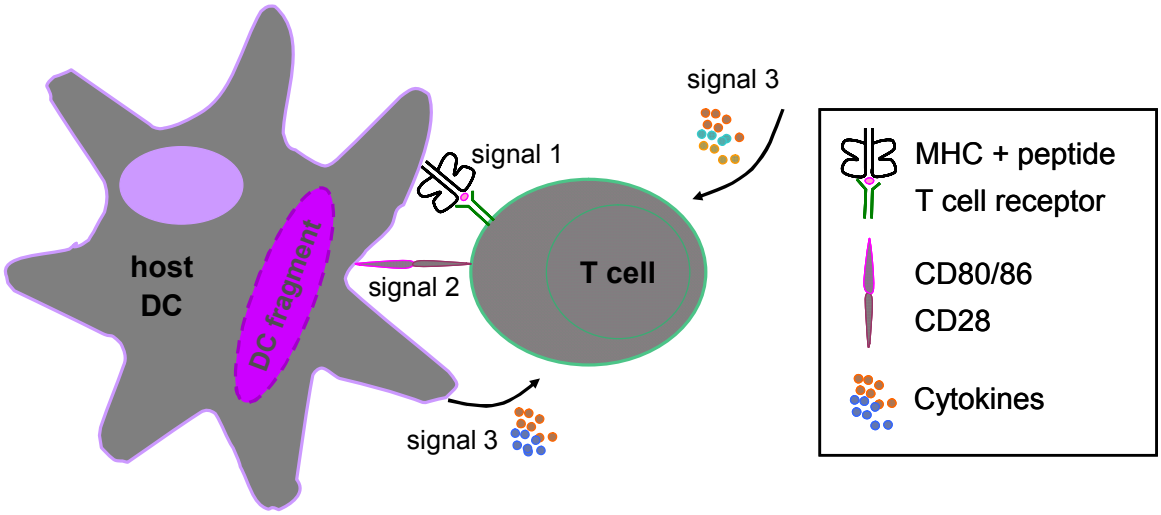

Fig. 31: Proposed mechanism of T cell activation after DC-based vaccination against leishmaniasis. DC of recipient mice take up fragments of i.v. injected antigen-loaded DC which are the source of antigenic determinants. These antigens are subsequently presented in the spleen towards T cells which in turn become activated and start to proliferate. Therefore the induction of protective immunity is managed.

Questions which might come up here are: How many of these fragments are taken up by host DC and how do these DC acquire the capability of full T cell activation?

A simple answer to the first question is: Enough! The amount of disrupted DC which was used for vaccination of BALB/c mice resembles the cell number that was used for vaccination against *Leishmania* parasites with viable BMDC. These *in vivo* experiments clearly demonstrate that enough of these fragments are taken up by host DC to allow

sufficient T cell stimulation. But if this uptake of these fragments by DC is somehow selective or targeted has to be elucidated in the future to provide a good basis for further development of the vaccine.

As already mentioned, membrane particles of disrupted DC resemble exosome like structures and show comparable activity in terms of T cell activation and anti-tumor activity in *in vivo* models (110). Therefore, already known mechanisms and properties of exosomes could explain the good efficiency of DC fragment-based vaccination. In contrast to exosomes, vesicle formation after disruption is not a mechanism that allows a defined composition of membrane-bound or enclosed molecules. Anyhow, membrane-bound molecules which were found on the surface of exosomes can account for the efficient uptake of injected cell fragments by host DC. Macrophage-1 antigen [Mac-1] (53), CD9 (99, 198) and milk fat globule-EGF factor 8 protein [MFG-E8/lactadherin] (78, 212) have already been brought into relation with exosome uptake by DC. Additionally, membrane vesicles are the typical prey of DC and MΦ in the periphery and the spleen. Very early after transfer, already 10 minutes post injection $CD11c^+$ host cells can be found in the spleen, which took up labelled fragments of i.v. injected disrupted DC [Data not shown]. As already shown (181), PFA-fixed DC are efficiently taken up by DC of the recipient mice. Fragments of these PFA-fixed DC can be found in vesicles which are associated to the $LAMP\text{-}1^+$ endolysosomal pathway and are therefore predetermined for efficient presentation via MHC molecules. Together with the here shown co-localization of splenic T cells and DC which took up cellular fragments of injected BMDC, these findings show a direct line which follows *Leishmania* antigen from loading of BMDC until presentation to host T cells [Fig. 31].

