Signaling via Interleukin-4 Receptor α chain during dendritic cell–mediated vaccination is required to induce protective immunity against *Leishmania major* in susceptible BALB/c mice

Die auf dendritischen Zellen basierende Immunisierungsstrategie gegen *Leishmania major* in BALB/c Mäusen ist abhängig von der Stimulation der Interleukin-4 Rezeptor α Kette

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1. Introduction

1.1 Leishmaniasis

Parasitic protozoa of the genus *Leishmania* are obligate intracellular, vector-borne parasites and are transmitted to humans in the Old World by sand flies of the species *Phlebotomus* and by flies of the genus *Lutzomyia* in the New World. Over 20 species and subspecies are reported to infect humans, while each *Leishmania* species causes different types of symptoms with significant morbidity and mortality. Mainly four types of debilitating and disfiguring clinical manifestations, collectively known as leishmaniasis are distinguished, depending on the parasite species, the host's immune response and genetic background [1,2].

1.1.1 Different forms of leishmaniasis

Leishmaniasis is endemic in 88 countries throughout the Old and New World and can be divided into the severe and life-threatening form of visceral leishmaniasis (VL) ("Kala-Azar") caused by *L. donovani* complex (*L. donovani infantum* in the Old World; *L. donovani chagasi* in the New World), mucocutaneous leishmaniasis (MCL) ("Espundia") caused by *L. braziliensis* complex, diffuse cutaneous leishmaniasis (DCL) which resembles disseminated and chronic skin lesions of leprosy and the self-limiting form of cutaneous leishmaniasis (CL) ("Oriental Sore") caused by *L. tropica, L. major* and *L. aethiopica* in the Old World and *L. mexicana* complex in the New World [3].

Human CL infections in the Old World are due to the zoonotic form of CL caused by *L. major* and represent a worldwide health problem with a high endemicity of ~ 90% in developing countries of the Middle East and sub-Saharan Africa. In

African countries as well as in India the spreading of *Leishmania* infections is correlated with seasonal or sex workers and refugees [4,5].

A further important aspect which aggravates the risk of an infection is malnutrition and immunosuppression. CL has emerged in North America as a consequence of infected soldiers returning from missions in the Middle East. From a European point of view, *Leishmania* infections led to a rising cause for concern, as emerging co-infection in HIV-infected patients. These overlapping diseases of leishmaniasis and AIDS were identified to be prevalent in intravenous drug addicts and were firstly reported 30 years ago [4,6,7].

1.1.2 Clinical outcome of leishmaniasis

During a bite of an infected sand fly, infective promastigotes forms of the parasite enter the human host and are ingested by reticuloendothelial cells with phagocytic capacities, e.g. macrophages [8]. Within these host cells parasites metamorphose into the amastigote form and multiply via binary fusion until the host cell bursts, allowing the freed parasites to infect other phagocytic cell types [9,10]. This is possible, as the parasite becomes adapted to the proteolytic enzymes of the phagocytic cell [11].

The clinic of leishmaniasis is characterized by red papulae which appear at the site of infection 2 to 8 weeks later, either causing localized skin ulcerations due to a hypersensitive response or causing the spread of the nodules over large areas of the skin, due to an anergic response (DCL). The CL tends to be resolved within 2-4 months after infection [12,13]. Once a patient mounts such a CL infection, life-long immunity to re-infections is guaranteed [6,14].

The ability to control CL infections depends on the host immune status [15] and the induction of a protective cytokine-driven immune response [16,17]. The

genetic background of the host plays another important role in resistance or susceptibility to infection with *Leishmania* [18]. Clinical studies of *Leishmania*-infected patients during the outbreaks of VL in Sudan revealed that one locus on chromosome 22q12 and possibly two other loci on chromosome 2q22-23 (both associated with schizophrenia in humans) may play an important role in controlling susceptibility to infection [19].

1.2. Immunology

1.2.1 Experimental leishmaniasis

Depending on the parasite species, resistance to infection is mostly associated with a T helper (Th) type 1 cell-mediated immune response, resulting in the degradation of intracellular parasites by the phagocytic capacities of macrophages. Resistance to infection is associated with a cytokine milieu dominated by tumor necrosis factor (TNF)-α and interferon (IFN)-γ, whereas susceptibility is correlated with an interleukin (IL)-4, IL-10 and IL-13-driven Th2-directed immune response [20,21].

The association of a Th1-biased immunity with resistance and a Th2-biased immunity with susceptibility to experimental *L. major* infection allowed the investigation of disease promoting or restraining factors within the host. The experimental *Leishmania* infection model is a very well established *in vivo* model to investigate the host immune response to an intracellular pathogen, as the genetic predisposition of different mouse strains either correlate with the dominance of IL-4-driven Th2 response in non-healer mice or IL-12-driven Th1 response in cured mice. Certain mouse strains, such as BALB/c mice develop chronic progressive and systemic disease pathology as a consequence of a non-protective Th2-driven immune response, mimicking the human forms of Kala-Azar or DCL. In contrast

mouse strains, such as C57BL/6 mice resist infections as a consequence of an directed Th1-driven immune response [17,22].

This association of Th1/Th2 with the outcome of *Leishmania* infection was gained from studies on the early immune response in *L. major*-infected genetically resistant C57BL/6 mice, showing the association of IL-12-driven Th1 response with high levels of IFN-γ and no IL-4 in mediating a healing phenotype, whereas susceptible BALB/c mice showed high levels of IL-4 early during infection but no Th1 cytokines were present, indicating a Th2-biased immune response [23,24,25,26]. As so often, this simple model of susceptibility and resistance is challenged as the mechanisms involved in the acquisition of immunity are questioned or even proofed to be more complex.

1.2.2 Prerequisite for resistance or susceptibility to infection

Differences between the clinical disease outcome in BALB/c and C57BL/6 mice are correlated with immunoregulatory factors, showing that the modulation of nitric oxide (NO) in macrophages of susceptible mice, which is a prerequisite to eliminate *Leishmania* parasites, promotes the proliferation of amastigotes. Upon macrophage rupture, high levels of free parasites spread to the infection-draining lymph nodes (LN) and other organs in BALB/c mice [16].

Genetically resistant mice develop an IFN-γ-driven and IL-12-mediated T cell response dominated by a $CD4^+$ Th1 phenotype, whilst in susceptible mice the dominant response is a IL-4-driven response dominated by IL-4, IL-13 and IL-10-secreting $CD4^+$ Th2 phenotype [25,26,27]. The critical role for IFN-γ in mediating resistance was demonstrated in IFN-γ-deficient (IFN-$\gamma^{-/-}$) mice which failed to cure from infection [28]. The association of IL-4 with susceptibility came from studies using IL-$4^{-/-}$ BALB/c mice [29,30] or mice treated with α-IL-4 monoclonal antibodies

(mAb), resulting in cure from infection with *Leishmania* [25,31]. Th2-polarized immune responses are driven by early IL-4, thus suppressing the differentiation of Th1 lymphocytes and macrophage activation while inhibiting their IFN-γ secretion.

1.2.2.1 The role of IL-12

Depletion of IL-12 demonstrated that genetically resistant mice became susceptible to infection [32] and the treatment of susceptible mice with recombinant murine IL-12 cures mice from infection [33]. Exogenous IL-12 redirects early Th2 response to infection in susceptible mice and promotes resistance [33], as in resistant mice exacerbating disease progression was observed in IL-$12^{-/-}$ C57BL/6 mice [32]. IL-12 production is delayed even in resistant mice, and this inability of the immune system to produce early IL-12 may explain the early induction of a Th2-dominated immune response, as IL-12 signaling through IL-12 receptor (IL-12R) is essential to sustain a Th1 response [34].

1.2.2.2 The role of IL-4 and IL-10

The cellular origin of *Leishmania*-promoting IL-4 in BALB/c mice is tracked down to a $CD4^+$ T cell population with a Vβ4Vα8 T cell receptor (TCR), recognizing the leishmanial homologue of the receptor for activated C kinase (LACK) [35,36] rendering T cells non-responsive to IL-12 and correlates with lesion development and disease exacerbation [37]. Dendritic cells (DC) are the main source of early IL-12 in *Leishmania* infections and studies in C57BL/6 mice demonstrated that skin-derived DC are the main source of early IL-12p40 whereas the secretion of DC-derived IL-12p70 requires IFN-γ stimulation [38,39]. LACK recognition by this $CD4^+$ T cell population in BALB/c mice was demonstrated to be involved in susceptibility, as LACK-reactive cells are biased to produce early IL-4 upon *L. major* infection, thus

determining susceptibility to infection in BALB/c mice. Studies in mice expressing green fluorescent protein (GFP)-labeled IL-4 showed similar frequencies of *L. major*-induced IL-4-producing cells that bind a LACK-major histocompatibility complex (MHC) class II tetramer in susceptible and in resistant mice [40]. This goes in line with previous reports postulating an early IL-4 response upon infection of resistant mice [41,42]. Concluding, the capacity of the host immune system to prevent susceptibility depends rather on the ability to redirect an early induced Th2 response, than controlling early IL-4 secretion.

Nonetheless, controlling the release of these Th2-promoting cytokines, which inhibit for example reactive nitrogen intermediates (RNI) and IFN-γ in activate macrophages, is crucial to establish a protective immunity [43]. Leishmanicidal activity of alternatively activated macrophages is suppressed upon IL-4-induced arginase-1 expression, consequently promoting disease progression, while IL-4-induced arginase-1-expressing DC promote progressive leishmaniasis during the onset of infection [44,45].

Another disease promoting cytokine in experimental as well as in human leishmaniasis is regulatory T cell (T_{reg})-derived IL-10, which has the ability to suppress the phagocytic capacities of macrophages and it suppresses the antigen-presenting functions of DC [21,46,47]. IL-10, IL-4 and transforming growth factor (TGF)-β are very strong macrophage-deactivator and inhibitor of DC functions [48,49], supporting their role in susceptibility to *Leishmania* infections. Susceptible mice treated with α-IL-10R Ab or IL-$10^{-/-}$ mice displayed a resistant phenotype and sterile cure, indicating the involvement of IL-10 in parasite persistence [27,50]. The sources of *Leishmania*-promoting IL-10 are T_{reg} cells, macrophages or DC [46,51], whereas the main source of *Leishmania* persistence-controlling IL-10 are $CD4^+$ $CD25^+$ T_{regs} [52].

1.2.3 Role of $CD4^+$ and $CD8^+$ T cells in the immunity to leishmaniasis

The development of a long-lasting immunity is mediated by $CD4^+$ memory and effector T cells. Effector T cells home to tissues, secrete cytokines and require persisting parasites to survive, whereas antigen-specific memory T cells proliferate and differentiate in secondary lymphoid organs into IFN-γ-secreting effector cells, stimulate DC maturation, and their persistence is independent of parasite occurrence [53,54].

Cure of *Leishmania* infection depends on the presentation of parasite-derived antigens via DC and macrophages to naïve lymphocytes. So primed $CD4^+$ and $CD8^+$ lymphocytes induce nitric oxide synthase (iNOS) and IFN-γ, thus mediating the production of NO and reactive oxygen species (ROS) in macrophages, ending up in killing of the intracellular parasite [17,55]. $CD8^+$ T lymphocytes are another source of IFN-γ, thus mediating immunity to *L. major* [56]. But $CD8^+$ T lymphocytes have also been reported to aggravate acute infection with *L. braziliensis* [57] and to enhance the manifestation of chronic *L. donovani* infection [58]. Persisting parasites were reported to be involved in sustaining a long-lasting immunity and resistance to subsequent infections [52]. Persisting parasites can be helpful to regulate a dynamic equilibrium between protective Th1 immune response and the immunoinhibitory effects of parasite-specific T_{regs} and IL-10. Only IL-$10^{-/-}$ mice achieved sterile cure demonstrating the requirement for IL-10 in establishing latency [59], as parasite persistence was not established in IL-$10^{-/-}$ C57BL/6 mice or in mice treated with α-IL-10R Ab [59].

1.2.4 DC and their role in anti-leishmanial immunity

An orchestra of immune reactions occurs from the time of infection until a parasite-specific immunity is established. During the initial infection, innate immune

cells play important roles in phagocyting and eliminating pathogens, presenting pathogen-derived antigens and producing cytokines. Immune cells, like macrophages, neutrophils, natural killer cells, mast cells or DC are important cell types to initiate protective immunity. DC are the most effective and outstanding cell type in instructing efficient anti-leishmanial immunity while presenting antigens to naïve T lymphocytes, thus priming $CD4^+$ Th cell subsets and NK cells [15,60]. Moreover, DC secrete the Th1-promoting cytokine IL-12 upon contact with *Leishmania* parasites, as migratory dermal DC are among the first host cells to take up invading pathogen, by recognizing parasite derived phosphoglycans (e.g. lipophosphoglycan, LPG) [61,62,63].

Even though the host immune system can combat infections, severe problems occur when facing pathogens with effective immune evasion mechanism. *Leishmania* for example developed multiple and powerful immune evasion mechanisms. The main evasion strategies are the inhibition of macrophage phagosomal killing or maturation, the inhibition of DC maturation and migratory capacities, the recruitment of inhibitory T_{reg} cells and most important, *Leishmania* inhibits the pivotal DC-derived IL-12 secretion, thus shaping a non-protective humoral immune response [22,50,55,60,64,65].

1.3 Treating *Leishmania* infections

1.3.1 Therapeutic treatments against leishmaniasis are facing problems

Systemic or intralesional antimonials are the gold standard used to treat human leishmaniasis. But a unique standard treatment is not established yet. The clinical outcome and the treatment strategies also depend on the causative *Leishmania* species, as the efficiency of treatment strategies varies between the different parasite species [66]. Species identification via genomic replication by

polymerase chain reaction (PCR) is not available in the endemic developing countries, resulting in the treatment of *Leishmania*-infected patients without the identification of the specific *Leishmania* species.

The major disadvantage of chemotherapeutics is owed to rising resistances to drugs, for example Amphotericin B, consequently resulting in the administration of a higher dosage of these toxic drugs [67,68]. The necessity to discover new drugs is favored, as most of the drug therapies are costly long-time treatments and resistances to current treatment strategies arise [12]. As mentioned previously, leishmaniasis is a complex disease based on the different parasite species causing varied disease outcome and an efficient approach to prevent patients from infection is not available, yet. The development of a cost-effective vaccination strategy could be a good exception to that, as it is also requested by the World Health Organization (WHO).

1.3.2 Prophylactic treatment

Designing an efficient vaccination strategy against leishmaniasis requires detailed understanding of the host-pathogen interactions to be able to induce parasite-specific immunity. Furthermore, new vaccines must correspond to the criteria of safety, reproducibility and efficacy. The most promising vaccine in dogs and in humans which is under evaluation in Phase I clinical trials in the United States of America is Leish-111f, is a tandemly linked multi-subunit recombinant *Leishmania*-vaccine formulated in monophosphoryl lipid A (MPL) in a stable emulsion [69,70,71].

Approaching a vaccine against leishmaniasis could be helpful to fight the infection listed as Category I Disease by the WHO, classifying leishmaniasis as an emerging and uncontrolled disease. The research of such a listed infection focuses on the acquisition of new knowledge and the design of new disease controlling tools

and systems. Nonetheless, leishmaniasis has been thought to be among the first infections where a vaccine would be feasible, owed to the induction of life-long protection upon recovery from CL [14].

The use of so called “leishmanization” allowed the deliberate introduction of lesions to children in endemic areas by using life and virulent *Leishmania* parasites from cutaneous lesions. This method has been practiced for centuries and is still used in some rural areas for example in Uzbekistan [14,72]. During the 1970s and 1980s large vaccination trials using live and virulent *L. major* promastigotes were developed and used in the Soviet Union and Israel, [14,73]. Although this approach was successful with regard to lesion development and subsequent immunity to *L. major* infection, safety concerns remained, reproducibility was not guaranteed and the viability and infectivity of the inoculated parasites varied resulting in delayed-type hypersensitivity in cases where the parasites were not virulent [73]. Observing harmful side effect, for example the development of large lesions and immunosuppression, terminated the use of leishmanization in many countries and switched the focus to killed vaccines [73,74] as recommended by the WHO. Even though, a lot of knowledge is gained regarding the genetics of the parasite and the pathoimmunology and significant effort was spent in generating a prophylactic vaccine, no control strategies are available against human leishmaniasis, yet [6,75].

1.3.3 Immunization strategies

These gained information supported the idea that the generation of a vaccine against different forms of leishmaniasis should be realistic [74,76]. Different vaccination strategies have been developed over the years, which can be divided into three categories. 1) Live virulent or attenuated *Leishmania* parasites [73,77]; knock-out parasites [76] or parasites containing a suicidal cassettes [74], 2) Killed

Leishmania parasites with or without adjuvant [73,76,78] and 3) Recombinant or synthetic DNA [79] or recombinant protein vaccine [80,81]. Vaccination strategies using killed parasites or recombinant parasite-derived proteins are favored, owed to the risky handling of live vaccines, even though a single administration of non-virulent *L. tarentolae* parasites into mice resulted in cross-reactive humoral and cellular immunity - also between species - [77] and live-attenuated parasites closely mimic natural infection [73].

Studies in the 1980s using killed *Leishmania* parasites revealed the importance of the route of administration, as protection was only achieved in mice injected intravenously or intraperitoneally [76]. This is of important notice as up to that point vaccination studies using killed *Leishmania* parasite were administered intramuscular or subcutaneous and failed to confer protection. Only as the importance of the site of administration was found, this vaccination strategy was successful in mice when injected intravenously [65,82]. Even nowadays, autoclaved *L. mexicana* parasites are used in Venezuela as immunotherapeutic to treat CL [73,76]. Independent of the success of killed vaccination strategies, there is also the disadvantage of lowered immunogenicity by destroyed parasite surface proteins, thus it is not mimicking a natural infection with regard to immunogenic reaction and pathogenesis [83].

DNA vaccination strategies, established in the 1990s, are postulated to be the most promising vaccination strategy. The intramuscular injection of purified recombinant antigenic proteins encoded on plasmid DNA resulted in the endogenous expression of parasite derived proteins *in vivo* [84,85,86]. The advantages of a DNA vaccine are obvious. Different *Leishmania* species-specific proteins can be encoded in one vaccine and the antigenicity is kept by the expression of unaltered or unchanged *Leishmania* antigens [87]. Furthermore, bacterial-derived DNA plasmids

are immunogenic *per se* due to their unmethylated cytosine-phosphate-guanosine (CpG) motifs in their backbone, inducing Th1 cell-mediated immunity which is known to be essential to confer protection to *Leishmania* infection [85,88].

Novel vaccine strategies include components of sand fly saliva or antigen-presenting cells (APC) as vaccine carrier [89,90]. Approaches with sand fly saliva use the knowledge that saliva enhances the infectivity of *Leishmania*, as for example the saliva component SP15 antigen conferred protection to *L. major* infection in mice [90,91,92].

1.4 Dendritic cells

Enormous amounts of knowledge was gained from DC and their role in immune response since they were firstly discovered in 1973 in peripheral lymphatic organs of mice [93]. DC are an important element of the immune system due to their ability to function as sentinels in the periphery and to initiate strong T lymphocyte response to the invading pathogen.

Mouse DC are classified either as plasmacytoid DC (pDC) or myeloid DC (mDC), like dermal DC and Langerhans cells (LC), which act as migratory sentinels [94], being rapidly recruited by the neutrophil-derived chemokine CCL3 to the site of infection [95,96,97].

DC are migratory APC which are highly specialized in uptake, processing and presentation of pathogen-derived antigens via MHC molecules to T cells [98] within lymphoid organs, resulting in the cytokine-regulated differentiation into Th1 or Th2 cells, thus initiating cellular immunity to infection [99,100].

Immature DC reside in peripheral tissues until they are activated by pathogens, thus resulting in structural and functional transformation [99]. The migratory capacity of DC upon activation to lymphoid organs is unique to DC and it is

not shared by other APCs [98,101]. The maturation process is characterized by increased expression of MHC and co-stimulatory molecules, down-regulation of phagocytic capacity and antigen-uptake, enhanced cytokine secretion, expression of chemokine receptors, enabling DC migration and recruitment of other cell types [102]. Activated DC are characterized by up-regulated and increased expression of MHC class I and II molecules, co-stimulatory molecules (CD80 and CD86), as well as enhanced IL-12 expression [103,104]. Thus activated, DC have the ability to present antigens via MHC class I to $CD8^+$ T lymphocytes as a result of Fcγ Receptor (FcγR)-mediated phagocytosis and via MHC class II to $CD4^+$ T lymphocytes as a result of complement receptor (CR)3-mediated phagocytosis of parasites [105]. Due to cytokine secretion upon activation, DC polarize naïve Th cell-proliferation and initiate an inflammatory response to infection.

The induction of $CD4^+$ T lymphocytes is the most important factor in mediating either susceptibility or resistance to *Leishmania* infections, depending on the predominant Th subset [17]. Resistance against leishmaniasis is associated with a cytokine milieu dominated by TNF-α and Th1-derived IFN-γ [106], whereas a Th2 immune response characterized by IL-4, IL-10, and IL-13 correlates with susceptibility [107].

1.5 Dendritic cells as vaccine carrier

As already mentioned above, arising resistances to chemotherapeutics and the high safety concerns of prophylactic treatment using virulent parasites (leishmanization), lead to the focusing on novel adjuvant-vaccination strategies in research and development in experimental leishmaniasis studies. It has been shown that the use of non-living *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) as an

adjuvant failed to confer substantial protection to humans against CL [108], assuming that no long-lasting vaccine-induced immunity was mediated [109].

DC-mediated vaccination or immunotherapy against infection is emerging, since DC are long-lived pivotal APC ensuring the maintenance of an efficient antimicrobial T cell stimulation in the lymphatic tissues [102,110]. DC are shown to have immunemodulatory capacities *in vivo* and this characteristic allows the use of DC as an adjuvant for immunization against *Leishmania* infections.

The experimental *Leishmania* model uncovered the importance of the route of antigen delivery in influencing T cell priming, as it was also observed in experimental tumor studies [82,111,112]. Sustained non-protective Th2 responses were obtained in otherwise resistant mice, when vaccine administration occurred intravenously or intranasal [113]. Critical parameter determining the efficiency of immunization depends crucially on an intravenous route of injection [114], even if some studies demonstrated a prophylactic effect against experimental murine VL upon intra muscular injection of radio-attenuated *Leishmania* parasites [115].

