DENDRITIC CELLS ACTIVATED BY CpG MOTIFS ARE POTENT INDUCERS OF A TH1 IMMUNE RESPONSE THAT PROTECTS MICE AGAINST LEISHMANIASIS

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CONTENT

INTRODUCTION

1. Leishmaniasis

1.1 Definition

Human infection with parasites of the genus *Leishmania* may lead to a variety of clinical syndromes collectively known as leishmaniasis. The name is related to W. B. Leishman who first isolated the organism from a British soldier suffering from an idiopathic febrile illness, associated its presence with the symptoms of the disease, and reported its morphological similarity to trypanosomes [1]. An increasing number of different *Leishmania* species are now implicated as causal agents of the disease which are transmitted by sandflies of the genus *Phlebotomus* [Old World] or *Lutzomyia* [New World] during biting.

1.2 The parasite

The genus *Leishmania* belongs to the Subkingdom Protozoa, Phylum Sarcomastigophora, Order Kinetoplastida, and Family Trypanosomatidae, which also includes the genus *Trypanosoma* that comprises several other human pathogens. Kinetoplastidae are unicellular organisms that possess a single flagellum and have a characteristic structure called the kinetoplast that extends to a single large mitochondrion. Although a variety of different morphological forms of the parasite has been described in the insect vector, they are collectively called promastigotes. Promastigotes are extracellular, elongated, motile and harbor a long flagellum emerging from the anterior end of the body [2]. In nature, they exist exclusively in the gut of the female phlebotomine sandfly, but can be propagated in vitro in culture medium conditions that mimic the insect environment. The second morphological form is the small amastigote that is spherically shaped, non-motile and lacks an external flagellum. Amastigotes are obligate intracellular parasites of macrophages and other cells of the reticuloendothelial system of the mammalian host. They can also be produced in vitro by infecting macrophage monolayers and, more recently, some *Leishmania* species have been propagated in vitro as axenic amastigotes by culturing parasites in conditions that mimic the endosomal compartment of the mammalian host [3, 4]. The life cycle of *Leishmania* parasites is described in the figure 1.

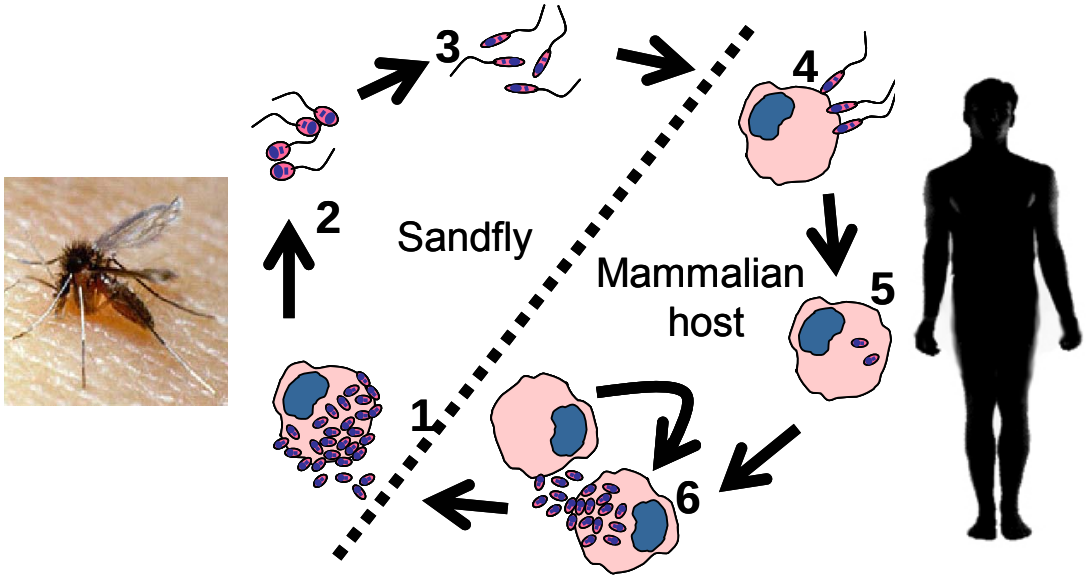

Fig.1. The life cycle of *Leishmania sp.* *Leishmania* parasites cycle between an invertebrate and a vertebrate host. When a sandfly acquires amastigotes during a bloodmeal [1], most of the parasites are destroyed within the first hours and those surviving transform into promastigotes [2]. After a few days, procyclic promastigotes replicate actively and colonize the midgut epithelium by using the flagellum [2]. Thereafter, promastigotes differentiate to infective metacyclic forms, and migrate to the pharynx where they can be transmitted to a new mammalian host during a subsequent bloodmeal [3]. The infected sandfly may inoculate 10-200 promastigotes into the dermis of a vertebrate host. Most of the free promastigotes will be destroyed by polymorphonuclear leucocytes but some attach to receptors on the surface of macrophages and other APC [4] and are phagocytosed [5]. Within the macrophage, promastigotes rapidly transform into amastigotes [5]. The amastigote form of *Leishmania* parasites is able to resist the intracellular anti-microbial mechanisms and readily multiplies [6]. Amastigote-filled macrophages burst, and the parasites re-infect other cells [6].

1.3 Clinical presentation of leishmaniasis

Symptomatic leishmaniasis in humans occurs in at least three major forms: cutaneous, mucocutaneous and visceral [Fig. 2]. The different presentations are the consequence of the pathologic reactions caused by the parasite in those sites and are believed to reflect the net effect of the parasite tropism and virulence and the immune status of the hosts. Cutaneous leishmaniasis [CL] is the most widespread form of the disease [Fig. 2A], found in Africa, the Indian subcontinent, Latin America, south west Asia and part of the Mediterranean basin. Although the clinical onset and evolution of CL may change according to the species and geographical localization, the lesion generally starts with an erythematous papule that appears between a few days to a few months after infection, and develops to a nodule. Most frequently, the nodule ulcerates and evolves to one of the several ulcerative forms of the disease, or develops to other nodular-type of lesions. In the Old World CL is mostly due to *L. major*,

which produces self-healing lesions that may be multiple and disabling. In most of the cases, healing of CL lesions leaves disfiguring scares that create a lifelong aesthetic stigma. CL due to *L. tropica* is usually more chronic and its most severe form, recidivans leishmaniasis, is very difficult to treat, often becoming long-lasting, destructive and disfiguring. In the New world, *L. braziliensis*, *L. panamensis*, *L. guyanensis* and *L. mexicana* produce CL lesions ranging from self-healing ulcers to chronic lesions which are difficult to treat. A fortunately less frequent cutaneous presentation of this disease is the diffuse cutaneous leishmaniasis [DCL]. It is typically caused by *L. aethiopica* in the Old World and by *L. amazonensis* or *L. mexicana* in the New World, and is associated with a *Leishmania*-specific defect in the cell-mediated immune response. In DCL patients the initial nodule does not ulcerate, and new nodules commonly develop on the face of the patient [Africa] and on the whole body [South America]. The entire face may be covered by nodules, which closely resemble lepromatous leprosy. DCL never heals spontaneously and is extremely difficult to treat [5].

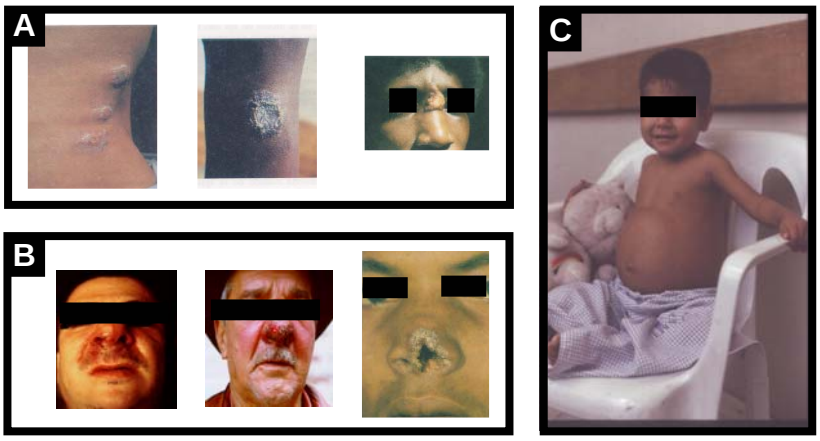

Fig. 2. Clinical presentations of leishmaniasis. A: cutaneous leishmaniasis, B: mucocutaneous leishmaniasis, C: visceral leishmaniasis.

Mucocutaneous leishmaniasis [MCL] is characterized by an extensive destruction of oronasal and pharyngeal cavities with hideous disfiguring lesions, mutilation of the face and great suffering for life [Fig. 2B]. An exacerbated destructive cell-mediated immune [CMI] response seems to be responsible of the pathology in MCL patients. Interestingly, MCL is believed to be a consequence of parasite reactivation, since mucosal lesions appear typically months or even years after the healing of the primary skin lesion in the absence of re-infection. It is mostly related to *Leishmania* species of

the New World such as *L. braziliensis*, *L. panamensis* and *L. guyanensis*, but mucosal lesions caused by *L. major*, *L. donovani* and *L. infantum* in immunosupressed patients have also been reported [5].

In visceral leishmaniasis [VL] or kala-azar, the parasite invades internal organs [spleen, liver, bone marrow, etc] inducing fever, loss of weight, splenomegaly, hepatomegaly, lymphoadenopathy and anemia [Fig. 2C]. It is the most severe form of leishmaniasis and nearly always fatal if left untreated. VL is endemic in several parts of Africa, India and Latin America, and is caused by *L. chagasi* in the New World and *L. donovani* or *L. infantum* in the Old World. Post kala-azar dermal leishmaniasis [PKDL] is usually manifested as a complication of VL, and is considered the major source of VL transmission in India. PKDL develops 1-5 years after the apparent clinical cure of the visceral disease with chronic nodular cutaneous lesions similar to DCL and usually requires a long and expensive treatment [5].

1.4 Epidemiology and public health

The World Health Organization [WHO] considers leishmaniasis as one of the most important human diseases to be targeted. Leishmaniases are present in four continents and are considered to be endemic in 88 countries, most of them being underdeveloped or developing countries. More than 90% of the VL cases in the world are reported from four countries and more than 90% of the CL cases occur in six countries. The number of people suffering from leishmaniasis is estimated to be 12 million and the population living in endemic areas and therefore at risk of infection is approximately 350 million world-wide. Currently the global annual incidence is estimated to be 1-1.5 million new cases of CL and 500,000 new cases of VL per year. Although these estimations are based on official reports, the real number of people affected by *Leishmania* parasites is thought to be higher for the following reasons: (1) numerous cases are undiagnosed, misdiagnosed or unreported due to the poor access of patients to medical facilities. (2), most of the official data are obtained through passive case detection only and not trough active search. (3), leishmaniasis is a notifiable disease only in 40 of the 88 endemic countries. (4), in many endemic areas, there is a significant number of infected but asymptomatic people that are even more relevant epidemiologically than leishmaniasis cases. In spite of the scarcity of reliable data, there is no doubt that the number of both cases of clinically apparent leishmaniasis as

well as infected asymptomatic people around the world is considerably greater than official reports. At least 20 different *Leishmania* species are known to cause leishmaniasis and are transmitted by at least 30 different species of sandflies. A variety of domestic and wild animals have also been described as reservoirs of the parasite. In most of the endemic areas *Leishmania* transmission is zoonotic and humans are infected only secondarily. In some other areas, however, leishmaniasis is anthroponotic and humans are believed to be the unique reservoir. Such a variety in the etiological agents, vectors and reservoirs explains the complexity of the epidemiological patterns of leishmaniasis in different areas and complicates the possibility to implement strategies of control [5, 6].

1.5 Experimental leishmaniasis

Many experimental models of leishmaniasis have been developed. A pioneer model used the naturally occurring infection by *L. enrietti* in guinea pigs and allowed to introduce the notion that CMI is responsible for resistance to leishmaniasis [7]. This model, however, started to be replaced by murine models in the 70's, when it was appreciated that CBA mice were immune whereas BALB/c mice were susceptible to infection with a *Leishmania* species that causes human disease [8, 9]. Since then, a number of mouse strains have been used as experimental models of infection with different *Leishmania* species including *L. major*, *L. tropica*, *L. mexicana*, *L. amazonensis*, *L. donovani*, *L. infantum* and *L. braziliensis*. Some other animals like the gold hamster, the dog, or non-human primates have been also used as models for visceral leishmaniasis by *L. donovani* or cutaneous leishmaniasis caused by *L. viannia* subgenus since they appear to better reproduce some aspects of the clinical presentation of those infections in humans [10]. However, difficulties and costs with manipulating those larger animals and the lack of relevant reagents for assessing important parameters of the immune response have hampered the use of those models. In contrast, genetically homogeneous inbred strains of mice are available and allow to evaluate the genetic contribution of the host in resistance and susceptibility. Reagents for mouse immune markers as well as mice selectively deficient for relevant immune molecules are readily available, allowing to assess the role of specific immune pathways in the development of resistance or disease. For these reasons, murine models of leishmaniasis represent currently the most powerful approach to dissect the

complexity of the host-parasite interaction in this infection and intracellular parasitism in general.

Experimental infection of inbred mice with *L. major* promastigotes is perhaps the best-studied model of a chronic infectious disease and many of the concepts derived from those studies have also been shown to hold true for other non-protozoal intracellular pathogens. After subcutaneous or intradermal infection with *L. major*, mice from the majority of inbred strains [C57BL/6, CBA/N, C3H/He, Sv129/Ev, etc.] develop only small lesions at the site of the inoculation that cure spontaneously after 6-12 weeks and become immune to re-infection. In contrast, mice from a few other strains such as BALB/c are susceptible to infection, since after the same protocol of infection they develop progressive lesions that never heal, and the parasite actively replicates and disseminates to internal organs leading to a systemic disease and death [11, 12]. This genetically determined predisposition for susceptibility or resistance is currently known to be the result of a selective expansion of different T cell populations that mediate an interleukin [IL]-4-dominated response that causes disease or an interferon [IFN]-γ-dominated response that promotes parasite clearance and healing.

2. The immunobiology of murine leishmaniasis

2.1 Cell- but not antibody-mediated immunity is crucial to resist *Leishmania* infection

Although early reports indicated a possible contribution of a *Leishmania*-specific antibody [Ab] response in the generation of protective immunity against leishmaniasis, a vast amount of data indicates that effective control of disease is dependent upon the development of cellular but not humoral immunity [13-15]. For instance, Ab levels do not correlate with resistance to disease in several strains of mice [16]; passive transfer of Ab fractions from immune mice do not affect infection of BALB/c mice [17]; mice deficient in Ab response are still resistant to *L. major* infection [18]; and whereas the delayed-type hypersensitivity [DTH] response is usually absent in infected BALB/c mice, the Ab titers are often higher than those observed in resistant mice [19]. In addition, B cell depletion failed to influence the

outcome of *L. major* infection in resistant mice [20] and reduced lesions in susceptible BALB/c mice [21]. In contrast, cellular immune response has been demonstrated to mediate protection against infection in resistant mice and also in susceptible mice that are protected from the disease by vaccination or immunological intervention. For instance, athymic nude mice [nu/nu] which are T cell-deficient, are susceptible to *L. major* or *L. tropica* infection even on a resistant CBA or C57BL/6 background [12, 22]; Adoptive transfer of syngeneic T cells to these mice restores the ability to resist infection [12]; resistant mice, rendered T cell-deficient by thymectomy and irradiation, also display reduced ability to resolve disease [8]. Protective immunity can be transferred to syngeneic naive mice by T cells from resistant mice that have cured a primary infection [11]. Similarly, T cells from susceptible mice that have been immunized against infection can transfer protection to naïve recipients [23]. These experiments solidly support the essential nature of the T cell responses in mediating protective immunity against murine leishmaniasis.

2.2 $CD4^+$ T cells are key players in resistance and regulation of immunity to *L. major* infection

The adoptive transfer of purified $CD4^+$ T cells was sufficient to restore resistance in T cell-deficient mice and to transfer immunity from immune to naïve resistant mice [11, 12, 23-25]. Similarly, resistance to infection can also be conferred to naïve BALB/c mice by transferring $CD4^+$ cells from immunized mice [23]. More interesting, susceptibility of BALB/c mice to *L. major* can be overcome by sublethal whole body γ-irradiation [26], injection with anti-CD4 Ab [27], or cyclosporine A treatment [28]. After resolution of infection in those mice, they are refractory to further *Leishmania* challenge and mount a strong DTH response to parasite antigen [29]. Moreover, $CD4^+$ cells from those mice can transfer DTH responsiveness and resistance to naïve mice [23, 29]. These results indicate that $CD4^+$ T cells are required for resistance and for the induction of protective immunity in susceptible mice. However, irradiation and anti-CD4 Ab experiments suggest that these cells are also actively implicated in susceptibility. Indeed, sublethally irradiated resistant mice are not able to clear the infection if they are reconstituted with hematopoietic cells derived from naïve susceptible mice [30]. Furthermore, the protective effect of irradiation, anti-CD4 Ab or cyclosporine A in BALB/c can be suppressed by transfer of naïve T cells [26] or $CD4^+$ cells [24] from susceptible BALB/c mice. Moreover, $CD4^+$ T cell lines derived

from BALB/c mice were shown to be able to suppress the generation of protective immunity in resistant [31] and immunized susceptible mice [32]. Therefore, $CD4^+$ T cells from susceptible mice not only fail to mediate protection but they also can suppress an ongoing protective response. In summary, these experiments demonstrated that both susceptibility and resistance are determined by a $CD4^+$ subset of T cells. These $CD4^+$ T cell subsets mediate either effector or suppressor functions and, therefore, the selective induction of one of them would determine the outcome of disease.

2.3 Th1/Th2 cells and resistance or susceptibility to *L. major*

In 1986, Mosmann and colleagues [33] demonstrated that $CD4^+$ T cells can be divided into at least two subsets according to their lymphokine secretion patterns. T cells that respond to antigen by secreting IL-2, IFN-γ and tumor necrosis factor [TNF]-β were designated T helper [Th] 1 cells, while T cells releasing IL-4, IL-5 and IL-10 were termed Th2 cells [34]. Th1 cells are known to mediate cellular immune functions such as DTH [35], whereas Th2 cells provide efficient help for the generation of humoral responses [36]. A large body of information implicating these subsets in the cure or progression of experimental leishmaniasis and other intracellular infections has been published since then. $CD4^+$ cells from lymph node [LN] and spleen of infected resistant mice contain high levels of IFN-γ and IL-2 mRNA and undetectable messages for IL-4 and IL-10, whereas susceptible mice exhibit an opposite pattern [37]. Serum levels of IgE are also elevated in infected BALB/c mice [38], reflecting the role of IL-4 in immunoglobulin [Ig] E switching. More convincing evidence for the regulation of *L. major* infection by Th1 and Th2 cells was subsequently provided when parasite-specific T cell lines and clones that secrete different pattern of cytokines in vitro upon T cell receptor [TCR] engagement were developed and used in transfer experiments. When T cell lines that respond in vitro to parasite antigen by secreting either IFN-γ and IL-2 or IL-4 were transferred to BALB/c mice, protection or exacerbation was observed, respectively [39]. Similar results were observed when cells lines were transferred to severe combined immunodeficient [SCID] mice [40]. In addition, a T cell clone that recognizes a soluble antigen from *L. major* and secretes IFN-γ and IL-2 could transfer protection to BALB/c mice [41]. Thus, Th1 or Th2 cells are sufficient to confer the entire phenotype of resistance or susceptibility to mice infected with *L. major*.

2.4 Th1- and Th2-related cytokines in resistance and susceptibility

A more straightforward evidence for the in vivo relevance of Th1 and Th2 cells comes from the functional analysis of the role of the cytokines they produce. The requirement of IFN-γ for the control of *L. major* infections is clearly demonstrated by the observation that resistant mice either treated with a monoclonal Ab [mAb] directed against IFN-γ or deficient in IFN-γ expression by gene disruption were unable to control leishmaniasis and developed a Th2-type of response [42, 43]. The administration of IFN-γ to susceptible mice did not affect the final outcome of disease [44, 45], indicating that this cytokine is necessary but not sufficient for protective immunity. The analysis of other Th1-related cytokines, such as IL-2, indicated a less relevant role in resistance although it seems to contribute to the development of Th2 cells at the initial stages of infection [46].