Unfortunately, splenocytes and LN cells of mice that were vaccinated and subsequently infected with *L.major* did not release consistent cytokine patterns. Nevertheless, protective vaccination with differently prepared disrupted antigen-loaded DC unequivocally resulted in reduced footpad swelling and reduced parasite load in draining lymph nodes [dLN] in comparison to PBS treated mice.

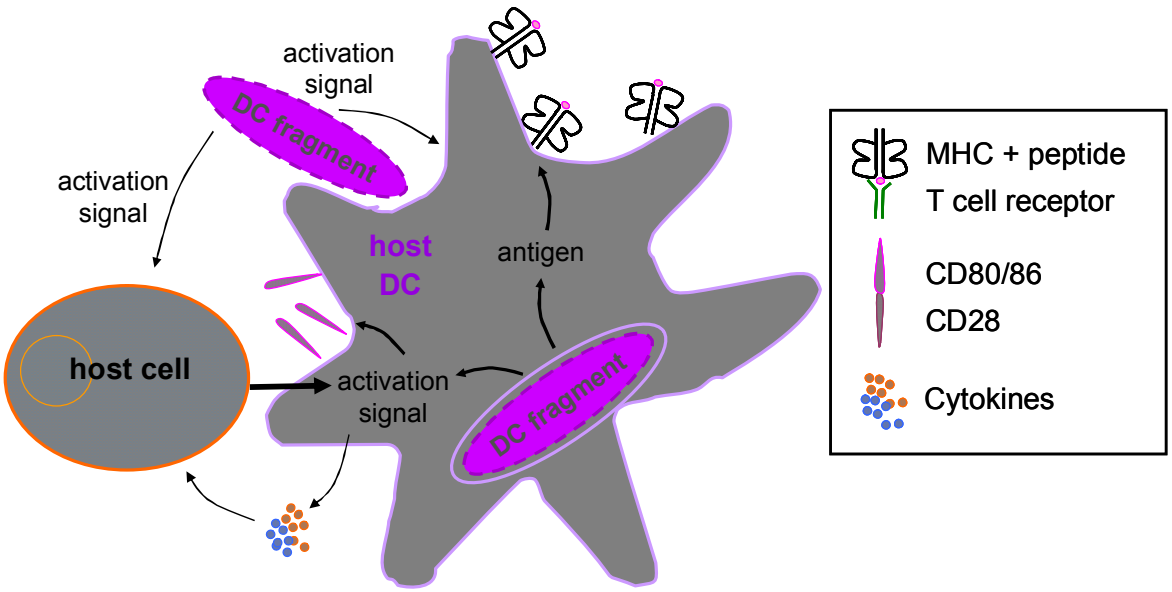

Fig. 32: Endocytosis of transferred DC fragments by host DC accompanied by subsequent activation. DC of the recipient mice take up fragments of i.v. injected antigen-loaded DC. These fragments serve as source for *Leishmania* antigen as well as of activating stimuli. The mechanism of host DC activation is not yet revealed. Support during activation may follow different pathways and may be initiated via several mechanisms.

But, to study this mechanism it was tried to set up *in vivo* assays which resemble the proposed mechanism. Cell compositions of DC, fragments of antigen-loaded DC and T cells of primed mice did not show any activity in terms of T cell proliferation. These proliferation assays did not show reasonable data unless an undefined composition of

splenic cells has been used to support the induction of T cell proliferation [see chapter 4.4.1]. Then, antigen-specific T cell proliferation of $CD4^+$ and $CD8^+$ T cells was monitored. This combination of activated $CD4^+$ and $CD8^+$ T cells is needed as source of IFNγ after onset of disease (22, 42). Further more, whenever antigen-specific proliferation was seen it was accompanied by the production of IL-2 and high amounts of IFNγ. The supportive activity of splenic cells in this setting might be contact-dependent activation and release of DC activating cytokines by NK cells or other cell types (65, 152, 168). Nevertheless, both cell types, DC and NK cells, have to become activated via certain stimuli.