The central role of DC in immunoregulation, their capacity to elicit strong T cell responses is exploited in DC-mediated vaccination strategies. In these strategies, DC are used as adjuvant to introduce pathogen-derived antigens to the host immune system. For this occasion, DC are isolated from mice or humans, loaded with antigens *ex vivo* and used to promote immunity or therapeutic effects not only in *Leishmania*-infected hosts but also in malignant diseases, like renal cell and prostate carcinoma or other bacterial and parasitic infections, like *Borrelia burgdorferi*, *Mycobacterium bovis* BCG, *Chlamydia trachomatis*, *Candida albicans* and *Toxoplamsa gondii* [116,117,118,119,120,121,122,123,124]. First studies demonstrated the possible role of LC as natural adjuvant in inducing immunity to subsequent experimental infection with *L. major* [125]. It has been shown that a

single i.v. immunization with *in vitro L. major* antigens (LmAg)–pulsed LC conferred protection against experimental *L. major* infection in BALB/c mice. Resistance was associated with reduced parasite burden at the site of infection and other infected tissues, and a shift towards a protective Th1 cell-mediated immunity [125]. Another study using *L. donovani* antigen-pulsed DC, engineered to secrete IL-12, conferred protection in BALB/c mice [126].

Whereas IL-12 secretion by macrophages is down-regulated, DC readily produce IL-12 upon infection with *L. major* which is known to be crucial for the differentiation of naïve T cells into parasite-specific Th1 lymphocytes [127,128]. The importance of DC-derived IL-12 in this vaccination strategy was shown in studies using LmAg-pulsed LC from IL-$12^{-/-}$ mice resulting in abrogated capacities to induce protection [62,129]. CpG-containing oligodeoxynucleotides (ODN) has been shown to be a strong DC stimulus, thus promoting a strong Th1 immunity and resistance to infection in DC-mediated vaccination strategies [130,131]. High levels of $CD11c^{+}$ DC were detected in the draining LN of so protected mice, which were potent inducers of Th1-promoting IL-12p70 and IFN-γ [131].

Moreover, tumor antigen-pulsed DC are highly efficient vaccines in inducing T cell-mediated anti-tumor immunity in patients and immunization with antigen-containing DC-targeting liposomes, like amphotericin B [116,117,118,132]. For this purpose, DC are generated *ex vivo*, loaded with tumor antigens and re-injection into the host, thus inducing a strong T cell responses upon migration of DC from the site of inoculation to the T cell area of lymphoid organs [82,116,118]. The *ex vivo* generation of such DC-based vaccines allows the appropriate loading with antigens on different needed DC maturation and activation stages and the fine-tuning using different cytokine-inducing stimuli prior to injection. Recent studies in cancer patients

and in experimental leishmaniasis showed that only mature DC induce tumor-specific or parasite-specific cell-mediated immunity [65,129,133,134].

Being able to establish a standard protocol to generate specifically modulated and primed DC-based vaccines, would simplify the evaluation of possible vaccine candidates in preclinical experimental studies.

1.4 Interleukin-4 and its Interleukin-4 Receptor

A distinct cytokine environment is essential for the differentiation of immune cells into either Th1 or Th2 cells. With regard to this, IL-4 counter-regulates Th1-mediated immunity and promotes disease progression, whereas IFN-γ in combination with IL-12 is a strong Th1 lymphocyte inducing cytokine [135]. Concluding from this, IL-4 plays a key role in inducing IL-4 production in $CD4^+$ T cells, determining for Th2 cell differentiation. Following these results and many others gained before, IL-4 was postulated as disease promoting cytokine, which is crucial for the development of Th2-mediated immune responses.

To investigate the functional role of IL-4-mediated signaling during *Leishmania* infection, various knock-out mice have been generated. IL-$4^{-/-}$ BALB/c mice, remained susceptible to infection with *L. major*, as a protective Th1 immune response has not been established [136]. Contrary results were observed in the same model, showing that IL-$4^{-/-}$ BALB/c mice became resistant to infections, but not when infected with virulent *L. major* strains [30,136,137,138]. Global IL-4Rα chain-deficient BALB/c (IL-4R$\alpha^{-/-}$) mice were generated as described elsewhere [139] to investigate the disease outcome of leishmaniasis in the absence of IL-4-mediated functions. These mice showed an unimpaired Th2 polarization in response to an infection with *L. major* [140]. IL-13 can substitute for IL-4 in mediating non-healing Th2 responses,

as IL-4 and IL-13 double knock-out mice and IL-4Rα$^{-/-}$ BALB/c mice were resistant to infection [137,138,141].

Cell-specific IL-4Rα$^{-/-}$ mice have been generated to investigate the impact of IL-4Rα-mediated signaling on various cell types during *Leishmania* infection. CD4^{+} T cell-specific Lck^{cre}IL4Rα$^{-/lox}$ BALB/c mice confirmed the detrimental role of IL-4-induced proliferation of Th2 lymphocytes. These results also indicated that IL-4Rα responsive cells are involved in protection against leishmaniasis [142] as these mice were resistant to infection with *L. major*, suggesting that another IL-4 responsive non-CD4^{+} T cell population mediates protection against experimental CL. Studies using macrophage specific LysM^{cre}IL4Rα$^{-/lox}$ BALB/c mice showed that IL-4Rα signaling in macrophages is rather involved in parasite dissemination and parasite growth, than in the induction of Th1 immunity to infection [44].

In contrast to the Th1/Th2 associated resistance or susceptibility to experimental *L. major* infections, studies using IL-4$^{-/-}$ BALB/c mice demonstrated the protective role of IL-4 during infection [143,144], as the disruption of IL-4 did not convert these mice resistant to infection with neither *L. major* nor *L. donovani*, but even worsened the disease outcome [144]. IL-4Rα$^{-/-}$ BALB/c mice were significantly more susceptible to *L. donovani* infections compared to IL-4$^{-/-}$ mice [143]. These studies show that other factors beside IL-4 contribute to disease progression and that the role of this key Th2 cytokine is more than controversial.

The IL-4 receptor is a heterodimer complex consisting of the IL-4Rα chain and a common γ chain (γc) [145]. The IL-4R type I, is composed of the IL-4Rα chain and the γc chain and can only be triggered by IL-4, whereas the IL-4R type II, consisting of the IL-4Rα chain and the IL-13Rα1 chain can be triggered by IL-4 as well as IL-13 [146]. Not only IL-4, but also IL-13 can signal through this IL-4Rα chain along with IL-

13Rα1, consequently resulting in abrogated IL-4- and IL-13-mediated functions in IL-4Rα$^{-/-}$ mice, while IL-13-mediated functions are preserved in IL-4$^{-/-}$ mice [139,147].

Previous studies comparing IL-4Rα$^{-/-}$ and IL-4$^{-/-}$ BALB/c mice led to the suggestion that IL-13, which shares the IL-4Rα subunit with IL-4, could promote hepatic granuloma formation and control parasite burdens in mice suffering from VL [148]. Data gained from comparative *L. donovani* infection-studies in IL-13$^{-/-}$ and IL-4Rα$^{-/-}$ BALB/c mice indicated the role of IL-13 in promoting anti-leishmanial Th1 cell-mediated immunity. As murine lymphocytes lack the IL-13Rα1, the authors suggested that IL-13 signals through DC, thus promoting protective immunity [148].

The results gained within the last decade reflect a substantial body of evidence for the contrary functions of IL-4. On the one hand, IL-4 is a Th2-promoting cytokine, but on the other hand, IL-4 can facilitate an anti-leishmanial immune response. These type-1 response-inducing capacities of IL-4 have not only been shown in experimental leishmaniasis, but also in experimental *Toxoplasma gondii* and *Candida albicans* infections [149,150], most probably by instructing IL-12 secretion of DC [151]. It has been demonstrated that the presence of IL-4 during DC activation, but not during T cell priming instructs DC to produce IL-12 and promotes Th1 cell-maturation and resistance to leishmaniasis. These data indicate that the contrary functions of IL-4 *in vivo* depend on the IL-4 sensitive cell type.

To investigate the potential role of IL-4 responsive DC and the IL-4 instruction on DC in facilitating protection against leishmaniasis, DC-specific CD11c^{cre}IL-4Rα$^{-/lox}$ BALB/c mice were generated (Hurdayal et al., manuscript under revision). Those mice are hyper-susceptible to infection with *L. major,* indicating the important role of IL-4 signaling on DC in the establishment of a protective Th1 cell-mediated immunity.

1.5 *Leishmania*-host cell interactions

Phagocytes represent some of the most potent players in innate and adaptive immunity to infection with *Leishmania*, as these cells have the capacity to eliminate the invading pathogen due to their microbicidal effector mechanism [15]. Research into the discovery of drugs and vaccines for leishmaniasis uncovered the complexity of the interaction of *Leishmania* with immunological cells, like neutrophils, macrophages or DC, as the parasite developed successful escape mechanisms to circumvent the phagocytic environment and also to manipulate the immune response of the host for their own benefit [152]. The majority of studies on *Leishmania*-host cell interactions are based on *in vitro* studies with murine cells or on *in vivo* experiments with *Leishmania*-infected mice.

Neutrophils are known as the “first line” defense against invading pathogens, as their granules contain antimicrobial proteins and lytic enzymes, and possess the ability to degrade the invading pathogen upon phagocytosis [153]. An immune escape mechanism by *Leishmania* allows the persistence of the parasite within neutrophils, which are the first cell type that phagocytose *Leishmania* [153,154] by inhibition of apoptotic functions of the neutrophils [155]. This is an efficient strategy of the parasite to avoid the recruitment and activation of macrophages, as *Leishmania* “hides” from the immune system by using neutrophils as “Trojan horses” [156,157].

Macrophages are the most frequented host cell of *Leishmania*. Macrophage-specific phagocytic capacities are inhibited by *Leishmania*, allowing the replication and survival within this hostile environment by escaping the lysosomal digestion and degradation within the phagolysosome [158]. The main leishmanicidal function of macrophages is the synthesis of NO by L-arginine oxidized by iNOS or by arginase 1 [159]. Interestingly, *Leishmania* parasites express arginase 1 themselves [160], highlighting one of many immune evasion strategies to ensure parasite survival. The

leishmanicidal activity of alternatively activated macrophages is suppressed upon IL-4-induced arginase 1 expression, consequently promoting early disease progression, while IL-4-induced arginase 1-expressing DC promote progressive leishmaniasis during the onset of infection [161,162].

1.5.1 *Leishmania*-DC interactions

Leishmania uptake by mDC is a highly dynamic process occurring in a FcγR I- and III- or CR3-mediated manner within the first few hours of infection [105,163,164], enabling the parasites to modulate DC functions as well as DC-induced immune response. *Leishmania* amastigotes are able to suppress DC activation and maturation by various mechanisms, termed “silent entry” [165]. *L. amazonensis, L. donovani, L. infantum, L. pifanoi* and *L. mexicana* circumvent DC activation by targeting the C-type lectin receptor, ICAM-3-grabbing nonintegrin (DC-SIGN) on the surface of DC [166]. Another potent immune evasion mechanism used by *Leishmania* is the manipulation of the migratory functions of DC. *L. major*-secreted products inhibit the motility of splenic DC [167] and the skin emigration of LC is diminished by purified *L. major* LPG [168], indicating the ability of *Leishmania* parasites to alter the transport of antigens to lymphoid tissues [169].

However, the DC maturation status influences the intracellular fate of *Leishmania*. It has been shown that *L. major* promastigotes reduce lysosomal degradation in immature DC as a consequence of decreased fusion activity of parasite-containing phagosomes with lysosomes, resulting in enhanced parasite survival within the host cell [170]. The fusion retardation in immature DC allows the differentiation of promastigotes into amastigotes, which are more adapted to the harsh conditions within lysosomal compartments. In contrast, parasite degradation in mature DC occurs after parasite-containing phagosomes acquire small GTPase Ras-

related protein (Rab) 7 and fuse with lysosomes [170]. *Leishmania* amastigotes are potent suppressors of DC functions. Rapid phosphorylation of mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK)1/2 is mediated by *L. amazonensis* amastigote-derived proteases or proteasomes, whereas phosphorylation of MAPK/ERK occurs with a 4 hours delay in DC infected with promastigotes, most likely associated with their differentiation into amastigotes [171]. In contrast to the activation of the MAPK/ERK pathway in *L. amazonensis*-infected DC resulting in the inhibition of IL-12 [172], *L. major* infection mediates the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and the up-regulation and nuclear translocation of interferon regulatory factor (IRF)-1 and IRF-8, thus promoting an IL-12- and type I IFN-induced protective Th1 response [173]. The phosphatidylinositol 3-kinase (PI3K) negatively regulates excessive IL-12 secretion by DC, thus preventing potential immunopathologic and pro-parasitic effects mediated by uncontrolled sensitization and disproportionate physiological responses [174].

These observations document that *Leishmania* parasites developed complex and wide-ranging immune evasion strategies to assure parasite survival within a hostile environment. The knowledge about *Leishmania*-host cell interactions collected in the murine model will facilitate the development of novel strategies to combat *Leishmania* infections in humans.

2. Aims of the study

Prevention of tissue damages at the site of *L. major* inoculation can be achieved if the BALB/c mice were systemically given LmAg-loaded bone marrow-derived DC (BMDC) that had been exposed to CpG ODN [65]. DC-mediated immunity to infection is achieved by the conversion of an IL-4-driven Th2 response, converting a progressive leishmaniasis into a protective Th1-mediated immunity, indicated by an IL-12- and IFN-γ-dominated cytokine pattern.

As previous studies allowed establishing that IL-4 was involved in the redirection of the immune response towards a type 1 profile [149,175], especially demonstrating that IL-4 instruct BMDC to secrete elevated levels of IL-12, I was interested in further exploring the role of IL-4Rα-mediated instruction of the vaccinating DC and the host DC during induction of protection against leishmaniasis. For this purpose, wild type (wt) BALB/c mice or DC-specific $CD11c^{cre}$IL-$4R\alpha^{-/lox}$ BALB/c mice were given either wt or IL-$4R\alpha^{-/-}$ LmAg-loaded BMDC exposed or not to CpG ODN prior to inoculation of 2 x 10^5 stationary phase *L. major* promastigotes into the BALB/c footpad.

The clinical outcome was observed and immunological analyses were performed to determine any possible differences in the effectiveness of the IL-4 responder and non-responder BMDC used as vaccine carrier.

Material and Methods

3. Material and Methods

3.1 Materials

Antibodies

Antibody	Manufacturer
AKP Streptavidin	BD Biosciences Pharmingen Co, Heidelberg, Germany
IgG1*, κ (A19-3)	BD Biosciences Pharmingen Co, Heidelberg, Germany
IgG1*, λ1 (G235-2356)	BD Biosciences Pharmingen Co, Heidelberg, Germany
IgG_{2a}, κ (G155-178)	BD Biosciences Pharmingen Co, Heidelberg, Germany
Rat anti-mouse CD11c (HL3)	BD Biosciences Pharmingen Co, Heidelberg, Germany
Rat anti-mouse CD16/CD32 (2.4G2)	BD Biosciences Pharmingen Co, Heidelberg, Germany
Rat anti-mouse CD4 (GK1.5)	BD Biosciences Pharmingen Co, Heidelberg, Germany
Rat anti-mouse CD4 (RM4-5	BD Biosciences Pharmingen Co, Heidelberg, Germany
Rat anti-mouse CD80 (16-10A1)	BD Biosciences Pharmingen Co, Heidelberg, Germany
Rat anti-mouse Dendritic Cells (33D1)	BD Biosciences Pharmingen Co, Heidelberg, Germany
Rat anti-mouse F4/80 (BM8)	Biolegend, Uithoorn, Netherlands
Rat anti-mouse I-A^d (AMS-32.1)	BD Biosciences Pharmingen Co, Heidelberg, Germany
Rat anti-mouse IFN-γ (XMG1.2)	BD Biosciences Pharmingen Co, Heidelberg, Germany
Rat anti-mouse IL-10 (JES5-16E3)	BD Biosciences Pharmingen Co, Heidelberg, Germany

Rat anti-mouse IL-12 (p40/p70) (C15.6)	BD Biosciences Pharmingen Co, Heidelberg, Germany
Rat anti-mouse IL-4 (11B11)	BD Biosciences Pharmingen Co, Heidelberg, Germany
Rat anti-mouse IL-4 Receptor (mIL4R-M1)	Genzyme, Neu-Isenburg, Germany
Recombinant mouse IL-13	BD Biosciences Pharmingen Co, Heidelberg, Germany
Recombinant mouse IL-4	BD Biosciences Pharmingen Co, Heidelberg, Germany
Streptavidin	AbD Serotec, Puchheim, Germany

Chemicals

Chemicals & Reagents	Manufacturer
1-Propanol	Carl Roth, Karlsruhe, Germany
2 log DNA ladder	New England BIOlabs, Frankfurt, Germany
2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethansulfonacid (Hepes)	Invitrogen, Darmstadt, Germany
2-Mercaptoethanol	Sigma-Aldrich, Schnelldorf, Germany
2-Propanol	Carl Roth, Karlsruhe, Germany
Acetic acid	Carl Roth, Karlsruhe, Germany
Acrylamide	Serva, Heidelberg, Germany
Agarose ultra pure	Gibco, Paisley, UK
Albumin-Fraction V	AppliChem GmbH, Darmstadt, Germany
Ampicillin sodim salt	Sigma-Aldrich, Steinheim, Germany
Ampuwa® sterile water	Fresenius Kabi, Bad Homburg, Germany
BSA	Sigma-Aldrich, Schnelldorf, Germany

Calium chloride	AppliChem GmbH, Darmstadt, Germany
Citric acid	Sigma-Aldrich, Schnelldorf, Germany
CpG ODN	Qiagen Operon, Köln, Germany
D (+) Glucose	Carl Roth, Karlsruhe, Germany
DABCO	Carl Roth, Karlsruhe, Germany
Dimethylsilfoxid	AppliChem GmbH, Darmstadt, Germany
DMEM	Gibco, Paisley, UK
dNTP Mix	Invitrogen, Darmstadt, Germany
Dulbecco´s Phasphate buffered Saline (PBS)	Gibco, Paisley, UK
EDTA Disodiumsalt	AppliChem GmbH, Darmstadt, Germany
Ethanol	Carl Roth, Karlsruhe, Germany
Ethidiumbromide	Sigma-Aldrich GmbH, Steinheim, Germany
FACS Clean	BD Biosciences Phamingen™, Heidelberg, Germany
FACS Flow	BD Biosciences Phamingen™, Heidelberg, Germany
FACS Rinse	BD Biosciences Phamingen™, Heidelberg, Germany
Fetal calf serum	PAA Laboratories, Pasching, Austria
Formaldehyde	Merck, Darmstadt, Germany
Gentamycin solution	Sigma-Aldrich, Schnelldorf, Germany
Giemsa	Sigma-Aldrich, Schnelldorf, Germany
Glutaraldehyde	Sigma-Aldrich, Schnelldorf, Germany
Hydrogen peroxide	Sigma-Aldrich, Schnelldorf, Germany

Hygromycin B	Sigma-Aldrich GmbH, Steinheim, Germany
Isofluran	Abbot GmbH & Co KG, Wiesbaden, Germany
L-Glutamine	BIOchrom AG, Berlin, Germany
Luglosch´solution	AppliChem GmbH, Darmstadt, Germany
MACS Bleach solution	Miltenyi Biotech, Bergisch Gladbach,Germany
MACSQuant Running Buffer	Miltenyi Biotech, Bergisch Gladbach,Germany
MACSQuant Storage and washing solution	Miltenyi Biotech, Bergisch Gladbach,Germany
Magnesium sulfate	AppliChem GmbH, Darmstadt, Germany
Methanol	Carl Roth, Karlsruhe, Germany
Monensin sodium salt	Sigma-Aldrich, Schnelldorf, Germany
N,N-Dimethylformamid	Carl Roth, Karlsruhe, Germany
Paraformaldehyde	AppliChem GmbH, Darmstadt, Germany
PBS	Gibco, Paisley, UK
Penicillin G sodium salt	Sigma-Aldrich, Schnelldorf, Germany
Phenol-Chloroform	Merck, Darmstadt, Germany
p-Nitrophenyl N-acetyl-b-D-Glucosaminide	Sigma-Aldrich, Schnelldorf, Germany
p-Nitrophenyl phosphate	Sigma-Aldrich, Schnelldorf, Germany
Primer	Eurofins MWG Operon, Ebersberg, Germany
Propidium iodide solution	Sigma-Aldrich, Schnelldorf, Germany
Protease Inhibitor Cocktail	Thermo Scientific, Waltham, USA
recombinant GM-CSF	PeproTech, London, UK

RPMI 1640	Invitrogen, Karlsruhe,Germany
SAV-AP Complex	BD Biosciences Pharmingen Co, Heidelberg, Germany
Saponin	Carl Roth, Karlsruhe, Germany
Sodium acetate	AppliChem GmbH, Darmstadt, Germany
Sodium carbonate	Merck, Darmstadt, Germany
Sodium deoxycholate	Sigma-Aldrich, Schnelldorf, Germany
Sodium hydrogen carbonate	AppliChem GmbH, Darmstadt, Germany
Sodium phosphate	Sigma-Aldrich, Schnelldorf, Germany
Sodium stibogluconate	Sigma-Aldrich, Schnelldorf, Germany
Streptomycin sulfate	Sigma-Aldrich, Schnelldorf, Germany
Taq DNA Polymerase	New England Biolabs, Frankfurt, Germany
Tris	Carl Roth, Karlsruhe, Germany
Tris-HCL	Sigma-Aldrich, Schnelldorf, Germany
Trypan Blue	Invitrogen, Darmstadt, Germany
Trypsin	Sigma-Aldrich, Schnelldorf, Germany
Tween 20	Sigma-Aldrich, Schnelldorf, Germany

Equipment

Equipment	Manufacturer
13R fuge	Heraeus Sepatech GmbH, Osterode, Germany
Ascent Software®	Thermo Electron Corporation, Erlangen, Germany
Biofuge 13	Heraeus Sepatech GmbH, Osterode,

	Germany
CellQuest Pro 2.0®	BD Biosciences Pharmingen Co, Heidelberg, Germany
ELISA Reader MRX	Dynatech laboratories, Chantilly, USA
FACS Calibur ™	BD Biosciences Pharmingen Co, Heidelberg, Germany
Fresco 21 centrifuge	ThermoScientific GmbH, Langenselbold, Germany
Gel documentation system INTAS	Intas, Göttingen, Germany
GraphPad Prism®	GraphPad Software, San Diago, USA
Incubator	Binder Labortechnik GmbH, Tuttlingen, Germany
Incubator	Heraeus Instruments GmbH, Osterode, Germany
MACSQuant™	Miltenyi Biotec, Bergisch Gladbach, Germany
Mastercycler	Eppendorf AG, Hamburg, Germany
Megafuge 1.0 R	Heraeus Sepatech GmbH, Osterode, Germany
Microscope Zeiss Axiolab	Carl Zeiss, Jena, Germany
Mini Spin	Eppendorf AG, Hamburg, Germany
Multifuge X1R	ThermoScientific GmbH, Langenselbold, Germany
Sterile bench Safe 2020	ThermoScientific GmbH, Langenselbold, Germany

3.2 Methods

3.2.1 Genotyping of IL-4Rα-deficient BALB/c mice

To confirm the loss of a functional IL-4Rα chain in the population of breeded BALB/c mice, tail biopsies were collected. These tail biopsies were used to isolate DNA following the isopropanol purification protocol. In detail, tail biopsies were lysed in 200µg/ml Proteinase K-containing lysis buffer (100mM Tris, 5mM ethylene diamine tetraacetic acid (EDTA), 0.2% SDS, 200mM NaCl, pH 8.5) over night under shaking conditions at 56°C. Upon centrifugation for 10 min at 13000 rpm the viscous DNA-containing supernatant was precipitated in isopropanol and centrifuged for 20 min at 13000 rpm at 4°C. The precipitated DNA-containing pellet was washed in 70% ethanol, centrifuged for 10 min at 13000 rpm and air dried. The DNA was resuspended in TE buffer (10mM Tris, 1mM EDTA, pH 8.0), incubated for four hours at 56°C and stored at 4°C until PCR analysis was performed.