The analysis of the role of Th2-related cytokines has been strongly focused to IL-4 since it seems to play a substantial role in the generation of a non-healer response. Susceptible BALB/c mice were completely protected against the disease and developed a Th1 response when an anti-IL-4 mAb was administrated during early infection [44]. In agreement with this, IL-4-deficient BALB/c mice were able to resolve lesions after infection with a particular strain of *L. major* [47, 48]. Delivery of recombinant IL-4 to the lesion site at an early stage of infection exacerbated the disease in BALB/c mice [49], whereas its injection into resistant mice reduced the Th1 response although it did not generate susceptibility [50]. Transgenic expression of IL-4 in the B cell compartment, however, rendered resistant mice susceptible as they were unable to control *L. major* infection [51]. Thus, IL-4 is necessary and sufficient to generate a non-healing response in experimental murine leishmaniasis. When several strains of mice exhibiting different degrees of resistance to *L. major* infection were analyzed for IL-4 and IFN-γ production, the expression of IL-4 mRNA correlated well with the severity of lesion formation, while the IFN-γ mRNA expression did not correlate with resistance [52]. Therefore, the production of IL-4 is more critical than IFN-γ production and seems to control lesion development.

Further evidence for the importance of Th cell polarization in vivo relies on the observation that both subsets appear to inhibit each others functions. For instance,

IFN-γ can inhibit the IL-2- or IL-4-induced proliferation of Th2 cells in vitro [53, 54], presumably due to the differential expression of IFN-γ receptor on the surface of those cells [55]. BALB/c mice injected with IFN-γ plus *L. major* have a reduced IL-4 production [45] and IFN-γ-deficient mice on a resistant background develop a functional Th2 response to infection [43]. This suggests that in normal mice IFN-γ may suppress the induction of Th2 cells. Similarly, IL-10 is known to inhibit cytokine secretion and proliferation of Th1 cells [56-58]. Thus, the initial generation of either cell subset will inhibit the generation and function of the other, causing a polarized response to the infection and, therefore, the initial events leading to subset activation are critical for the outcome of disease [Fig. 3].

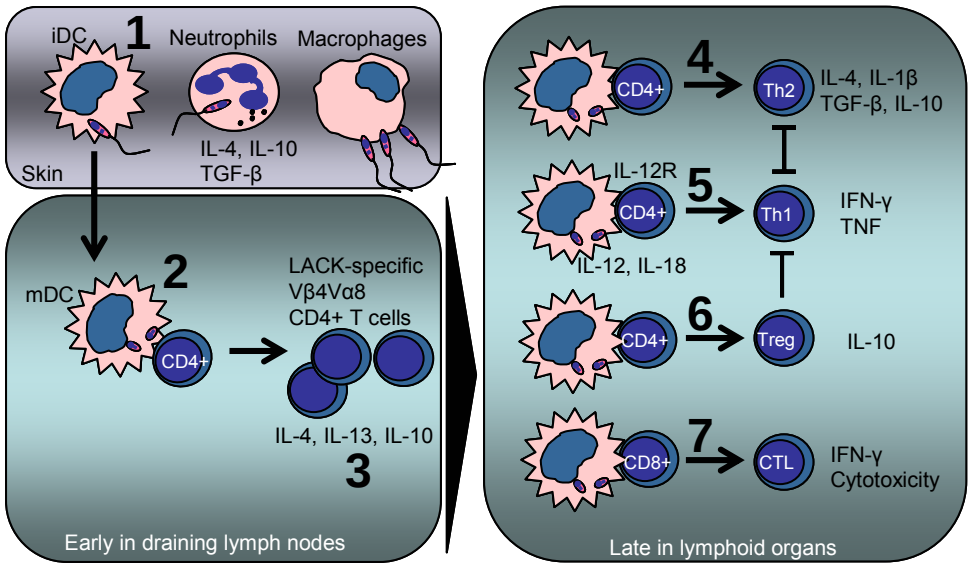

Fig. 3. Immune responses in mice after *L. major* infection. After *L.major* deposition in the skin, neutrophils, macrophages and dendritic cells [DC] take up parasites [1]. Infected DC undergo maturation and migrate to draining lymph nodes [2]. In both resistant and susceptible mice, an early production of IL-4 and other type-2 cytokines is observed in lymph nodes and appears to be due to the selective amplification of an oligoclonal population of T cells expressing a Vβ4Vα8 TCR that recognizes the LACK peptide [3]. In susceptible mice, the early Th2 response is not redirected and a stable commitment towards a Th2 response is observed [4]. This inability of redirecting the Th response is due in part to the instable expression of the IL-12R β2-chain on activated $CD4^+$ T cells and possibly to the action of type 2-biased DC in different lymphoid organs after dissemination of parasites to the viscera. In resistant mice, the parasite is restricted to the draining lymph nodes and an IL-12-dependent redirection towards a stable Th1 response is observed [5]. Th1 and Th2 cells co-exist in both resistant and susceptible mice and negatively affect each others development. However, some weeks after infection, the overall response is dominated by Th1 and Th2 cells in resistant and susceptible mice, respectively. Even in the presence of an effective Th1 response, a subpopulation of IL-10-producing regulatory T cells are produced that prevent immunopathology and promote parasite persistence [6]. Other cells like $CD8^+$ T cells are also activated during *L. major* infection and appear to contribute to resistance [7], whereas neutrophils help to the development of susceptibility by producing type-2 cytokines [1]. iDC: immature DC, mDC: mature DC, TGF-β: tumor growth factor-β, TNF: tumor necrosis factor, Treg: regulatory T cell, CTL: cytotoxic lymphocyte.

2.5 The induction of $CD4^+$ T cell subsets

Th1 and Th2 cells are believed to develop from a common $CD4^+$ T precursor cell termed Th0 which produces cytokines of both types [59-62]. The currently known factors influencing polarization of $CD4^+$ cells include: (1) the dose, route and type of antigen, (2) the type of antigen-presenting cell [APC] stimulating the T cell, (3) the "strength" of the signal, i.e. the affinity of the TCR for the major histocompatibility complex [MHC]-antigen complex plus receptor density and duration of receptor ligation and (4) the presence of immunologically active hormones such as glucocorticoids [63]. However, all these factors seem to operate by modifying the local cytokine milieu and, therefore, the cytokine environment surrounding the newly activated T cell is a fifth factor believed to be the most important force driving $CD4^+$ T cell polarization. In fact, a considerable number of models indicated that priming of naïve $CD4^+$ cells in the presence of IL-4 or IL-12 induces the development of a Th2 or Th1 response, respectively, and that polarization takes place soon after $CD4^+$ T cell activation [63]. The role of these two cytokines in the initiation of a protective or disease-promoting immune response to *L. major* in mice is well established. The experiments outlined above showed that IL-4 production shortly after infection is critical for disease development. The decisive role of IL-12 for the initiation of a protective Th1 response was subsequently demonstrated by the observation that resistant C57BL/6 mice genetically deficient in IL-12 expression develop an aberrant Th2 response with high levels of IL-4 and succumb to *L. major* infection [64]. A similarly sustained Th2 response and disease exacerbation was observed in wild-type resistant mice chronically treated with anti-IL-12 Ab [46, 65]. Moreover, exogenous administration of IL-12 to BALB/c mice early after infection is able to redirect the normal Th2 response and promote resistance [46, 66] [Fig. 3].

A careful analysis of the kinetics of cytokine expression in LN after subcutaneous [s.c.] infection with *L. major* has been performed by several groups, and, although some conflicting results were obtained, the most important conclusions were common [45, 67-69]. As soon as 16 hours after infection, BALB/c mice start to produce large amounts of IL-4 and the high levels are maintained during disease progression [70, 71]. The cellular source of this early IL-4 burst was later identified as $CD4^+$ cells in depletion and purification experiments [72]. Interestingly, the analysis of the TCR usage of those cells in infected mice demonstrated that all of the IL-4 mRNA was

produced by $CD4^+$ T cells expressing the Vβ4 and the Vα8 TCR chains, and that these cells recognized the *Leishmania* antigen LACK [*Leishmania* homolog of receptor for activated C kinase] [73]. Elegant experiments, in which a Th1 curative response was induced in BALB/c mice that were either deficient in Vβ4 $CD4^+$ T cells or tolerant to the LACK antigen as a result of the transgenic expression of the protein, led to the conclusion that this oligoclonal Vβ4 Vα4 $CD4^+$ population is responsible for the early disease-promoting IL-4 response in BALB/c mice [74, 75]. The analysis of the affinity of the TCR of LACK-specific $CD4^+$ cells for peptide-MHC led to the hypothesis that susceptible mice display low-affinity LACK-reactive TCR that are biased to produce IL-4 early after infection whereas LACK-reactive $CD4^+$ T cells from resistant mice display high-affinity TCR and lack of this IL-4 response [76, 77] [Fig. 3].

Other experimental evidence, however, argues against a determining role of the early IL-4 production by LACK-reactive $CD4^+$ T cells in susceptibility to *L. major* infection. First, Vβ4 Vα8 TCR usage in *L. major*-infected BALB/c and C57BL/6 mice is similar [78]. Second, LACK-reactive T cells also produce a burst of IL-4 in resistant B10.D2 mice [79]. Third, a more refined model that uses the green fluorescent protein [GFP]-IL-4 reporter mice indicated that the frequency and kinetics of LACK-specific IL-4-producing $CD4^+$ cells induced early by *L. major* infection in resistant and susceptible is similar [80]. And fourth, an early, although transient, IL-4 response after *L. major* infection has been also detected in some resistant strains of mice [65, 67, 69, 81, 82]. These observations suggest that the ability of resistant mice to develop a protective response may predominantly be determined by the capacity to redirect an early Th2 response rather than by the absence of an initial IL-4-related Th2 response.

2.6 A sustained Th2 response in BALB/c mice

Irrespective of whether every resistant strain of mice does or does not produce IL-4 early after infection, a common observation is the low level of this cytokine after 4-5 days of infection, as compared to susceptible mice [70, 71]. In BALB/c mice, high levels of IL-4 are maintained during the whole course of disease, although the cellular sources appear to be different in a later stage of infection [71]. $CD4^+$ cells producing IL-4 after 5 days of infection are no longer restricted to those that use the Vβ4 Vα8

TCR and their antigen specificities are not confined to LACK [71, 83]. However, BALB/c mice systemically deleted for Vβ4, but not Vβ6 $CD4^+$ cells, were not capable of generating early IL-4 transcripts, developed a Th1 response and controlled *L. major* infection [73]. Conversely, administration of exogenous IL-4 to Vβ4-deficient BALB/c mice only during the first few days of infection restored the Th2 cell development at a later stage and the resulting susceptibility to *L. major* infection [74]. Moreover, as mentioned above, the treatment of BALB/c with neutralizing anti-IL-4 Ab within the first 24 [44] but not 48 or 72 hours [73] after infection was able to inhibit the IL-4 mRNA expression of $CD4^+$ cells at later times and subsequently to redirect towards a Th1 response [71]. Taken together, these results strongly suggest that the IL-4 rapidly produced in BALB/c mice by LACK-specific Vβ4 Vα8 $CD4^+$ T cells in response to infection instructs the irreversible commitment of *L. major*-specific $CD4^+$ T cells towards the Th2 phenotype in less than 48 hours.

As mentioned above, IL-12 is a unique cytokine in its ability to redirect the early Th2 response to *L. major* in BALB/c mice and to promote resistance. Not surprisingly, a number of experiments trying to connect a failure in the IL-12 pathway with the early IL-4 production in BALB/c mice have been reported in the last years. IL-12 administration one day before, but not 48 hours after infection, readily suppressed the early IL-4 burst, abolished the subsequent Th2 response and protected BALB/c mice from leishmaniasis, indicating a state of unresponsiveness to IL-12 after the IL-4 burst [72]. In vitro experiments that reproduced this effect and used IFN-γ production as a read-out showed that this state of unresponsiveness to IL-12 occurred only in $CD4^+$ T cells that had recognized a *L. major*-derived epitope [84]. Interestingly, the induction of this unresponsive state both in vivo and in vitro was totally precluded by neutralizing the IL-4 burst during the first days of infection [84]. The down-regulation of the IL-12 receptor β2 [IL-12Rβ2] chain was proposed to be responsible for the selective loss of IL-12 signaling during priming in vitro in naïve $CD4^+$ T cells from transgenic BALB/c mice [85]. The following observations suggest that the state of unresponsiveness to IL-12 in specific $CD4^+$ T cells from *L. major*-infected BALB/c mice is also due to the loss of IL-12Rβ2 chain expression and subsequent lack of IL-12 signaling: (1) $CD4^+$ T cells from BALB/c mice up-regulated the expression of IL-12Rβ1 and IL-12Rβ2 subunits one day after infection, (2) from 48 hours to 8 days after infection the expression of IL-12Rβ2 is not longer detected whereas IL-12Rβ1

expression was maintained, (3) neutralization of the early burst of IL-4 in BALB/c mice during the first days of infection resulted in maintenance of the IL-12Rβ2 chain expression, (4) resistant mice that don't mount an early IL-4 mRNA burst following infection with *L. major* [72] maintain the expression of the IL-12Rβ2 chain on their specific $CD4^+$ cells, which remain responsive to IL-12, and (5) the protective effect of the IL-4 neutralization in BALB/c mice is IL-12-dependent [71, 84, 86-88]. Thus, the maintenance of IL-12Rβ2 chain expression seems critical for Th1 cell commitment in resistant mice, whereas IL-4-mediated down-regulation of IL-12Rβ2 chain expression is an important step in stable commitment of BALB/c $CD4^+$ T cells to the Th2 pathway following infection with *L. major* [Fig. 3]. However, the role of the IL-12Rβ2 expression stability in susceptibility to *L. major* has been recently challenged by the finding that BALB/c mice that express an IL-12Rβ2 transgene maintain a non-healing phenotype, despite stable IL-12 signaling [89].

2.7 The Th1 response in resistant mice

IFN-γ is the signature cytokine of type 1 responses and experiments in knock-out mice have evidenced its essential role in the induction of immunity in resistant mice [43]. Similarly, genetic ablation of molecules involved in the generation of IFN-γ responses [i.e. IL-12, CD40, CD40 ligand], as well as down-stream components of the IFN-γ signaling pathways [IFN-γR, STAT4, T-bet] leads to uncontrolled leishmaniasis and usually to the development of a default Th2 pathway in naturally resistant mice [64, 90-94]. IL-18 has been shown to cooperate with IL-12 to direct a Th1 response but the final control of *L. major* infection in IL-18-deficient mice demonstrated a non-essential role [95, 96]. Interestingly, recent evidence suggests that IL-12, in addition to its role in the initiation of a protective immune response, is also important for the maintenance of this protective response. Some of the IL-12-deficient mice that were treated transiently with IL-12 developed progressive lesions [97]. Similarly, treatment with anti-IL-12 Ab can reactivate lesions in resistant mice that had healed cutaneous lesions and harbored persistent parasites [98]. Moreover, primed Th1 cells from healed mice cannot transfer immunity to IL-12-deficient mice [97].

2.8 Effector mechanisms

Several laboratories have confirmed that control of *Leishmania* infection is nitric oxide [NO]-dependent. Leishmanicidal activity in vitro and in vivo is dramatically

reduced when macrophages or mice are treated with competitive inhibitors of the NO synthase [NOS] [99-105]. When activated in vitro, macrophages from resistant mice express significantly higher levels of NOS and produce larger amounts of NO compared to macrophages from susceptible mice [106]. Most convincingly, disruption of the inducible NOS [iNOS] gene in mice gave rise to mutant mice that were highly susceptible to *L. major* infection in spite of developing a strong Th1 immune response [107]. Th1 cell-derived cytokines [in particular IFN-γ plus TNF-α] are potent inducers of iNOS with subsequent production of reactive nitrogen intermediates [RNI] and parasite killing [108]. The synergistic role of TNF-α in the induction of parasite killing is additionally demonstrated by the finding that treatment of infected mice with recombinant TNF-α reduces lesions whereas anti-TNF-α treatment exacerbates the disease [103, 109]. More strikingly, TNF-α-deficient mice [110] as well as IFN-γ-deficient mice are unable to control *L. major* infection. Recently, an additional effector mechanism has been proposed that influences parasite control. Mice deficient in Fas [CD95] or Fas ligand, cannot eliminate *L. major* despite enhanced production of NO [111, 112], suggesting that macrophage apoptosis through the Fas-Fas ligand pathway might contribute to host resistance [Fig. 4].

Conversely, Th2 cytokines, such as IL-4 and IL-10, can down-regulate macrophage activation and therefore restrict parasite killing. Both cytokines inhibit iNOS enzyme expression and NO production by IFN-γ-activated macrophages [113-115]. Other cytokines such as tumor growth factor [TGF]-β and IL-13 have also been shown to inhibit macrophage activation and intracellular killing of *L. major* [115, 116]. The in vivo role of IL-4 has been addressed extensively [see above] and appears to be critical for Th2 cell development and maintenance, in addition to macrophage inactivation. IL-10 has been more appreciated lately as a susceptibility factor in *L. major* infection. Although treatment of BALB/c mice with anti-IL-10 Ab showed little effect [117, 118], IL-10-deficient mice were more resistant to *L. major* infection than wild-type BALB/c mice [119]. In addition, IL-10R blockade conferred partial resistance to *L. major* in BALB/c mice [95]. Also, resistant mice expressing an IL-10-encoding transgene under the control of the MHC class II promoter, which directs the expression of IL-10 mainly to APC that display MHC class II molecules, were more susceptible to *L. major* infection [120]. TGF-β was able to exacerbate *L. major* infection and anti-TGF-β Ab were able to enhance resistance by increasing the

production of NO by macrophages in parasitized lesions [121]. In a similar way, experiments using both IL-13-deficient as well as IL-13-transgenic mice have implicated this cytokine as an additional factor contributing to Th2 response and susceptibility [122, 123]. Thus, although the relative contribution of these Th2-related cytokines to the priming and differentiation of Th2 cells is still to be determined, it seems clear that the perturbation of the parasite-killing mechanisms is a major part of their biological effects [Fig. 4].

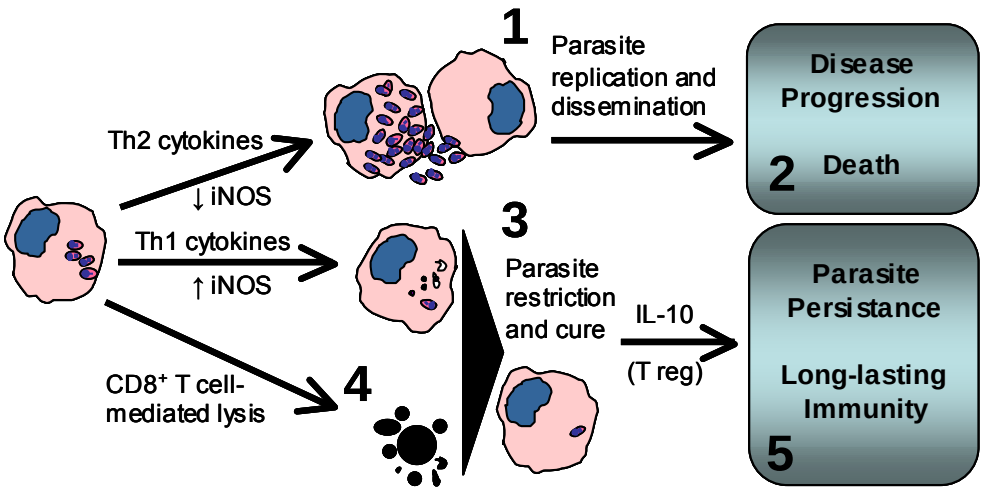

Fig. 4. Effector mechanisms in murine leishmaniasis. A Th2 response in susceptible mice renders macrophages unresponsive to activation signals required for NO-dependent killing and allow parasite replication and dissemination [1]. This leads to disease progression and eventually death [2]. Th1 cytokines potently up-regulate the expression of iNOS and activate infected macrophages for intracellular killing [3]. Parasite replication is therefore restricted and lesions remain small and heal. $CD8^+$ T cells cooperate to control infection by producing type 1 cytokines and by their lytic activities [4]. In the presence of a protective Th1 response, IL-10-producing regulatory T cells are also generated and mediate the persistence of low numbers of parasites, allowing sustained antigenic stimulation and immunity [5].