In experiments presented here [see chapter 4.5.1], suspensions of disrupted antigen-loaded DC were separated into the sedimenting membranous fraction and the soluble fraction. After i.v. injection of these fractions, only the membranous fraction was able to induce sufficient immunity against infections with *L. major* parasites. This data points towards membrane-bound stimuli on the surface of exosome-like vesicles which are responsible for DC activation. Additionally, DC disruption is maintained via crude methods. Exosome-like vesicles, which are formed after disruption of DC's membranes, will resemble more or less vesicles of necrotic cells rather than those of apoptotic cells. More, it is well known that DC are able to endocytose necrotic cell debris and in turn become activated by danger-associated molecular patterns (62) like uric acid (186), High mobility group box protein 1 (133) and Hsp70 (12, 217). In consequence, exosome-like vesicles which originate from disrupted *Leishmania* antigen-loaded DC provide antigen for the activation of *Leishmania*-specific T cells as well as proper signals which are able to induce DC maturation.

In order to develop a cell-based vaccine towards a rather simple composition of antigen, adjuvant and a good shuttle the efficiency and the molecular mechanisms have to be monitored quite closely during development. Therefore, the production and release of cytokines by splenic and lymphatic cells was assessed after termination of *in vivo* experiments and during several *in vitro* assays.

Since several years it is known that Th_1-related immune responses are necessary for the clearance of *Leishmania* parasites during acute infection (126). For the establishment of such a Th_1-related immune response IL-12p70 and IFNγ are the key cytokines. IL-10 and IL-4 are the major opponents which might induce Th2-related immune responses or suppress Th1-related immune responses respectively. On account of this, splenocytes of naïve mice were looked at *in vivo* to monitor release of these four cytokines together with IL-6 and IL-12p40 upon stimulation with the different DC-based vaccines. Here, it is shown that disrupted CpG-activated BMDC already show Th_1-stimulatory capacity independent of *Leishmania* antigen. Slight levels of IL12-p70 and IL-2 point towards induction of T cell proliferation and instruction of a Th_1-related phenotype. This could lead to an unspecifically activated and pre-determined immune system which develops proper Th_1-related immune responses against *Leishmania* parasites upon infection one week after vaccination. However, antigen-loading of BMDC is absolutely necessary for induction of antigen-specific T cell proliferation [see chapter 4.4.1]. Much more, vaccination with disrupted DC - without antigen-loading - and subsequent infection with *L. major* leads to exacerbation of the disease [see Fig. 12]. In the context of effective vaccination and induction of protective immunity against leishmaniasis with disrupted antigen-loaded DC this mixture of cytokines fits to the current paradigm of T cell activation via DC-based vaccination.

One step ahead, one week after successful vaccination the immune system of the respective mice has to face the *Leishmania* parasites. As it is predicted, for induction of a protective immune response the expansion of specific Th_1 cells and the consequential production of IFNγ for the activation of MΦ is needed. Splenocytes of mice which were vaccinated with disrupted antigen-loaded CpG-activated BMDC produce exactly the right mixture of cytokines *in vivo* after stimulation with either viable parasites or crude *Leishmania*-antigen. Large amounts of IL-2 indicate T cell proliferation and high titres of IFNγ are able to induce MΦ-activation for the subsequent destruction of *Leishmania* parasites in the parasitophorus vacuoles (122). Increased levels of IL-6 suggest an inflammatory environment whereas IL-6 plays a dual role via immunosuppressive activity (226) as well as via anti-Treg activity (173). Surprisingly, elevated amounts of IL-10 were found. But, since a while IL-10-producing *Leishmania*-specific T cells are known (103). Beyond, recent studies give more evidence of IL-10 production in late stages of *T. gondii* or *L. major* infections (147). But, not the anticipated preformed Th_2 cells or misguided naïve T cells that were directed to the Th_2-related phenotype produced this Th_1-opposing cytokine. Th_1 cells release IL-10 in the context of immunoprotection, whenever an immune response has to be shut down. This gives a hint to a more complex role of IL-10, than it was proposed so far in the Th_1/Th_2-model. Overall, these two sets of data perfectly demonstrate the induction of antigen-specific T cell activation together with the establishment of a protective immune response to *Leishmania* parasites that is characterized by IL-12 production in the beginning of T cell priming and IFNγ production as activation stimulus for parasite killing by MΦ.