To amplify the targeted DNA by PCR, specific primer pairs have been used. Amplification of DNA with IL-4R wt sense (GTA CAG CGC ACA TTG TTT TT) and IL-4R wt antisense (CTC GGC GCA CTG ACC CAT CT) primer resulted in a 153bp wt fragment, whereas the amplification with IL-4R KO E10 reverse (ACC TGT GCA TCC TGA ATG AT) and IL-4R KO intron 6-2 (CCC TTC CTG GCC CTG AAT TT) primer resulted in the amplification of a 1300bp IL-4R$\alpha^{-/-}$ fragment. All primers were purchased as unmodified oligos from eurofins mwg operon (Ebersberg, Germany) at a synthesis scale of 0.05µmol and HPSF purified. The PCR reaction mixture contains the ingredients listed in Table 1.

Table 1. PCR for detection of IL-4Rα

reagent	final concentration	final volume
ddH_2O		
wt PCR		35.75 µl
IL-4Rα$^{-/-}$ PCR		37.75 µl
10x Buffer	2 mM	5 µl
dNTPs	2 pmol/µl	4 µl
Primer sense	2 pmol/µl	1 µl
Primer antisense		1 µl
DNA template		
wt PCR		3 µl
IL-4Rα$^{-/-}$ PCR		1 µl
Taq-Polymerase		0.25 µl
Total volume		**50µl**

Table 2 shows the PCR program used for the amplification of DNA:

Table 2. PCR program for genotyping of IL-4Rα$^{-/-}$ and wt mice.

temperature	duration	cycles
94°C	3 min	
94°C	45 sec	
Wt primer:		
59°C	30 sec	
IL-4Rα$^{-/-}$ primer:		
54°C	30 sec	
72°C	1 min 45 sec	36
72°C	3 min	
4°C	forever	

For detection of DNA, 1% agarose gels were prepared in TAE buffer (40mM Tris, 1mM EDTA, 4mM acetic acid, pH 8.0). DNA samples were diluted in 6x sample buffer. Gels were run at a current of 100mA and a voltage of 80V prior to DNA staining in ethidium bromide solution (0.5 µg/ml) and visualized using UV transilluminators.

3.2.2 Mice

Sex- and age-matched wt BALB/c (Charles River Breeding Laboratories, Sulzfeld, Germany) and CD11c^{cre}IL-4R$\alpha^{-/lox}$ BALB/c mice (Hurdayal et al., manuscript under revision) were 6-8 weeks old at the onset of the experiments. IL-4R$\alpha^{-/-}$ BALB/c mice were kindly provided by Prof. Dr. Gottfried Alber (University of Leipzig, Germany) [176]. All mice were kept under specific pathogen-free conditions. Mice experiments were performed in strict accordance with the German Animal Welfare Act 2006 (TierSchG) and the animal protocol was approved by the government of Lower Franconia (permission no. 55.2-2531.01-16/09) and by the Animal Research Ethics Committee of the University of Cape Town, South Africa (license no. 009/042).

3.3.3 Generation of bone marrow-derived dendritic cells

DC were generated from bone marrow progenitors as described previously [177]. Briefly, isolated bone marrow cells from 6-8 weeks-old female BALB/c or IL-4R$\alpha^{-/-}$ mice were cultured in RPMI 1640 medium (Invitrogen, Karlsruhe, Germany) in the presence of 200 U/ml recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF; PeproTech, London, United Kingdom). Fresh medium supplemented with GM-CSF was added to the culture on days 3 and 6. After 10 days, non-adherent cells were harvested and used for further experiments. These cells were shown to have the typical mDC morphology [65].

3.3.4 *L. major* parasites and preparation of *L. major* antigens (LmAg)

The virulent *L. major* isolate (MHOM/IL/81/FE/BNI) was maintained by continuous passage in BALB/c mice. Amastigotes were isolated from lesions as previously described [178]. Promastigotes were grown *in vitro* in blood-agar cultures. For the preparation of LmAg, stationary-phase promastigotes were subjected to three

cycles of rapid freezing and thawing and diluted to a final concentration of 1 x 10^9 /ml in phosphate-buffered saline (PBS).

3.3.5 Stimulation of BMDC used as vaccine carrier

After 10 days of culture, BMDC were incubated for 4 hours in the presence of either 25 µg/ml CpG ODN 1668 (5´- TCCATGACGTTCCTGATGCT - 3´, Qiagen Operon, Cologne, Germany), 20 ng/ml recombinant mouse IL-4 (rIL-4; BD Biosciences, Heidelberg, Germany) or 20 ng/ml recombinant mouse IL-13 (rIL-13; BD Biosciences, Heidelberg, Germany), or a combination of CpG ODN and one recombinant cytokine, prior to the addition of 30 µl/ml LmAg for 18 hours. The cells were incubated at 37°C and 5% CO_2. Thereafter, stimulated BMDC were washed and resuspended in PBS.

3.3.6 Enzyme-linked immunosorbent assay (ELISA)

The supernatant of differentially stimulated BMDC or lymphocytes were analyzed for their cytokine composition by sandwich ELISA. Briefly, 96-well plates were coated with purified capture anti-cytokine mAb diluted in carbonate buffer overnight at 4°C. To reduce unspecific binding, plates were incubated with blocking solution (10% bovine serum albumin (BSA) in PBS-Tween) for 4 hours prior to the addition of cell supernatants for overnight. Recombinant IL-4, IL-10, IL-12p70 and IFN-γ (all Ab purchased from BD Biosciences, Heidelberg, Germany) was plated as serial 1:2 dilutions and used to set up a standard curve. Incubation with the respective biotin-conjugated capture Ab was followed by the incubation with horseradish peroxidase (HRP)-conjugated streptavidin (1:1000 in PBS; Dako Diagnostica GmbH, Hamburg, Germany), each for one hour and developed with p-nitrophenyl phosphate (1 mg/ml, Sigma) dissolved in diethanolamine buffer. The

optical density at 490 nm and 405 nm was determined with an ELISA reader (MRX, Dynatech laboratories, Chantilly, USA) and cytokine concentrations were calculated by extrapolating the OD values to the linear regression of the standard curve.

3.3.7 Treatment of mice

BALB/c and CD11c^{cre}IL-4R$\alpha^{-/lox}$ BALB/c mice were treated with 5 x 10^5 BMDC intravenously (i.v.) into the tail vein. Control mice were treated with PBS. One week post vaccination the mice were infected subcutaneously into the right hind footpad with 2 x 10^5 stationary-phase *L. major* promastigotes in a final volume of 30µl in PBS. The course of infection was monitored weekly by measuring the increase in footpad size of the infected versus the noninfected footpad.

One, three or six weeks post infection, mice were sacrificed and single cell suspensions from the infected footpads as well as the draining popliteal LN were obtained. The parasite burden was determined by limiting dilution assays as described previously [179].

3.3.8 Flow cytometry

3.3.8.1 Extracellular Fluorescence activated cell sorting (FACS)

Lymphocytes were fixed with paraformaldehyde (PFA, 4%) and resuspended in FACS buffer (0.1% NaN_3 and 2.5% heat inactivated fetal calf serum (FCS)) or MACS buffer (0.5% BSA and 2 mM EDTA) containing anti-Fc receptor Ab (purified rat-anti mouse CD16/CD32) together with the appropriate combinations of the following surface marker: Biotin-streptavidin (SAV)-HRP-conjugated anti-CD11c (HL3); fluorescein isothiocyanate (FITC)-conjugated anti-I-A^d (AMS-32.1); and phycoerythrin (PE)-conjugated CD80 (16-10A1) (all Ab were purchased from BD Biosciences, Heidelberg, Germany). Staining with isotope-matched control antibodies

was performed. Data was obtained using either the FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany) or MACSQuant Analyzer (Miltenyi Biotech, Bergisch Gladbach, Germany) and analyzed using FlowJo (Tree Star Inc., CA, USA).

3.3.8.2 Intracellular FACS

1 x 10^6 lymphocytes were activated for 2 hours with 25 ng/ml phorbol myristate acetate (PMA) and 1 µg/ml ionomycin (both from Sigma-Aldrich, Deisenhofen, Germany). Cultures were supplemented with 1µM monensin for the final 4 hours of culture. Cells were stained with biotin-SAV-HRP-conjugated anti-CD11c (HL3) or FITC-conjugated anti-CD4 Ab and fixed in 4% PFA, permeabilized with 0.2% saponin and stained using PE-conjugated anti-IL-12 (c15.6), anti-IFN-γ (XMG1.2) or anti-IL-4 Ab. IgG1 was used as isotope control (all Ab were purchased from BD Biosciences, Heidelberg, Germany).

3.3.9 LmAg-stimulated cytokine release

5 x 10^6 lymphocytes were cultured in the presence of LmAg (parasite-to-cell ratio 30:1) or left untreated for 72 hours. The levels of IL-4, IL-12p70 and IFN-γ in the cultured supernatants were determined by sandwich ELISA using Ab pairs purchased from BD Biosciences, Heidelberg, Germany according to the manufacturer´s instructions.

3.3.10 Statistical analysis

Values are given as mean ± SD and significant differences were determined using Student´s *t* test (GraphPad Prism version 5, San Diego, CA, USA).

Results

4. Results

4.1 Breeding of IL-4Rα-deficient BALB/c mice

Experimental murine leishmaniasis is a well established model to investigate the correlation of the type of Th cell-mediated immune response and the outcome of the disease. A healer phenotype to murine leishmaniasis (e.g. C57BL/6 mice) is correlated with the development of a Th1-dominated immune response, characterized by high levels of IFN-γ and low levels of IL-4, whereas susceptibility (e.g. BALB/c mice) is correlated with the development of a Th2-dominated immune response, characterized by high levels of IL-4 and low levels of IFN-γ [180]. *In vitro* studies showed that IL-4 promotes the differentiation of naïve Th cells into the Th2 phenotype [25]. Furthermore, *Leishmania*-susceptible mice convert into a resistant phenotype when IL-4 is depleted *in vivo* by mAb [31].

Following these results and many others gained before, IL-4 was postulated as disease promoting cytokine, which is crucial for the development of Th2-mediated immune responses. IL-4$^{-/-}$ BALB/c mice, which have been generated to investigate the role of IL-4 *in vivo*, remained susceptible to infection with *L. major*, as a protective Th1 immune response has not been established [136]. Contrary results were observed in the same model, showing that these mice resist infection to *L. major* [30].

The IL-4R type I, which is composed of the IL-4Rα chain and the γc chain can only be triggered by IL-4, whereas the IL-4R type II, consisting of the IL-4Rα chain and the IL-13Rα1 chain can be triggered by IL-4 as well as IL-13 [146].

Global IL-4Rα$^{-/-}$ BALB/c mice were generated as described elsewhere [139] to observe the disease outcome of leishmaniasis in the absence of IL-4-mediated functions. In brief, Mohrs et al generated IL-4Rα$^{-/-}$ mice by homologous and site-

specific recombination in BALB/c-derived embryonic stem cells under the Cre/loxP system. The authors showed impaired IL-4- and IL-13-mediated signaling as consequence of this recombination by *in vitro* testing of cells harvested from IL-4Rα$^{-/-}$ mice, indicating that IL-4Rα chain is part of the receptor for IL-4 as well as IL-13. The phenotype of *L major*-infected IL-4Rα$^{-/-}$ BALB/c mice was described as reduction of footpad swelling and parasite burden within the infected organs during the first three month of infection. During the onset of infection IL-4Rα$^{-/-}$ mice developed enormous footpad swelling accompanied with necrotic lesions and elevated parasite dissemination into organs, finally resulting in high mortality during the chronic phase of infection. The results by Mohrs et al. demonstrated an IL-4 mediated mechanism of susceptibility in BALB/c mice during the acute phase of infection and a possible immunity to infection-maintaining effect of IL-13 signaling through its receptor, as IL-4$^{-/-}$ BALB/c mice controlled the infection also during the chronic phase, but IL-4Rα$^{-/-}$ mice failed to do so [139].

I decided to use BMDC from IL-4Rα$^{-/-}$ mice for the immunization studies to investigate the role of IL-4Rα signaling in BMDC used as vaccine carrier in mediating protection against experimental leishmaniasis. For this purpose, two male IL-4Rα$^{-/-}$ BALB/c mice, which were a generous gift from Prof. Dr. Gottfried Alber, Leipzig (Germany), were used to generate a population of IL-4Rα$^{-/-}$ mice, while pairing them with female wt BALB/c mice (Figure 1).

The heterozygous F1 generation was mated further until homozygous IL-4Rα$^{-/-}$ mice were confirmed via PCR analysis in the F2 and F3 generation. Homozygous IL-4Rα$^{-/-}$ male as well as female mice were paired to generate homozygous progenies. The disruption of the IL-4Rα gene locus (153 kb band) is irreversible but following

generations and all mice used for further experiments were analyzed individually via PCR and examined for their deficiency of IL-4Rα (1300 kb band).

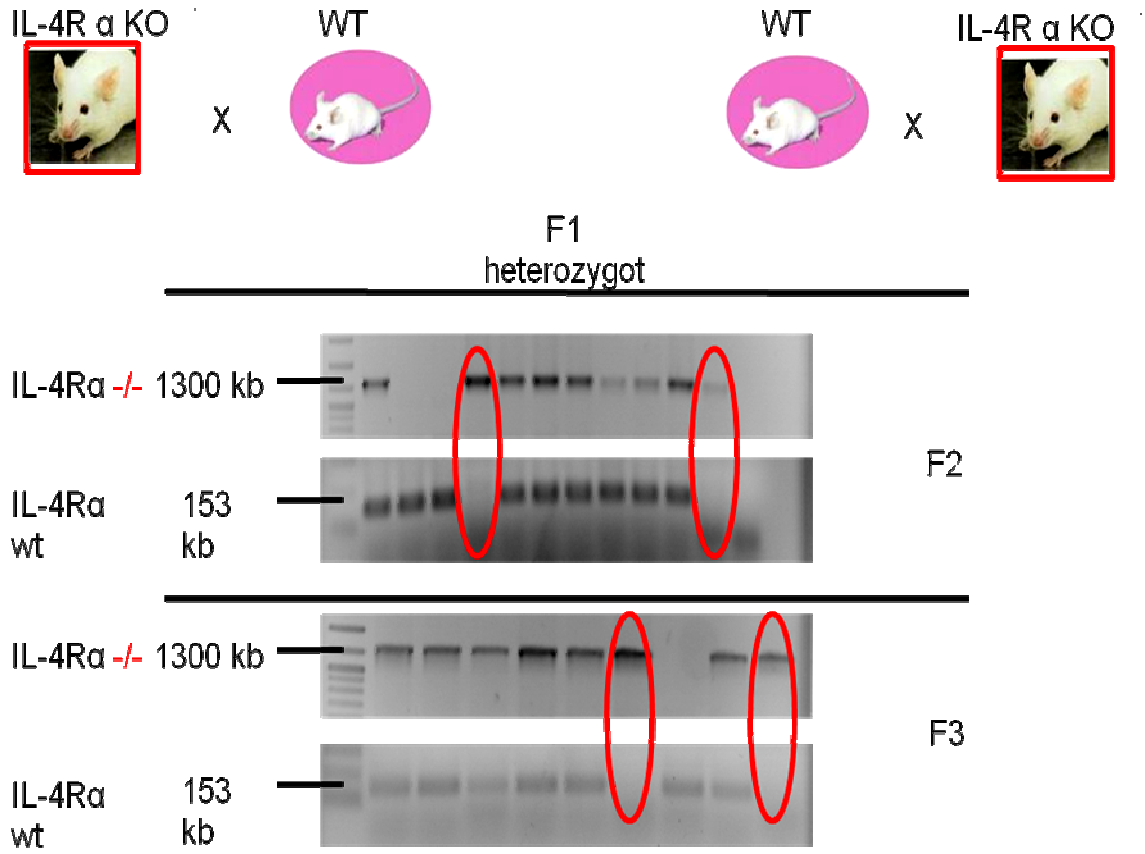

Figure 1. Breeding of IL-4Rα-deficient BALB/c mice.
Male IL-4Rα$^{-/-}$ BALB/c mice were paired with female wt BALB/c mice to generate a heterozygous F1 generation which was analyzed via PCR using IL-4R wt sense (GTA CAG CGC ACA TTG TTT TT) and IL-4R wt antisense (CTC GGC GCA CTG ACC CAT CT) primer resulting in the amplification of a 153bp IL-4Rα wt fragment and IL-4R KO E10 reverse (ACC TGT GCA TCC TGA ATG AT) and IL-4R KO intron 6-2 (CCC TTC CTG GCC CTG AAT TT) primer resulting in the amplification of an 1300bp IL-4Rα$^{-/-}$ fragment. Homozygous IL-4Rα$^{-/-}$ female and male mice of the F2 and F3 generation were paired to generate a stable population of homozygous IL-4Rα$^{-/-}$ progenies as confirmed by PCR analysis.

4.2 Comparison of wt and IL-4Rα-deficient BMDC *in vitro*

DC are potent APC when it comes to orchestrate appropriate immune responses to pathogens. The induction of antigen-specific immune responses depends on the maturation status of DC, as immature DC exhibit low levels of co-stimulatory cytokines to activate and induce the cell differentiation of naïve T cells

[134]. Mature DC, on the other hand, are highly immunogenic as their secreted co-stimulatory cytokines are strong activators of antigen-specific T cells, which in turn stimulate the production of IL-12 by DC tremendously [181]. This IL-12 production by DC is pivotal to induce a strong Th1 response, which is gained after appropriate stimulation of DC *in vitro* [182].

Important knowledge was gained in *in vitro* studies of differentially stimulated DC with regard to their production of IL-12. As already mentioned, IL-12 plays a crucial role in the differentiation of naïve T cells into resistance to *Leishmania*-inducing Th1 cells and consequently the levels of DC-derived IL-12 has been used as an indicator for the ability to induce Th1 immunity. The stimulation of DC with the strong TLR9 ligand CpG ODN resulted in enhanced release of DC-derived IL-12 [183].

4.2.1 Elevated IL-12 secretion by wt BMDC was observed upon stimulation with CpG ODN and recombinant IL-4

As the pivotal role of DC-derived IL-12 in the induction of *Leishmania*-specific immunity has been shown in many *in vitro* as well as *in vivo* studies, the levels of DC-derived IL-12 was determined in differentially stimulated wt and IL-4Rα-deficient BMDC and shown in the present study.

Once homozygous IL-4R$\alpha^{-/-}$ BALB/c mice were gained, BMDC were cultured for 10 days in GM-CSF-conditioned RPMI medium and stimulated overnight with CpG ODN, recombinant IL-4 or IL-13 alone, or a combination of CpG ODN and rIL-4 or rIL-13 and compared to treated wt BMDC. The supernatant was analyzed using sandwich ELISA for IL-12 (Figure 2).

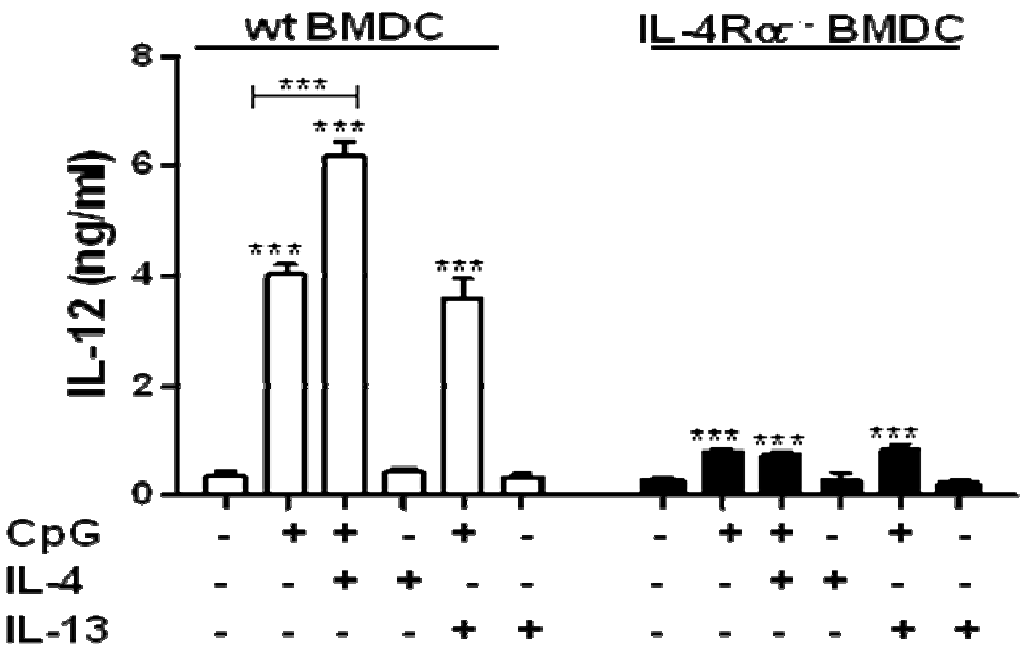

Figure 2. Increased IL-12 secretion upon additional stimulation with rIL-4 in wt BMDC.
BMDC generated from wt (white bars) or IL-4Rα$^{-/-}$ BALB/c mice (black bars) were harvested at day 10 after culture and stimulated over night with CpG ODN, recombinant IL-4 or IL-13, or combinations of these, as indicated above. Levels of IL-12 in the supernatant of cultured cells of five independent experiments were determined by sandwich ELISA. ***, p< 0.0005 compared to untreated cells or two groups were compared as indicated by |—|.