2.9 Other cell types may play a role in resistance and susceptibility

With regard to the T cell subsets that are involved in resistance to *L. major*, the crucial role of $CD4^+$ T cells has been consistently proven [82, 124, 125]. For $CD8^+$ T cells, however, some new findings have suggested a possible role depending on the model used for infection. Early studies showed that $CD8^+$ T cells were dispensable for resistance to primary infection [25, 126, 127] but important for immunity to re-challenge [128] when the typical high dose and s.c. infection model was used. In a model that better resembles the natural infection by using a low dose of parasites and intradermal [i.d.] infection, $CD8^+$ T cells were required for the control of primary infection [129]. Some studies in humans indicating that $CD8^+$ T cells are present in patients with active leishmaniasis and during healing are consistent with these

findings [130, 131]. Although the role of this subpopulation of T cells in the induction of resistance is still enigmatic, it has been proposed that through the release of IFN-γ into the lesions as well as the killing of infected cells that are ineffective in intracellular killing, $CD8^+$ T cells contribute to resistance to *L. major* infection [95] [Figs. 3 and 4]. Other T cell subpopulations that might contribute to protective immunity to leishmaniasis are the γδ T cells. They are present in lesions and blood of patients with active leishmaniasis [132, 133] as well as in mice infected with *L. major* [134]. However, since the majority of γδ T cells do not express CD4 or CD8 molecules and resistance to *L. major* infection can be transferred by $CD4^+$ T cells alone, they are considered to be of secondary importance [15].

Natural killer [NK] cells are a component of the innate immune system which have also been implicated in the development of protective Th1 responses, primarily through their ability to produce IFN-γ early after infection [68]. More recent reports, however, have shown that mice selectively deficient in NK cells efficiently produce IFN-γ and heal their lesions after *L. major* infection [135], indicating that they are ultimately not required for resistance. It was recently appreciated that other cells of the innate immune system may contribute to the sustained Th2 induction and susceptibility to *L. major* in mice. Neutrophils are the first type of leucocytes that migrate to the skin after *L. major* inoculation into the footpads [136]. Interestingly, the neutrophil infiltrate in resistant C57BL/6 mice is transient, whereas in BALB/c mice a high number of them are observed for several weeks [137, 138]. Depletion of neutrophils at the time of *L. major* challenge in BALB/c mice inhibited the IL-4 response and promoted partial resistance [138]. Thus, neutrophils appear to contribute to the early IL-4 response in BALB/c mice and therefore to susceptibility [Fig. 3].

2.10 Persistence of *L. major* infection

There is a considerable amount of evidence indicating that *Leishmania* parasites establish a life-long persistent infection, that is usually asymptomatic, after the resolution of a primary infection in humans [139, 140]. In resistant mice, the healing of a cutaneous lesion is followed by a striking immunity to re-infection although viable parasites can still be detected one year after cure [141]. The restriction of parasite growth during this silent stage requires $CD4^+$ T cells, $CD8^+$ T cells, IFN-γ, IL-12 and iNOS, because impairment of these responses leads to the reactivation of

infection and appearance of lesions [98, 142, 143]. Similarly, the observation that resistant mice that were deficient for the expression of IL-10 or treated with anti-IL-10R Ab during the chronic phase after cure developed sterile immunity [143] indicated that this cytokine is crucial to maintain a persistent infection. This conclusion is further supported by studies with humans showing that IL-10 continues to be produced after healing of cutaneous or visceral leishmaniasis [144-146] and gives a plausible explanation for why those individuals fail to achieve sterile cure. Interestingly, the sterile cure observed in resistant mice that lack IL-10 signaling pathways [143], or in susceptible mice that were infected with a very low dose of *L. major* [147] is followed by the inability to resist a re-challenge infection. These results indicate that a persistent subclinical infection with *L. major* is required for maintaining long-lasting immunity and that IL-10-producing cells may be crucial for the induction of this persistence [Figs. 3 and 4]. Both fibroblasts and dendritic cells [DC] have been proposed to be host cells for viable *L. major* parasites in latent infections [148, 149]. Moreover, some evidence indicates that DC that harbor parasite antigens for long periods of time are able to stimulate T cells in the LN and could be important in maintaining T cell memory in asymptomatic chronically infected mice [149]. Similarly, the IL-10-producing cells that appear to mediate parasite persistence have been recently described in C57BL/6 as a population of $CD4^+CD25^+CD45RB^{low}$ regulatory T cells [150]. Interestingly, these regulatory T cells are reminiscent of the suppressive $CD4^+$ T cell subset found in BALB/c mice [118]. It appears that a sub-population of $CD4^+$ T cells that mediates parasite persistence and probably also prevents immunopathology in resistant mice would either expand to generate the disease-promoting Th2 cells or would lead to an imbalance in the expansion and activity of Th2 cells in susceptible mice, allowing an uncontrolled growth of the pathogen.

3. Dendritic cells

3.1 Dendritic cells and the immune response

Millions of years of evolution have endowed mammals with a complex immune system that efficiently copes with a myriad of invading microbes, including viruses, bacteria, fungi and parasites. Soon after the invasion of mammalian tissues by a

particular microbe, a number of cells and soluble factors are activated and delivered in order to avoid its colonization and deleterious effects. Such mediators that efficiently function without prior exposure to microbes are also present in invertebrates and plants and are collectively known as the innate immune system. In mammals, this rapid innate response is largely mediated by epithelial cells, neutrophils, macrophages, NK cells, DC, cytokines and complement factors, and is characterized by being unspecific, short-lived, and triggered by germline-encoded receptors expressed in those cells. These receptors are termed pattern recognition receptors [PRR] since they recognize the so-called pathogen-associated molecular patterns [PAMP], which are molecular structures that are present and frequently shared by large groups of microorganisms but are not present in the host. Recognition of these structures thereby allows the immune system to distinguish infectious non-self from non-infectious self. Evolution since vertebrates, however, faced microbial challenges that required a sustained, specific and long-lived response, and the result was the appearance of the adaptive immune system. This adaptive immune response is mediated by clonal populations of T and B lymphocytes through the specific recognition of molecular details in microbial antigens and is triggered by receptors that have undergone somatic gene rearrangement. This response develops over the lifetime of an individual by facing antigen challenges, is delayed in time, but provides fine specificity and immunological memory of infection. An adaptive immune response, however, cannot be initiated without the participation of cells of the innate system [151].

The capacity of the immune system to mount an effective and durable response against pathogenic microbes is ensured by means of the coordinated action of the innate and the adaptive immune systems. Early after infection, the innate immune response not only restricts pathogen establishment but also instructs the adaptive immune system about the nature of the pathogenic challenge [151]. A group of cells of the innate immune system, the APC, are able to take up, process and present microbial-derived peptides to T cells and therefore are highly specialized for the activation of the adaptive response. Among the APC, the DC represent a unique system of sensors of invading pathogens. They are the only cells that are able to induce primary immune responses by activating naïve T cells, thus permitting the establishment of immunological memory [152]. In addition, DC are equipped with

different PRR as well as endocytic receptors for the uptake of microbes and dying cells, a feature that allows to detect the presence of the pathogen and to determine its identity. In this way, DC make the decision of whether an adaptive immune response is initiated or not and determine the quality and magnitude of the response. For these reasons, DC are currently considered to be crucial players in the initiation and regulation of immune responses [151-153].

3.2 The dendritic cell system

DC were first described by Paul Langerhans who identified "dendritic-shaped cells" in the skin more than a century ago [154]. These skin cells were named Langerhans cells [LC] and for a long time they were considered to belong to the nervous system. In 1977, two groups independently reported the expression of "Ia" antigen on LC, and therefore their antigen-presenting capacity [155, 156]. At about the same time, a new population of cells with similar properties was found in lymphoid organs by Steinman and Cohn [157]. Since then, an enormous amount of information about the immunobiology of these cells has been published.

DC represent a heterogeneous cell population residing in most peripheral tissues, particularly at sites of interface with the environment [skin and mucosae], where they represent 1-2% of the total cell numbers [152, 158]. They are continuously produced from haematopoietic stem cells within the bone marrow with the help of FMS-related tyrosine kinase 3 ligand [Flt3L] and granulocyte-macrophage colony-stimulating factor [GM-CSF] and delivered to the tissues through the blood. In the absence of ongoing inflammatory responses, DC constitutively patrol through the blood, peripheral tissues, lymph and secondary lymphoid organs. In peripheral tissues DC take up self and non-self antigens. Internalized antigens are then processed into proteolytic peptides, and these peptides are loaded onto MHC class I and II molecules. In steady-state conditions, i.e. in the absence of immune responses, DC remain in an immature state and do not differentiate to carry out the functions of initiators of immunity. Instead, some recent evidence indicates that immature DC are able to silence T cells either by deleting them or by expanding regulatory T cells. Thereby, DC have been proposed to play a relevant role in the induction of peripheral tolerance [159]. Conversely, upon pathogen encounter, DC undergo a developmental process, called maturation, which comprises a very refined series of events that allows

DC to differentiate from an efficient cell for antigen uptake to a highly specialized cell for antigen presentation and lymphocyte activation [160]. Microbial products as well as inflammatory cytokines and apparently some other self-molecules, induce DC maturation through direct interaction with specific receptors expressed on the surface of DC. T cells and epithelial cells also contribute to the final DC maturation through direct cell-to-cell contact and the secretion of cytokines [161]. The maturation process also triggers the targeted migration of DC from peripheral tissues, through the lymph, towards secondary lymphoid organs, where they activate antigen-specific T cells and initiate an adaptive anti-microbial response [Fig. 5].

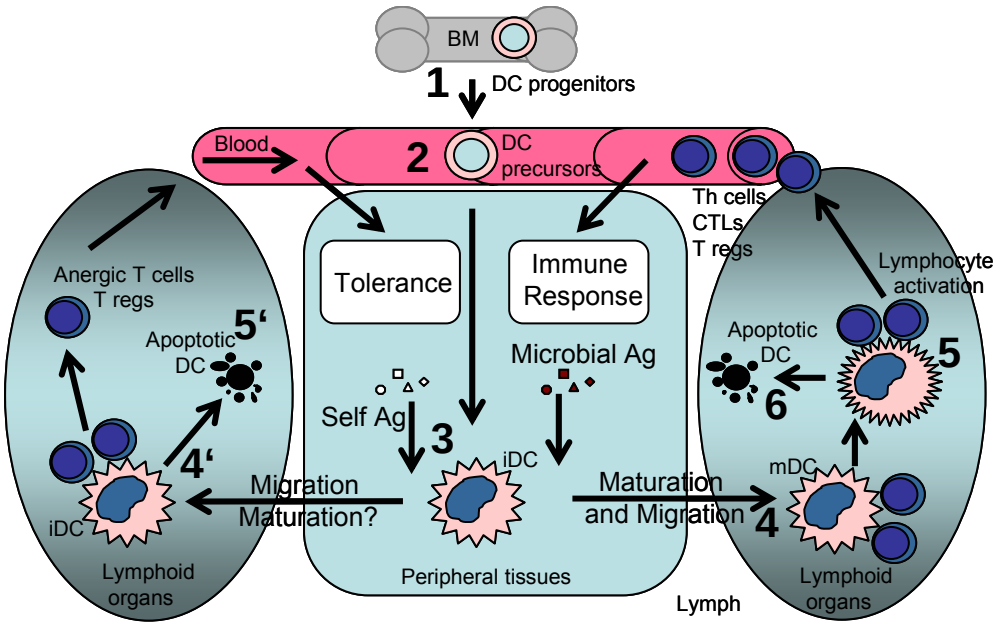

Fig. 5. The life cycle of DC and the immune response. Proliferating DC precursors are generated in bone marrow [1] and differentiate to non-proliferating DC precursors [2]. DC precursors are delivered to the blood [2] and enter tissues as immature DC [3]. In the periphery, immature DC take up foreign antigens and begin to mature in response to pathogen products and inflammation. Maturating DC travel through afferent lymphatics towards lymphoid organs, where they encounter and activate pathogen-specific T cells [4]. Activated T cells help DC in terminal maturation [5], which allows lymphocyte expansion and differentiation into cytokine-producing Th cells, CTL and regulatory T cells. Activated T cells migrate and reach the original site of antigen deposition where they vigorously respond to cells that are presenting those antigens. Under steady state conditions, DC circulate and migrate to lymphoid organs as immature DC or semi-mature DC, where self-antigens are presented to auto-reactive T cells [4']. Interaction of immature DC with T cells leads to T cell anergy, apoptosis or induction of regulatory T cells and allow the maintenance of peripheral tolerance to self-antigens. After interaction with lymphocytes, DC die by apoptosis [6, 5']. Ag: antigen, BM: bone marrow, iDC: immature DC, mDC: mature DC, T regs: regulatory T cells, CTL: cytotoxic lymphocyte, Th: T helper cell.

3.3 Dendritic cell lineages and subsets

In addition to the interactions with T cells in the lymphoid organs, DC interact with other cell types, including B cells and NK cells, resulting in the mobilization of other components of the innate and adaptive immune systems. Taking into account the variety of functions attributed to DC, it is not surprising that a growing number of DC subsets are being described. Complex and frequently controversial reports, regarding the number and the development of different DC lineages and their functional consequences, have been published in the last years. A first theory, the "specialized lineage model", proposes that different DC subsets are derived from early divergences in the developmental pathway, producing several distinct haematopoietic sublineages. Those lineages would develop into independent precursors that are functionally committed. A second theory, the "functional plasticity model", argues that specialized DC subtypes represent different activation states of a single lineage and that the functional differences depend entirely on local environmental signals [162, 163]. Many authors currently accept that a combination of both models could explain the variety and complexity of DC functions [153, 162-164].

3.4 Myeloid and lymphoid dendritic cells: the murine subsets

DC subsets have been intensively studied in the lymphoid tissues of uninfected mice. Early studies classified mouse DC into two major subsets, the myeloid $CD8^-$ DC and the lymphoid $CD8^+$ DC. This classification was based on the observation that $CD8^-$ DC developed from a myeloid-restricted precursor, whereas the $CD8^+$ DC were derived from a lymphoid-restricted precursor. These DC subpopulations were shown to be segregated in the microanatomical sites of the lymphoid organs [$CD8^+$ DC are in the T cell-rich areas of the spleen and LN, whereas $CD8^-$ DC are in the marginal zones] and to have different functional properties [see below] [162, 165]. However, later reports clearly demonstrated that both myeloid- and lymphoid-restricted precursors could produce both DC subsets [166-169]. Moreover, most recently, a common precursor population, yielding $CD8^+$ and $CD8^-$ DC but devoid of myeloid or lymphoid differentiation potential, has been characterized [170]. Thus, the terms lymphoid and myeloid DC should be used only to associate one specific DC subset with the presence or absence of the CD8 marker but do not reflect the haematopoietic origin of the subset. In spite of this, but for reasons of simplicity, the terms lymphoid and myeloid DC will be used in the present work, i.e. the term lymphoid DC denotes $CD8^+$ DC and the term myeloid DC denotes $CD8^-$ DC.

Besides the CD8α homodimer, other surface markers like CD4, CD11b and CD205 have been used to define five DC subpopulations in mice, some of which are present in LN but not in spleen. Lymphoid DC form the predominant subset in the thymus, whereas myeloid DC dominate the spleen. The distribution of DC subpopulations in the LN is more equilibrated and the presence of two additional DC subsets has been reported: first, the $CD4^-CD8^-CD11b^+CD205^+$ DC that are present in all LN and are believed to be the mature form of the tissue interstitial DC, and second, a myeloid-like DC subset which is present only in LN that drain the skin and is characterized by the strong expression of CD205 and low levels of CD8. These DC typically express high levels of Langerin, a characteristic marker of epidermal LC, suggesting that they are the mature form of the LC [171-173].

3.5 Myeloid and plasmacytoid dendritic cells: human subsets

The identification of homologous mature DC subpopulations in lymphoid tissues in humans has been hampered by the difficulty to obtain sufficient amounts of cells and by the observation that the lymphoid-related marker CD8 is not expressed on human DC. However, the very few studies performed with human spleen, tonsils and thymus suggest a similar heterogeneity in mature DC subpopulations as well as the presence of myeloid- and lymphoid-like subsets [174, 175]. Therefore, most of the knowledge we hold about human DC subsets, their origins and functions, comes from the in vitro studies that use umbilical-cord blood or bone marrow $CD34^+$ haematopoietic progenitor cells and peripheral blood DC precursors. $CD34^+$ progenitor cells, upon culture in the presence of GM-CSF and TNF-α, generate two separate lineages of DC. One lineage generates intermediates that express CD1a and the skin-homing receptor cutaneous leukocyte antigen [CLA] and leads to the development of cells that have Birbeck granules and express LC-associated antigens [Lag] which are typical of skin LC. A second lineage produces $CD1a^-CLA^-$ intermediates and generates the typical interstitial DC [Birbeck granules-negative, Lag^-, $CD9^+CD68^+$Factor $XIII^+$] [163, 176, 177].

Two additional DC precursors [pDC] can be found in human peripheral blood, the monocytes and the plasmacytoid cells. Monocytes may differentiate into DC that promote Th1 responses [type 1 DC, DC1] whereas plasmacytoid cells differentiate

into Th2-promoting cells [type 2 DC, DC2; see below, section 3.13]. Monocytes [or pDC1] and plasmacytoid cells [or pDC2] are considered to be precursors and not immature DC because of their lack of the typical veiled morphology, their inability to induce significant naive T cell activation and their poor mobility in vitro. In addition, those cells have been more directly involved in innate immunity against microbes [163]. These pDC express different sets of PRR and are radically different in phenotype. pDC1 express myeloid markers, whereas pDC2 express lymphocyte-related molecules. When pDC1 are cultured in the presence of GM-CSF and IL-4 for 6 days, they generate a typically-shaped immature DC termed myeloid DC1. When pDC2 are cultured with IL-3 or are activated by viral infection they differentiate into immature plasmacytoid DC or immature DC2. Both DC1 and DC2 share typical characteristics of immature DC and can be fully matured by different stimuli. They also respond to T cell-mediated activation by performing segregated functions. For instance, DC1 produce IL-12 but not type I IFN [IFN-α and IFN-β], whereas DC2 are potent producers of type I IFN [162, 163].

Myeloid and plasmacytoid DC derived from the blood have been also described in mice more recently [162]. Moreover, pDC2 have been found in lymphoid tissues as an additional subset that was previously missed, because it expresses the lymphocyte marker B220, leading to their elimination along with B cells during the selection-sorting procedure. These murine pDC2 closely resemble the human counterpart, for example in their apparent lymphoid origin, their surface phenotype and their ability to differentiate to typically-shaped DC2 that produce massively type I IFN upon virus stimulation [178-180].

3.6 The link between antigen uptake and presentation: dendritic cell maturation

A major distinction between DC and other APC is the particularity that DC exhibit in handling antigens. For instance, after antigen capture, DC usually undergo a tightly regulated developmental process termed maturation, which enables them to distinguish self from non-self and, if necessary, orchestrate an appropriate immune response. It is believed that the decision of whether an immune response should be initiated or not, as well as its quality and intensity, is made by DC and transmitted to the adaptive system. The qualitative and quantitative variations in the maturation

process that DC undergo in response to the different maturation signals appear to represent the code that DC use to instruct the adaptive response [160].

3.7 Antigen uptake by immature dendritic cells

Immature DC are extraordinarily efficient in antigen uptake. They internalize pathogens, infected cells, dead cells or products derived from them to use for antigen presentation. Three different types of endocytosis have been described for DC: Macropinocytosis, phagocytosis and receptor-mediated endocytosis [160, 181]. Macromolecules are taken up by a variety of endocytic receptors expressed on human and murine DC, such as Fc receptors, complement receptors, scavenger receptors and heat-shock protein [HSP] receptors. Similarly, a growing number of receptors of the C-type lectin family, some of which are DC-specific, are being implicated in antigen internalization. Type I lectins present in DC include the macrophage mannose receptor and DEC205. The type II lectins Langerin and DC-specific intercellular adhesion molecule-3-grabbing nonintegrin [DC-SIGN] are expressed by LC and interstitial DC, respectively. Langerin is involved in the formation of Birbeck granules, whereas DC-SIGN has been implicated as the DC receptor for some important human pathogens including the human immunodeficiency virus, *Mycobacterium tuberculosis* and *Leishmania* [181-184]. Particulate and soluble antigens are efficiently internalized by phagocytosis and macropinocytosis in DC. In contrast to macrophages, macropinocytosis in immature DC is constitutive and represents a critical antigen uptake pathway allowing DC to rapidly and non-specifically sample large amounts of surrounding fluid. DC can also phagocytose a large number of microbes as well as necrotic and apoptotic cells by using a variety of receptors and pathways [181].