Much more surprisingly, using the purified fractions of disrupted antigen-loaded CpG-activated BMDC for stimulation of naïve splenocytes or vaccination of mice respectively

the pattern of cytokines did not show this perfect emphasis on Th_1-related cytokines. Although elevated, of IL-2 expression after stimulation with these fractions was not significantly hightened whereas a biological significance already was shown for the membranous fraction in *in vivo* experiments [see Fig. 22]. More, after stimulation of membranous fraction-primed splenocytes with *Leishmania*-antigen a clear up-regulation of IL-2 production can be seen, which points towards antigen-specific T cell activation and proliferation. But, it also shows the immunostimulatory capacity of the soluble fraction, which resembles the cytokine release after stimulation with not-loaded disrupted BMDC [see Fig. 20]. Unfortunately, no significant amount of IL-12p70 was found in these co-cultures to complete the set of Th_1-related cytokines during early T cell priming. Co-cultures of splenocytes from membranous fraction-primed mice lack the tremendous amounts of IFNγ after stimulation of splenocytes with *Leishmania*-antigen or viable parasites, although there is biological evidence from the *in vivo* experiments. However, these co-culture experiments have not been individually modified to let them generate comparable results. In fact, no significant concordance can be found between splenocytes of mice that were vaccinated with complete disrupted DC solution or the purified membranous fraction regarding Th_1-related cytokine release [see Fig. 26].

During development and simplification of cell-based vaccines it is very important to monitor the efficacy of the vaccine as well as the consistency of molecular mechanisms which are involved in induction of immune responses, respectively. The origin, where this development for an anti-*Leishmania* vaccine was started, is an antigen-loaded CpG-activated BMDC-based vaccine. Now we know that after disruption of these BMDC neither lack of CpG-mediated pre-activation nor different MHC background, nor purification via ultra centrifugation, nor exchange of BMDC for MΦ causes loss of

biological activity of this vaccine. Taking all this together, the basic composition of an effective vaccine would be the membranous part of cells - not necessarily autologous cells - which endocytosed sufficient amounts of *Leishmania*-antigen. However, regarding molecular mechanisms, these vaccine preparations differ in their ability to induce the release of Th_1-related cytokines, at least in this *in vivo* model. Of course rather unexpected but, this is a good occasion to study the Th_1/Th_2 paradigm more in detail. Nevertheless, as biological efficacy ranks much higher than *in vitro* data, the here presented data clearly demonstrates the possibility of transforming classical cell-based vaccination strategies towards simple as well as effective vaccine formulations.

6. Abstract

Dendritic cell-based vaccination is a well established technique for preventive and therapeutic instruction of the immune system where conservative vaccine formulations fail to cure or prevent diseases, respectively. Efficiency of this technique already was demonstrated in infectious diseases as well as for cancer in animal or human studies. Well controlled manipulation and antigen-loading of immature DC is most beneficial to this technique. But, time-consuming and cost-extensive procedures for preparation of DC precursors, expansion and stimulation of DC and inpatient administration are big disadvantages regarding vaccine development for pandemic infectious diseases that occur mainly in underdeveloped countries. Therefore vaccines are needed that are pathogen-tailored and able to induce equal immune responses as their DC-based vaccine models.

For vaccination against *Leishmania* parasites such a DC-based vaccine is feasible and its efficacy to induce protective Th_1-based immune responses was already demonstrated in several animal studies. But, one of our own studies indicated supportive activity of host cells exceeding the allocation of T cells to become activated by transferred DC. IL-12, an important cytokine for the induction of Th_1-related immune responses, has to be produced by host cells. Therefore, the aim of this study was to investigate the mechanism of BMDC-based vaccination with regard to simplification of the vaccine formulation. Key questions that have been addressed are: Which cells process the information that is transferred by the injected DC and what are the key components of this information? Further more, it was looked at whether altered vaccine formulations are able to induce protective immunity and whether they share equal molecular mechanisms.

The current paradigm of BMDC-based vaccination proposes direct interaction of transferred BMDC with host T cells. These BMDC have to be antigen-loaded for

stimulation via antigen-peptide-MHC molecule-complexes and they have to be activated for proper co-stimulation of T cells. Here, this study demonstrates that neither activation for co-stimulation nor direct interaction with adequate MHC molecules is needed for the induction of protective immunity against infection with *Leishmania*-parasites.

Disrupted antigen-loaded BMDC are able to induce protective immunity in BALB/c mice without pre-stimulation via CpG ODN. Beyond, if BMDC were used with a different MHC-background than recipient mice then the vaccine still would be efficient in terms of reduction of footpad swelling and parasite load in draining lymph nodes. Even more, DC-specific features are no key component that leads to protective immunity as vaccination with disrupted antigen-loaded MΦ shows equal properties than before mentioned vaccine formulations. Further more, it was found that host DC play a major role in transforming the incoming signal, received from transferred antigen-loaded DC, into Th_1-related stimuli and *Leishmania*-antigen-specific T cell activation.