CpG ODN-stimulated wt BMDC are characterized by high levels of IL-12 (4.03 ng/ml) compared to non-stimulated control BMDC (0.37 ng/ml). Interestingly, IL-12 levels of CpG ODN-stimulated IL-4Rα-deficient BMDC are merely elevated (0.79 ng/ml) compared to non-stimulated control BMDC (0.28 ng/ml). IL-12 secretion by wt as well as IL-4Rα-deficient BMDC is not affected by the single stimulation with rIL-4 or rIL-13. Enhanced IL-12 secretion by wt BMDC upon double stimulation with CpG ODN and rIL-4 has already been reported by Biederman et al. [175] and this observation is also confirmed by our data, as CpG ODN and rIL-4 stimulated wt BMDC secrete 6.18 ng/ml IL-12 compared to CpG ODN stimulated wt BMDC (4.03 ng/ml). Only the combination of CpG ODN and rIL-4 resulted in elevated levels of IL-12 secreted by wt BMDC, as rIL-4 alone had no increasing effect.

Evaluating the results gained from CpG ODN and rIL-4-stimulated IL-4Rα-deficient BMDC lead to the result that this additional activation of IL-12 secretion in wt BMDC is mediated by IL-4 triggering of IL-4Rα, as IL-4Rα-deficient BMDC stimulated with CpG ODN and rIL-4 did not show an increasing secretion of IL-12.

4.2.2 IL-13 signaling through IL-4Rα on BMDC has no IL-12 instructing effect

To rule out the possibility that IL-13 could be involved in this IL-12 enhancing effect while triggering IL-4Rα, the results demonstrate that stimulation with rIL-13 *per se* has no IL-12 promoting effect on neither wt nor IL-4Rα-deficient BMDC and the double stimulation with CpG ODN and rIL-13 has no IL-12 enhancing effect when compared to only CpG ODN stimulated BMDC, too (Figure 2). In general it can be postulated that IL-4 non-responsive BMDC secrete lower levels of IL-12 upon stimulation than wt BMDC. These results allow the statement that IL-4 signaling through its IL-4Rα receptor on BMDC in combination with another strong BMDC activator signal (CpG ODN) initiates the secretion of Th1-promoting IL-12.

4.2.3 Additional stimulation with recombinant IL-4 instructs only wt BMDC to secrete elevated levels of IL-12

The generation of IL-4Rα$^{-/-}$ BALB/c mice was a prerequisite to culture IL-4Rα-deficient BMDC, which can be used to examine the effect of IL-4Rα triggering in DC used as vaccine carrier and the mediation of immunity to murine leishmaniasis. In this immunization model CpG ODN-activated BMDC are used to present LmAg to the host immune system, thus allowing the establishment of *L. major*-specific immunity to subsequent infection. To achieve the presentation of LmAg via BMDC to the host immune system, LmAg is loaded onto CpG ODN-activated BMDC *in vitro*. To

examine whether LmAg-loading onto BMDC has a cytokine-inducing effect *per se* the vaccine generation protocol has been used to stimulate wt or IL-4Rα-deficient BMDC prior to ascertaining the levels of IL-10 as an inhibitor, and IL-12 as a promoter of Th1 cell proliferation in the supernatant of cultured cells (Figure 3).

IL-4 responding BMDC stimulated with CpG ODN and loaded with LmAg secrete low levels of IL-10 (0.25 ng/ml) (Figure 3A) and elevated levels of IL-12 (2.92 ng/ml) (Figure 3B). The additional stimulation with rIL-4 resulted in elevated levels of IL-10 (0.43 ng/ml) (Figure 3A) as well as IL-12 (5.34 ng/ml) (Figure 3B). This combines the two reported functions of IL-4. On the one hand, IL-4 is a key cytokine promoting and sustaining a Th2 response indicated by elevated levels of IL-10, and on the other hand, IL-4 promotes an IL-12-driven Th1 response, as indicated by elevated levels of IL-12.

IL-4 non-responsive BMDC secrete *per se* high levels of IL-10 (Figure 3A) and low levels of IL-12 (Figure 3B) compared to wt BMDC in both conditions. The results observed with IL-4 non-responding BMDC indicate that the IL-12 enhancing effect of rIL-4 acting on wt BMDC is specific to rIL-4 signaling through IL-4Rα chain, as the IL-12 boosting effect has not been observed in IL-4 non-responding BMDC.

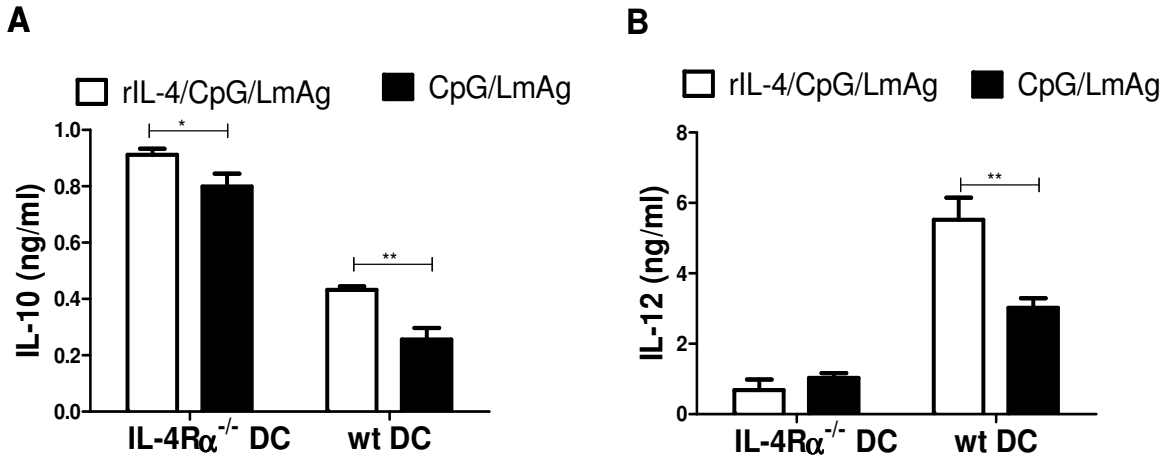

Figure 3. Recombinant IL-4 induces IL-10 secretion in both types of BMDC, but IL-12 secretion only in wt BMDC.
BMDC generated from wt or IL-4Rα$^{-/-}$ BALB/c mice were harvested at day 10 and stimulated overnight with CpG ODN and LmAg in the presence or absence of recombinant IL-4, as indicated above. Levels of IL-10 (A) and IL-12 (B) in the supernatant of cultured cells of three independent experiments were determined via sandwich ELISA. *, $p<0.05$; **, $p< 0.005$ comparison between BMDC stimulated in the presence or absence of rIL-4.

In general, Figure 3B reflects the results already observed in Figure 2, showing that IL-4Rα triggering by IL-4 (in this setup by recombinant IL-4) induces high levels of IL-12, as elevated levels of IL-12 are observed in wt BMDC but not in IL-4 non-responsive BMDC.

The results shown above demonstrate that IL-4Rα-deficient BMDC fail to induce the secretion of elevated IL-12 levels due to their unresponsiveness to IL-4 as a consequence of their IL-4Rα chain-deletion. These *in vitro* results already gave the first indication that immunization studies with IL-4Rα-deficient BMDC presumably would have negative effect in mediating immunity to infection, as the induction of a strong IL-12-driven Th1 immune response is a prerequisite to mediate resistance to leishmaniasis.

4.3 Clinical outcome of leishmaniasis in differentially immunized mice

It is well established that the main inducer of a Th2 response in *Leishmania*-susceptible BALB/c mice is IL-4 [184]. On the other hand, it has been shown that IL-4 has the ability to instruct a Th1 response and resistance against *L. major* in these mice. The presence of IL-4 during the initial phase of DC activation results in an increased IL-12-driven Th1 response [175] and the above results show that IL-4 non-responding BMDC fail to secrete elevated levels of IL-12 in an IL-4Rα-dependent manner.

To investigate the functional role of IL-4-mediated signaling during *Leishmania* infection, various knock-out mice have been generated. IL-$4^{-/-}$ [30], as well as IL-$4R\alpha^{-/-}$ BALB/c mice [139] are resistant to infection with *L. major*. Cell-specific IL-4Rα-deficient mice have been generated to investigate the impact of IL-4Rα-mediated signaling on various cell types during *Leishmania* infection. $CD4^+$ T cell-specific $Lck^{cre}IL4R\alpha^{-/lox}$ BALB/c mice show a resistant phenotype, whereas DC-specific $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ BALB/c mice are hyper-susceptible (Hurdayal et al., manuscript under revision). Hyper-susceptibility in $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ BALB/c mice is characterized by increased footpad swelling; the development of severe necrotic lesions and high parasite dissemination into organs, demonstrating that IL-4Rα signaling in DC is a necessity to control severe *Leishmania* infection.

4.3.1 IL-4Rα signaling in BMDC used as vaccine carrier plays an important role in the induction of resistance against *L. major* infection

It has been shown that the Th2 key cytokine IL-4 can induce protective Th1-mediated immunity in *L. major*-susceptible BALB/c mice, as characterized by the secretion of high levels of DC-derived IL-12 [175]. In order to investigate whether IL-

4Rα signaling in DC used as vaccine carrier is required to induce protection against leishmaniasis, BMDC were generated from IL-4Rα$^{-/-}$ BALB/c or wt BALB/c mice. The BMDC were activated with the TLR 9 ligand CpG ODN and pulsed with LmAg prior to i.v. injection into naïve BALB/c mice. Immunized BALB/c or control mice were challenged with *L. major* one week after vaccination and the course of disease was monitored weekly.

In accordance with our previous study [65], BALB/c mice immunized with CpG ODN-activated and LmAg-pulsed wt BMDC were able to control leishmaniasis (Figure 4A). However, a significant progression of *L. major* infection was observed in mice immunized with CpG ODN-activated and LmAg-pulsed BMDC generated from IL-4Rα$^{-/-}$ donors. Even though these mice were able to restrict footpad swelling during the first three weeks, an uncontrolled lesion development was observed in the advanced phase of infection (Figure 4A). Unprotected control mice showed a

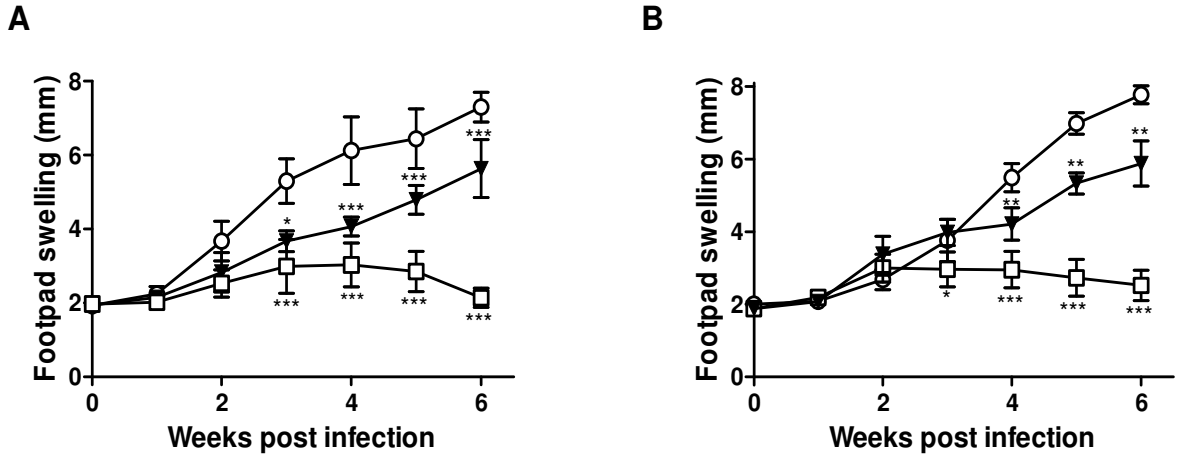

Figure 4. CpG ODN-activated and LmAg-loaded IL-4Rα-deficient BMDC fail to induce protection against leishmaniasis
BALB/c mice (A) or CD11c^{cre}IL-4Rα$^{-/lox}$ mice (B) were immunized i.v. with *in vitro* CpG ODN-activated and LmAg-loaded wt (□) or IL-4Rα$^{-/-}$ (▼) BMDC one week prior to infection with *L. major* promastigotes. Control mice received PBS (O) before infection. The increase in size of the infected compared with the noninfected footpad was measured weekly. The results are expressed as mean ± SD of 10 animals. *, p< 0.05, ***, p< 0.0005 compared to PBS group (O).*, p < 0.05, ***, p< 0.0005 compared to the respective PBS-treated control group.

progressive course of disease with massive footpad swelling and the development of necrotic lesions, which was not observed in mice immunized with conditioned IL-4Rα-deficient BMDC. The lack of necrotic lesions in these mice can most likely be explained by the delayed course of disease.

4.3.2 Successful immunization depends on IL-4Rα-mediated instruction of vaccinating BMDC

To investigate whether DC of the host also require IL-4Rα signaling during vaccination, DC-specific $CD11c^{cre}$IL-$4R\alpha^{-/lox}$ BALB/c mice were immunized and infected (Figure 4B). The treatment of mice with properly conditioned wt BMDC induced protection, independent of whether DC of the host organism are IL-4 responder (Figure 4A) or not (Figure 4B). In contrast, immunization with conditioned IL-4Rα-deficient BMDC was not capable to induce the control of infection, as indicated by uncontrolled lesion development. Controlled footpad swelling was observed in $CD11c^{cre}$IL-$4R\alpha^{-/lox}$ mice treated with PBS or BMDC until three weeks post infection.

The results obtained in *in vitro* studies as well as in immunized BALB/c mice showed the importance of IL-4Rα-mediated instruction of DC used as vaccine carrier in mediating resistance to *L. major* infection.

4.3.3 Activation of LmAg-pulsed BMDC with rIL-4 alone does not confer the potential to induce protective immunity

The experiments shown above indicate that IL-4Rα responsiveness of the vaccinating BMDC is a prerequisite to confer resistance against leishmaniasis in BALB/c mice. However, whether the presence of IL-4 during vaccine generation is

required or whether the protection inducing effect of wt BMDC is due to IL-13 signaling through IL-4Rα chain cannot be inferred from those results, shown in Figure 4.

To clarify the hypothesis that IL-4Rα signaling is critical for the ability of DC to induce resistance against leishmaniasis and to address the possible combinations of how to activate BMDC used in our vaccination strategy, BMDC generated from wt BALB/c mice were stimulated with either rIL-4 alone or a combination of rIL-4 and CpG ODN prior to loading with LmAg. These differently treated BMDC were injected into wt (Figure 5A) or CD11c^{cre}IL-4Rα$^{-/lox}$ mice (Figure 5B) one week prior to infection with *L. major*. Wt BALB/c mice were additionally immunized with rIL-13 and CpG ODN-stimulated and LmAg-loaded BMDC. The course of lesion development was monitored weekly.

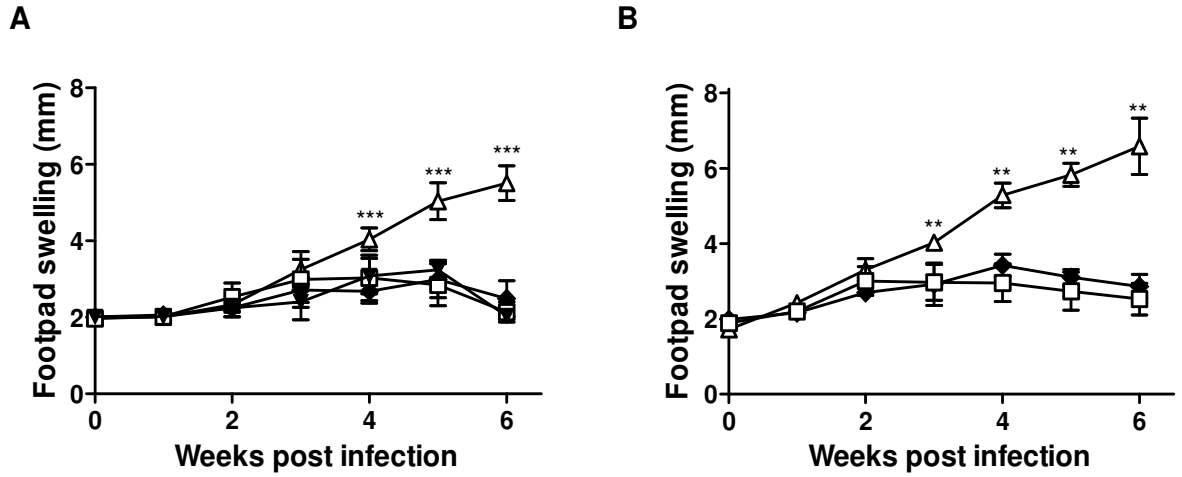

Figure 5. Stimulation of BMDC with rIL-4 prior to LmAg-loading is insufficient to induce protection against leishmaniasis.
BALB/c (A) or CD11c^{cre}IL-4Rα$^{-/lox}$ mice (B) were immunized i.v. with rIL-4-stimulated and LmAg-loaded wt BMDC (wt DC/IL-4/LmAg) (△), a combination of rIL-4 and CpG ODN-activated and LmAg-loaded wt BMDC (wt DC/IL-4/CpG/LmAg) (◆) or CpG ODN-activated and LmAg-loaded wt BMDC (wt DC/CpG/LmAg) (□) one week prior to infection with *L. major*. 5 BALB/c mice (A) were additionally immunized i.v. with rIL-13- and CpG ODN-stimulated and LmAg-loaded BMDC (■) one week prior to infection. The footpad swelling was measured weekly. The results are expressed as mean ± SD of 5 CD11c^{cre}IL-4Rα$^{-/lox}$ mice or 10 BALB/c animals. **, p< 0.005, ***, p< 0.0005 compared to positive control (wt DC/CpG/LmAg) (□).

The results show that in the absence of CpG ODN stimulation, rIL-4-treated and LmAg-pulsed BMDC did not have the potential to induce protective immunity with regard to the footpad swelling in neither wt (Fig. 5A) nor CD11c^{cre}IL-4R$\alpha^{-/lox}$ (Fig. 5B) mice. No differences in the course of disease were observed in mice immunized with CpG ODN-activated BMDC generated in the presence or absence of rIL-4 or rIL-13. Those groups of mice were clinically protected as indicated by controlled footpad swelling. The results show that additional stimulation of wt BMDC with rIL-4 or rIL-13 during vaccine generation seems not to be essential to mediate immunity to *L. major,* and from these results it cannot be concluded that IL-4Rα triggering on BMDC used as vaccine carrier is specifically mediated by IL-4 or whether this effect is mediated by IL-13. But Figure 2 showed that enhanced IL-12 release by DC has only been observed in rIL-4/CpG ODN-stimulated BMDC (see also below, Figure 14A) and that the stimulation with rIL-13 had no IL-12 boosting effect.

4.3.4 Only properly conditioned wt BMDC mediate protection against murine leishmaniasis

To rule out the possibility that BMDC used as vaccine carrier have an immune stimulatory effect *per se*, wt and CD11c^{cre}IL-4R$\alpha^{-/lox}$ mice were immunized with pure IL-4 responder or non-responder BMDC and CpG ODN-stimulated and LmAg-pulsed wt BMDC, serving as protective positive control or PBS, serving as non-protective negative control, one week prior to infection with *L. major* promastigotes into the right hind footpad. The clinical manifestation was documented weekly (Figure 6).

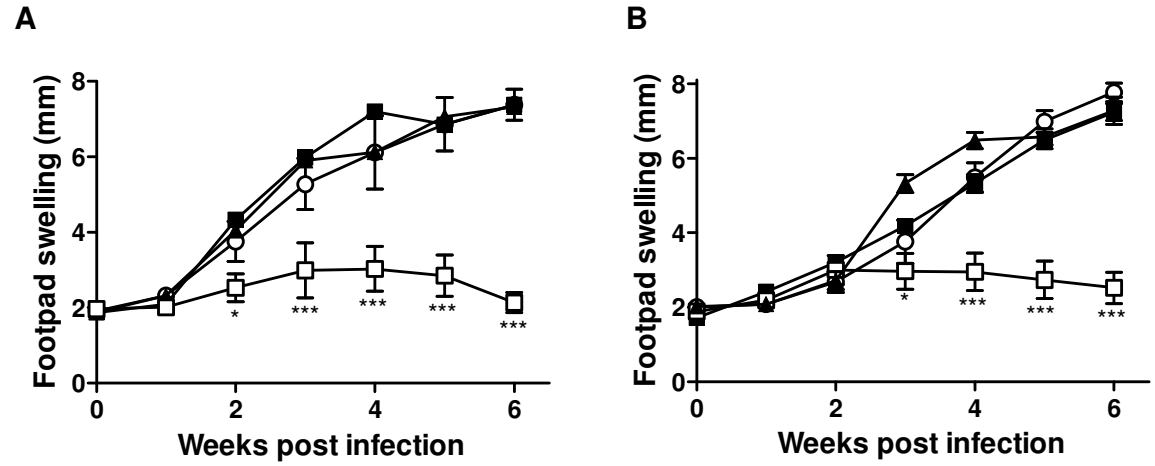

Figure 6. Pure BMDC used as vaccine have no immunity mediating effect *per se*.
BALB/c mice (A) or CD11c^{cre}IL-4R$\alpha^{-/lox}$ mice (B) were immunized i.v. with *in vitro* CpG ODN-activated and LmAg-loaded wt (□) BMDC or pure wt (■) or IL-4R$\alpha^{-/-}$ (▲) BMDC one week prior to infection with *L. major* promastigotes. Control mice received PBS (○) before infection. The increase in size of the infected compared with the noninfected footpad was measured weekly. The results are expressed as mean ± SD of 10 animals. *, $p < 0.05$, ***, $p < 0.0005$ compared to PBS group (○).*, $p < 0.05$, ***, $p < 0.0005$ compared to the respective PBS-treated control group.