As compared to mature DC, immature DC express moderate levels of surface MHC class II products, and low levels of surface MHC class I and co-stimulatory molecules [e.g. CD86]. The limited surface expression of MHC class II molecules is mainly due to intracellular sequestration rather than reduced synthesis. In fact, MHC class II molecules are actively synthesized and targeted to endosomes and lysosomes in immature DC. Since antigen degradation is inefficient in immature DC, the loading of MHC class II molecules is limited and they are retained in endosomal compartments [181]. A rapid turnover of the surface MHC class II-peptide complexes to endosomal

compartment has been also reported as a mechanism contributing to their limited surface expression on immature DC [181] [Fig. 6].

3.8 Antigen presentation by mature dendritic cells

After the detection of microbial products or exposure to proinflammatory cytokines, immature DC transform into mature DC, cells with an exceptional capacity for T cell stimulation. This transition is accompanied by dramatic changes in the cell morphology, motility, endocytic capacity and distribution and expression of molecules associated with antigen presentation. A down-regulation of the endocytic capacity is observed soon after the sensing of a maturation signal, leading to a subsequent restriction in the range of antigens that DC will be able to present after leaving peripheral tissues. This down-modulation of antigen uptake is based on two independent mechanisms: a decrease in cell surface expression of most antigen receptors and the reduction of both micropinocytosis and phagocytosis [185, 186]. One of the hallmarks of DC maturation is the redistribution of MHC class II molecules from intracellular compartments to the cell surface as well as the up-regulation of co-stimulatory molecules [CD80, CD86], T cell adhesion molecules [e.g. CD48 and CD58], and MHC class I molecules. Surface MHC class II molecules can increase up to 20-fold while CD86 increases up to 100-fold [160]. Increased surface expression of the MHC class II molecules is not the result of neo-synthesis, since the mRNA levels are only slightly and transiently increased during maturation [187, 188]. Instead, it is largely due to the increase in the protease activity in the endosomal compartments with the subsequent degradation of antigens and loading onto preformed MHC class II molecules. MHC class II-peptide complexes are then rapidly assembled and transported to endosomal vesicles where they co-localize with co-stimulatory and MHC class I molecules before being delivered to the cell surface as clusters of molecules involved in T cell stimulation [189-191]. The reduction in the endocytic activity upon maturation also leads to a significant reduction in the transport of internalized MHC molecules to lysosomes for degradation, resulting in stabilization of MHC class II-peptide complexes at the cell surface [187, 192]. Later on, MHC class II synthesis is down-regulated, and association of peptides with the newly synthesized MHC molecules becomes very inefficient, focusing the range of peptides displayed at the mature DC surface to those arising from antigens encountered before or during the induction of maturation [Fig. 6]. In addition, obvious

morphological changes are also evident when DC are induced to mature. The cells extend long "dendritic" processes, consisting actually of folded portions of the membrane that may increase the opportunities of interaction with T cells.

3.9 Dendritic cell migration

Concomitant with the modification in their antigen-presenting abilities, maturation also induces massive migration of DC out of peripheral tissues [152]. Profound changes in the cytoskeleton organization as well as modifications in the expression of chemokine receptors and adhesion molecules contribute to the migration of DC through the lymph to the T cell-rich areas of the secondary lymphoid organs [152]. For instance, chemokine receptors associated with the homing to normal or inflamed tissue, such as CCR2, CCR6, CCR1 and CCR5 are down-regulated, whereas chemokine receptors responsible for the attraction of cells to LN, such as CCR7, are up-regulated in mature DC [193] [Fig. 6].

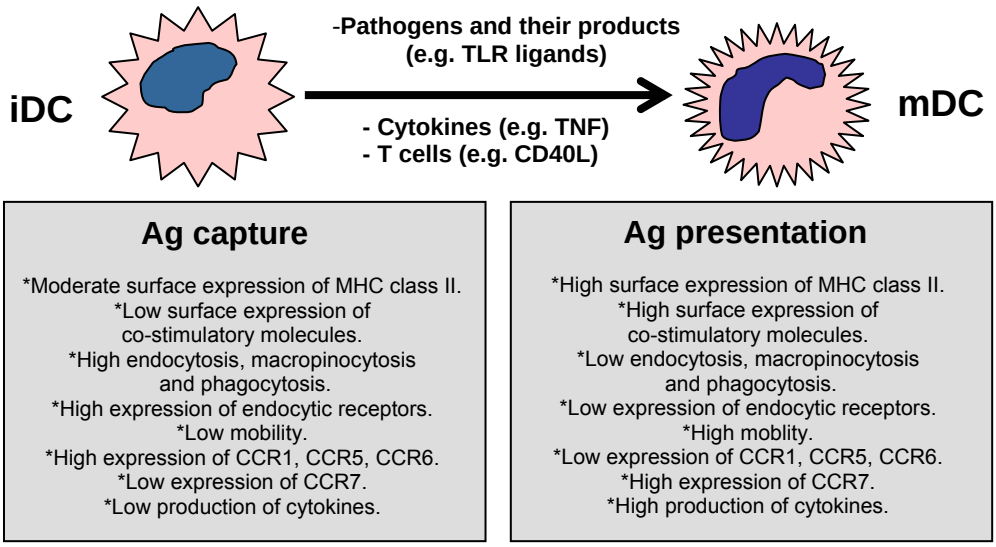

Fig. 6. Maturation of DC. The characteristics of immature and mature DC [iDC and mDC], as well as the stimuli inducing DC maturation are listed. TLR: Toll-like receptors, TNF: tumor necrosis factor. CCR1, CCR5, CCR6 and CCR7 are chemokine receptors.

3.10 What induces dendritic cell maturation?

From a physiologic point of view, the molecules triggering DC maturation can be grouped in three major categories. First, DC can be directly and potently activated by pathogens through the recognition of a number of molecules commonly produced by them, collectively known as PAMP. Second, several pro-inflammatory cytokines, usually produced by cells of the innate immune system secondarily to infection or

inflammation also trigger DC maturation. And third, after interacting with T cells in lymphoid organs, DC maturation can be enhanced by feedback signals provided by those recently activated T cells. DC are well equipped with the appropriate receptors enabling them to recognize those ligands [Fig. 6].

Toll-like receptors [TLR] are the prototype of DC receptors for PAMP. The microbial products they recognize are among the most potent DC activators and include lipopolysaccharide [LPS], CpG motifs and double-stranded RNA. Different members of the TLR family recognize different PAMP. For instance, TLR4 recognizes LPS, a molecule present in a variety of Gram-negative bacteria; TLR2 is involved in the response to different Gram-positive cell wall components [including peptidoglycans], to bacterial lipoproteins and to *Klebsiella pneumoniae* Omp A protein; TLR5 recognizes flagellin from both Gram-positive and Gram-negative bacteria; and TLR9 binds to unmethylated CpG motifs [194]. Different DC subsets express different, but complementary, TLR and respond by undergoing maturation upon activation by the specific TLR ligands [163]. DC also express receptors for the inflammatory cytokines TNF-α and IL-1β, and maturation is rapidly triggered by these ligands [152, 195]. Similarly, $CD4^+$ T cells induce DC maturation through the engagement of receptors on DC. The most frequently studied and probably most potent pathway is triggered by interactions between CD40 ligand [CD40L] expressed on T cells and CD40 expressed on DC. T cell-mediated CD40-independent maturation of DC has been also reported [152, 181]. The signal transduction pathways activated upon engagement of TLR, cytokine receptors and CD40 on DC are complex and largely unknown. However, irrespective of the receptor engaged, the final activation of the nuclear factor-κB is always observed and represents a hallmark of DC maturation [160, 181].

In the last years, some endocytic receptors have been also implicated in mediating DC maturation. In particular, the engagement of some Fc receptors by immune complexes, or by anti-Fc receptor Ab, have been reported to induce DC maturation [196-198]. Similarly, necrotic cells and possibly apoptotic cells can also lead to DC maturation [199-202]. The nature of the molecules mediating this effect is unknown, although the delivery of HSP by necrotic cells has been proposed to mediate this maturation through unknown receptors [203, 204].

3.11 Dendritic cells and the initiation of the immune response

A substantial amount of evidence supports the concept that DC are the main professional APC mediating T cell priming. The surface expression of MHC and co-stimulatory molecules is far higher in DC than in B cells or macrophages [158]. In vitro priming of alloreactive, naïve TCR-transgenic T cells or the expansion and activation of antigen-specific naïve precursor from polyclonal populations indicated that DC are the major inducers of T cell priming among APC [158]. Moreover, when antigen-pulsed DC were injected into naïve mice, a potent induction of primary $CD4^+$ and $CD8^+$ T cell responses was observed [205, 206]. The antigen-presenting capacity of DC in situ has been also assessed: in mice infected with parasites [207], virus [208], or immunized with soluble proteins or DNA [209-214], the ex vivo recovered DC were probed with T cells or specific Ab and the presence of specific MHC-peptide complexes at the surface of the cells was demonstrated. Direct visualization of the interaction between antigen-specific transgenic T cells and antigen-loaded DC on LN sections has been also reported [215].

The decision of whether an antigen-specific immune response is initiated or not seems to be made by DC and is intimately related with the process of maturation. Immature DC exhibit low levels of MHC-peptide complexes and co-stimulatory molecules on the surface and therefore are not able to provide the threshold of signaling required by naïve T cells to become activated and undergo cell division. These immature DC are present in the LN in low numbers as a consequence of their normal circulation between lymphoid and non-lymphoid tissues under steady-state conditions [159] or, alternatively, they represent a population of DC exhibiting a certain limited degree of spontaneous maturation that allows migration [216]. These cells have been frequently termed non-immunogenic or tolerogenic DC and appear to be not only unable to induce immune responses, but also to actively silence self-reactive T cells and therefore participate in the maintenance of peripheral tolerance [159]. In contrast, fully mature DC, that are the result of an inflammatory or infectious process in the periphery, migrate massively to LN and become highly immunogenic. They accumulate long-lived MHC-peptide complexes and co-stimulatory molecules on the surface and thereby assure a strong and sustained stimulation of specific T cells, leading to their rapid proliferation and differentiation [216]. Activated T cells subsequently trigger further DC maturation via CD40L and cytokines, improving their

T cell-stimulatory capacity, boosting IL-12 production and prolonging their lifespan [216, 217].

3.12 Dendritic cells instruct the type of immune response initiated

$CD4^+$ T cell activation is a crucial event in the establishment of an integral antigen-specific immune response. Activated Th cells are known to regulate a variety of processes involving other cells of the immune response, such as the development and activation of Ab-secreting B cells, cytotoxic cells, and other effector components. Similarly, recent evidence indicates that subpopulations of $CD4^+$ T cells, for instance the $CD25^+CD4^+$ T cells, might down-modulate a range of T cell effector functions and are critical for homoeostasis [218]. For these reason, $CD4^+$ T cells have been described as the "directors" of the immune "orchestra". The pivotal role that DC play in the activation of the adaptive arm of the immune system is not restricted to the initiation. DC can also "give shape" to the response by instructing $CD4^+$ T cells about the type of immune functions required. Thus, in the orchestra of the immune system, $CD4^+$ T cells direct, but DC are the composers of the symphony. Although the fact that DC activate $CD4^+$ T cells to develop into the Th1 or Th2 phenotype appears to be beyond doubt, the mechanisms underlying such a differentiation are still controversial. Pioneer papers describing murine DC subsets raised the very attractive possibility that the type of Th response was a function of the subset of activating DC. This hypothesis was proven to be true in several systems [165]. However, more recent findings have shown that every DC subset exhibits a considerable plasticity in their $CD4^+$ T cell differentiation capacity [164].

3.13 Dendritic cell subsets and Th1/Th2 development

Two groups independently reported in 1999 that $CD8^+$ DC direct a Th1 response whereas $CD8^-$ DC lead to Th2 development after in vitro pulsing and adoptive transfer into naïve mice [219, 220]. The induction of a Th1 response by $CD8^+$ DC was shown to be dependent of the production of IL-12 by the donor cells [220], confirming the reports indicating that this DC subset produces the highest IL-12 levels after optimal in vitro stimulation [221]. Similarly, in vitro studies with co-cultures of human DC subsets and naïve T cells indicated functional specialization. Monocyte- or plasmacytoid-derived DC that were activated by CD40 ligation primed Th1 or Th2 cells, respectively, and the ability to prime Th1 cells was also associated with the

production of IL-12 [222]. Although numerous recent reports strongly challenge the idea of functional specialization of DC [see below], two recent publications support the concept that different DC populations may be segregated functionally in both humans [223] and mice [224]. The expression of PRR as well as the reactivity to their microbial ligands are dramatically different and complementary in pDC1 and pDC2. Monocyte-derived DC express TLR1, TLR2, TLR4, TLR5 and TLR8, and respond to the appropriate microbial ligands, including peptidoglycan, lipoteichoic acid and LPS, whereas plasmacytoid-derived DC express TLR7 and TLR9 and are responsive to CpG motifs. Moreover, in response to appropriate microbial stimuli, DC1 produce TNF-α and IL-6, whereas DC2 rapidly secrete type I IFN [163]. Although the consequences related to Th1/Th2 induction are still to be determined, these observations indicate that in terms of recognition and immediate response, some degree of specialization appears to be determined by DC lineage [Fig. 7A].

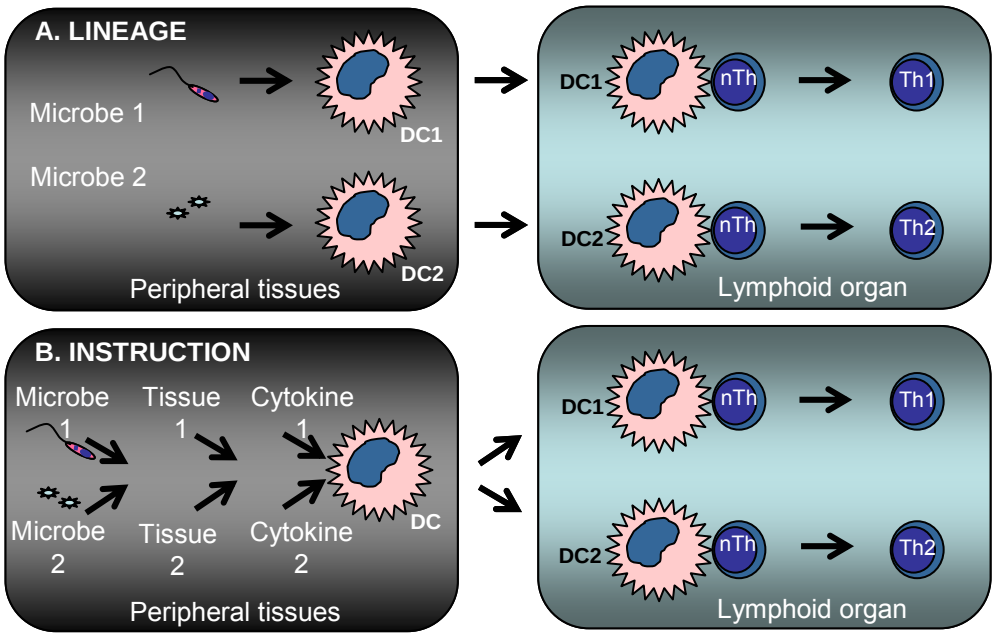

Fig. 7. Models of T helper cell differentiation by DC. A, Preexisting DC1 and DC2 are triggered by different microbial stimuli to elicit Th1 or Th2 responses. B, DC are conditioned to become DC1 or DC2 by environmental factors. In this model the nature of the microbial stimulus, the anatomical localization where the DC-microbe interaction takes place, and the presence of polarizing cytokines will determine the type of Th cell response elicited. nTh: naïve Th cell.

3.14 The type of dendritic cell stimulation and Th1/Th2 development

A large body of experimental evidence supports the concept that microbial signals as well as other environmental factors instruct DC and are major determinants of the type of Th cell response elicited. Thus, DC exhibit a striking plasticity in the way to

respond to different stimuli. Early observations showed that $CD8^+$ DC but not $CD8^-$ DC induce apoptosis and limited T cell responses and present high levels of self-peptides in the T cell areas of lymphoid organs [225-227], indicating a role in the induction of tolerance rather than stimulation. In fact, growing evidence indicates that $CD8^+$ DC are specially suited for cross-presentation of self-antigens [228, 229] and that they represent a special subset of tolerogenic or regulatory DC [230-232]. These observations indicate that different and even contrasting functional properties can be attributed to murine $CD8^+$ DC in different experimental settings. Additionally, $CD8^+$ DC can induce either Th1 or Th2 responses when stimulated with LPS from *Escherichia coli* or *Porphyromonas gingivalis*, respectively [233], indicating that the induction of Th1 responses is not a pre-determined outcome after priming with $CD8^+$ DC.

Similarly, in a number of reports, $CD8^-$ myeloid DC, formerly linked to Th2 induction, were shown to induce both Th1 and Th2 cells depending on the type of microbial signals used for maturation. In a TCR transgenic model, Whelan and collaborators showed that bone marrow-derived DC [BMDC], which are typical myeloid $CD8^-$ DC, differentially promoted Th1 or Th2 cells in vitro when activated with LPS or a nematode-secreted glycoprotein, respectively [234]. MacDonald et al. confirmed those in vitro findings, but also provided experimental evidence in vivo: BMDC pulsed with typical Th1 or Th2 inducers, such as *Propionibacterium acnes* or a *Schistosoma mansoni* antigen, were able to induce a Th1 or Th2 response after adoptive transfer [235]. Interestingly, the same microbe could instruct DC to induce a Th1 or Th2 response depending on the developmental stage of the pathogen: the yeast stage of *Candida albicans* activated BMDC to induce protective Th1 responses in vitro and in vivo, whereas the hyphae stage induced a Th2 response and no protection [236]. Moreover, the replicative status of the microbe may also influence the response, since $CD8^-$ myeloid DC treated with live or inactivated influenza virus primed either Th1 or Th2 responses, respectively, upon in vivo transfer [237]. Thus, similar to $CD8^+$ lymphoid DC, the induction of a predetermined Th cell response by myeloid $CD8^-$ DC is not the most common observation. Instead, the type of microbe or microbial products used for DC activation appears to be more important than lineage. Recent reports directly comparing $CD8^+$ and $CD8^-$ DC in the same experiment confirmed this hypothesis. First, Schlecht and colleagues reported that

freshly isolated peptide-pulsed DC from both lineages induced a non-polarized Th response in vivo [238]. Second, by using an in vitro model with TCR transgenic T cells, Manickasingham and collaborators demonstrated that both $CD8^+$ and $CD8^-$ DC were comparable in their ability to prime Th1 or Th2 cells depending on the microbial signals used for DC activation. In the absence of activating signals, both DC subsets induced a non-polarized response. Under these conditions, the antigen dose, the DC:T cell ratio, the protocol of T cell purification, but not the DC lineage, could further promote some degree of polarization [239] [Fig. 7B].

Further support for the instructive model comes from the studies of IL-12 regulation by DC in response to different stimuli. Given the crucial role played by IL-12 in the sensitization of Th1 cells, the production of this cytokine by DC has been used as a surrogate of their ability to prime Th1 cells. The in vitro and in vivo production of bioactive IL-12p70 by DC in response to CD40 ligation was shown to be dependent on microbial priming signals, such as treatment with a *Toxoplasma gondii* extract or CpG motifs [240]. After in vivo treatment with a number of different Th1-promoting microbial substances, both $CD8^+$ and $CD8^-$ DC are able to strongly produce IL-12p70 although some differences are observed depending on the type of stimulus [240, 241]. In a more recent report, the same group also showed that different murine DC subsets can be similarly induced to produce either IL-12 or IL-10 in vitro by a number of Th1- or Th2-promoting microbial substances and that CD40 ligation triggers the amplification of those pre-determined signals [242]. Thus, a boosted cytokine response that may influence the Th cell priming, is not necessarily attributed to a single DC subset but rather to the microbial signal and the T cell feedback.

Similar findings have been reported for human DC subsets. Monocyte-derived myeloid DC were potent inducers of IL-12 and Th1 responses when treated with type 1 microbial compounds [such as LPS, CpG, a *Bordetella pertussis*-derived antigen or a double-strand RNA mimetic], whereas the same cells efficiently promoted Th2 development when activated by substances derived from type 2 microbes [such as *S. mansoni*-derived extracts or a toxin from *Vibrio cholerae*] [195, 243-245]. Plasmacytoid-derived human DC, formerly associated with Th2 responses [see above] have been also reported to efficiently induce Th1 responses when activated by virus infection [246]. Very recently, O'Garra's group published that the newly described

murine plasmacytoid DC are as potent as the conventional myeloid BMDC in directing Th1 responses when activated trough the PRR they express [e.g. by CpG treatment] [224].