Suspensions of disrupted antigen-loaded DC resemble a combination of laid off soluble molecules together with exosome-like vesicles that formed after disruption of membranes. Here it was shown that separation of the membranous and soluble fractions and subsequent transfer into BALB/c mice will lead to protection of these mice against infection with *L. major* promastigotes only if the membranous fraction is used as vaccine. More, this vaccine formulation takes advantage of easy storage at -80°C with no need of fresh production. This clearly demonstrates that the immunity-inducing principle of disrupted DC-based vaccination lies within the membrane enclosed fraction.

On a molecular level, disrupted antigen-loaded DC induce Th_1-related cytokines during vaccination and as response on pathogen encounter. *In vivo* assays revealed IL-12 production and antigen-specific T cell proliferation among splenocytes that were

stimulated with disrupted antigen-loaded DC. Splenocytes of accordingly vaccinated mice produce tremendous amounts of IFNγ after stimulation with *Leishmania* parasites. In summary, disrupted antigen-loaded BMDC fulfil all characteristics of DC-based vaccination against *Leishmania major*. But, while purification of membranes of antigen-loaded DC and subsequent transfer to BALB/c mice leads to control of the disease in the animal model, only slight levels of Th_1-related cytokines are seen in the *in vivo* assays. Whether this points towards a loss of vaccine activity on unseen levels or unknown sites where Th_1-related immunity is induced by both, complete solution and purified membranes, still has to be determined.

Consequently, modifications of classical BMDC-based vaccines against infections with *L. major* have been found. These modified vaccines provide easier manufacturing procedures accompanied by statistically and biologically significant efficacy regarding prevention of leishmaniasis in BALB/c mice. Moreover, it was shown that a vaccine would be possible that does not have to consist of autologously prepared DC together with no need of pre-activation.

This study demonstrates the transformation of a DC-based vaccine against *L. major* into a convenient DC-targeted technique and can guide the way to affordable and efficient vaccines against leishmaniasis.

7. Zusammenfassung

Die Impfung mittels Antigen-beladener dendritischer Zellen [DZ] ist mittlerweile eine gut etablierte Technik, die dann zum Einsatz kommt, wenn Standard-Impftechniken versagen, vor Krankheiten zu schützen beziehungsweise diese zu heilen. Die Effizienz dieser Technik konnte bereits für diverse Infektionskrankheiten und Krebserkrankungen in experimentellen Tiermodellen sowie am Menschen gezeigt werden. Hierbei ist die Möglichkeit zur wohldefinierten Manipulation und Antigenbeladung der DZ ein großer Vorteil gegenüber den konventionellen Ansätzen. Jedoch ist vor allem bei der Anwendung im klinischen Bereich die Präparation, Herstellung und Manipulation dieser autologen DZ mit einem erheblichen technischen, zeitlichen sowie finanziellen Aufwand verbunden. Hinsichtlich einer Präventivimpfung gegen eine pandemische Infektionskrankheit, die in hauptsächlich unterentwickelten Ländern vorkommt, wird dieser Aufwand sicherlich ein Hindernis darstellen. Daher muss für solche Fälle ein maßgeschneiderter Impfstoff entwickelt werden, der sich am Vorbild des effektiven DZ-basierten Impfstoffs orientiert.

Für die Impfung gegen die *Leishmania* Parasiten besteht so ein DZ-basierter Impfstoff bereits. Dessen Wirkung, eine T-Zell Antwort vom Typ Th_1 zu induzieren, wurde bereits in mehreren Veröffentlichungen demonstriert. Zusätzlich hat aber eine unserer Studien gezeigt, dass das typische Th_1-bezogene Zytokin IL-12 zur Differenzierung naiver T-Zellen nicht von den injizierten DZ bereitgestellt werden muss, sondern von der geimpften Maus. Dies gab erste Hinweise auf eine stärkere Beteiligung des Wirts-Immunsystems als zuvor angenommen.

Daher sollte hier vertieft der Mechanismus dieser DZ-basierten Impfung untersucht werden, wobei modifizierte Impfstoff-Ansätze zum Einsatz kommen sollten. Dabei wurden die Fragen nach der vom Impfstoff transportierten Information und dem Empfänger dieser Information berücksichtigt.