The results document once again that untreated BMDC are not suitable to confer protection against leishmaniasis, as expected. Independent, whether BMDC used as vaccine or whether the DC of the immunized host are IL-4 responder (Figure 6A) or non-responder (Figure 6B), pure BMDC are not able to induce immunity against leishmaniasis in BALB/c mice. It has been reported previously that LmAg-pulsed BMDC are not capable to confer protection against murine leishmaniasis caused by *L. major* in BALB/c mice [65], as antigen-pulsing does not induce the maturation of BMDC, which is a critical prerequisite to act as potent inducer of T cell activation [185]. As expected, wt BMDC loaded with only CpG ODN or LmAg had no protective effect (data not shown).

Combining the results shown above and the previously established knowledge about the importance of the particular type of BMDC activation to acquire the ability to induce immunity, the conclusion can be drawn that CpG ODN-stimulated and

LmAg-pulsed BMDC are potent inducer of immunity to leishmaniasis in BALB/c mice, as long as the vaccine carrier is sensitive to IL-4 signaling through its IL-4Rα chain.

4.4 Clinical protection induced by properly conditioned IL-4Rα responsive BMDC correlates with reduced parasite burden and dissemination

The parasite burden at the site of infection, the draining popliteal LN as well as the spleen was analyzed to confirm the correlation between the lesion size and the parasite replication and dissemination into organs of immunized mice. For this purpose, mice from the most relevant groups of the experiments shown above were sacrificed at week one, three or six post infection and the infected footpad, the infection site-draining popliteal LN and the spleen was analyzed for their parasite burden via limiting dilution assays. The determination of the parasite burden at week one and three post infection was chosen to investigate the effect of the vaccinating BMDC on the early immune response to infection. As no significant differences between the vaccinated groups have been observed one week post infection, these data are not shown.

4.4.1 Reduced parasite burden at the site of infection and draining popliteal LN in clinically protected mice

Figure 7 shows the parasite burden in individual BALB/c mice at week three or six post infection, as indicated. The parasite burden at the site of infection was reduced about 10^5-fold and within the draining LN about 10^2-fold six weeks post infection in protected BALB/c mice immunized with wt DC/CpG/LmAg compared to unprotected mice (Figure 7). BALB/c mice immunized with IL-4 non-responsive BMDC develop severe and progressive leishmaniasis, even though the footpad

swelling did not reach the levels of PBS-treated mice (Figure 4A), as indicated by uncontrolled parasite replication at the site of infection and the parasite dissemination into the draining popliteal LN. Further conclusions can be drawn from these data when comparing the parasite burden in mice immunized with additionally rIL-4- or rIL-13-stimulated BMDC. The parasite burden at the site of infection as well as the dissemination into the draining popliteal LN was significantly reduced in these mice six weeks post infection.

The reduction of the parasite burden and the clinical protection of BALB/c mice correlates with the injection of CpG ODN-activated IL-4 responsive BMDC, independent of the additional stimulation with either rIL-4 or rIL-13.

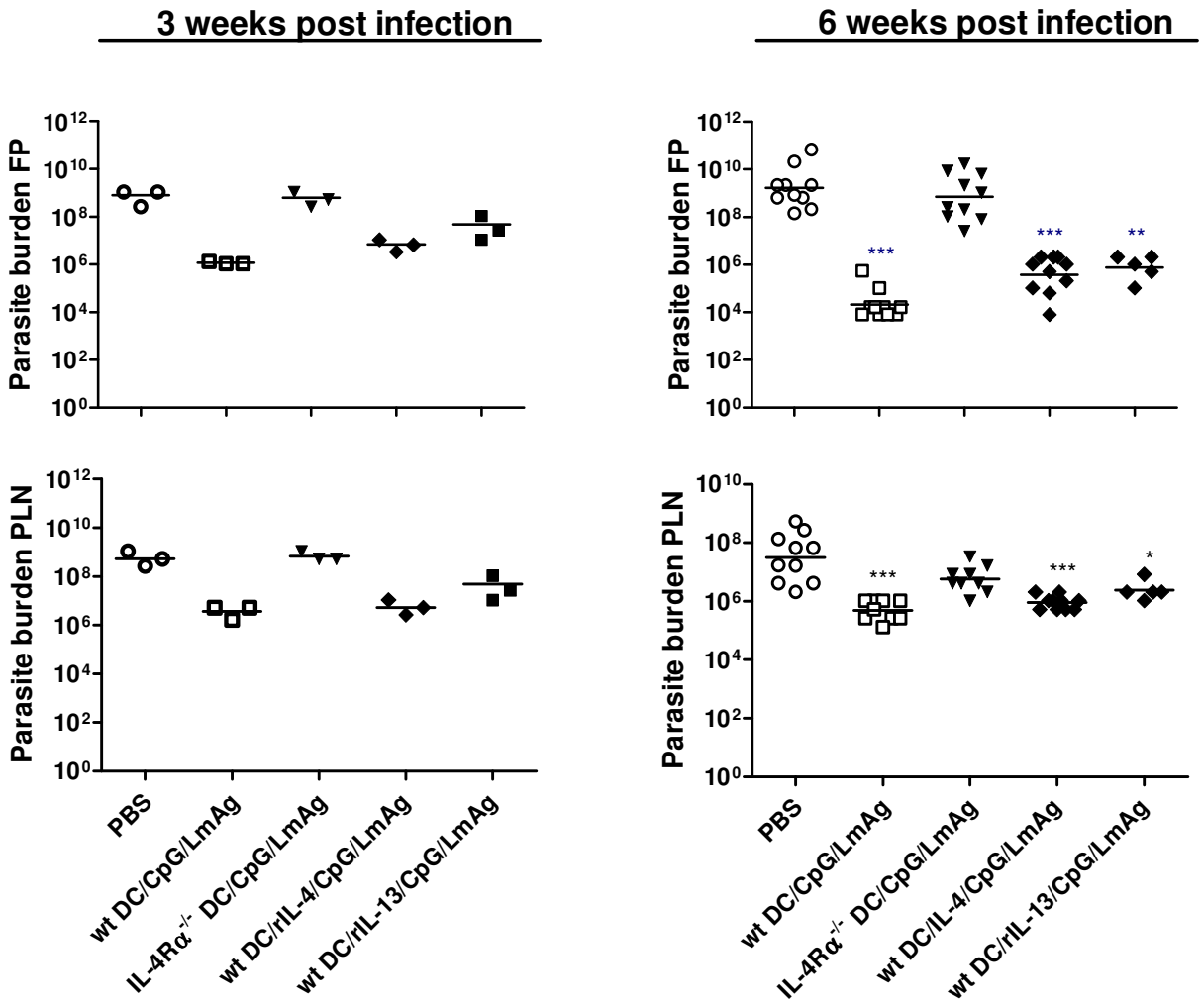

Figure 7. The parasite burden correlates with the clinical outcome of disease in BALB/c mice immunized with properly conditioned IL-4 responsive BMDC.
BALB/c mice were immunized as indicated on the x-axis one week prior to infection with *L. major* promastigotes into the hind footpad. The parasite burden of the infected footpad and the draining popliteal LN of BALB/c mice was determined three or six weeks after infection as indicated. The results of 3 mice (3 wks p.i.) or 10 mice (6 wks p.i.) are shown. **, $p < 0.005$; ***, $p < 0.0005$ compared to the respective negative control (PBS) (O).PLN, popliteal lymph node. wt DC/CpG/LmAg, wild type DC activated with CpG ODN and pulsed with *L. major*-antigen. IL-4Rα$^{-/-}$ DC, interleukin-4 receptor alpha chain-deficient dendritic cells.

4.4.2 IL-4Rα signaling on host DC prevents uncontrolled parasite dissemination into draining popliteal LN

In general, immunization with conditioned IL-4Rα-deficient BMDC was not capable to induce the control of infection, neither in BALB/c (Figure 7) nor in

CD11c^{cre}IL-4Rα$^{-/lox}$ mice (Figure 8), as indicated by uncontrolled parasite replication at the site of infection. Controlled footpad swelling was observed in CD11c^{cre}IL-4Rα$^{-/lox}$ mice treated with PBS or BMDC until 3 weeks post infection (Figure 4B).

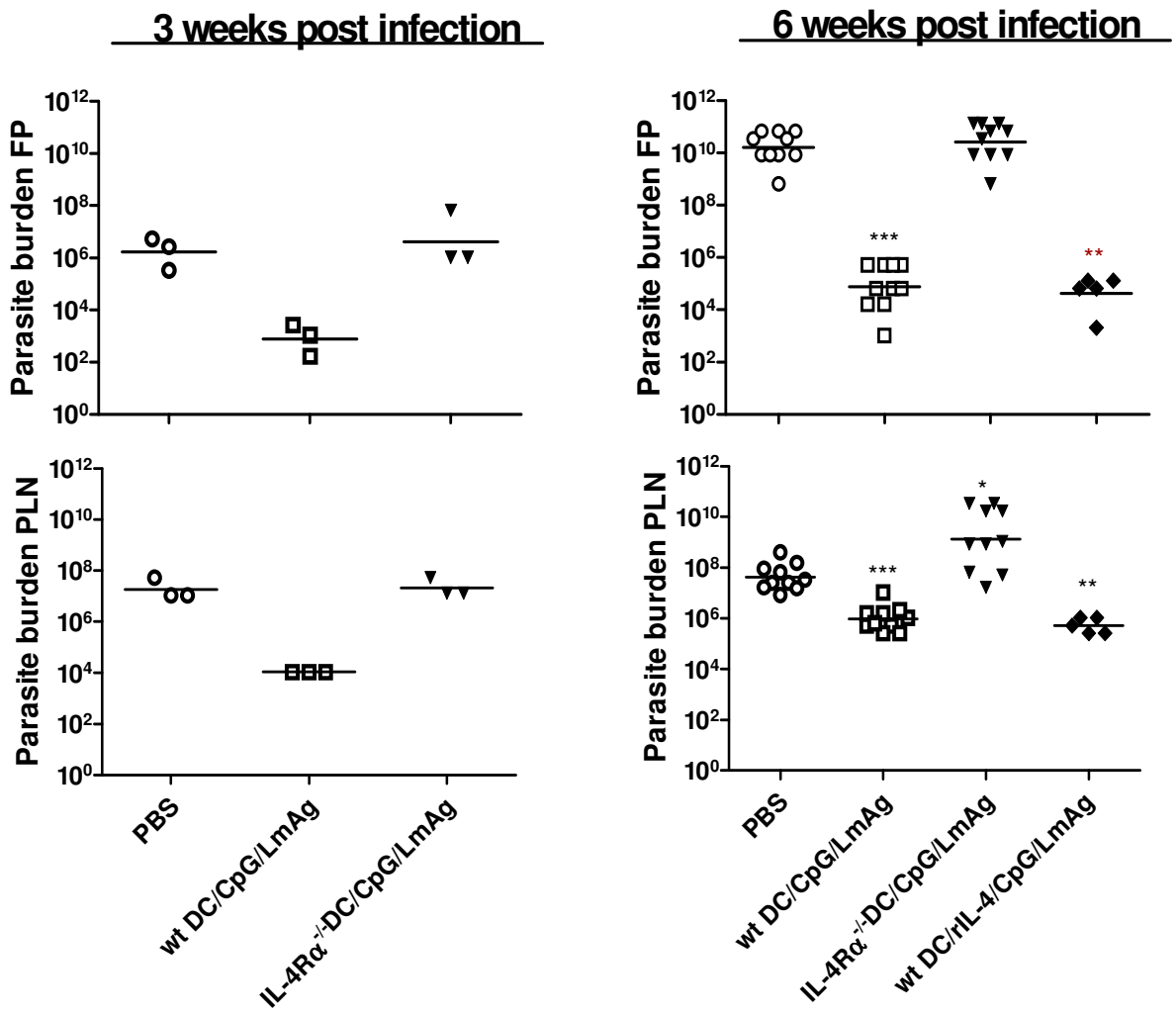

Figure 8. The parasite burden correlates with the clinical protection in CD11c^{cre}IL-4Rα$^{-/lox}$ BALB/c mice immunized with properly conditioned IL-4 responsive BMDC.
CD11c^{cre}IL-4Rα$^{-/lox}$ BALB/c mice were immunized as indicated on the x-axis one week prior to infection with *L. major* promastigotes into the hind footpad. The parasite burden of the infected footpad and the draining popliteal LN of CD11c^{cre}IL-4Rα$^{-/lox}$ mice was determined three or six weeks after infection. The results of 3 mice (3 wks p.i.) or 10 mice (6 wks p.i.) are shown. *, $p < 0.05$, ***, $p < 0.0005$ compared to the respective negative control (PBS) (O).

Delayed lesion development was accompanied by reduced parasite burden and a high IFN-γ response by LmAg-stimulated draining LN cells three weeks post infection, but a Th1-biased immunity was not established during the onset of infection (see below, Figure 15). In a complete IL-4Rα-deficient system (neither vaccine

carrier nor recipient DC are IL-4 responder), an uncontrolled parasite replication in the infected LN (10^2-fold increase compared to PBS treated group) was observed (Figure 8).

This observation is in contrast to the 10-fold reduction of the parasite burden within wt BALB/c mice immunized with IL-4Rα-deficient BMDC (Figure 7), indicating that the inhibition of IL-4Rα signaling on host DC is detrimental and leads to increased dissemination of parasites into draining popliteal LN. Equivalent results were obtained three weeks post infection (Figure 7 and 8).

4.4.3 Clinical outcome and parasite burden is independent of the presence of recombinant IL-4 during vaccine generation

Furthermore, no differences in the course of disease were observed in mice immunized with CpG ODN-activated BMDC generated in the presence or absence of rIL-4 (Figure 7 and 8). Both groups of mice were clinically protected as indicated by controlled footpad swelling (Figure 5) and parasite burden in the examined tissues.

These results show that additional stimulation of IL-4 responsive BMDC with rIL-4 during vaccine generation seems not to be essential to mediate immunity to *L. major*, but that the boosting effect of additional rIL-4 (Figure 2 and 3; see below, Figure 14A) depends on properly conditioned BMDC.

4.4.4 Parasite dissemination into the spleen is reduced in protected mice

Further analyses of the parasite dissemination into the spleen were performed six weeks post infection to determine the parasitic load within this organ in immunized mice (Figure 9). The results show that wt BALB/c mice immunized with properly conditioned IL-4Rα-deficient BMDC reduced parasite dissemination into the spleen (Figure 9A), but $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice failed to control the parasite

replication and dissemination upon immunization with properly conditioned IL-4 non-responder BMDC (Figure 9B). The inability of IL-4Rα-deficient BMDC to reduce the parasite burden and dissemination is even worse in a complete IL-4Rα-deficient system (neither vaccine nor host DC are IL-4 responder) (Figure 8; Figure 9B).

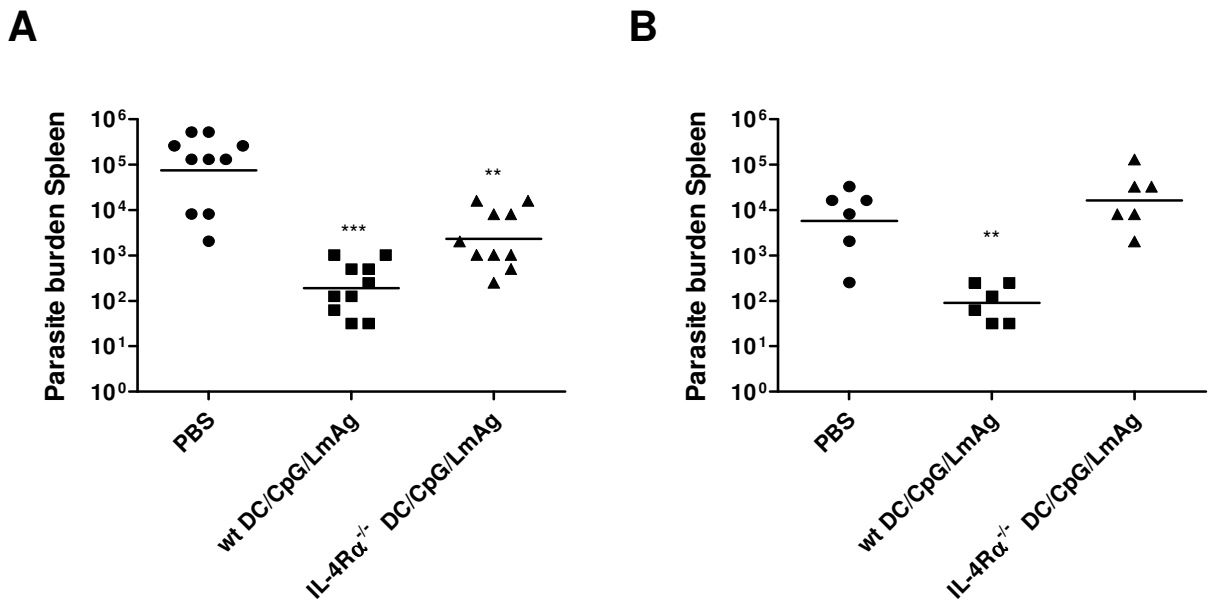

Figure 9. Protected mice show reduced parasite burden in the spleen.
BALB/c (A) or CD11c^{cre}IL-4Rα$^{-/lox}$ BALB/c mice (B) were immunized as indicated on the x-axis one week prior to infection with *L. major* promastigotes into the hind footpad. The parasite burden of the spleen of BALB/C and CD11c^{cre}IL-4Rα$^{-/lox}$ mice was determined six weeks after infection. The results of 10 BALB/c and 6 CD11c^{cre}IL-4Rα$^{-/lox}$ mice are shown. **, $p < 0.005$, ***, $p< 0.0005$ compared to the respective negative control (PBS) (O).

Uncontrolled parasite dissemination into the spleen was observed and the parasite burden within the draining popliteal LN was significantly increased in a complete IL-4Rα-deficient system. The clinical protection of mice immunized with properly conditioned wt BMDC correlates with the control of parasite replication at the site of infection and the parasite dissemination into the infection site-draining LN and the spleen, as shown in Figures 4 - 9.

These results indicate the importance of IL-4Rα-mediated instruction of DC used as vaccine carrier to mediate protection against leishmaniasis, as IL-4Rα-

deficient BMDC were unable to induce parasite clearance in the host organism. But furthermore, these limiting dilution assays indicate that the inhibition of IL-4Rα signaling in host DC is detrimental and leads to increased dissemination of parasites into organs, as a complete IL-4Rα-deficient DC set-up caused uncontrolled parasite dissemination into the draining LN and spleen.

4.5 IL-4Rα-deficient BMDC are less capable of inducing high levels of activated and mature DC in the draining lymph nodes of infected mice

Immature DC are present in nearly all tissues, serve as pathogen sensors for their host and are critical to elicit T cell immunity in leishmaniasis. Host-derived DC migrate to the site of infection, take up and process antigens in a very dynamic process, which are then loaded onto MHC class I or II molecules already within the first few hours of infection [63]. DC activation is associated with parasite uptake-induced up-regulation of MHC class I and II molecules and the expression of co-stimulatory molecules, for example CD80, and in comparison to neutrophils or macrophages, DC are the most potent inducer of an immune response while activating naïve T cells within lymphoid organs [101]. Thus activated, DC differentiate into mature DC and initiate the immune response while migrating to the local draining LN, where they cross-talk with other cells of the immune system to initiate a *Leishmania*-specific $CD4^+$ T cell differentiation [186,187].

4.5.1 Decreased levels of activated and mature host DC in the draining popliteal LN of mice immunized with IL-4Rα-deficient BMDC

To determine the presence of DC at the site of T cell priming, the activation and maturation status of $CD11c^+$ cells in the lesion-draining LN with regard to MHC class II and CD80 expression were analyzed (Figure 10).

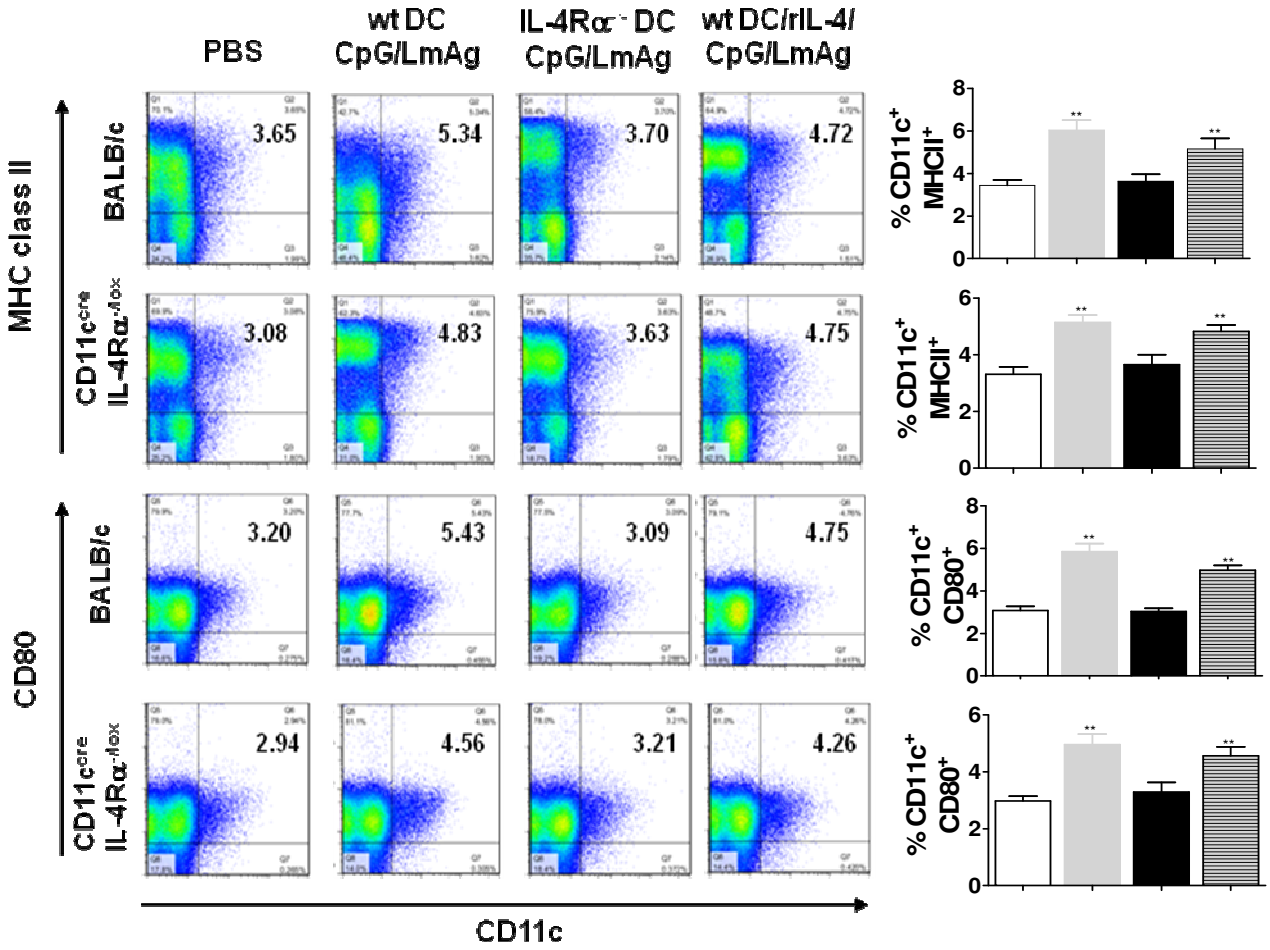

Figure 10. Decreased levels of activated DC in the infected popliteal lymph nodes in mice vaccinated with IL-4Rα$^{-/-}$ BMDC.
The lymphocytes of the draining popliteal LN of 5 BALB/c or 5 CD11c^{cre}IL-4Rα$^{-/lox}$ mice, treated as indicated, were collected six weeks post infection with *L. major* promastigotes, surface-stained for CD11c, MHC class II and CD80 expression to determine the proportion of activated and mature DC in the LN and analysed using FACSCalibur. The x-axis of the dot blots label CD11c and the y-axis MHC class II or CD80, as indicated. The numbers indicate % of gated cells within the distinct quadrant. The mean ± SD of 5 mice each is shown as bar graphs (white column: PBS-treated group, grey column: wt DC/CpG/LmAg immunized group, black column: IL-4Rα$^{-/-}$ DC/CpG/LmAg immunized group, lined column: wt DC/rIL-4/CpG/LmAg immunized group). **, $p < 0.005$ compared to the respective PBS-treated control group.