In addition to microbial products, a number of cytokines have been demonstrated to skew DC to induce Th1 or Th2 responses in mice and humans [195, 247]. In humans for instance, monocyte-derived DC that were matured in the presence of IFN-γ were potent producers of IL-12 and primed Th1 cells, whereas culture of cells in the presence of prostaglandin E2 or TGF-β led to the development of mature DC that did not produce IL-12 and that primed Th2 cells [195, 248]. The effect of DC-skewing cytokines may be particularly important when considering the heterogeneity that different peripheral tissues impose to the initial activation of DC in situ. In mice, $CD11c^+$ cells purified from Peyer's patches or lung, but not spleen, produce IL-4 and IL-10 and preferentially induce Th2 polarization [195, 249, 250]. How local inflammatory mediators and tissue-specific or tissue–regulated molecules would modulate the ability of DC to polarize the T cell response is unknown. A very interesting report indicated that TLR expression and responsiveness is significantly reduced in epithelial intestinal cells and that a Toll-inhibitory factor is expressed in those cells [251]. This could lead to a very refined tissue-specific modulation of DC functions that would impact Th cell activation [Fig. 7B].

Taken together, all these findings suggest that the ability of DC to initiate an effective Th1 or Th2 immune response is the result of a complex network of interactions among the DC subsets, the type of microbial signals, the type of inflammatory and anti-inflammatory signals, and other environmental and tissue-related factors.

3.15 Use of dendritic cells for immunotherapy

The analysis of the mechanisms underlying antigen presentation and T cell stimulation by DC provided the rationale for developing a novel strategy of immune intervention. As outlined above, DC not only initiate the immune response, but also control and regulate the type of response. These unique properties have been appreciated increasingly in the last years and there is significant hope that the better knowledge of DC biology will help to manipulate immune responses. Indeed, DC are

currently being used for the treatment of serious human pathological conditions such as cancer.

In a seminal report, Inaba and colleagues demonstrated that injection of DC, charged with antigen ex vivo, could sensitize normal mice to protein antigen [252]. Since then, many reports showed that DC are able to induce potent antigen-specific anti-tumor responses and may protect mice against a lethal challenge with tumor cells [205, 253-256]. In some cases, DC-based vaccination was effective to induce regression of established murine tumors [257, 258], and paved the way for their use in cancer therapy. Most of these studies were based on the in vitro generation of DC from bone marrow precursors, followed by in vitro loading with tumor-associated antigens [TAA] in the form of tumor cells, soluble antigens, peptides, or by transfecting DC with TAA-encoding DNA or even RNA, and injection into naïve or tumor-challenged mice [259]. Some modifications in the ex vivo DC pulsing, such as the inclusion of Th cell epitopes, exosomes, or the induction of maturation with TLR ligands or cytokines, have been implemented in order to improve efficacy [260-264].

In humans, clinical trials using DC-based immunotherapy have been initiated or completed in melanoma, lymphoma, myeloma, prostate and renal cancer patients [265]. Most of these trials used monocyte-derived DC and some studies employed proliferating $CD34^+$ precursors [266]. The results of most published trials demonstrated safety and a variable level of efficacy [181, 259, 266]. The use of mature DC is currently recommended since immature DC were shown to be poor immunogens and induce regulatory T cells [266]. Maintaining and enhancing DC survival also appears to promote immunogenicity [266]. In general, although experiences in DC-based therapy of cancer are encouraging, the approach is still at an early stage and a number of points need to be carefully addressed in the future: First, animal models have shown that the desirable abrogation of tumor-induced tolerance might be accompanied by the induction of autoimmunity. Second, the variability in the design of the different studies makes it difficult to compare the results and to draw conclusions. It is therefore necessary to reach a consensus with respect to the clinical monitoring and following up in order to properly validate the effect of the therapy. In particular, there is a necessity of establishing surrogate markers that allow the prediction of the clinical outcome. Third, future work is required to define

standardized conditions for reproducible large-scale DC preparation as well as the optimal conditions for DC pulsing and activation. This includes studies designed to directly compare different DC subsets and different antigen formulations and methods of delivery. Finally, since in vitro and in vivo studies suggested that DC dose, route of injection and frequency of the DC stimulations may influence the type of response, it is also necessary to determine the optimal conditions for those parameters [266, 267].

4. Interaction of dendritic cells and *Leishmania* parasites

Different types of cells have been described to be infected in vivo after *L. major* deposition in the skin by a sandfly, or by experimental injection. Among them, macrophages have attracted special attention for a number of reasons. First, macrophages appear to be the primary target of the parasite as indicated by the predominance of *Leishmania*-harboring macrophages in skin lesions, lymphoid organs and also in vitro. Second, macrophages are known to be crucial effector cells in the clearance of the infection. And third, macrophages are APC that were thought to be responsible for the initiation of anti-*Leishmania* immune responses. Today, however, we know that the key players in sensitizing *Leishmania*-specific T cells are DC and not macrophages.

A set of pioneering reports demonstrated that LC appear to be recruited into murine leishmaniasis lesions, that LC in epidermal cell suspensions selectively ingested *L. major* amastigotes and that DC containing *L. major* could be identified in LN draining the inoculation site [207, 268, 269]. In addition, upon migration and antigen transport from the infected skin to the LN, only DC were able to provide the sensitizing signals for the activation of naïve T cells [207, 268, 269]. After *L. major* infection in vitro, LC retain parasite antigen in immunogenic form for prolonged periods, due to the increased stability of MHC class II-peptide complexes [270], and may thus account for a sustained stimulation of parasite-specific T cells that maintain protective immunity [269, 271]. The infection of LC-like fetal skin-derived DC [FSDC] by *L. major* amastigotes has been also reported. In contrast to primary LC, FSDC might be kept in an immature stage in vitro, allowing to assess how infection affects DC maturation. In contrast to macrophages, FSDC up-regulated MHC and co-stimulatory

molecules upon infection with *L. major* amastigotes and, more interestingly, they also up-regulated the expression of IL-12 and other pro-inflammatory cytokines [272]. Infection of spleen DC with *L. major* promastigotes also led to IL-12 production without inducing further maturation [273]. Additional support to these findings comes from the in vivo observation that following systemic infection with *L. donovani* in mice, DC and not macrophages were the main source of IL-12 in the spleen [274]. Thus, whereas APC functions and IL-12 production in macrophages are impaired by *Leishmania* infection [275-279], infected DC exhibit an IL-12-producing mature phenotype that fulfill the requirements for the initiation of an effective specific T cell response. Indeed, infected spleen DC were able to induce naïve T cells to proliferate [273] and infected FSDC were able to activate naïve T cells to produce IFN-γ in vitro [280]. Interestingly, DC derived from both resistant and susceptible mice were similarly competent to produce IL-12 and to activate naïve T cells to proliferate [273] or to produce IFN-γ [280] in vitro, in agreement with the reported detection of IL-12 in the LN of resistant and susceptible mice soon after infection [70].

Human monocyte-derived DC have been also infected with *L. major* metacyclic promastigotes and undergo subsequent maturation. Infected DC produced IL-12 in a CD40L-depedent manner and were able to induce a secondary T cell proliferation and IFN-γ production [281]. However, the ability of human DC to produce IL-12 appears to be *Leishmania* strain- and species-specific, since *L. tropica* or *L. donovani*-infected DC were unable to produce IL-12p70 in response to CD40 ligation [282]. Such a complexity in the DC-*Leishmania* interaction is also emerging for murine DC. A recent report indicated that after infection with *L. mexicana* promastigotes or amastigotes, BMDC do not undergo maturation and fail to produce IL-12 but rather appear to establish a silent infection [283]. This silent infection of DC is different to the active suppression of antigen presentation and co-stimulatory functions observed in *Leishmania*-infected macrophages, because infected DC were still responsive to other activators such as LPS and IFN-γ [283]. Other authors reported that, although infection of murine BMDC with *L. amazonensis* induced maturation in cells from both resistant and susceptible strains of mice, the cytokine production and immune response upon adoptive transfer was different. DC from resistant C3H mice produced IL-12 and induced a Th1 response in syngeneic recipients, whereas DC from susceptible BALB/c mice produced IL-4 in addition to IL-12, and induced a Th2

response [284]. Thus, both in humans and in murine models, each *Leishmania* species appears to interact differently with DC, and therefore influences the immune responses elicited by different hosts in a distinct manner. Additionally, as indicated by a recent report, different DC subsets may exhibit different susceptibilities to infection, and may also produce different levels of cytokines in response to *Leishmania* infection [285].

The macrophage receptors mediating *Leishmania* uptake as well as their parasite ligands have been extensively characterized. For DC, however, the cell receptors mediating *Leishmania* internalization are poorly documented. A very early report implicated the complement receptor 3 as major molecule on the surface of LC mediating the internalization of *L. major* amastigotes [268]. More recently, by using axenic amastigotes and therefore excluding the possibility of opsonization with host components, the type II lectin DC-SIGN was identified as the first direct receptor for *L. pifanoi* amastigotes in human DC [182]. Although the specific *Leishmania* ligand for DC-SIGN is currently unknown, its ability to bind highly glycosylated molecules from other human pathogens, such as *M. tuberculosis*, points to the abundant *Leishmania* glycoconjugate lipophosphoglycan [LPG] as a potential candidate.

An intriguing aspect of the biology of *Leishmania*-DC interaction to be addressed in the future is the identification of the DC receptors and the parasite ligands that mediate DC activation and IL-12 production. The best characterized molecules mediating microbial recognition and triggering DC maturation are the TLR. It is possible that members of the TLR family are involved in the recognition of protozoan parasites and some evidence supporting this idea is provided by studies that implicated TLR2 in the recognition of *Trypanosoma cruzi* glycosylphosphatidylinositol anchors [286]. Moreover, mice that are functionally deficient in TLR signaling have an impaired production of IL-12 and are more susceptible to *T. gondii* and *L. major* infection [287, 288], indicating that TLR are important for the recognition of these protozoan parasites. Which receptors and how TLR-associated pathways are linked to *Leishmania* and *Toxoplasma*-mediated DC activation is unknown. The future identification of the parasite structures that are recognized by those receptors will also significantly contribute to the understanding of the mechanisms mediating the initiation of anti-parasitic response and will open new possibilities for immune

intervention. A molecule cloned from *L. braziliensis* [the *Leishmania* homolog of eukaryotic ribosomal elongation and initiation factor 4a, LeIF] has been reported to be a potent inducer of IL-12 production [289, 290]. This molecule was shown to stimulate IFN-γ production by spleen cells from SCID mice in an IL-12-dependent manner [290]. No receptors have been identified that recognize LeIF, but TLR4 has been excluded because cells from C3H/HeJ mice, which lack TLR4, respond to LeIF stimulation [290].

5. Use of dendritic cells as adjuvants for vaccination against infectious diseases

The special properties exhibited by DC are also being exploited for improving anti-infectious vaccine efficacy. DC are crucially involved in the generation and amplification of virtually every type of antimicrobial effector function, including the induction of Th cells, killer cells [NK cells; cytotoxic T cells, CTL] and Ab production. More critically, DC also elicit T cell memory, a critical goal of vaccination. The in vivo activation of DC is known to be crucial for an effective anti-microbial response that leads to subsequent immunity and, therefore, DC have been called "natural" adjuvants. Moreover, the classical vaccines that use attenuated organisms, such as measles and smallpox vaccines, may have unknowingly exploited the adjuvant roles of DC. Similarly, novel and promising approaches, such as DNA vaccination or the use of novel adjuvant formulations, appear to exert their effects through the selective activation of DC [291]. Although targeting DC in vivo has shown to enhance the immunogenicity of antigens significantly [292], this cannot always be achieved and may be difficult to be controlled. A way of circumventing the variability of DC targeting in vivo is to use them directly as a vaccine after antigen loading in vitro.

In contrast to the field of tumor therapy, the use of DC-based immune interventions in infectious disease settings has just begun. The first study was published in 1997 and used a murine model of bacterial infection [293]. DC are now being examined as adjuvant and vaccine delivery system in a wide spectrum of infectious diseases caused by various viral, bacterial, fungal and parasitic pathogens. Most notably, despite the

enormous heterogeneity of the pathogens tested, antigen-pulsed DC are remarkably efficient in inducing both humoral and T cell-mediated responses which often result in complete protection against disease. For example, DC-based vaccinations were shown to elicit protective immunity against bacterial infections caused by *Borrelia burgdorferi* [293], *Chlamydia trachomatis* [294] and *M. tuberculosis* [295], against viral infectious caused by lymphocytic choriomeningitis virus [296], influenza virus [297], or genital herpex simplex virus [298] and against fungal and parasitic infections caused by *C. albicans* [236], *T. gondii* [299], *L. major* [300] and *L. donovani* [301]. A number of immunological parameters that are associated with the induction of protection by DC-based immunization were studied. In the model of experimental murine leishmaniasis, it was found that a single injection of ex vivo antigen-pulsed LC was sufficient to induce protective immunity against a single or repeated parasite challenges, and that protection correlated with the induction of a Th1-type cytokine profile [300]. Interestingly, protection was dependent on the route of immunization indicating a critical role of DC homing after immunization [300]. In a later report, antigen-pulsed DC that were genetically manipulated to over-express the Th1-inducing cytokine IL-12 were used in a vaccination trial against *L. donovani* infection and a significant level of protection was observed. Interestingly, the same report showed that genetically manipulated DC were also able to reduce the parasite burden when administrated after *Leishmania* challenge [301], indicating a potential use for immunotherapy. In summary, DC-based anti-infective vaccines are currently a novel and growing field with a promising future. Although a number of aspects, such as the availability of homogeneous DC for in vitro pulsing, and their practical use in clinical settings, will need to be addressed in the future, it is clear that the better understanding of the mechanisms underlying protection in DC-vaccinated animals will help to develop efficient anti-microbial immunoprophylactic and immuno-therapeutic strategies.

AIMS OF THE STUDY

The use of DC as adjuvants has emerged as a powerful strategy for antigen delivery that may be applied for the prophylaxis and treatment of infectious diseases. Previous observations in our laboratory indicated that LC pulsed with *L. major* lysate protect against murine leishmaniasis. However, the isolation of large numbers of pure LC from mouse skin is very difficult. Therefore, the possibility to use this strategy for immune interventions in humans requires the identification of a different DC source. For these reasons, it was highly desirable to implement an easy protocol to obtain murine DC in large numbers and high purity. Moreover, since the immunogenicity and efficacy may be improved by genetic manipulation of DC, it is desirable to have access to a source of proliferating DC or proliferating DC precursors.

Bone marrow samples are suitable sources of DC for those purposes. A protocol for generating murine BMDC has been published and applied in other disease models. DC are generated in vitro from proliferating precursors, allowing genetic manipulation, and are easily obtained in large amounts, overcoming the limitations of LC. Since human myeloid DC precursors can be easily obtained from peripheral blood or alternatively from bone marrow samples, the standardization of a protocol for BMDC preparation that induce protective immunity against parasite infection in mice will facilitate a subsequent testing in human. For these reasons, in the present project, the following questions were addressed:

1. Are antigen-pulsed BMDC, like LC, able to protect against *L. major* infection in mice?

2. Is there any requirement of activating signals for conferring a protective phenotype in BMDC?

3. Do protected mice develop a Th1 immune response?

4. What are the mechanisms of protection?

MATERIALS AND METHODS

1. Reagents for cell culture and plastic material

The cell culture medium was Click-RPMI 1640 [Biochrom AG, Berlin, Germany], supplemented with 10% heat inactivated fetal calf serum [FCS; PAA Laboratories GmbH, Linz, Austria], 2 mM L-glutamine [Biochrom], 10 mM Hepes buffer [Biochrom], 60 µg/ml penicillin [Sigma, Taufkirchen, Germany], 20 µg/ml gentamycin [Sigma] and 0.05 mM 2-mercaptoethanol [Sigma]. Sterile phosphate-buffered saline [PBS] was from Invitrogen [Karlsruhe, Germany]. The counting of viable cells was done with 0.4% trypan Blue [Invitrogen] and the digestion of mouse tissue was performed with sterile trypsin [ICN, Frankfurt, Germany]. Plastic 50 ml and 15 ml tubes were from Sarstedt [Nümbrecht, Germany]. Microfuge tubes [1.5 and 0.5 ml], ELISA plates and pipet tips were from Eppendorf [Hamburg, Germany]. Petri dishes [60 x 15 and 90 x 16 mm] and cell scrapers were from Greiner [Kremsmünster, Austria]. Culture plates [6-, 12-, 24- and 96-well] and plastic pipets were from Corning [New York, USA]. 1 ml syringes were from Terumo [Leuven, Belgium] and 2 ml, 5 ml, 10 ml and 20 ml syringes were from Henke Sass Wolf GmbH [Tuttlingen, Germany]. Cell strainers were purchased from BD Biosciences Pharmingen [Heidelberg, Germany].

2. Reagents for treatment of cells and mice

Recombinant murine GM-CSF, TNF-α and IL-12 were from Pepro Tech [London, UK]. *E. coli*-derived LPS was from Sigma and anti-CD40 mAb [HM40-3, low endotoxin] from BD Biosciences Pharmingen. The CpG-containing oligodeoxynucleotide [ODN] 1668 [CpG ODN, 5′ TCCATGA**CG**TTCCTGATGCT 3′] and the control AT-rich oligodeoxynucleotide [non-CpG ODN, 5′ ATTATT-ATTATTATTATTAT 3′] were manufactured by MWG [München, Germany] and Qiagen Operon [Cologne, Germany]. The synthetic peptide LACK-T cell epitope [LTE; amino acids 158-173, FSPSLEHPIVVSGSWD] was derived from the LACK protein and used in antigen presentation assays. The Gene B protein [GBP]-derived peptide [PKEDGHTQKNDGDGPKEDGHTQKNDGDG] was derived from the *Leishmania* GBP protein [302] and used as control in those experiments. The peptides were purchased from Jerini AG [Berlin, Germany] and Bio-synthesis [Lewisville, Texas, USA]. Neutralizing rat anti-mouse IL-12 mAb [IgG2a, hybridoma C17.8] was

created in the laboratory of Dr. Giorgio Trinchieri [Wistar Institute, Philadelphia, USA]. Rat IgG control Ab were purchased from Sigma.

The recombinant antigens LeIF, M15, and MM6H were kindly provided by Dr. Yasir Skeiky, Corixa Corporation, Seattle, USA. These antigens were produced in *E. coli* as His-tagged proteins and purified by NTA-agarose affinity and ion-exchange chromatography. The purity was higher than 90% and the level of endotoxin less than 100 EU/mg protein. LeIF and M15 are immunodominant *Leishmania* antigen that were identified by cDNA library screening with sera from CL patients. Both antigens have been shown to induce partial protection in mice [303, Y. Skeiky, personal communication]. MM6H is a recombinant chimeric protein consisting of a linear arrangement of the immunodominant regions of three major *Leishmania* antigens [MAPS, M15 and 6H] that were also isolated by immunoscreening [Y. Skeiky, personal communication].

3. Mice

Female BALB/c and C57BL/6 mice were purchased from Charles River Breeding Laboratories [Sulzfeld, Germany]. IL-12p40 and IL-12p35-deficient BALB/c mice were kindly provided by Dr. Gottfried Alber, University of Leipzig [304, 305]. C57BL/6 mice deficient for IL-12p35, MHC class I and class II molecules and IL-2 as well as BALB/c IL-2 knock-out mice were provided by Drs. Klaus Erb, Ursula Bommhardt and Anneliese Schimpl, University of Würzburg, respectively. Animals were 6 to 8 weeks old at the onset of experiments and were kept under conventional conditions.

4. *L. major* parasites and preparation of antigen

The *L. major* isolate MHOM/IL/81/FE/BNI used in this study has been described previously [306]. Parasites were maintained by passage in BALB/c mice. Promastigotes were grown in conventional biphasic medium consisting of a solid base of rabbit-blood agar plus a liquid phase of Click RPMI. Parasites used for in vitro and in vivo infection were passaged 3 to 7 times in vitro to maintain maximal infectivity. For the preparation of total *L. major* antigen [LmAg], stationary-phase promastigotes were collected, washed three times with PBS, resuspended at 1 x 10^9/ml in PBS and

subjected to three cycles of freezing and thawing at -80^{o}C and 25^{o}C, respectively. LmAg was stored at -80^{o}C and aliquots were thawed not more than twice.