Das aktuelle Paradigma zur DZ-basierten Impfung besagt, dass transferierte DZ im direkten Kontakt mittels dreier Signale T-Zellen stimulieren und aktivieren. Dafür müssen diese DZ mit dem entsprechenden Antigen beladen und aktiviert worden sein um das Antigen-Peptide mittels MHC Molekül im Kontext der Co-Stimulation präsentieren zu können. Jedoch zeigt diese Studie hier, dass weder eine Aktivierung der DZ noch die Präsentation des Antigens mittels passender MHC Moleküle notwendig ist für die Induktion einer protektiven Immunantwort gegen *Leishmania* Parasiten.

Aufgeschlossene, mit Antigen beladene DZ müssen nicht vor dem Transfer mit CpG ODN aktiviert worden sein, um entsprechende Immunität zu verleihen. Ebenso hat der MHC Typ in diesem Falle auch keinen Einfluss auf die Effektivität des Impfstoffs. Da im Weiteren aufgeschlossene mit *Leishmania*-Antigen beladene Makrophagen nach Impfung die gleiche Wirkung erzielen, wie vorangegangene DZ-basierte Impfstoffe, können keine DZ spezifischen Mechanismen Schlüsselkomponenten der Induktion einer protektiven Immunität sein. Darüber hinaus konnte gezeigt werden, dass die DZ der geimpften Mäuse, eine maßgebliche Rolle bei der Verarbeitung transferierter Signale spielen.

Suspensionen aufgeschlossener DZ stellen eine Kombination aus freigesetzten löslichen Molekülen sowie Membranvesikeln dar, die sich nach dem Aufschluss gebildet haben. Nach Auftrennung dieser beiden Fraktionen konnte gezeigt werden, dass ausschließlich die Membran-Fraktion nach Verimpfung eine geeignete Immunantwort zum Schutz vor *Leishmania* Parasiten induzieren kann. Als Vorteil dieser Aufreinigung erweist sich zudem die stabile Lagermöglichkeit bei -80°C. Somit ist klar gezeigt, dass die Immunität-verleihende Einheit dieser Impfstoffvarianten in der Membran-Fraktion liegt.

Verfolgt man die Induktion Th_1-zugehöriger Zytokine in *in vivo* Experimenten so ergibt sich im Falle der Gesamtsuspension aufgeschlossener, mit *Leishmania*-Antigen beladener

DZ ein klares Bild. Diese Suspension erzeugt das volle Spektrum der DZ-basierten Impfung gegen *Leishmania* Parasiten. Es kann sowohl Produktion von IL-12 und IL-2 als auch eine antigenspezifische T-Zell Proliferation nach Stimulation von Splenozyten mit der entsprechenden Suspension verzeichnet werden. Außerdem produzieren Splenozyten von entsprechend geimpften Mäusen nach Stimulation mit *Leishmania*-Antigen erhebliche Mengen des entscheidenden Zytokins IFNγ. Obwohl jedoch die Verimpfung aufgereinigter Membranvesikel dieses Ansatzes im Tierversuch zu biologisch sowie statistisch signifikanten Ergebnissen führt, lassen sich die entsprechend Th_1-bezogenen Zytokine im *in vivo* Ansatz nur in geringen Maße nachweisen. Ob dies jedoch für einen *in vivo* unbemerkten Aktivitätsverlust des Vakzins oder für andere lymphatische Organe als Ort der T-Zell Instruktion spricht, ist noch unbekannt und muss noch geklärt werden.

Folglich konnten Abwandlungen des ursprünglichen und klassischen DZ-basierten Impfstoffes gegen *Leishmania major* gefunden werden, die sowohl Vorteile in der Herstellung bzw. Lagerung als auch BALB/c Mäusen gleichwertigen Schutz vor Leishmaniose bieten. Darüber hinaus konnte gezeigt werden, dass ein Impfstoff möglich wäre, der nicht aus autologen DZ herzustellen wäre und dementsprechend auch keine Voraktivierung benötigt.

Somit ist die Möglichkeit gegeben aus dem DZ-basierten Impfstoff eine einfache und effektive Technik zur Herstellung eines auf DZ abgestimmten Impfstoffes abzuleiten.