Wt or CD11c^{cre}IL-4Rα$^{-/lox}$ mice that had been immunized with properly conditioned IL-4Rα-deficient BMDC were less capable of inducing high levels of

activated and mature DC in the draining LN (Figure 10, black bars). In wt BALB/c mice this is indicated by low levels of $CD11c^+$ $MHCII^+$ (3.63%) and $CD11c^+$ $CD80^+$ cells (3.04%) which are comparable to the levels of activated and mature DC within the LN of PBS-treated mice (white bars). No significant differences have been observed in the levels of $CD11c^+$ $MHCII^+$ (3.65%) or $CD11c^+$ $CD80^+$ (3.29%) cells within the LN of $CD11c^{cre}$IL-4R$\alpha^{-/lox}$ mice upon immunization with IL-4Rα-deficient BMDC or PBS.

Clinically protected mice (immunized with wt DC/CpG/LmAg, grey bars, and wt DC/rIL-4/CpG/LmAg, lined bars) showed significantly higher percentages of $CD11c^+CD80^+$ and $CD11c^+MHCII^+$ cells compared to mice that had been immunized with IL-4R$\alpha^{-/-}$DC/CpG/LmAg (black bars). Resistant BALB/c mice (grey bars) showed elevated levels of $CD11c^+$ $MHCII^+$ (6.05%) as well as $CD11c^+$ $CD80^+$ (5.85%) cells and $CD11c^{cre}$IL-4R$\alpha^{-/lox}$ mice mimic these results by elevated levels of $CD11c^+$ $MHCII^+$ (5.15%) as well as $CD11c^+$ $CD80^+$ (4.96%) cells within the LN.

Immunization with wt BMDC additionally stimulated with recombinant IL-4 resulted in clinically protected mice, as indicated by controlled footpad swelling and reduced parasite burdens. Independent whether the vaccine was generated in the presence or absence of rIL-4, no differences have been observed with regard to the levels of activated and mature DC within the LN between those two groups (in the absence of rIL-4, grey bars; in the presence of rIL-4, lined bars).

These results were indicated in BALB/c mice as early as one week post infection with a peak of activated and mature DC within the LN three weeks post infection (Figure 11).

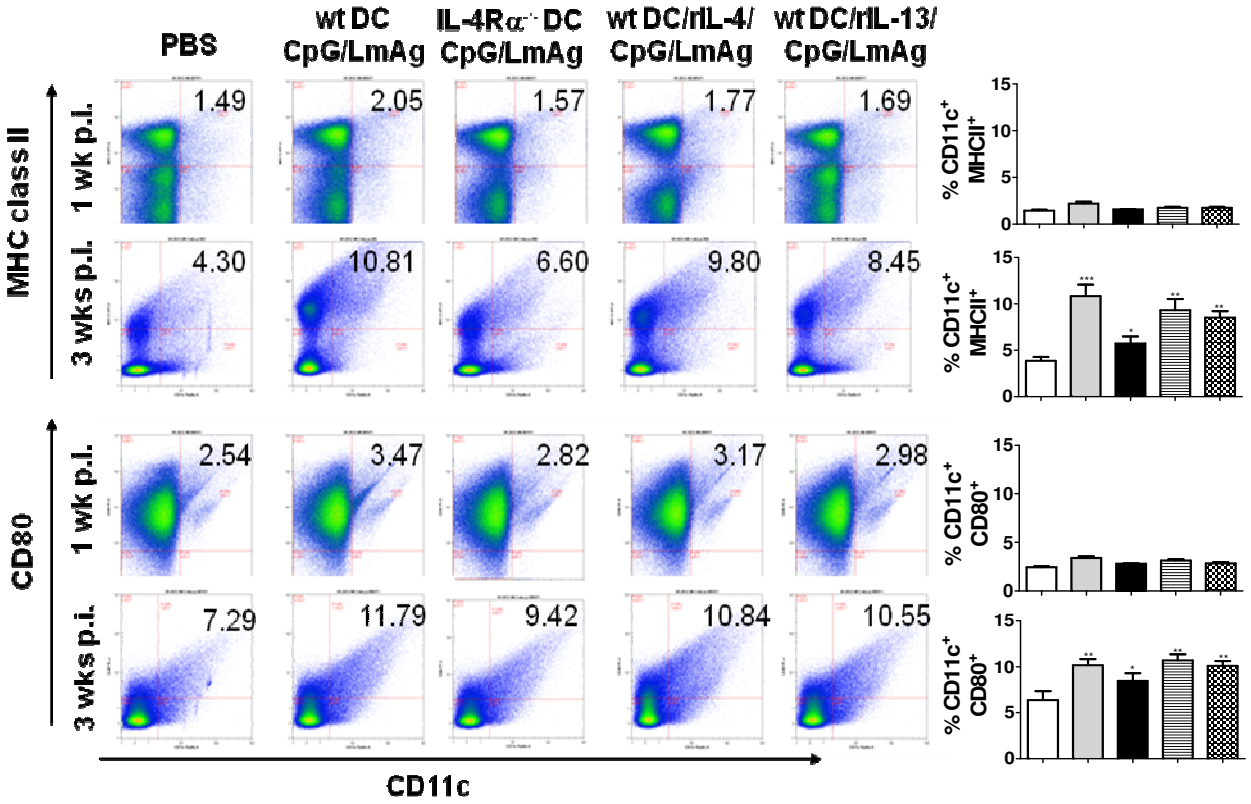

Figure 11. Decreased levels of activated DC in the infected draining LN in susceptible mice already three weeks post infection.
The lymphocytes of the draining popliteal LN of 5 BALB/c or 5 CD11c^{cre}IL-4Rα$^{-/lox}$ mice, treated as indicated, were collected one or three weeks post infection with *L. major* promastigotes, surface-stained for CD11c, MHC class II and CD80 expression to determine the proportion of activated and mature DC in the LN and analysed using FACSCalibur. The x-axis of the dot blots label CD11c and the y-axis MHC class II or CD80, as indicated. The numbers indicate % of gated cells within the distinct quadrant. The mean ± SD of 3 mice each is shown as bar graphs (white column: PBS-treated group, grey column: wt DC/CpG/LmAg immunized group, black column: IL-4Rα$^{-/-}$ DC/CpG/LmAg immunized group, lined column: wt DC/rIL-4/CpG/LmAg immunized group, checkered column: wt DC/rIL-13/CpG/LmAg immunized group). *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$ compared to the respective PBS- treated control group.

Independent of the presence of recombinant IL-4 (lined bars) or IL-13 (checkered bars) at the time of vaccine generation, mice showed similar pattern of activated and mature DC within the LN comparable to mice immunized with the standard vaccine (wt DC/CpG/LmAg, grey bars).

The presence of elevated levels of activated and mature DC within the LN of *Leishmania*-infected mice was observed, as expected. Previous reports have shown, that DC within the LN of infected mice do not harbor *L. major* parasites nor parasite-derived antigens before three weeks post infection, even though parasites were found in the LN already a few hours post infection [188]. Only upon elevated levels of activated parasite-presenting DC are present in the LN, T cell priming occurs around 3-4 weeks post infection [41]. The results shown in Figure 11 at three weeks post infection go in line with these previously published observations.

Concluding, these results demonstrate that upon immunization with IL-4-responsive BMDC, clinically protected mice do not only show controlled footpad swelling and parasite burden within the infected tissues, but also elevated levels of activated and mature recipient DC in the LN, indicating a prerequisite to induce strong *Leishmania*-specific T cell priming.

4.6 Vaccination with IL-4Rα-deficient BMDC did not induce a shift towards a protective Th1 response in BALB/c mice

The generation of *Leishmania*-reactive $CD4^+$ T lymphocytes depends on the ability of DC to present parasite-derived antigens to naïve T cells, thus shaping the type of immune response directed against leishmaniasis [189,190]. Activated DC can shape the type of immune response either to a type 1 or type 2 direction, depending on the cytokine environment at the site of antigen uptake but also at the site of T cell priming within the lymphoid organs. Murine as well as human $CD4^+$ T lymphocytes can be divided into polarized Th1 and Th2 subsets based on their cytokine secretion [191]. In experimental leishmaniasis, resistance to infection is characterized by the presence of IL-12- and IFN-γ-driven Th1 lymphocytes, whereas an IL-4-secreting Th2 cell-dominated immunity results in susceptibility to infection [192]. DC-derived IL-12 is

a key cytokine to direct the T cell differentiation into a type 1 phenotype and DC are the primary source of IL-12 in lymphoid tissues [193], demonstrating their pivotal role in shaping a protective cell-mediated immunity to experimental *L. major* infections.

4.6.1 IL-4 non-responding BMDC are unable to induce high levels of IL-12 secreting host DC in the draining popliteal LN

The secretion of IL-4, IL-12 and IFN-γ by $CD11c^+$ and $CD4^+$ cells in the LN draining the lesions was analyzed as indicators for either a Th1 or Th2 directed immunity. Intracellular FACS staining of PMA/ionomycin-stimulated lymphocytes revealed that clinically protected mice show higher levels of Th1 cytokines and low levels of IL-4 in $CD11c^+$ and $CD4^+$ cells (Figure 12A-D).

Both types of vaccinating DC (conditioned wt BMDC in the presence or absence of rIL-4) led to a higher IL-12 secretion by $CD11c^+$ cells in the infected LN compared to unprotected mice (Figure 12C). Protected wt BALB/c mice (black bars) had 4.42% (wt DC/CpG/LmAg) or 4.59% (wt DC/rIL-4/CpG/LmAg) IL-12 secreting DC compared to unprotected mice, where the percentage of IL-12 secreting DC was 2.44% (PBS) or 1.4% (IL-4Rα$^{-/-}$ DC/CpG/LmAg). The results observed in $CD11c^{cre}$IL-4Rα$^{-/lox}$ mice mimicked the results gained in BALB/c mice, showing that IL-4 non-responding DC used as vaccine are unable to mediate high levels of IL-12 secreting DC into the LN of infected mice, which is a prerequisite to induce a protective Th1-mediated immunity. The graph shown in Figure 12D also indicates differences with regard to the secreted levels of IL-12 in the different mouse strains. Comparing the PBS-treated groups of the two mouse strains, higher levels of cytokines were secreted per se in wt BALB/c (2.43%) mice than in $CD11c^{cre}$IL-4Rα$^{-/lox}$ mice (1.08%).

$CD11c^+$ cells of protected mice also displayed lower levels of IL-4 (Figure 12D). Both mouse strains immunized with properly conditioned IL-4 responsive

BMDC showed reduced levels of IL-4 (1.28% wt BALB/c and 1.03% CD11c^{cre}IL-4Rα$^{-/lox}$ mice). In comparison to wt BALB/c mice (2.8%, PBS-treated group), CD11c^{+} cells of CD11c^{cre}IL-4Rα$^{-/lox}$ mice showed *per se* higher levels of IL-4 (3.47%, PBS-treated group), demonstrating their tendency to induce a Th2-dominated susceptibility rather than an IL-12-driven Th1-mediated resistance (also indicated in Figure 12C).

4.6.2 CD11c^{cre} IL-4Rα$^{-/lox}$ mice secrete *per se* higher levels of IL-4 and lower levels of IFN-γ upon contact with LmAg

CD4^{+} cells secreted higher amounts of IFN-γ in protected mice (2.32 - 2.52%), compared to unprotected control mice or mice immunized with conditioned IL-4Rα-deficient BMDC (Figure 12A). No differences have been observed with regard to the levels of IFN-γ between the analyzed mouse strains. CD4^{+} cells of mice vaccinated with BMDC, independent whether these were IL-4 responder or not, secreted reduced levels of IL-4 (Figure 12B). Again, differences of the IL-4 levels secreted by CD4^{+} cells were observed between the two mouse strains. Wt BALB/c mice secreted *per se* lower levels of IL-4 (0.96%, wt DC/CpG/LmAg) in contrast to the elevated levels of IL-4 secreted by CD4^{+} cells of CD11c^{cre}IL-4Rα$^{-/lox}$ mice (2.55%, wt DC/CpG/LmAg).

Mice immunized with IL-4Rα-deficient BMDC controlled the IL-4 secretion by CD4^{+} cells to a certain level (Figure 12B), but failed to control IL-4 secretion by CD11c^{+} cells (Figure 12D) and failed to induce high levels of Th1 cytokines by CD11c^{+} (Figure 12C) or CD4^{+} cells (Figure 12A).

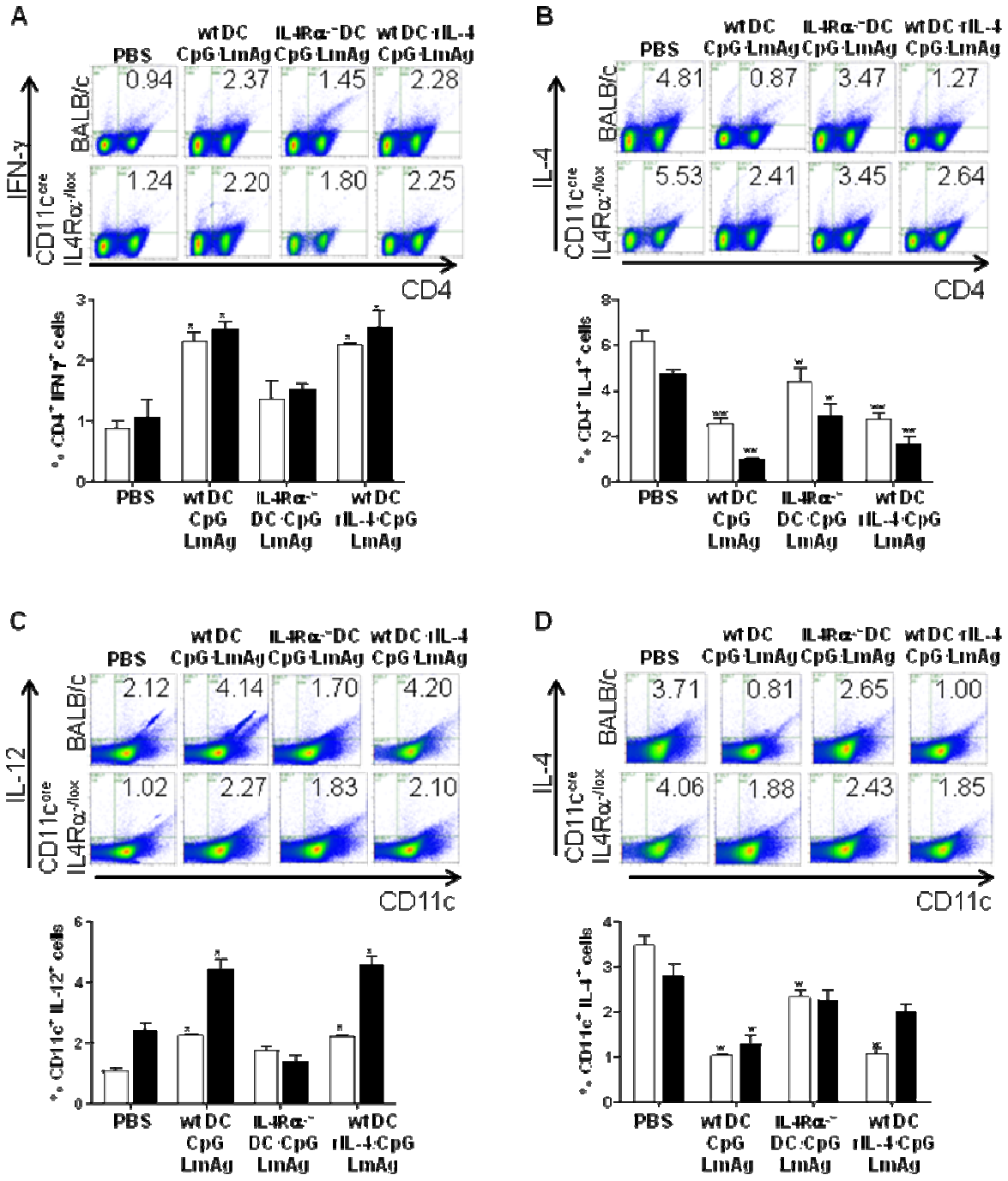

Figure 12. IL-4Rα triggering of vaccine carrier leads to the control of IL-4 production by host lymphocytes.

Lymphocytes of the draining popliteal LN of 5 BALB/c or CD11c^{cre}IL-4Rα$^{-/lox}$ mice, treated as indicated, were collected six weeks post infection and activated for 2 hours with PMA-ionomycin before adding monensin for the final 4 hours of culture. The cells were stained for CD4 and IFN-γ (A) or IL-4 (B) and for CD11c and IL-12 (C) or IL-4 (D) and analysed using FACSCalibur. The x-axis of the dot blots label CD11c or CD4 and the y-axis IL-4, IL-12 or IFN-γ as indicated. The numbers indicate % of gated cells within the distinct quadrant. The bar graphs show the percentage of gated cytokine-secreting lymphocytes as the mean ± SD of 5 BALB/c (black column) or CD11c^{cre}IL-4Rα$^{-/lox}$ mice (white column). *, $p < 0.05$, **, $p < 0.005$, compared to the respective PBS-treated control group.

4.6.3 A peak of activated and mature CD11c$^+$ cells at three weeks post infection

Figure 13 shows the levels of IL-4-secreting CD4$^+$ cells and IL-12-secreting CD11c$^+$ within the LN of immunized and infected wt BALB/c mice at weeks one and three post infection. A peak of activated and mature DC in the LN of protected mice was already detected three weeks post infection (Figure 11) going in line with previous reports that LmAg-loaded BMDC were not detected earlier in the LN of infected mice.

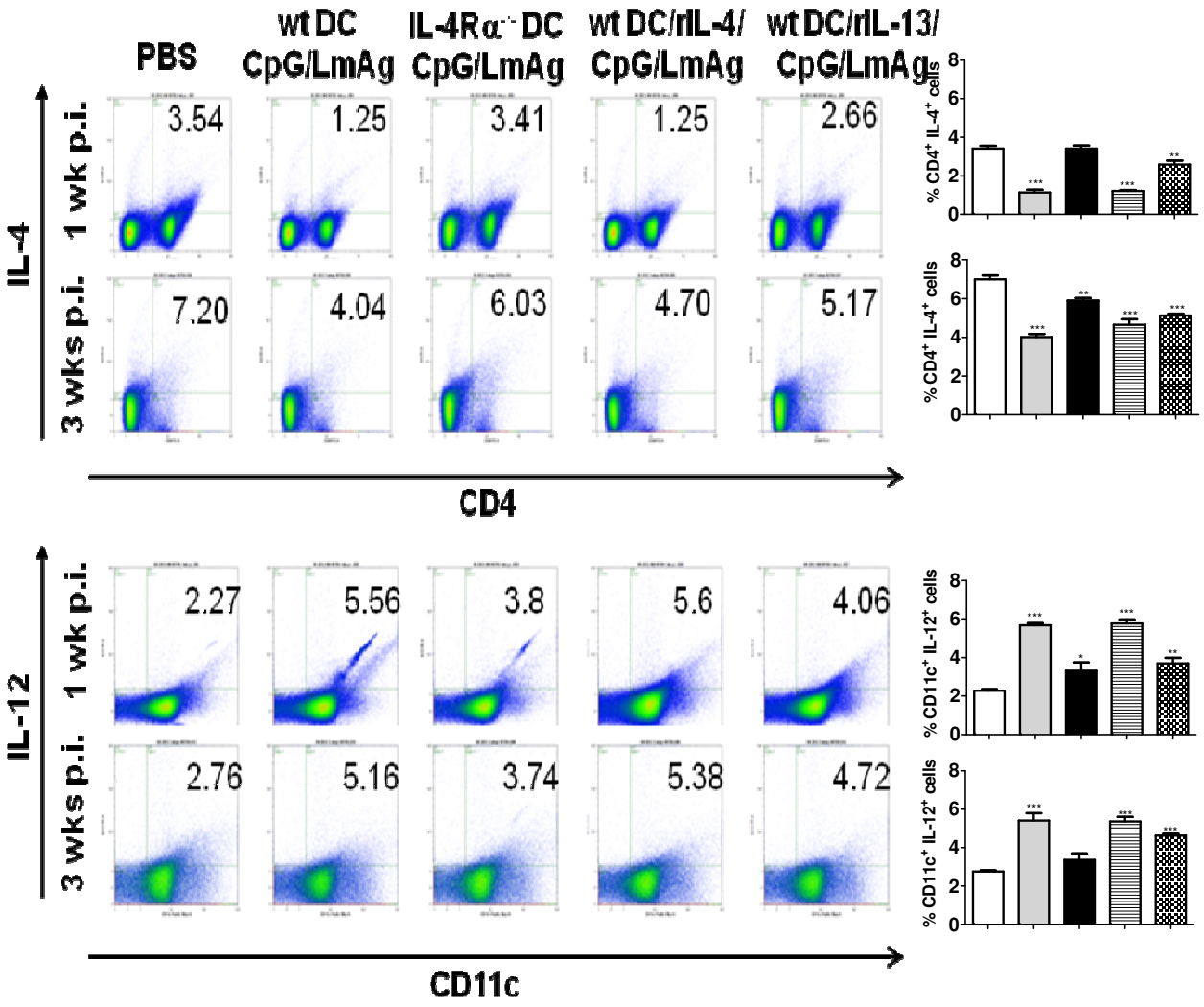

Figure 13. IL-4Rα triggering of vaccine carrier leads to the control of IL-4 production by host lymphocytes already early after infection.
Lymphocytes of the draining popliteal LN of 5 BALB/c mice, treated as indicated, were collected six weeks post infection and activated for 2 hours with PMA-ionomycin before adding monensin for the final 4 hours of culture. The cells were stained for CD4 and IL-4 and for CD11c and IL-12 and analysed using FACSCalibur. The x-axis of the dot blots label CD11c or CD4 and the y-axis IL-12 or

IFN-γ as indicated. The numbers indicate % of gated cells within the distinct quadrant. The bar graphs show the percentage of gated cytokine-secreting lymphocytes of 5 mice (white column, PBS immunized group; grey column, wt DC/CpG/LmAg immunized group; black column, IL-4Rα$^{-/-}$ DC/CpG/LmAg immunized group; lined column, wt DC/rIL-4/CpG/LmAg; checkered column, wt DC/rIL-13/CpG/LmAg). *, $p < 0.05$, **, $p < 0.005$; ***, $p < 0.0005$ compared to the respective PBS-treated control group

The massive presence of IL-12-secreting DC in protected mice initiates the priming of *Leishmania*-specific T cells and reduces the secretion of IL-4 by CD4$^+$ T cells. Concluding from the observed peak of activated DC and levels of cytokines secreted by T cells at three weeks post infection, this is the time point of massive DC-T cell interaction and most probably the period of *Leishmania*-specific T cell priming.