5. BMDC preparation

DC were generated from bone marrow progenitors by following the protocol published by Lutz et al. [307], with minor modifications. Briefly, total bone marrow cells were obtained from femurs and tibiae after flushing with a syringe containing PBS. The cell suspension was washed and resuspended in Click RPMI culture medium. At day zero, 2 million cells were seeded in bacteriological petri dishes in a total volume of 10 ml culture medium containing 200 U/ml GM-CSF. Additional 5 ml of culture medium containing 200 U/ml GM-CSF were added at days 3 and 6. For most of the experiments, nonadherent cells were harvested after 10 days of culture and resuspended at 1 x 10^6 cells/ml in fresh culture medium. For some experiments BMDC were collected at days 7, 8 or 9. To analyze the morphologic characteristics of the cells, 10 µl of the cell suspension were smeared onto a glass slide, stained with Eosin G/Thiazine "Diff-Quik II" dye [Dade Behring, Marburg, Germany] and observed in a conventional light microscope.

6. Preparation of peritoneal macrophages

Naïve mice were injected with 2-3 ml of thioglycolate [Sigma] intraperitoneally [i.p.] and were sacrificed after 5 days. The abdominal skin was removed and, with the help of a 20 ml syringe containing 8 ml of cold culture medium, the peritoneal cavity was washed several times. The culture medium containing peritoneal cells was extracted and the cell suspension washed 3 times by centrifugating at 1600 rpm for 10 minutes at 4^{o}C. The cells were then counted and cultured in 10 ml Click RPMI culture medium at a density of 1 x 10^6/ml in petri dishes at 37^{o}C for 2 hours. After macrophages were attached, the supernatant was removed and the monolayer washed twice with culture medium. Cell scrapers were then used to collect the attached macrophages and cells were resuspended at 1 x 10^6/ml for further use.

7. Preparation of Langerhans cells

Epidermal LC suspensions were prepared from mouse ear skin as previously described [300, 308]. The mice were sacrificed and the ears removed and washed with ethanol. Each ear was split into the dorsal thin and the ventral thick halves and

incubated with 0.6% or 1% trypsin [dissolved in PBS], respectively. The trypsin digestion was performed at 37°C for 45 minutes for the thin ear halves and 90 minutes for the thick ear halves. After careful aspiration of the trypsin, the epidermal sheets were peeled and transferred to a petri dish containing Click RPMI and a tea sieve. The sieve was then shaken for 4 minutes and the epidermal sheets discarded in order to collect the cell suspension. Cells were washed once with culture medium and resuspended at 4 x 10^6/ml. These preparations contained 3–5% of LC that constitutively express MHC class II as well as MHC class II-negative keratinocytes and were devoid of macrophages [308].

8. Enrichment of T cells by nylon wool passage

The passage of cells through a nylon column is an effective procedure to enrich T cells. By this process, macrophages, most B cells as well as other adherent cells are depleted. 1.8 g of teased and loosely connected nylon wool was packed to 18 ml in syringes of 20 ml volume. The column was rinsed with RPMI [about 40 ml] and air bubbles were removed. A final wash with RPMI supplemented with 5% FCS was done prior to 45 minutes incubation at 37ºC. Mesenteric, inguinal, popliteal and axillary LN cells from naïve mice were collected and adjusted to 5 x 10^7 cells/ml in RPMI supplemented with 10% FCS. The suspensions were added to the columns and the cells were allowed to enter the nylon wool. The column was incubated at 37ºC for 45 minutes to allow the adherence of cells. Then, nonadherent cells were eluted at a rate of about 1 drop every 3 seconds by washing with 40 ml RPMI-10%FCS pre-warmed at 37ºC. The T cell-enriched suspension was washed once with Click RPMI and subsequently used for mixed lymphocyte reactions [MLR]. Fluorescence-activated cell sorting [FACS] analysis of these T cell enriched suspensions always indicated a purity of more than 90% [not shown].

9. Mixed lymphocyte reactions

The antigen presentation capacity of BMDC was tested by performing allogeneic and syngeneic MLR. BMDC and peritoneal macrophages were prepared from BALB/c and C57BL/6 mice, treated with mitomycin C [50 μg/10^6 cells, Sigma] for 30 minutes, washed with RPMI-5%FCS 3 times and resuspended in Click RPMI culture medium. 2 x 10^5 enriched T cells from naïve BALB/c and C57BL/6 mice were co-incubated with 10^2 to 10^4 mitomycin C-treated syngeneic BMDC, allogeneic BMDC,

or allogeneic macrophages in 96-well round-bottom tissue culture plates, in a final volume of 200µl. Triplicate cultures were set up and incubated for 5 days in a humidified CO_2 incubator at 37°C. ^{3}H-Thymidine [0.5 µCi/well, Amersham, Braunschweig, Germany] was added for the last 18 hours. Triplicate cultures with T cells only were also included. The cells were harvested onto filter strips and incorporation of ^{3}H-Thymidine was analyzed in a microplate β-reader [Wallac, PerkinElmer Life Sciences, Boston, USA]. T cell proliferation was expressed as counts per minute [cpm] after subtracting the background incorporation in the absence of APC.

10. Proliferation assay with a *L. major*-specific T cell hybridoma

The antigen-presentation capacity of BMDC was also tested by using a *Leishmania*-specific T cell proliferation assay. A $CD4^+$ T cell hybridoma [LMR 8.1, kindly provided by Dr. Nicolas Glaichenhaus, University of Nice, Nice, France] that reacts with the LTE immunodominant peptide in an I-A^d-restricted manner was used. 96-well culture plates were used to co-incubate 1 x 10^5 LMR 8.1 cells with titrated numbers of BALB/c BMDC or LC in a final volume of 200 µl in the absence or presence of the LACK epitope [50 µg/ml], the irrelevant GBP peptide [50 µg/ml], LmAg [equivalent to 10 parasites per cell], or live *L. major* promastigotes [10 parasites per cell]. Cultures were frozen after 24 hours of incubation and the concentration of IL-2 in supernatants was determined as a read-out of T cell proliferation.

11. FACS analysis

BMDC were collected after 10 days of generation and the surface expression of the following markers was determined: MHC class II, CD86, CD11c, CD11b, CD4, DEC-205/NLDC-145, B220 and CD8α. The induction of MHC class II and CD86 expression in BMDC that had been treated overnight with LPS [1 µg/ml], TNF-α [500 U/ml], LmAg [the equivalent to 30 parasites per cell], or live parasites [30 parasites per cell], was also determined by flow cytometry. 2 x 10^5 cells were incubated on ice in 0.1 ml of PBS supplemented with 0.1% [w/v] bovine serum albumin [BSA; Sigma], 0.02% sodium azide and 5 µg/ml of a purified anti-Fc receptor [purified rat anti-mouse CD16/CD32, BD Biosciences Pharmingen] Ab for 10 minutes at 4°C. The following Ab at saturated concentrations [0.5-5 µg/ml] were then added to the cells

and incubated at 4°C for 15 minutes: R-Phycoerytrin [PE]-conjugated anti-mouse CD86 [B7.2], PE-conjugated anti-mouse I-A^d [MHC class II], fluorescein isothiocyanate [FITC]-conjugated anti-mouse CD11c, PE-conjugated anti-mouse CD4, Cy-chrome-conjugated anti-mouse CD45R/B220, FITC-conjugated anti-mouse CD8α, biotin-conjugated anti-mouse CD11b [BD Biosciences Pharmingen] and purified rat anti-mouse NLDC-145/DEC-205 [BMA Biomedicals, Geneva, Switzerland]. For the expression of CD11b and NLDC-145/DEC-205, a second step of incubation with FITC-conjugated streptavidin [Serotec GmbH, Düsseldorf, Germany] and FITC-conjugated goat anti-rat IgG Ab [Jackson ImmunoResearch Laboratories, West Grove, USA], respectively, was included. Cells were washed between incubations and specificity of staining was confirmed using the following isotype-matched control mAb: PE-conjugated rat IgG2a κ, PE-conjugated mouse IgG2a κ, FITC-conjugated hamster IgG1 λ, PE-conjugated rat IgG2a κ, Cy-chrome-conjugated rat IgG2a κ, FITC-conjugated rat IgG2a, biotin-conjugated rat IgG2b κ and purified rat IgG2a [BD Biosciences Pharmingen], respectively. Analysis was performed with a FACscalibur flow cytometer [BD Biosciences Pharmingen] by gating cells according to the light scatter distribution in order to exclude dead cells. Data were analyzed with CellQuest 2.0 software.

12. Dendritic cell pulsing, vaccination and infection of mice

BMDC were adjusted to a concentration of 1 x 10^6 cells/ml in medium containing 200 U/ml GM-CSF and pulsed with 30 μl of total LmAg/ml culture [approx. equivalent to 30 parasites per cell]. Some experimental groups were concomitantly treated with well-established inducers of BMDC maturation during pulsing [LPS: 1 μg/ml; CpG and non-CpG ODN: 25 μg/ml; anti-CD40 mAb: 5 μg/ml; and TNF-α: 500 U/ml]. Cultures with untreated BMDC, as well as BMDC that were treated with CpG, non-CpG ODN or LPS in the absence of LmAg were also included. Cells were incubated overnight [approx. 18 hours], washed to remove remaining parasite antigen and maturation stimuli, and resuspended in PBS. In one experiment, LC-containing epidermal cell suspensions were cultured in parallel to BMDC with LmAg [equivalent to 10^7 parasites per 4 x 10^6 cells per ml], washed and resuspended in PBS.

5 x 10^5 BMDC or 3 x 10^5 LC were administered intravenously [i.v.] into a tail vein of naive mice in a volume of 100 µl. Control mice were treated with 100 µl PBS. One week later, mice were infected s.c. with 2 x 10^5 [BALB/c mice] or 2 x 10^6 [C57BL/6 mice] stationary-phase *L. major* promastigotes into the right hind footpad using a syringe with a particularly fine needle [30G]. The course of infection was monitored weekly by measuring the footpad swelling, compared with the uninfected contra-lateral footpad, and by determining the amount of parasites in the footpads at defined time points.

To determine the number of BMDC required for the induction of protection, different amounts of CpG-activated and LmAg-pulsed BMDC ranging from 10^4 to 10^6 cells were injected i.v. To study the influence of the route of BMDC administration, 5 x 10^5 CpG-activated and LmAg-pulsed BMDC were injected s.c., i.p., or i.v. into naïve BALB/c mice. The parasite challenge was performed one week later and the lesion development was monitored as described above. Finally, in some experiments, BMDC were pulsed with single recombinant antigens [LeIF, 20 µg/ml or 100 µg/ml; M15, 100 µg/ml; MM6H, 25 µg/ml] and 5 x 10^5 cells were used in a vaccination trial in BALB/c mice.

13. Analysis of the duration of the protective effect and of the protection against re-infection

In some experiments, BALB/c mice that were protected against the primary challenge were reinfected. Mice were reinfected with 5 x 10^5 or 8 x 10^5 parasites s.c. into the left hind footpad at 9 or 10 weeks after the primary infection. As controls, naive mice were challenged with the same doses of parasites and the development of lesions was determined weekly. The parasite load in the footpads of those animals was also determined. To analyze the duration of the protective effect induced by BMDC in BALB/c mice, naïve mice were vaccinated i.v. with 5 x 10^5 CpG-matured LmAg-pulsed BMDC and the infective challenge was given 16 weeks later [2 x 10^5 parasites]. Protection was determined by measuring the footpad swelling and by quantifying the amount of parasites in the footpad.

14. Analysis of the therapeutic effect

For therapeutic immunization, BALB/c mice were first infected s.c. with 2 x 10^5 stationary-phase *L. major* promastigotes and subsequently, i.v. injections of CpG-activated LmAg-pulsed BMDC were given at different time points. In one experiment, mice received a single treatment with BMDC, whereas in a second trial, mice were injected with two doses of cells. The amount of BMDC inoculated i.v. into the tail vein was 5 x 10^5 per mouse in 100 µl of PBS. Disease evolution was followed by weekly measurements of footpad swelling and compared to non-treated infected mice.

15. Determination of the parasite load

In order to determine whether effective anti-*Leishmania* mechanisms were taking place at the site of infection, we determined the amount of viable parasites in the footpads by a limiting dilution method [309]. After 5-6 weeks of infection, the right foot was removed, washed with ethanol and rinsed three times with PBS. The soft tissue surrounding the site of infection was obtained by making some slits with a sterile scalpel and by macerating the foot in a cell strainer. Cell suspensions were then passed through a 30G needle in order to assure the delivering of intracellular parasites and subsequently centrifuged 5 minutes at 100g in order to separate tissue clumps and debris. Serial dilutions ranging from from 1:5 to 1:4.69 x 10^{12} were prepared with the supernatant and 100 µl/well were seeded in 96-well bood-agar microculture plates. For each dilution, replicates of 20 wells were set up. After 10 days of incubation at 28^{o}C in a humidified atmosphere with 5% CO_2, the cultures were scored for the presence of parasites using an inverted microscope. The estimation of the number of parasites per footpad was done by multiplying the reciprocal of the last dilution showing at least one positive well with the initial dilution factor. For some experimental groups, 10 µl of the footpad cell suspension were smeared onto a glass slide, stained with Eosin G/Thiazine "Diff-Quik II" dye [Dade Behring] and analyzed in a conventional light microscope for the presence of amastigotes.

16. Analysis of cytokine production by lymph node cells

LN draining the infected footpads were removed 5 weeks after infection. After preparation of single-cell suspensions, 1 x 10^6 cells were cultured in 1 ml volume [24-well plates] in the absence or presence of 10 µl LmAg for 72 hours. Thereafter,

culture supernatants were harvested for the determination of the cytokines IL-2, IL-4, IL-10 and IFN-γ by sandwich ELISA

17. Analysis of cytokine production by BMDC

BMDC were generated for 10 days and cultures containing 10^6 cells/ml were treated with LmAg and/or different maturation inducers and incubated for additional 24 hours. In some experiments, different concentrations of the maturation inducers were tested. Cells were then separated from culture supernatants by centrifugation at 2000 rpm for 10 minutes and the supernatants were used for measuring the concentration of IL-1β, IL-6, IL-10, TNF-α and IL-12 by ELISA. In some experiments, cell fractions were used for measuring the cytokine mRNA expression by RNAse protection assay [RPA] and reverse transcriptase-polymerase chain reaction [RT-PCR] assays.

18. Analysis of chemokine and chemokine receptor expression by BMDC

BMDC were generated for 10 days and cultures containing 10^6 cells/ml were treated with LmAg and/or different maturation inducers at the concentrations described above [see DC pulsing, section 12] and incubated for 24 hours. Cultures were centrifuged and the cell pellet was used for quantifying mRNA levels of some chemokine and chemokine receptors by RPA and for some experiments by RT-PCR.

19. Cytokine determination by ELISA

Supernatants of LN cells and BMDC cultures were used to determine the concentration of cytokines by sandwich ELISA. Plates were coated with purified capture anti-cytokine mAbs diluted in carbonate buffer and incubated overnight at 4^oC. The concentrations of the mAb used were: rat anti-mouse TNF-α, 4 μg/ml; rat anti-mouse IFN-γ, 1 μg/ml; rat anti-mouse IL-4, 1 μg/ml; rat anti-mouse IL-2, 2.5 μg/ml; rat anti-mouse IL-12p70, 2.5 μg/ml; rat anti-mouse IL-10, 5 μg/ml; rat anti-mouse IL-6, 4 μg/ml [BD Biosciences Pharmingen] and rat anti-mouse IL-1β, 4 μg/ml [R & D systems GmbH, Wiesbaden, Germany]. After washing 3 times with PBS-Tween [0.05%] plates were incubated with 200 μl blocking solution [10% BSA in PBS-Tween, Sigma] for 4 hours at room temperature [RT] and washed again 3 times. 50 μl of cell supernatants were then added to the plates and incubated overnight at 4^oC. Standard solutions of recombinant TNF-α, IFN-γ, IL-2, IL-12p70, IL-6 [BD Biosciences Pharmingen], IL-1β, IL-10 and IL-4 [R & D systems] diluted in 1%

BSA-PBS-Tween were added and serial 1:2 dilutions were also set up for a standard curve. After washing 3 times with PBS-Tween, plates were incubated for 1 hour at RT with the following detection mAb diluted in 1% BSA-PBS-Tween: biotin-conjugated rat anti-mouse TNF-α, 0.5 μg/ml; biotin-conjugated rat anti-mouse IFN-γ, 1 μg/ml; biotin-conjugated rat anti-mouse IL-4, 1 μg/ml; biotin-conjugated rat anti-mouse IL-2, 2.5 μg/ml; biotin-conjugated rat anti-mouse IL-12p70, 2.5 μg/ml; biotin-conjugated rat anti-mouse IL-10, 1 μg/ml; biotin-conjugated rat anti-mouse IL-6, 2 μg/ml [BD Biosciences Pharmingen] and biotin-conjugated rat anti-mouse IL-1β, 0.1 μg/ml [R & D systems]. Three washes were then followed by incubation with alkaline phosphatase-conjugated streptavidin [Dako Diagnostika GmbH, Hamburg, Germany] 1:1000 in PBS for 1 hour at RT and development with 100 μl of p-nitrophenyl phosphate [1 mg/ml, Sigma] dissolved in diethanolamine buffer. The optical density [OD] at 490 nm and 405 nm was determined with an ELISA reader [MRX, Dynatech laboratories, Chantilly, USA] and the concentrations of cytokines were calculated by extrapolating the OD values of the samples to the linear regression of the standard curve. The detection thresholds were: TNF-α: 15.6 ng/ml, IFN-γ: 0.49 ng/ml, IL-2: 1.95 ng/ml, IL-12p70: 0.03 ng/ml, IL-6: 0.98 ng/ml, IL-1β: 0.098 ng/ml, IL-10: 0.39 ng/ml and IL-4: 0.049 ng/ml.

20. Detection of anti-*Leishmania* IgG, IgG1 and IgG2a antibodies by ELISA

Mice were sacrificed 5 weeks after parasite infection and anti-*Leishmania* IgG, IgG1 and IgG2a serum Ab levels were assayed by ELISA. To this end, plates were coated with LmAg [equivalent to 5 x 10^5 parasites/well] overnight and washed with PBS. After blocking with 10% BSA in PBS-Tween for 4 hours at RT and washing with PBS-Tween, plates were incubated overnight with 100 μl diluted sera at 4^{o}C [dilutions: 1:100 for total IgG; 1:50 for IgG1 and IgG2a; in 1% BSA-PBS-Tween]. For total IgG detection, a secondary Ab [alkaline phosphatase-conjugated anti-mouse IgG, 1:500; BD Biosciences Pharmingen] was added for 1 hour and developed with p-nitrophenyl phosphate [Sigma]. For IgG1 and IgG2a Abs, plates were incubated with an isotype-specific secondary Ab [biotinylated rabbit anti-mouse IgG1 and IgG2a, at 0.5 mg/ml in 1% BSA-PBS-Tween; Biosciences Pharmingen] for 1 hour, and after washing and incubating with alkaline phosphatase-conjugated streptavidin [1:1000 in PBS; Dako Diagnostika GmbH] wells were developed similarly to total IgG. Relative levels of Ab are presented in OD at 490 nm.

21. Western blot

Total LmAg was centrifuged at 1000g for 10 minutes at 4°C, and 150 µl of the supernatant was boiled with 150 µl of sample buffer [100 mM Tris-HCl, 4 % sodium dodecyl sulfate, 0.2 % Bromophenol Blue, 20 % Glycerol, 200 mM Dithiothreitol] for 5 minutes. The resulting 300 µl sample was separated in a preparative 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE] gel by using a Miniprotean II unit [BioRad Laboratories GmbH, München, Germany] at 100 V. Separated antigens were transferred to nitrocellulose membranes [0.45 µm, BioRad] with a Mini Trans Blot cell [BioRad] during 1 hour at 250 mA. After blocking with 5% non-fat dry milk in PBS overnight and washing 3 times with PBS-Tween [0.05%], the membranes were mounted in a Mini-Protean Multiscreen apparatus [BioRad]. Serum samples were diluted 1:50 with blocking solution and 500 µl were individually added to each slot of the multiscreen device and incubated for 2 hours with agitation at RT. After washing 3 times with PBS-Tween, membranes were incubated with a biotinylated anti-mouse IgG2a or IgG1 diluted 1:125 [equivalent to 4 µg/ml] in blocking solution under agitation for 1 hour and washed 3 times. 1:1000 alkaline phosphatase-conjugated streptavidin was then added to the blots, incubated for 1 hour and washed with PBS 3 times. After dismounting from the multiscreen apparatus, membranes were developed with the precipitable chromogenic substrate 5-Bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium [BCIP/NBT, Promega GmbH, Mannheim, Germany].