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9. Annex

9.1. Abbreviations

Ab	Antibody
APC	Antigen-presenting cell
BMDC	Bone marrow-derived dendritic cell
BSA	Bovine serum albumin
CD	cluster of differentiation
CD40L	CD40 ligand
cDC	conventional DC
CL	Cutaneous leishmaniasis
CpG ODN	CpG-containing olygodeoxynucleotide
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DCL	Diffuse cutaneous leishmaniasis
DNA	Deoxyribonucleic acid
dLN	Draining lymph node
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
Flt3-L	FMS-related tyrosine kinase 3 ligand
GM-CSF	Granulocyte/macrophage colony-stimulating factor
HSP	Heat shock protein
IFN	Interferon

Ig	Immune globulin
IL	Interleukin
iNOS	inducible NOS
i.p.	Intraperitoneal
IPC	type I interferon producing cell
i.v.	Intravenous
L.	Leishmania
LmAg	*L. major* antigen
LN	Lymph node
LPS	Lipopolysaccharide
LT	Lymphotoxin
mAb	Monoclonal Ab
mDC	myeloid DC
MCL	Mucocutaneous leishmaniasis
MHC	Major histocompatibility complex
MΦ	Macrophage
NK	Natural killer
n.d.	Not detected
NO	Nitric oxide
NOS	NO synthase
n.s.	not significant
n.t.	not tested
OD	Optical density
PAMP	Pathogen-associated molecular patterns

PBS	Phosphate-buffered saline
pDC	Precursor of DC
PKDL	Post kala-azar dermal leishmaniasis
PRR	Pattern recognition receptors
s.c.	Subcutaneous
SD	standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
son.	sonicated
TCR	T cell receptor
TGF	Tumor growth factor
Th	T helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
VL	Visceral leishmaniasis
WHO	World health organization

9.2. Publications

Article

Schnitzer, J. K., S. Berzel, M. Fajardo-Moser, K. A. Remer, and H. Moll. 2010. Fragments of antigen-loaded dendritic cells (DC) and DC-derived exosomes induce protective immunity against *Leishmania major*. Vaccine 28:5785-5793

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Oral Presentation

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J. K. Schnitzer, M. Fajardo-Moser, K. A. Remer, H. Moll. Mechanism of dendritic cell - based vaccination against *Leishmania major***.** 3rd Network Meeting of GK520, GK592 and GK794,Wildbad,Rothenburg, Germany, 07/2008

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Poster

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9.3. Curriculum Vitae

PERSONAL INFORMATION

Name:	Johannes K. Schnitzer
Date of Birth:	21.04.1978
Place of Birth:	Ebern, Germany
Citizenship:	German

EDUCATION

05/2006 – 03/2010	**Doctoral thesis** "Mechanism of dendritic cell-based vaccination against *Leishmania major*", Prof. Dr. Heidrun Moll, Institute for molecular infection biology, University Würzburg
October, 19th, 2005	**Diploma certification**
01/ 2005 – 10/ 2005	**Diploma thesis** "Characterization of a BMP TypII receptor associated protein", Prof. Dr. Petra Knaus, Institute for biochemistry, Free University of Berlin,
10/ 1999 – 10/ 2005	**Studies** at the Biocenter of the Julius-Maximilians Universitaet Wuerzburg, Germany Biology, Diploma Main subject: Microbiology Minor subjects: Physiological Chemistry, Genetics
June, 16th, 1998	**A-levels**
1993 - 1998	Clavius Gymnasium Bamberg, Germany
1988 - 1993	Gymnasium Roth, Germany
1984 - 1988	Primary school „Kupferplatte" in Roth, Germany

EMPLOYMENTS

since 06/ 2010	Institut Virion\Serion GmbH, Würzburg
05/ 2009 – 03/ 2010	Institute for molecular infection biology, AG Moll, University Würzburg
05/ 2006 – 05/ 2009	Graduate College "Immunomodulation" (GK520, DFG Fellowship)
11/ 2005 – 01/ 2006	Institute for biochemistry, Free University of Berlin
11/ 1998 – 08/ 1999	Military service

Würzburg, 15. Januar 2013

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9.5. Affidavit

I hereby confirm that my thesis entitled „Mechanism of Dendritic Cell-based Vaccination against *Leishmania major*" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and/or materials applied are listed and specified in the thesis.

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

Würzburg, 15 January 2013

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation „Mechanismus der Dendritische Zelle-basierten Impfung gegen *Leishmania major*" eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

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

Würzburg, 15. Januar 2013