These results demonstrate that IL-4Rα signaling in BMDC used as vaccine carrier enables host DC to secrete high levels of protective IL-12 and to reduce the levels of IL-4, indicating a switch from a non-protective IL-4-driven Th2 response to a protective IL-12- and IFN-γ-dominated Th1 response. Once again, higher levels of IL-4 were observed in mice with IL-4 non-responding DC, demonstrating the imbalance in favor of a non-protective Th2 immune response and might be one aspect to explain the hyper-susceptible phenotype of CD11c^{cre}IL-4Rα$^{-/lox}$ mice.

4.7 IL-4Rα signaling in BMDC used as vaccine carrier is important for the *L. major*-induced release of Th1 cytokines

Protection against leishmaniasis is associated with a Th1 immune response characterized by high levels of IL-12 and low levels of IL-4. To analyze the potential of BMDC-based vaccines to mediate a *L. major*-stimulated Th1 response, total lymphocytes of the most relevant groups were collected six weeks post infection and stimulated for 72 hours with LmAg. Subsequently, the cytokine levels of IL-4, IL-12 and IFN-γ were measured by sandwich ELISA.

4.7.1 The presence of rIL-4 during vaccine generation increases the *L. major*-induced IL-12 release by lymphocytes *in vivo*

LmAg stimulation of lymphocytes (black bars) from protected BALB/c mice caused the secretion of high levels of IL-12 (7.57 ng/ml, wt DC/CpG/LmAg) and IFN-γ (53.31 ng/ml), but low levels of IL-4 (0.06 ng/ml), whereas mice immunized with IL-4Rα-deficient BMDC showed a reversed cytokine pattern (Figure 14A).

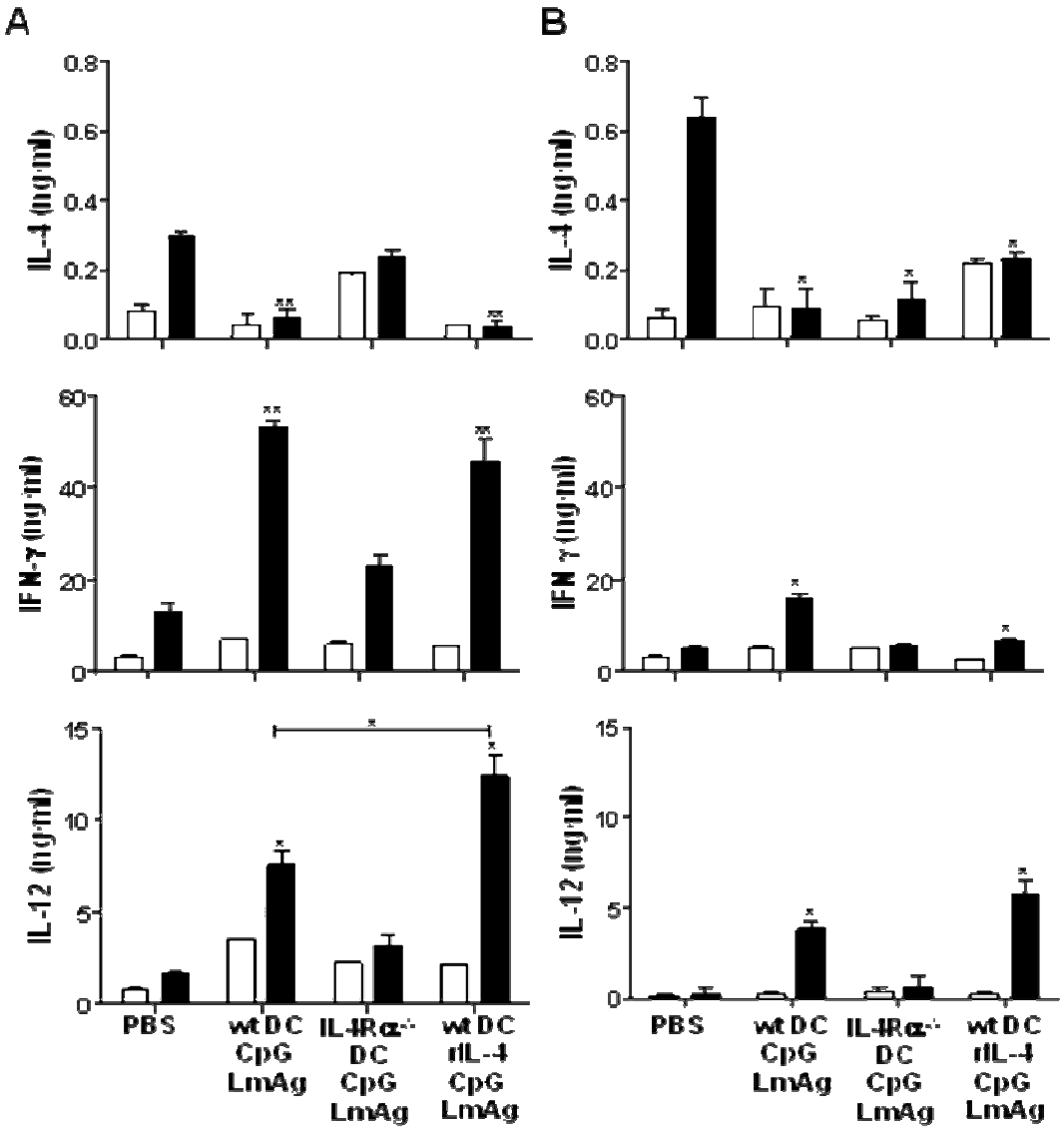

Figure 14. IL-4Rα triggering of BMDC increases *L. major*-stimulated IL-12 secretion *in vivo*.
BALB/c (A) or CD11c^{cre}IL-4Rα$^{-/lox}$ mice (B) were immunized with 5 x 10^5 BMDC prepared as indicated and infected one week later with 2 x 10^5 *L. major* promastigotes. Total lymphocytes of the draining popliteal LN were collected six weeks post infection and incubated for 72 hours in the absence (white bars) or presence (black bars) of LmAg. The levels of IL-4, IFN-γ and IL-12 were measured by ELISA in the collected supernatants. The results of 5 mice are shown. *, $p < 0.05$, **, $p < 0.005$ compared to the respective PBS-treated control group.

Interestingly, the additional stimulation of properly activated IL-4 responsive BMDC with rIL-4 resulted in elevated levels of IL-12 (12.36 ng/ml) upon *L. major* infection *in vivo* in BALB/c mice.

Low levels of IL-4 were observed in $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice independent of the presence (0.09 ng/ml) or absence (0.11 ng/ml) of IL-4Rα on DC used for immunization, as in contrast to BALB/c mice, also conditioned IL-4Rα-deficient BMDC were able to inhibit the release of IL-4 upon *L. major* stimulation (Figure 5B).

In line with the results obtained with BALB/c mice, increased levels of IFN-γ were observed in mice immunized with properly conditioned wt BMDC (16.1 ng/ml in the absence of rIL-4; 6.5 ng/ml in the presence of rIL-4), resulting in resistance to *L. major*. Immunization with conditioned IL-4Rα-deficient BMDC was unable to induce the production of IL-12 (0.62 ng/ml) and IFN-γ (5.59 ng/ml), even though the *L. major*-stimulated IL-4 secretion (0.11 ng/ml) was controlled.

4.7.2 A Th1-biased profile is already observed in protected mice early after infection

A Th1-dominated cytokine profile secreted by lymphocytes in response to LmAg-stimulation has been observed as early as one week post infection (white bars) in BALB/c mice immunized with properly conditioned IL-4 responding BMDC (Figure 15). One week post infection, LmAg-stimulated lymphocytes released 3.8 ng/ml IFN-γ , whereas the levels of IFN-γ in protected mice was drastically increased (17.27 ng/ml) during the time of massive T cell priming at three weeks post infection (Figure 15B).

A Th2-dominated cytokine pattern was observed in mice immunized with IL-4Rα-deficient BMDC as well as mice treated with PBS (Figure 15A). One week post

infection, IL-4 levels of 0.96 ng/ml have been observed upon lymphocyte-restimulation with LmAg, and 2.2 ng/ml of IL-4 was detected three weeks post infection. Low levels of IFN-γ (Figure 15B) in unprotected mice support the establishment of a Th2-biased immune response.

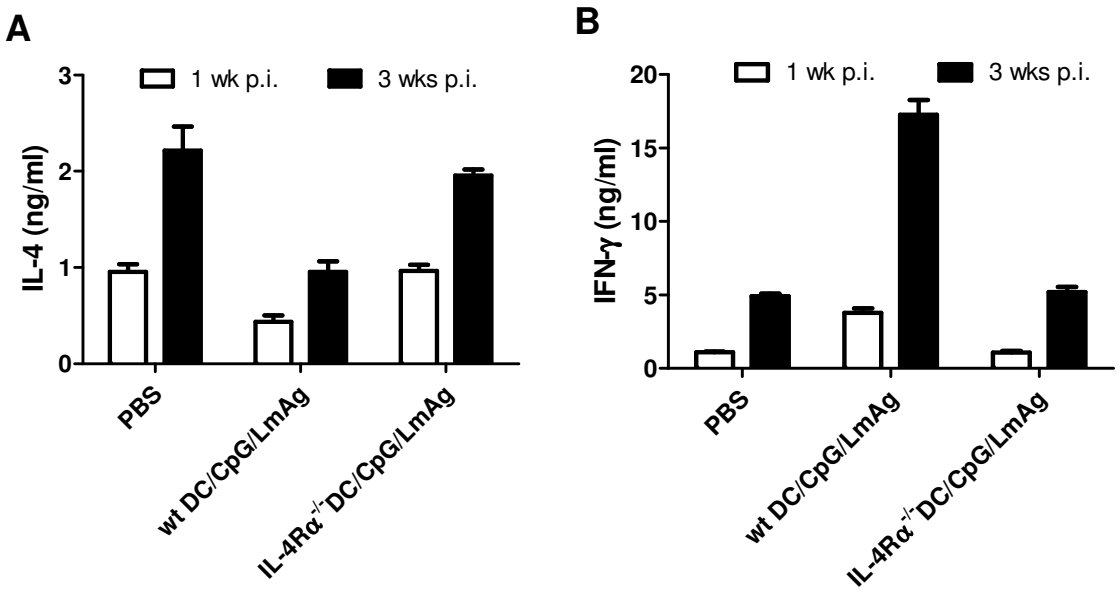

Figure 15. IL-4Rα-deficient BMDC fail to trigger protective Th1-dominated immunity in BALB/c mice already early after infection.
BALB/c were immunized with 5 x 10^5 BMDC prepared as indicated and infected one week later with 2 x 10^5 *L. major* promastigotes. Total lymphocytes of the draining popliteal LN were collected one (white bars) or three weeks (black bars) post infection and incubated for 72 hours presence of LmAg. The levels of IL-4 (A) and IFN-γ (B) measured by sandwich ELISA in the collected supernatants. The results of 3 mice are shown.

Elevated levels of IL-12 were only observed in wt BALB/c mice upon immunization with properly activated and additionally rIL-4-stimulated BMDC (Figure 14A), hence indicating the role of IL-4 responding host DC in the induction of IL-12 release upon *L. major* infection. As already shown in Figure 12, wt BALB/c mice secrete higher levels of Th1 cytokines, whereas CD11c^{cre}IL-4Rα$^{-/lox}$ mice secrete higher levels of IL-4. A complete IL-4Rα-deficient set-up (vaccine and host DC) showed that IL-4Rα-mediated instruction of DC is important to enhance protection

against leishmaniasis, as IL-4Rα-deficient BMDC were not capable of mediating resistance in CD11c^{cre}IL-4Rα$^{-/lox}$ mice.

Discussion

5. Discussion

Experimental CL is a very well established model to investigate the genetic predispositions determining the outcome of disease. Genetically homogenous inbred strains of mice have been used to determine the contribution of the mechanism and factors regulating the development of $CD4^+$ T cell-mediated immunity to infection [180]. The presence of and the responsiveness to IL-12 seems to be a prerequisite to overcome leishmaniasis, as the IL-12R β chain in susceptible BALB/c mice is down-regulated, whereas resistant C57BL/6 mice are strong producer of this receptor and are in turn also strong responder to IL-12 [194,195]. The crucial role of IL-12 has also been shown in studies treating C57BL/6 mice with anti-IL-12 mAb or BALB/c mice with rIL-12 converting the expected phenotypes [33,196].

The responsiveness to IL-4 and the predominated presence of IL-4 in experimental leishmaniasis is associated with a Th2-biased *Leishmania*-promoting immune response [23]. Elevated levels of early IL-4 in response to LACK-reactive $CD4^+$ T cells expressing Vβ4Vα8 TCR chains drive the differentiation of Th2 cells and the highly progressive disease outcome in BALB/c mice [184,197,198].

To determine the functional role of IL-4 during *Leishmania*-infections *in vivo,* IL-4-defcient BALB/c mice have been generated [30]. Disrupting the IL-4 gene in BALB/c mice rendered these mice resistant to acute and chronic infection with *L. major,* but complete parasite clearance was not observed. The authors suggest that an early IL-4 response is crucial for the progressive disease outcome in susceptible BALB/c mice, indicating that early IL-4 could antagonize with Th1-inducing IL-12.

In line with the observed phenotype in IL-4-deficient BALB/c mice, IL-4Rα-deficient BALB/c mice were also able to control *Leishmania*-infections, demonstrating the pivotal role of IL-4 in mediating susceptibility to leishmaniasis. Additional

molecules acting through IL-4Rα have been suggested to be necessary for anti-leishmanial immunity, as IL-4Rα-deficient BALB/c mice developed progressive and fatal leishmaniasis during the chronic phase of infection (~3-6 months post infection) [139]. The IL-4Rα chain is not only a component of IL-4, but also of an IL-13 receptor, indicating a possible role of IL-13 signaling through IL-4Rα chain in maintaining immunity during the chronic phase of leishmaniasis [139].

The aim of the present study was to determine, whether DC-mediated immunity to *L. major* infection in susceptible BALB/c mice is affected by the absence of IL-4Rα signaling in BMDC used as vaccine carrier. For this purpose, IL-4Rα-deficient BMDC from IL-4Rα$^{-/-}$ BALB/c mice were used as vaccine carrier in *L. major*-susceptible mice and compared to IL-4 responding vaccine.

The Th1/Th2 paradigm, postulating that IL-12 necessarily triggers Th1-mediated resistance and IL-4 necessarily triggers Th2-mediated susceptibility to leishmaniasis, was challenged by studies demonstrating the promoting role of IL-4 in the establishment of Th1-cell mediated immunity. Human DC, generated in the presence of low concentrations of IL-4, promoted the differentiation of Th2 lymphocytes, whereas high concentrations of IL-4 induced the release of DC-derived IL-12, thus initiating the expansion of Th1 lymphocytes [199]. Controversially to this Th1/Th2 paradigm, IL-4 was shown to possess the ability to induce elevated IL-12 release by DC, but only when IL-4 is present during the initial activation of DC. This indicated that IL-4 instructs DC to secrete elevated levels of Th1-promoting IL-12, whereas the presence of IL-4 during the period of T cell priming promotes the expansion of IL-4-secreting Th2 lymphocytes [175].

Cell-specific IL-4Rα-deficient mice have been generated to determine the decisive IL-4 responsive cell type directing the development of a non-protective Th2-biased immunity to leishmaniasis. CD4^{+} T cell-specific Lck^{cre}IL-4Rα$^{-/lox}$ BALB/c mice

show a resistant phenotype [142], whereas DC-specific CD11c^{cre}IL-4R$\alpha^{-/lox}$ BALB/c mice are hyper-susceptible (Hurdayal et al., manuscript in preparation). Hyper-susceptibility in CD11c^{cre}IL-4R$\alpha^{-/lox}$ mice is characterized by increased footpad swelling; the development of severe necrotic lesions and high parasite dissemination into organs, demonstrating that IL-4Rα signaling in DC is a necessity to control severe *Leishmania* infections. The studies mentioned above showed that the presence of IL-4 during DC activation induces the release of IL-12 and that DC-specific IL-4 non-responder mice are hyper-susceptible to infection with *L. major*.

In vitro analysis of wt and IL-4Rα-deficient BMDC shown in the present study, demonstrated significant differences in the release of IL-12 upon stimulation. As DC-derived IL-12 is critical to induce a strong Th1 response *in vivo,* the secretion of IL-12 by differentially stimulated BMDC was determined in this study. In general, IL-4 non-responsive BMDC secrete lower levels of IL-12 upon stimulation compared to wt BMDC, indicating that IL-4 signaling through IL-4Rα on BMDC in combination with contemporaneous stimulation with CpG ODN initiates the secretion of Th1-promoting IL-12. To prove the IL-4-specific effect of IL-4Rα triggering, IL-4 responsive BMDC were stimulated with rIL-13. The results promote the conclusion that the IL-12-boosting effect of BMDC additionally stimulated with rIL-4 is a specific effect, as stimulation with rIL-13 was not able to induce enhanced release of IL-12. Even though, IL-13 signals through IL-4Rα chain, it can be excluded that IL-13 signaling through this receptor is essential to induce IL-12 secretion, as indicated by the here shown results. The observations are in line with the above mentioned studies.

Knowing that stimulated IL-4Rα-deficient BMDC are not able to secrete high levels of IL-12, which is a prerequisite to establish a Th1 immunity to infection, the cytokine release upon stimulation and loading with LmAg has been determined, to

rule out the possibility that LmAg-loading affects the cytokine secretion by these cells. The loading with LmAg has no significant effect on the secreted cytokines, but the presence of rIL-4 boosted the release of IL-12 in IL-4 responsive BMDC. Analyzing the IL-10 release of treated BMDC indicated that IL-4 stimulation led to significantly enhanced IL-10 release. In case of wt BMDC, high levels of Th2-inducing IL-10 is counterbalanced by enormous levels of Th1-inducing IL-12, resulting in a Th1-biased immunity. As IL-4Rα-deficient BMDC secrete elevated levels of IL-10 and reduced levels of IL-12, the establishment of a Th2 immunity is favored, possibly explaining the inability of these BMDC to mediate protection in susceptible BALB/c mice. The hyper-susceptibility of $CD11c^{cre}$ IL-$4R\alpha^{-/lox}$ mice to infection with *L. major* and the *in vitro* results shown in the present study, gave the first indications that immunization studies with IL-4Rα-deficient BMDC presumably would be at least less efficient in mediating protection against leishmaniasis.

DC-mediated vaccination strategies aim towards priming immunological memory to induce a rapid and efficient immune response against subsequent infections. The strategy behind this model is based on the knowledge that natural immunity to re-infections with *L. major* occurs following recovery from a primary infection. The solid immunity mediated by persisting parasites has been used to protect humans from CL by inoculating live parasites to covered parts of the body ("leishmanization") with the aim that healing from a primary infection provides solid protection against subsequent infections [14]. Shaping the adaptive immunity by the presentation of pathogen-derived antigens via DC represents the basis for DC-mediated vaccination strategies [200,201].

DC, as the most potent APC, have the ability to induce long lasting immunity by activating $CD4^+$ and $CD8^+$ T cells and shaping cell-mediated immune responses due to their pivotal role as IL-12 donor, thus supporting the use of DC as adjuvant for

vaccines [98,196]. Protection against challenge with *L. major* was mediated by LmAg-loaded DC, like LC or BMDC [125,129] when used to immunize mice one week prior to infection. Furthermore, *L. major*-infected murine skin-derived DC released enhanced levels of IL-12 implicating an anti-*Leishmania* directed immunity [39]. DC-derived IL-12 is critical to shape a *Leishmania*-effective Th1 cell-dominated immunity, as mice were protected against VL upon inoculation of IL-12 over-expressing DC [126].

In the present study, the importance of IL-4Rα triggering during DC-mediated vaccination against the protozoan parasite *L. major* was investigated. The results show that complete protection against otherwise lethal leishmaniasis required immunization of BALB/c mice with IL-4 responsive BMDC, while IL-4Rα$^{-/-}$ BMDC failed to induce the restriction of lesion development. Even though the footpad swelling was restricted during the first three weeks of infection, a progressive course of disease, with the development of severe and necrotic lesions was observed at later stages of infection. *In vitro* studies showed that IL-4Rα-deficient BMDC secrete lower amounts of IL-12 and higher amounts of IL-10 upon stimulation with CpG ODN and LmAg compared to wt BMDC, which is most probably the reason for the failure of immunization with IL-4Rα-deficient BMDC, as no differences were observed regarding the activation status of wt or IL-4Rα-deficient BMDC (data not shown). Importantly, the levels of *Leishmania*-stimulated IL-12, the most potent inducer of immunity to *L. major* [109,202], were significantly increased in the LN of wt BALB/c mice immunized with properly conditioned wt BMDC that had been additionally activated with rIL-4. Elevated levels of IL-12 upon stimulation with LmAg were only observed in IL-4 responder recipients (wt BALB/c), but not in CD11c^{cre}IL-4Rα$^{-/lox}$ mice, demonstrating that IL-4 instruction of host DC is required to induce elevated levels of IL-12 during *L. major* infection. BMDC activated with rIL-4 alone were not

able to mediate protection against *L. major*, but induced elevated *Leishmania*-stimulated IL-4 levels *in vivo* (data not shown), skewing $CD4^+$ T cells towards a Th2 cell phenotype and promoting susceptibility in BALB/c mice.