22. RT-PCR

After treatment with LmAg and the different maturation inducers, total RNA was isolated from BMDC using the RNeasy total RNA extraction kit [Qiagen, Hilden, Germany] and 2 µg of RNA were reverse transcribed with the Omniscript RT kit [Qiagen]. 2 µl of cDNA were used in a PCR reaction with the appropriate primer pairs and conditions to estimate the relative amount of the respective mRNA. The Taq DNA polymerase was from Promega and the Mastercycler gradient thermocycler from Eppendorf. The following primer pairs were used to amplify CCR7, IL-12p35 and IL-12p40 cDNAs: CCR7 antisense: 5'-TGA CGT CAT AGG CAA TGT TGA GCT G-3', CCR7 sense: 5'-ACA GCG GCC TCC AGA AGA ACA GCG G-3'; IL-12p35 antisense: 5'-GCC GGC TAT CCA GAC AAT TA-3', IL-12p35 sense: 5'-CTA

CCA AGG CAC AGG GTC AT-3'; IL-12p40 antisense: 5'-ATG GCC ATG TGG GAG CTG GAG-3', IL-12p40 sense: 5'-TTT GGT GCT TCA CAC TTC AGG-3' [MWG]. A β-actin primer pair [antisense: 5'-TGT GAT GGT GGG AAT GGG TCAG-3' and sense: 5'-TTT GAT GTC ACG CAC GAT TTC C-3'; MWG] was used to normalize the amount of template RNA. PCR conditions were as follows: 94°C for initial denaturation [2 minutes], amplification with 35 cycles of 94°C [1 minute] for denaturation, 58°C [1 minute] for annealing, 72°C [1 minute] for elongation, and final extension at 72°C [10 minutes]. PCR products were run in a conventional 1% agarose gel and visualized by staining with ethidium bromide [Sigma].

23. RNAse protection assay

Quantification of the mRNA for cytokines, chemokines and chemokine receptors in BMDC was performed by using the "Riboquant Ribonuclease protection assay kit" [BD Biosciences Pharmingen]. The following multi-probe template sets were used: mCK-2b template set for mouse cytokines, the mCR-5 set for mouse chemokine receptors and the mCK-5 set for mouse chemokines [BD Biosciences Pharmingen]. The assay was performed according to the manufacturer's recommendations. Total RNA was isolated from BMDC using the RNeasy total RNA extraction kit [Qiagen] and resuspended in RNAse-free water at -70^{o}C until use. Probe synthesis was done by incubating the template set, [α-^{32}P]UTP [Amersham], nucleotide pool and T7 RNA polymerase at 37^{o}C for 1 hour. The reaction was terminated by adding DNAse and incubation at 37^{o}C for 30 minutes. The polymerized RNA was then purified by the phenol-chloroform-isoamyl alcohol-ethanol method, dried and resuspended in 50 µl hybridization buffer. Incorporation of radioactivity was quantified in a 1 µl sample by measuring the cpm values without scintillation fluid in a β-counter. The incorporation always ranged from 2-30 x 10^5 Cherenkov counts/µl. Probes were stored at -20^{o}C until needed.

For the hybridization, 20 µg total RNA was dissolved in 8 µl of hybridization buffer by vortexing and quick spin in a microfuge. The synthesized probe was also diluted in hybridization buffer in order to reach the optimal concentration [5 x 10^5 cpm/µl]. 2 µl of diluted probe was added to the RNA sample and mixed by pipetting. A drop of mineral oil was then added to each tube and incubated in a pre-warmed heat block at 90^{o}C. Immediately, temperature was turned down to 56^{o}C [allowing the temperature

to decrease slowly] and samples incubated for 12-16 hours. After this, a 15 minutes incubation at 37^{o}C prior to RNAse treatment was included. For the RNAse treatments, 100 µl of RNAse cocktail was added into the aqueous layer of each sample, quickly spinned and incubated for 45 minutes at 30^{o}C. 18 µl of proteinase K cocktail [666 µg/ml proteinase K, 133 µg/ml yeast tRNA, BD Biosciences Pharmingen] were incubated in separate tubes with the oil-free aqueous layer of the samples and after vortexing, the mix was incubated for 15 minutes at 37^{o}C. Nucleic acid was subsequently purified from the mix by the phenol-chloroform-isoamyl alcohol-ethanol method, air-dried and resuspended in 5 µl of 1x loading buffer [50 % Glycerol, 1 mM EDTA, 0.4 % Bromophenol Blue, BD Biosciences Pharmingen]. Samples were then heated for 3 minutes at 90^{o}C and placed immediately in an ice bath before gel separation.

Finally, samples were loaded onto a sequencing 5% acrylamide gel mounted in a Sequi-Gen sequencing cell [BioRad] and run at 50 W constant power until the leading edge of the bromophenol blue [front dye] reached 30 cm. As size markers, 2000 cpm of the probe set diluted in loading buffer were also run in every gel. After running, the gel mold was disassembled, adsorbed to filter paper, covered with Saram wrap, layered between two additional pieces of filter paper and vacuum-dried for 1 hour at 80^{o}C. Dried gels were then placed onto a X-ray retina film [Fotochemische Werke GmbH, Berlin, Germany] in a cassette with an intensifying screen and developed at -70^{o}C for 24-96 hours. To identify the protected bands in the autoradiograms, which represent the different “protected” mRNA, a semi-log curve plotting the migration distance of the “unprotected marker” versus the nucleotide length was drown and used as standard curve.

RESULTS

1. BMDC preparation and characterization

1.1 Generation of murine BMDC

In the past, a major limitation for studying the biology of the DC system was the difficulty to isolate sufficient amounts of cells from tissues and lymphoid organs with an appropriate purity. In 1992, however, Inaba and colleagues published a protocol for generating DC by culturing bone marrow precursors in the presence of GM-CSF and IL-4 [310]. More recently, an improved version of the protocol of Inaba et al. has been described in which considerably larger quantities of reasonably pure BMDC can be obtained [307]. This protocol is based on the culture of unselected total bone marrow cells at lower density for 8-10 days in the presence of GM-CSF as a sole growth factor, making it more simple and convenient. For this reason, as starting point of the present work, the method of Lutz et al. was implemented for the generation of BMDC and the quantity and quality of the obtained population of cells was analyzed before using it for further in vitro and in vivo experiments.

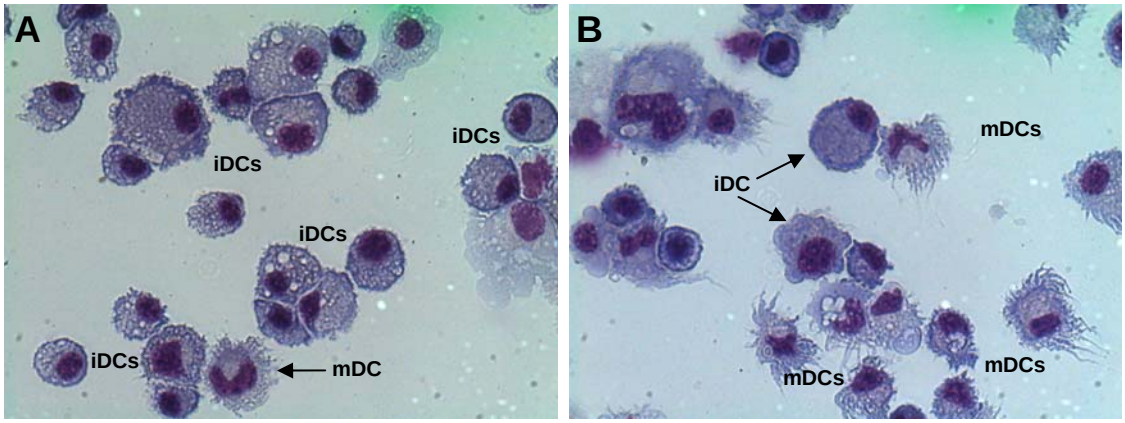

Fig. 1. BMDC. Bone marrow cells were cultured for 9 days with GM-CSF and the cells in suspension were re-plated in the presence of GM-CSF [A] or GM-CSF plus LPS [B]. The cells were fixed and stained with "Diff-Quik". The typical immature [iDC] and mature DC [mDC] are indicated [arrows]

We usually obtained 4-7 x 10^7 total bone marrow cells from the femurs and tibiae of 5 to 8 weeks-old BALB/c or C57BL/6 mice. Bone marrow cells were cultured at low density with GM-CSF as described in the section Material and Methods, and after 3-4 days an increase in the number of cells was evident. The typical adherent DC-generating clusters appeared at days 4-5 and were observed until days 8-9, when the majority of cells were in suspension. "Diff-Quik" staining of cells in the supernatant of 10 days-old cultures showed a dominant population of cells with small cytoplasmic

processes and excentric nuclei, compatible with immature DC, and also the typical mature DC, with long cytoplasmic processes and indented excentric nuclei [Fig. 1A]. The presence of LPS during the last day of culture gave rise to abundant mature DC [Fig. 1B]. Rounded cells with multi-lobular nuclei typical of granulocytes as well as small cells without any characteristics of differentiated leukocytes, presumably myeloid precursor cells, were also observed in some preparations [not shown] [307].

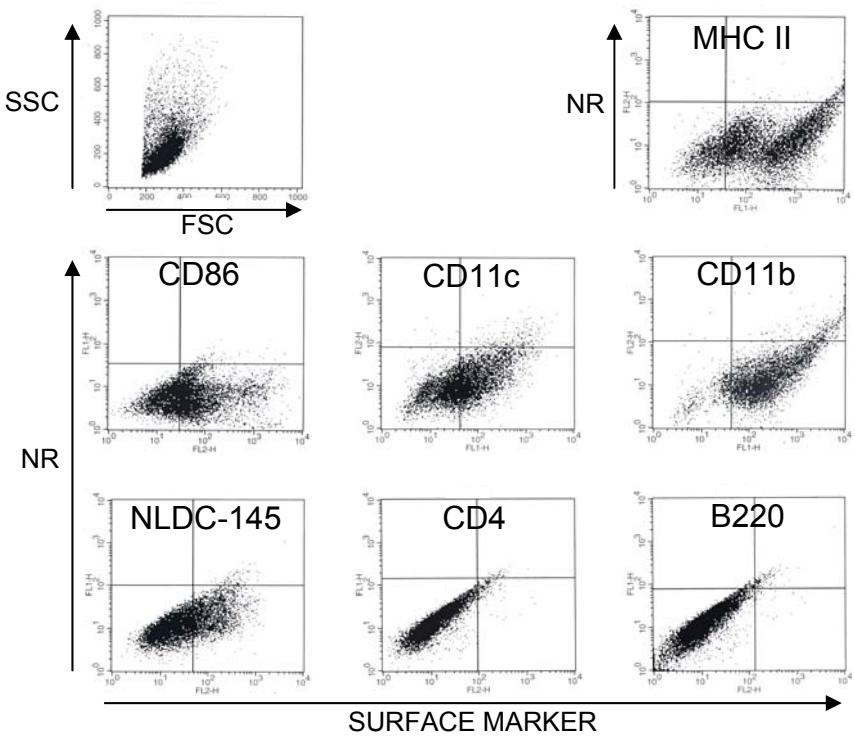

Fig 2. Surface phenotype of BMDC. BMDC were generated for 10 days, stained with Ab directed against the indicated surface markers and analyzed by flow cytometry. NR: not relevant, SSC: side scatter, FSC: forward scatter.

1.2 Surface phenotype of BMDC

Staining of the cells for some surface markers by FACS also indicated a typical DC-like phenotype. As shown in Figure 2, the predominant population of cells in the supernatant is expressing MHC class II, CD86, CD11b, CD11c and NLDC145. Cells were negative for CD8α [not shown] indicating a myeloid-like DC phenotype. As expected, these cells were able to up-regulate the surface expression of MHC class II and co-stimulatory molecules when treated with recognized inducers of DC maturation like LPS, TNF-α or *L. major* infection [Fig. 3]. Those treatments were also able to up-regulate the expression of other maturation markers, such as CD40 and ICAM-1 on the DC surface [not shown].

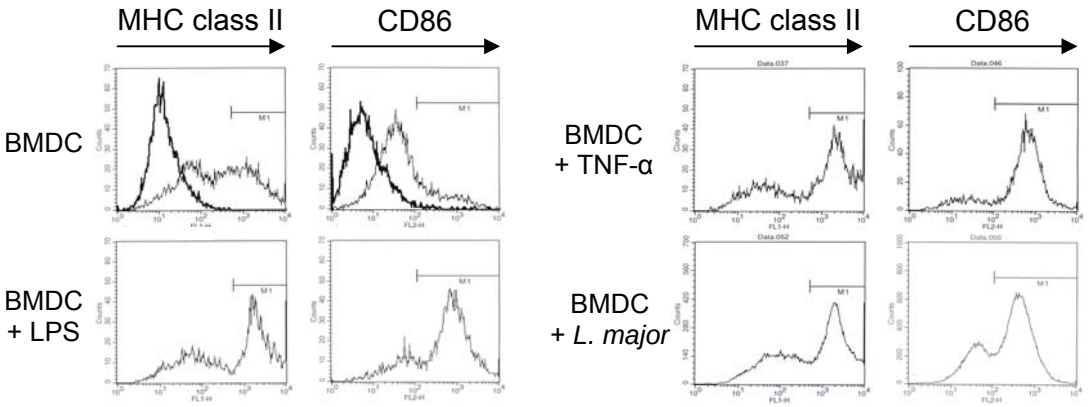

Fig 3. Induction of BMDC maturation. BMDC were incubated overnight with LPS [1 µg/ml], TNF-α [500 U/ml] or *L. major* parasites [30 parasites per cell] and the surface expression of MHC class II and the co-stimulatory molecule CD86 was determined by flow cytometry. Thick line: isotype control.

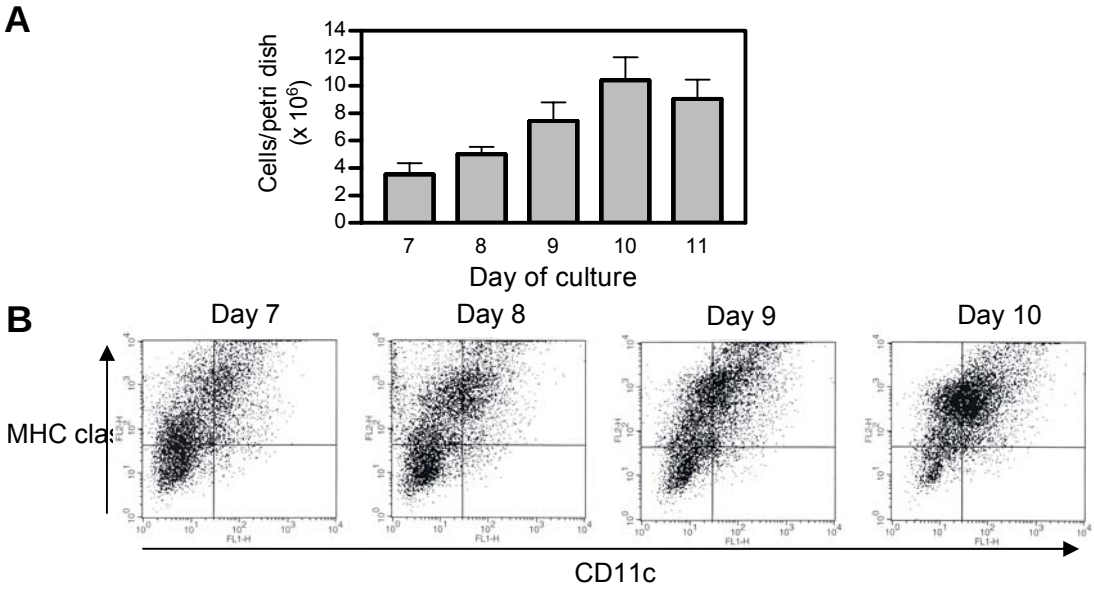

Fig 4. Kinetics of DC generation in bone marrow cultures. BMDC were generated in vitro for the indicated time and the number of viable cells was determined [A]. The expression of MHC class II and CD11c was also analyzed by two-color flow cytometry at different days of generation [B].

The yield of cells in the supernatants was maximal by day 10 of culture and usually ranged from 7 x 10^6 to 1.3 x 10^7 cells/petri dish [Fig. 4A]. The viability of the cells was always higher than 90% at day 10 of in vitro generation with GM-CSF and decreased after days 12-13 of culture [not shown]. An analysis of the expression of MHC class II and CD11c during the time of culture in vitro also indicated that maximal proportions of DC are obtained at day 10 of generation [Fig. 4B]. For those reasons the majority of the in vitro and in vivo experiments reported here were performed with BMDC that were generated in cultures with GM-CSF for 10 days.

1.3 Functional properties of BMDC

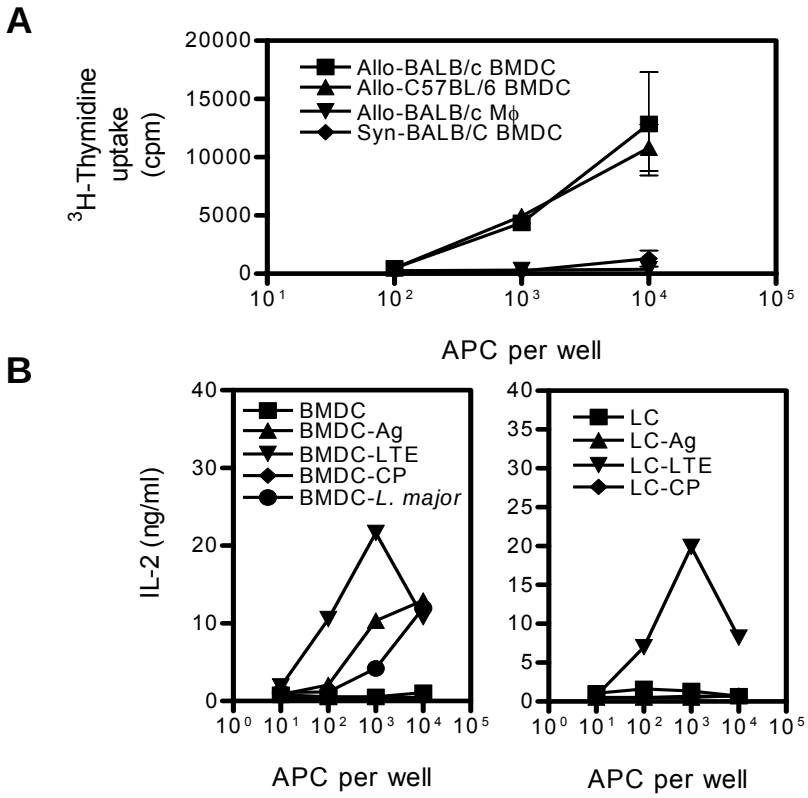

Fig 5. Antigen presentation by BMDC. A: MLR were set up by culturing purified T cells with titrating amounts of allogeneic BMDC or macrophages, or syngeneic BMDC, and T cell proliferation was determined. B: Titrated amounts of BMDC [left] or LC [right] were treated with LTE [50 µg/ml], CP [50 µg/ml], LmAg [10 µl/ml] or *L. major* [10 parasites per cell] and incubated with the LACK-specific T cell line. Non-treated cells were used as controls. After 24 hours the IL-2 secretion was measured in supernatants. Allo: allogeneic, Syn: syngeneic, Mφ: macrophages, CP: non-relevant control peptide, LTE: LACK T cell epitope.

To prove that cells obtained by the protocol described here exhibited also typical DC functions, we performed two different assays in order to evaluate the antigen presentation capacity. In the first, typical primary MLR with allogeneic and syngeneic enriched T cells were set up and the T-cell proliferation was measured by determining the ^{3}H-thymidine uptake. As indicated in Figure 5A, both BMDC generated from BALB/c and C57BL/6 mice exhibited a strong T cell-stimulatory capacity in allogeneic MLR and, as expected, were far more potent than macrophages or syngeneic cells.