At the site of infection, neutrophils instruct DC recruitment and activation, leading to Th1 cell-activation and immunity to microbial infection [203]. Previous reports have shown that LN-residing DC of infected mice do not harbor *L. major* parasites nor parasite-derived antigens before three weeks post infection, even though parasites were found in the LN already a few hours post infection [188]. Quantitative and qualitative antigen-specific T cell priming within the LN occurs 2-3 weeks post infection, indicating the immunity-driving function of antigen-loaded DC within the lymphoid tissues during that time of T cell priming [41]. The results shown in the present study at three weeks post infection, go in line with these previously published observations. Our results extend these findings by showing that upon immunization with IL-4-responsive DC, higher percentages of activated and mature recipient DC are observed in the LN draining the site of infection. Concluding, these results demonstrate that upon immunization with IL-4-responsive BMDC, clinically protected mice do not only show controlled footpad swelling and parasite burden within the infected tissues, but also elevated levels of activated and mature recipient DC in the LN, indicating a prerequisite to induce strong *Leishmania*-specific T cell priming.

The Th1/Th2 paradigm of experimental leishmaniasis is associated with IL-12- and IFN-γ-mediated resistance or IL-4-mediated susceptibility to *L. major* infection [17]. It is commonly accepted that IL-4 is the hallmark cytokine mediating the differentiation of naïve Th0 cells into the Th2 phenotype. However, the point that an IL-4-mediated Th2 response renders mice necessarily susceptible has never been proven for VL [204]. Furthermore, several data revealed a Th1-promoting effect of IL-

4, while being capable to prime for bioactive IL-12. For example, treating human peripheral blood mononuclear cells (PBMC) with IL-4 enhanced their IL-12 response to lipopolysaccharide or *Staphylococcus aureus* [205]. IL-12 production by human monocytes during interaction with T cells was increased upon IL-4-stimulation [206] and IL-4 provided a negative feedback causing murine as well as human DC to produce IL-12 [151]. IL-4 was furthermore reported to be required for the induction of protective Th1 cell responses to fungal infections, such as *Candida albicans* [149].

A protective role of IL-4 has also been shown for *L. major* infection in susceptible BALB/c mice [175]. It is important to note that the resistance-promoting role of IL-4 was only achieved when IL-4 was strictly present during the initial activation of DC upon infection. The presence of IL-4 during T cell priming resulted in the development of Th2 cells, which even rendered resistant TCR V_β4-deficient BALB/c mice susceptible to leishmaniasis. IL-4, acting on DC, induced the generation of a protective Th1 immune response against leishmaniasis in BALB/c mice [175].

Furthermore, it has been demonstrated that endogenous IL-4 is necessary for effective drug therapy with sodium stibogluconate against VL in BALB/c mice, as IL-4-deficient mice responded poorly to this treatment and showed increased parasite burdens in infected tissues [144]. Another example for IL-4-promoted healing has been documented in BALB/c mice vaccinated with a liposomal formulation against *L. donovani*, where an initially vaccine-induced mixed Th1/Th2 response, characterized by high levels of IFN-γ and IL-4, instructed an efficient Th1-mediated resistance [207]. Our data are consistent with these studies, showing that IL-4Rα signaling is important, enabling DC to induce a protective immune response in the recipient mice, hallmarked by high levels of *L. major*-induced IL-12 production in the LN of infected IL-4 responder mice.

Elevated IL-4 levels during the late phase of *L. major* infection in resistant C57BL/6 mice were associated with the maintenance of an existing protection [29], whereas susceptible BALB/c mice showed elevated IL-4 levels only during the early phase of infection [208]. These findings suggested a role of IL-4 in sustaining protection during the chronic phase of leishmaniasis.

The results of the present study indicate a direct link between IL-4Rα triggering of BMDC used for immunization and the induction of elevated levels of IL-12 upon *L. major* infection in BALB/c mice, which mediated complete protection against otherwise lethal leishmaniasis. Resistance in leishmaniasis has been reported to depend on DC-derived IL-12 [129], the inhibition of *Leishmania*-specific IL-4-secretion by Vβ4Vα8 $CD4^+$ T cells and the induction of a Th1-dominated immune response *in vivo* [209]. Another aspect to give consideration to is that immunizing $CD11c^{cre}$IL-4Rα$^{-/lox}$ mice with IL-4Rα-deficient BMDC resulted in progressive leishmaniasis, showing the importance of IL-4Rα signaling not only in the immunizing BMDC, but also in the host DC. This complete IL-4Rα-deficient DC set-up caused uncontrolled parasite dissemination into the draining LN, indicating that the inhibition of IL-4Rα signaling in host DC is detrimental and leads to increased dissemination of parasites into organs. In general, $CD11c^{cre}$IL-4Rα$^{-/lox}$ mice secrete elevated levels of IL-4 and decreased Th1 cytokines compared to wt BALB/c mice and the effect of increased IL-12 secretion upon immunization with additionally rIL-4 stimulated wt BMDC was not observed in these mice, showing the important role of IL-4Rα signaling in host DC for IL-12 production during *L. major* infection. Immunized $CD11c^{cre}$IL-4Rα$^{-/lox}$ mice showed controlled IL-4 levels independent of the type of vaccine, while IL-4Rα-deficient BMDC failed to induce a protective Th1 cytokine profile, resulting in a non-protective Th2 immune response. These results showed

that IL-4Rα signaling in the DC vaccine carrier is more critical than the IL-4 responsiveness of host DC.

The present study enhances our understanding of the role of IL-4Rα signaling in DC during cell-mediated vaccination against an intracellular pathogen, as triggering this receptor is essential to confer protection. Vaccination strategies against Th2-related diseases, such as allergies or parasitic infections, should not only concentrate on inhibiting anti-inflammatory Th2 responses by inducing a strong Th1 phenotype, but need to consider the proinflammatory effect of IL-4 as adjuvant on the vaccine efficiency. An important aspect to be considered is that IL-4 as well as IL-13 can signal through the common IL-4Rα chain. *In vitro* results showed that wt BMDC stimulated with CpG ODN and IL-4, but not IL-13, induced the secretion of elevated IL-12 levels compared to CpG ODN stimulated wt BMDC and that additional stimulation with IL-4 or IL-13 failed to induce elevated levels of IL-12 secreted by IL-4Rα-deficient BMDC. These *in vitro* results strongly suggest that the elevated secretion of DC-derived IL-12 is induced by IL-4 instruction of DC and not by IL-13 instruction. The results of the present study underline the importance of IL-4 signaling during vaccine design, as IL-4Rα signaling in the DC vaccine carrier is more important than IL-4Rα signaling in the host DC. The results document the crucial role of IL-4Rα signaling in DC-based vaccination against leishmaniasis by promoting a protective Th1 immune response.

DC are also studied as natural adjuvant or antigen-presenter to evoke T cell-mediated antitumor immunity *in vitro* [116] and *in vivo* [210]. The immunogenicity of antigens delivered by DC increased the clinical survival for advanced hepatocellular carcinoma (HCC) patients [211] and DC-mediated vaccination provided a well grounded motivation for the cell-mediated immunotherapy of chronic hepatitis C virus (HCV) [212] as well as HIV-1 infections [213]. Successful phase III clinical trials

strengthened the focus on cell-mediated vaccination strategies, leading to highly efficient cancer vaccines [214]. With regard to this, further studies should be aimed to design efficient and cross-reactive DC-mediated vaccines, which mediate a strong and long-lasting immunity to pathogenic infections.

An abundance of studies show the importance of DC as an adjuvant in the vaccine delivery system, allowing proper antigen-presentation and the specific genetic manipulation of these DC *in vitro* with an immunity-shaping combination of compounds [129,131,214,215,216]. The transfer of DC-mediated vaccination strategies to human leishmaniasis depends on the development of cost-efficient standard operating protocols for the isolation; culture and treatment of human DC or allogenic DC. Homing of *in vitro* generated and manipulated DC within the host organism, has to be approached to direct the strict homing of the vaccine carrier to the site of lymphoid organs, thus inducing a prompt antigen-specific immune response. A very interesting approach, based on the strong and unique potential of antigen-presentation of DC to induce protective immunity to infections is, to develop strategies aiming to target DC *in vivo* with *in vitro* modified antigens [217], circumventing the laborious and costly step of isolating and manipulating human DC.

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6. References

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7. Abstract

Cutaneous leishmaniasis is endemic in tropical and subtropical regions of the world. Effective vaccination strategies are urgently needed because of the emergence of drug-resistant parasites and severe side effects of chemotherapy. The research group of Heidrun Moll previously established a DC-based vaccination strategy to induce complete and long-lasting immunity to experimental leishmaniasis using LmAg-loaded and CpG ODN-activated DC as a vaccine carrier.

Prevention of tissue damages at the site of *L. major* inoculation can be achieved if the BALB/c mice were systemically given LmAg-loaded BMDC that had been exposed to CpG ODN. The interest in further exploring the role of IL-4 aroused as previous studies allowed establishing that IL-4 was involved in the redirection of the immune response towards a type 1 profile. Thus, wt BALB/c mice or DC-specific $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ BALB/c mice were given either wt or IL-4Rα-deficient LmAg-loaded BMDC exposed or not to CpG ODN prior to inoculation of 2×10^5 stationary phase *L. major* promastigotes into the BALB/c footpad. The results provide evidence that IL4/IL-4Rα-mediated signaling in the vaccinating DC is required to prevent tissue damages at the site of *L. major* inoculation, as properly conditioned wt DC but not IL-4Rα-deficient DC were able to confer resistance. Furthermore, uncontrolled *L. major* population size expansion was observed in the footpad and the footpad draining LN in $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice immunized with CpG ODN-exposed LmAg-loaded IL-4Rα-deficient DC, indicating the influence of IL-4Rα-mediated signaling in host DC to control parasite replication. In addition, no footpad damage was observed in BALB/c mice that were systemically immunized with LmAg-loaded wt DC doubly exposed to CpG ODN and recombinant IL-4. Discussing these findings allow the assumption that

triggering the IL4/IL4Rα signaling pathway could be a precondition when designing vaccines aimed to prevent damaging processes in tissues hosting intracellular microorganisms.

8. Zusammenfassung

Die kutane Leishmaniose ist vor allem in den tropischen und subtropischen Regionen endemisch. Die Notwendigkeit der Erforschung und Etablierung einer Impfstoffstrategie basiert auf dem Auftreten von starken Nebenwirkungen während einer medikamentösen Behandlung, als auch auf die Entwicklung von Resistenzen des Parasiten gegenüber herkömmlichen Behandlungsmethoden.

Die Arbeitsgruppe um Heidrun Moll etablierte eine auf dendritischen Zellen (DZ) basierende Immunisierungsstrategie, welche langlebige Immunität gegen experimentelle Leishmaniose vermittelt. Dabei dienen CpG ODN-stimulierte DZ als Adjuvans für *L.-major*–Antigene (LmAg). Die durch Infektion mit *Leishmania*-Parasiten hervorgerufene Gewebeschädigung kann in BALB/c-Mäusen verhindert werden, vorausgesetzt eine systemische Verabreichung von LmAg-beladenen und CpG ODN-aktivierten DZ erfolgte eine Woche vor der Infektion. Es konnte gezeigt werden, dass der Schutz durch die Induktion einer von Interleukin (IL)-12 und Interferon (IFN)-gamma dominierten T-Helfer (Th)1-Immunantwort herbeigeführt wurde und kranke Kontrollmäuse eine IL-4-dominierte Th2 Immunantwort aufwiesen. Mittlerweile zeigen zahlreiche Studien, dass IL-4 nicht ausschließlich eine krankheitsfördernde Funktion innehat, sondern auch die Fähigkeit zur Einleitung eine Typ-1-Immunantwort besitzt. Auf Grund dieser Studien wurde das Augenmerk auf die Rolle von IL-4 in der DZ-basierten Immunisierung gegen Leishmaniose in BALB/c Mäusen gelegt.

In der vorliegenden Arbeit wurde die Notwendigkeit der Stimulation der IL-4 Rezeptor alpha (IL-4Rα) Kette auf DZ, während einer DZ-basierten Immunisierung gegen Leishmaniose in BALB/c Mäusen gezeigt. Um dies zu erreichen, wurden Wildtyp (wt)-BALB/c-Mäuse oder DZ-spezifische $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ BALB/c Mäuse

entweder mit wt oder IL-4Rα-defizienten LmAg-beladenen DZ mit oder ohne Aktivierung durch CpG ODN, eine Woche vor der Infektion mit $2x10^5$ *L. major* Promastigoten in den Hinterfuß, immunisiert.

Die in dieser Doktorarbeit gezeigten Ergebnisse lassen den Schluss zu, dass die Stimulation der IL-4Rα-Kette auf den als Adjuvans eingesetzten DZ erforderlich ist, um eine Gewebsschädigung an der Infektionsstelle zu verhindern, da konditionierte wt DZ, nicht aber IL-4Rα-defiziente DZ in der Lage sind, Schutz gegen Leishmaniose zu vermitteln. Des Weiteren konnte eine unkontrollierte Ausdehnung von *Leishmania*-Parasiten im infizierten Fuß und in den angrenzenden Lymphknoten von $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ Mäusen beobachtet werden, welche mit CpG ODN-aktivierten und LmAg-beladenen IL-4Rα-defizienten DZ immunisiert wurden. Dieser Befund zeigt den Einfluss der Stimulation der IL-4Rα-Kette auf wirtsansässigen DZ im Hinblick auf die Eindämmung der Parasitenreplikation und Parasitenverbreitung. Zusätzliche Analysen in BALB/c-Mäusen, welche mit LmAg-beladenen, CpG ODN- und rekombinanten IL-4-stimulierten DZ immunisiert wurden, zeigten einen resistenten klinischen Verlauf der Infektion.

Die hier gezeigten Ergebnisse lassen die Vermutung zu, dass die durch die IL-4/IL-4Rα-Kette ausgelösten Signale in den DZ eine Grundvoraussetzung für eine erfolgreiche Immunisierung sind und sollten deswegen unbedingt bei der Entwicklung eines Impfstoffes gegen die gewebsschädigenden Folgen einer Leishmaniose oder anderer durch intrazelluläre Mikroorganismen verursachten Infektionen berücksichtigt werden.

9. Affidavit

I herby confirm that my thesis entitled “Signaling via Interleukin-4 Receptor α chain during dendritic cell–mediated vaccination is required to induce protective immunity against *Leishmania major* in susceptible BALB/c mice” 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 confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Würzburg, 02.10.2012 Anita Masic

10. Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation “Signaling via Interleukin-4 Receptor α chain during dendritic cell–mediated vaccination is required to induce protective immunity against *Leishmania major* in susceptible BALB/c mice” eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters angefertigt und keine andere 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, 02.10.2012 Anita Masic

11. Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
APC	Antigen-presenting cell
BCG	Bacillus Calmette-Guerin
BMDC	Bone marrow-derived DC
BSA	Bovine serum albumin
CCL3	Chemokine (C-C motif) ligand 3
CD	Cluster of differentiation
CL	Cutaneous leishmaniasis
CpG ODN	Cytosine-phosphate-guanosine oligodeoxynucleotide
CR3	Complement receptor 3
DC	Dendritic cell
DCL	Diffuse cutaneous leishmaniasis
DC-SIGN	DC-specific intracellular adhesion molecule 3-grabbing nonintegrin
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
F2/F3	Filial generation
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluoresceinisothiocyanat
GFP	Green fluorescent protein
GM-CSF	Granulocyte/macrophage colony-stimulating factor
GTP	Guanosintriphosphate
HIV	Human Immunodeficiency Virus

HPSF	High purity salt free
HRP	Horseradish peroxidase
i.v.	Intravenous
IFN-γ	Interferon gamma
IL	Interleukin
IL-12R	Interleukin-12 Receptor
iNOS	Inducible nitric oxide synthase
IRF	Interferon regulatory factor
L. major	*Leishmania major*
LACK	*Leishmania* homologue of eukaryotic ribosomal elongation and initiation factor 4a
LC	Langerhans cell
LmAg	*Leishmania major*- Antigen
LN	Lymph node
LPG	Lipophosphoglycan
mAb	monoclonal Antibody
MAPK	Mitogen-activated protein kinase
MCL	Mucocutaneous leishmaniasis
mDC	myeloid DC
MHC	Major histocompatibility complex
MPL	Monophosphoryl lipid
NF-κB	Nuclear factor kappa B
NK	Natural killer cell
NO	Nitric oxide
OD	Optical density
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pDC	plasmacytoid DC

PE	Phycoerythrin
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol 3-kinase
PMA	Phorbol myristate acetate
Rab	Ras-related protein
rIL	recombinant IL
RNI	Reactive nitrogen intermediates
ROS	Reactive oxygen species
SAV	Streptavidin
SD	Standard deviation
TCR	T cell receptor
Th	T helper
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
T_{reg}	Regulatory T cells
VL	Visceral leishmaniasis
WHO	World Health Organization
wt	wild type

12. Acknowledgement

First of all I would like to thank Prof. Heidrun Moll for the supervision of my PhD project and for the chance to be a member of the IRTG 1522 “HIV/AIDS and associated infectious diseases in Southern Africa“, for the freedom and possibility to attend and present my work on international meetings, for the possibility to meet many outstanding scientists, for her encouragement to plan and organize my PhD project and my working stays in Cape Town independently, for her support during the preparation of the publication and the doctoral thesis, but above all for exciting my passion for infection immunology.

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I was privileged to be a member of the IRTG 1522 “HIV/AIDS and associated infectious diseases in Southern Africa” headed by Prof. Axel Rethwilm (Würzburg, Germany) and Prof. Wolfgang Preiser (Stellenbosch, South Africa). The IRTG 1522

gave me the opportunity to work in an international environment, to gain insights into different fields of research and most of all, it allowed me to stay in our cooperating laboratory in Cape Town, twice. As part of our annual IRTG 1522 meetings and international symposia I was able to present and discuss my project with many outstanding scientists. The nice and warm atmosphere between the PhD students and PIs made me always feel very comfortable and I am very glad that I joined this program.

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13. Publications and presentations

Publications

Masic A, Hurdayal R, Nieuwenhuizen NE, Brombacher F, Moll H (2012)
Dendritic cell-mediated vaccination relies on Interleukin-4 Receptor signaling to avoid tissue damage after *Leishmania major* infection of BALB/c mice. PLoS Negl Trop Dis 6(7): e1721

Schurigt U, **Masic A,** Moll H. (2012)
Interaction of *Leishmania* parasites with host cells and its functional consequences. In T. Jäger, O. Koch, and L. Flohé (ed.), Drug Discovery for Trypanosomatid Diseases. Wiley-VCH, Weinheim. 1. Auflage April 2013.

Poster

Masic A. and Moll H. (2012)
Hurdayal R, Nieuwenhuizen NE, Brombacher F.
"The role of IL-4Rα signaling on dendritic cells during cutaneous leishmaniasis" Poster presentation at the IRTG 1522 "HIV/AIDS and associated infectious diseases in Southern Africa"; DFG/NRF review meeting, 26th June 2012 in Würzburg, Germany

Masic A, Hurdayal R, Révaz-Breton M, Brombacher F, Moll H. (2010)
"Role of IL-4Rα on Dendritic Cells during Vaccination against *Leishmania major*" Poster presentation at the 40th Annual Meeting of the DGfI, 23rd September 2010 in Leipzig, Germany

Abstracts

Masic A, Hurdayal R, Nieuwenhuizen NE, Brombacher F and Moll H. (2012) **"Interleukin-4 Receptor Signaling during Dendritic Cell-Mediated Vaccination is Required to Induce Protection against *Leishmania major* in susceptible BALB/c Mice"** Meetingabstract; DGP Meeting 2012; Deutsche Gesellschaft für Parasitologie, 14. – 17.03.2012, Heidelberg, Germany

International Symposia

24. – 26. 06. 2012; Annual Meeting of the IRTG 1522 "HIV/AIDS and associated infectious diseases in Southern Africa", IRTG 1522, Würzburg, Germany
Contribution: Talk "DC-mediated vaccination relies on Interleukin-4 Receptor signaling to avoid tissue damage after *Leishmania major* infection in BALB/c mice"

14. – 17. 03. 2012; DGP Meeting 2012; Deutsche Gesellschaft für Parasitologie; Heidelberg; Germany
Contribution: **Talk "Interleukin-4 Receptor Signaling during Dendritic Cell-Mediated Vaccination is Required to Induce Protection against *Leishmania major* in susceptible BALB/c Mice"**

19. – 20. 01 2012; IRTG 1522 International Meeting "HIV/AIDS and associated infectious diseases in Southern Africa"; IRTG 1522; Würzburg, Germany
Contribution: **Talk "Role of IL-4Rα on Dendritic Cells during Vaccination against *Leishmania major*"**

24. – 26. 02. 2011; 3rd International Symposium of the IRTG 1522; IRTG 1522 "HIV/AIDS and associated infectious diseases in Southern Africa"; Stellenbosch, South Africa
Contribution: **Talk "The role of IL-4Rα during DC-mediated vaccination against *Leishmania major*"**

22. – 25. 09. 2010; 40th Annual Meeting of the DGfI; Deutsche Gesellschaft für Immunologie; Leipzig, Germany

Contribution: **Poster „The role of Interleukin-4 Receptor alpha chain (IL-4Rα) on dendritic cells during DC-mediated vaccination against *Leishmania major*"**

14. – 16. 05.2010; IRTG 1522 Launch Symposium; IRTG 1522 "HIV/AIDS and associated infectious diseases in Southern Africa"; Kloster Banz, Germany

Contribution: **Talk "Role of IL-4Rα on Dendritic Cells during Vaccination against *Leishmania major*"**

27. – 28.02.2009; IRTG 1522 International Launch Symposium; IRTG 1522 „HIV/AIDS and associated infectious diseases in Southern Africa"; Stellenbosch, South Africa

Contribution: **Talk "The role of Dectin-1 and Interleukin-4 Receptor alpha chain (IL-4Rα) on Dendritic cells in Leishmaniasis"**