In the second antigen presentation assay, a *Leishmania*-specific LACK-reactive T-cell hybridoma was used as responder cells in co-cultures with BMDC that were infected with *L. major* parasites or pulsed with total LmAg or the synthetic LTE peptide. The

production of IL-2 in the supernatants of co-cultures was determined as read-out of T cell proliferation and activation and compared to cultures where LC were used as stimulators. As shown in Figure 5B, BMDC that were infected with *L. major* parasites, or pulsed with total LmAg or LTE, were potent APC in vitro. BMDC that were either untreated or pulsed with a non-relevant synthetic peptide did not induce any IL-2 production. BMDC were also able to process a recombinant LACK protein and present the LTE to the T cell hybridoma [not shown]. In contrast, LC were potent in presenting the LTE to the T cell hybridoma but very poor stimulators when pulsed with total LmAg [Fig. 5B]. This indicates that the BMDC generated as described here exhibit potent APC functions.

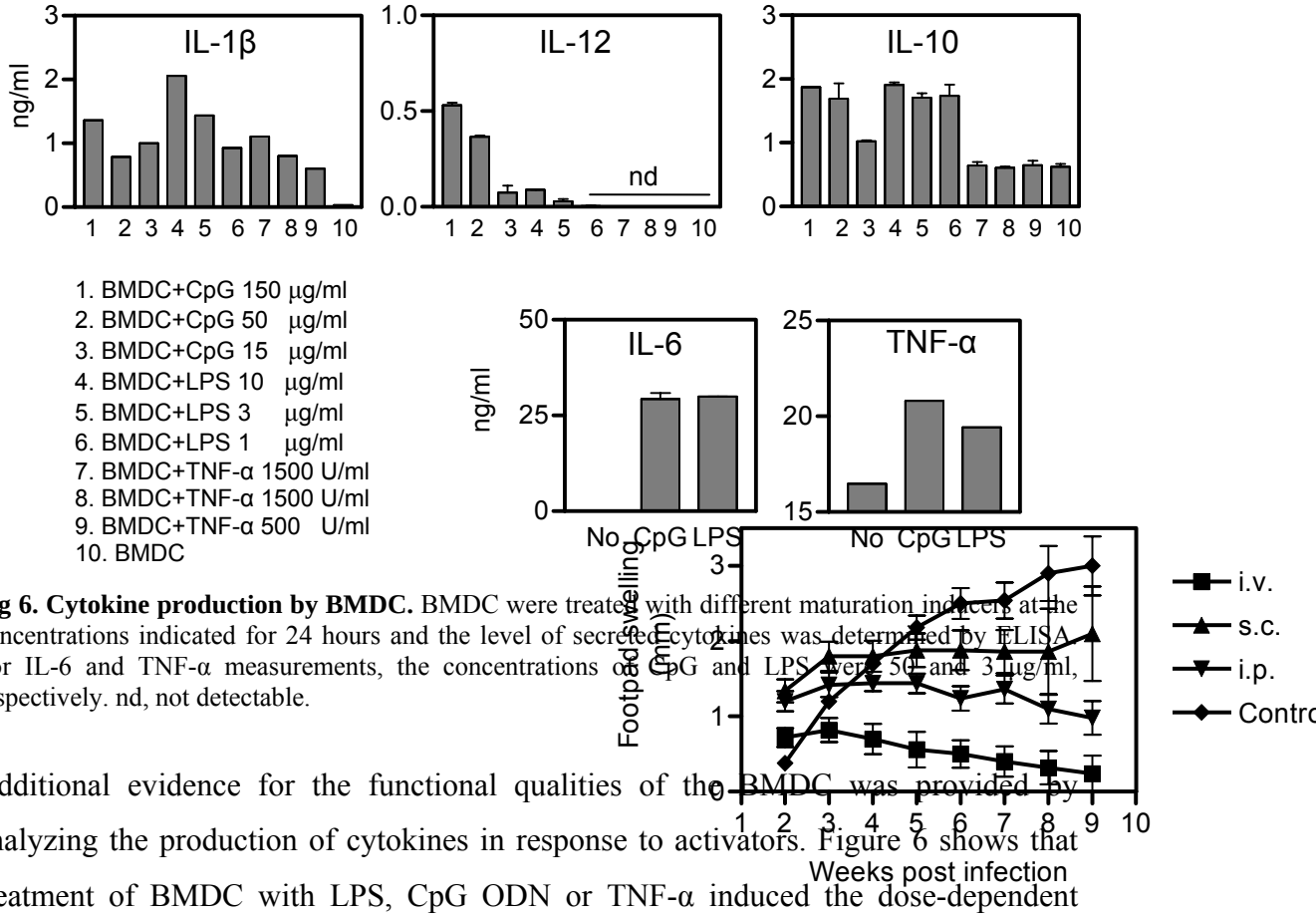

Fig 6. Cytokine production by BMDC. BMDC were treated with different maturation indicers at the concentrations indicated for 24 hours and the level of secreted cytokines was determined by ELISA. For IL-6 and TNF-α measurements, the concentrations of CpG and LPS were 50 and 3 µg/ml, respectively. nd, not detectable.

Additional evidence for the functional qualities of the BMDC was provided by analyzing the production of cytokines in response to activators. Figure 6 shows that treatment of BMDC with LPS, CpG ODN or TNF-α induced the dose-dependent secretion of IL-12, IL-10 and IL-1β. Up-regulation of TNF-α and IL-6 was also observed when BMDC were treated with LPS and CpG ODN. This demonstrates that cells generated as described here were able to secrete the cytokines typically produced by DC and that those cells were responsive to known DC activators.

Taken together, these results show that the protocol published by Lutz and colleagues [307] and implemented here allows to generate sufficient amounts of DC from bone marrow cultures, with the morphology, surface phenotype, APC functions, cytokine production and response to activators that are typical of DC. I therefore started to use these cells for further in vivo and in vitro experiments.

2. Antigen-pulsed BMDC are not suitable for vaccination against leishmaniasis

2.1 Antigen-pulsed BMDC do not protect against murine leishmaniasis

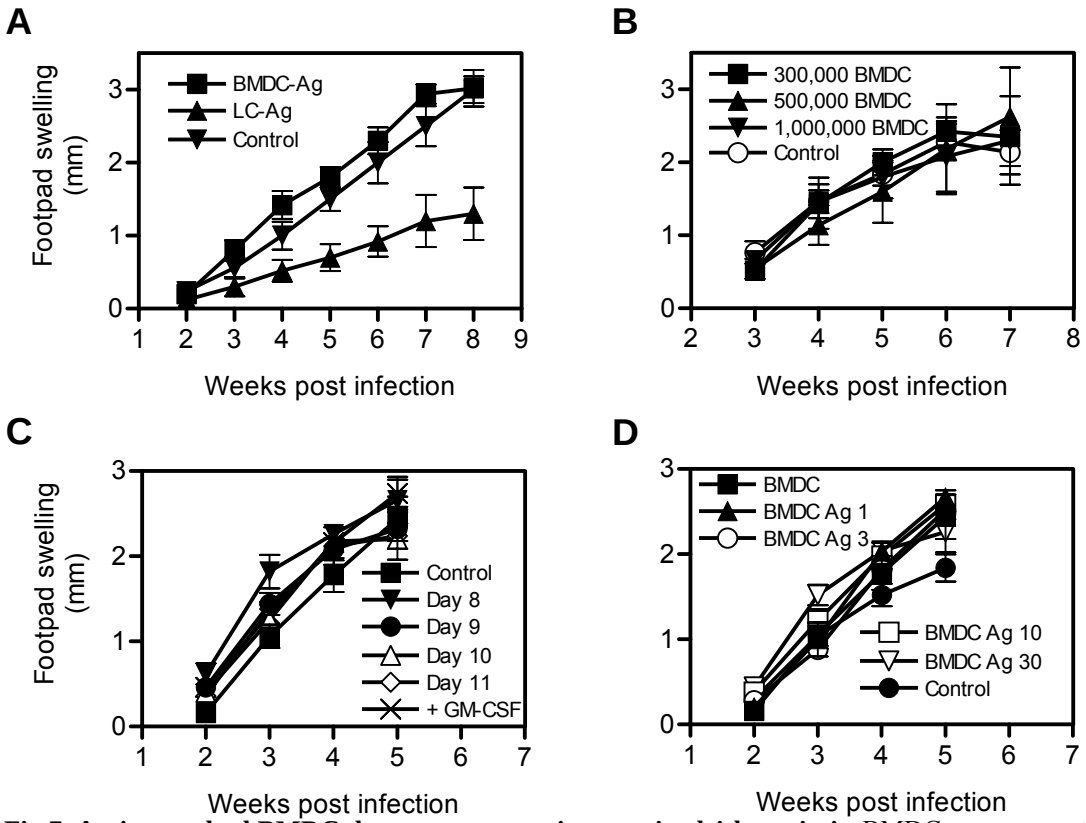

Fig 7. Antigen-pulsed BMDC do not protect against murine leishmaniasis. BMDC were generated for 10 days [A, B, D] or the indicated number of days [C], pulsed with 30 µl/ml [A, B, C] or the indicated amount of LmAg [in µl/ml, D], and 5 x 10^5 [A, C, D] or the indicated number of cells [B] were injected i.v. LC were also prepared, LmAg-pulsed and i.v. injected [A]. Mice were infected one week later with *L. major* parasites and the lesion development was determined by measuring footpad inflammation. Ag: antigen.

LC pulsed with total *Leishmania* lysate or recombinant antigens have been shown to confer protection against *L. major* infection in mice [300, 308]. It was important to know whether a similar protective function can be observed after treatment of susceptible mice with BMDC. For this reason, naïve mice were immunized with

LmAg-pulsed BMDC i.v. and one week later infected with *L. major* promastigotes. LmAg-pulsed LC were used as a protective positive control and PBS as negative control. As shown in Figure 7A, LmAg-pulsed LC, as expected, were able to mediate a significant reduction of the clinical manifestations of leishmaniasis when compared with the PBS-injected group. However, mice injected with LmAg-pulsed BMDC showed a lesion development that was comparable to the control group.

A number of different variables could affect the potential of BMDC to induce a protective immune response. Therefore, a series of experiments was designed where modifications in the protocol for DC generation, treatment and immunization were carried out. First, it was thought that the number of injected LmAg-pulsed BMDC could be critical for the induction of protection. LmAg-pulsed LC are usually protective when 200,000-300,000 cells are injected i.v. [300, 308]. We therefore treated groups of BALB/c mice with 3, 5, and 10 x 10^5 LmAg-pulsed BMDC and the protection against a subsequent challenge with parasites was analyzed. As shown in Figure 7B, the lesion development in all the experimental groups was comparable to control mice, indicating that the number of cells inoculated is not responsible for the failure in inducing protection. It was then reasoned that the developmental state of BMDC generated here could substantially differ from that of LC and this could be crucial for the induction of protection. For this reason, a new experiment was set up in which mice were treated with BMDC that were harvested from the bone marrow cultures on days 8, 9, 10 or 11. It was previously shown that BMDC harvested on days 8-9 are more immature and therefore more active in taking up antigens whereas those collected on days 10-12 are more mature and therefore more potent APC [307]. However, as shown in Figure 7C, the duration of DC generation again did not explain those results since none of the experimental groups was protected. As an additional potential explanation it was thought that the antigen concentration during pulsing may be a critical factor. This is based on some reports indicating that the ability of DC to induce the activation and differentiation of naive T cells can strongly be influenced by the dose of antigen [224, 239]. In addition, other reports have suggested that high concentrations of antigen can induce an “exhaust” DC phenotype by abrogating their capacity of inducing a Th1 immune response [259]. Therefore, an experiment with BMDC that were pulsed with different concentrations of LmAg [ranging from the equivalent of 1:1 to 30:1 parasites:BMDC] was performed. However, as demonstrated

in Figure 7D, all experimental groups developed lesions that were comparable in size to the control group. A number of other modifications in the treatment of BMDC prior to immunization were done. For example, as GM-CSF is known to prolong DC survival, we thought that the presence of this growth factor during pulsing may improve DC function and be important for inducing protection. We therefore immunized a group of mice with 500,000 BMDC that were pulsed with LmAg in the presence of 200 U/ml GM-CSF and evaluated protection against *L. major* infection. But again, the presence of GM-CSF did not affect the inability of LmAg-pulsed BMDC to change the course of *L. major* infection [Fig. 7C]. Similar results were obtained when IL-4 was added in addition to GM-CSF during pulsing [not shown].

Thus, taken together, those results show that in contrast to LC, BMDC that are pulsed with LmAg are not capable to confer protection against murine leishmaniasis caused by *L. major* in BALB/c mice.

2.2 Antigen-pulsing does not induce BMDC maturation

A large body of information demonstrated that maturation is a process critically required by DC in vitro and in vivo to become potent inducers of T cell activation. Furthermore, the ability of DC to promote a Th1 or Th2 immune response in vitro seems to be intimately related to their maturation state [152, 195]. With the protocol of BMDC generation implemented here, spontaneous maturation in a small subpopulation of cells can be observed in 10 days-old cultures [see morphology and MHC class II and CD86 expression in non-treated BMDC, Figs. 1 and 3]. It was then thought that in order to acquire a protective phenotype, BMDC should undergo maturation in vitro upon antigen pulsing. In order to test whether in vitro pulsing with LmAg induces BMDC maturation, the surface expression of MHC class II and CD86 molecules in BMDC that were used for immunization was analyzed. Untreated cells were also analyzed as a negative control, and cells that had been cultured in the presence of LPS or TNF-α were used as positive controls of maturation. As expected, the majority of untreated BMDC expresses intermediate or low levels of MHC class II and CD86 molecules indicating an immature phenotype [Fig. 8]. Cells that were treated with LPS or TNF-α up-regulated the surface expression of both markers indicating that they were mature. However, cells that were LmAg-pulsed overnight

did not up-regulate the expression of MHC class II or co-stimulatory molecules indicating that LmAg per se does not induce BMDC maturation [Fig. 8].

As expected, live *L. major* promastigotes were readily able to induce maturation indicating that the viability of the parasite is critical for DC maturation [Fig. 8]. The inability of LmAg to induce BMDC maturation was additionally proved by the observation that the production of pro-inflammatory cytokines like IL-1β, IL-6, TNF-α and IL-12 was not induced by BMDC pulsing [not shown]. These results provide a plausible explanation as to why protection was never achieved with LmAg-pulsed BMDC and suggest that induction of additional maturation during antigen pulsing might be required to activate their protective potential.

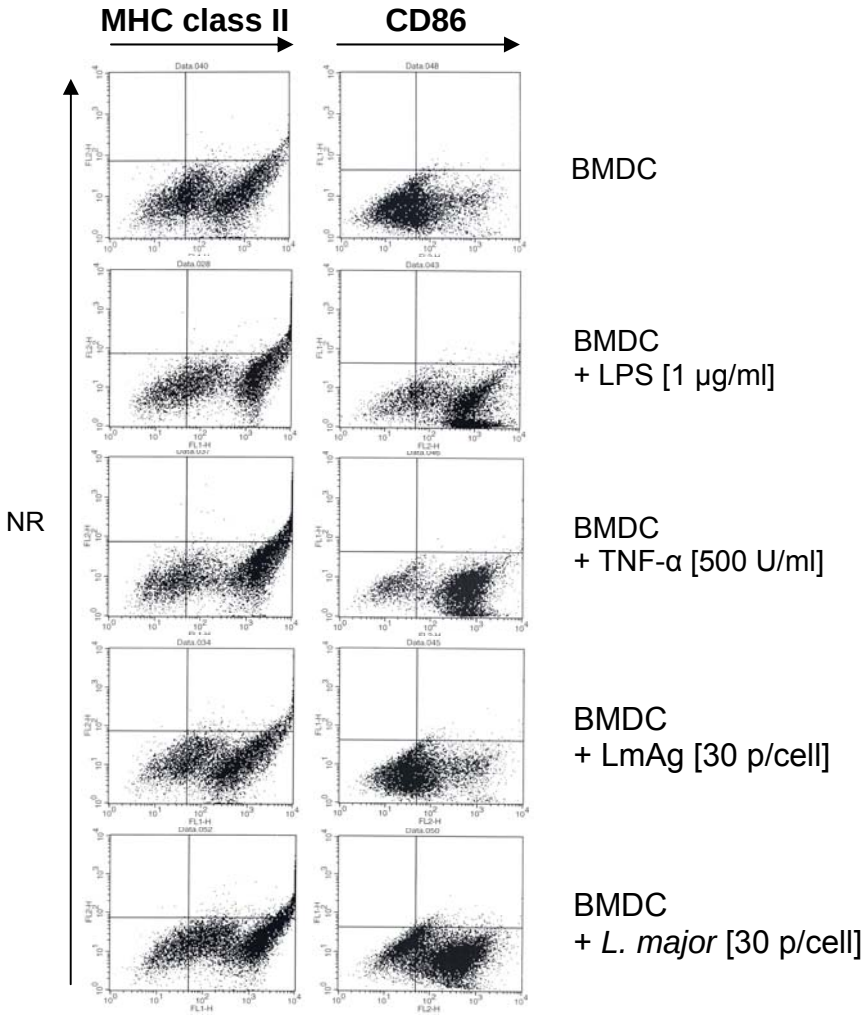

Fig 8. Antigen pulsing does not induce BMDC maturation. BMDC were prepared and treated overnight as indicated. The expression of MHC class II and the co-stimulatory molecule CD86 was determined by flow cytometry. NR: not relevant, p/cell: parasites/cell.

3. Antigen-pulsed BMDC require particular activation signals for acquiring the ability to induce a protective Th1 response against leishmaniasis

3.1 Activation with particular maturation inducers is required by BMDC to acquire the ability to protect against leishmaniasis

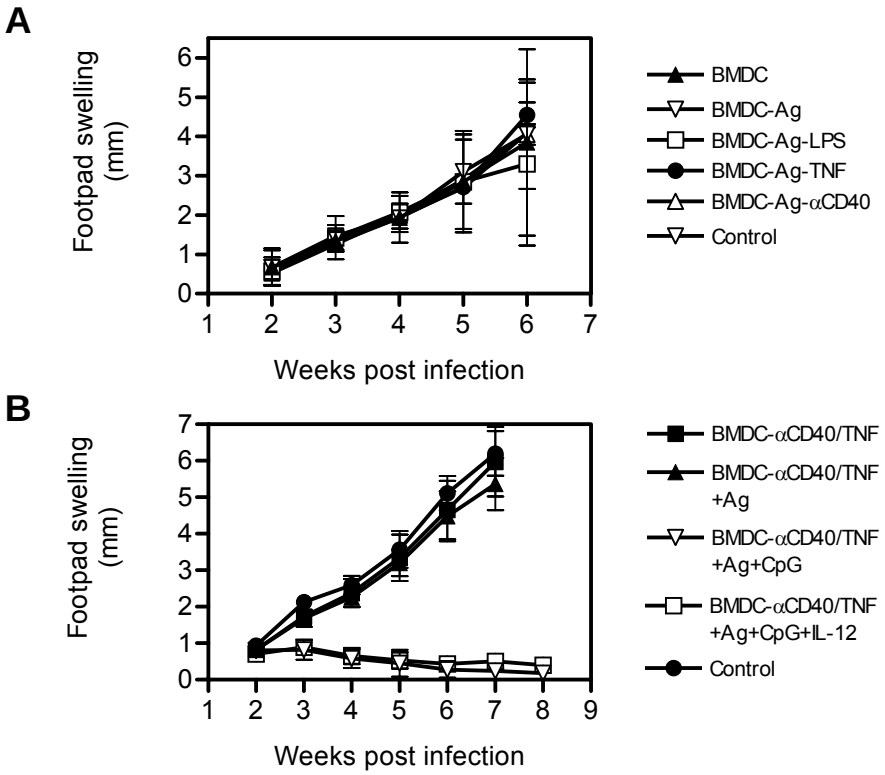

Fig. 9. Antigen-pulsed BMDC activated with particular maturation inducers protect against murine leishmaniasis. BMDC were prepared, incubated overnight with the indicated treatment and used in a vaccination trial. Two independent experiments are shown. Ag: antigen, αCD40: anti-CD40 Ab.

The observation that LmAg-pulsing fails to induce a mature phenotype of BMDC prompted me to evaluate whether further treatment with maturation inducers can confer the ability to protect BALB/c mice against *L. major* infection. In a set of experiments, BMDC were not only pulsed with LmAg but also treated with LPS, TNF-α, anti-CD40 Ab or CpG ODN as described in Material and Methods. Two independent experiments are shown in Figure 9. As previously observed, LmAg-pulsed BMDC were not able to induce protection against leishmaniasis [Figs. 8 and 9]. When LmAg-pulsed BMDC were additionally treated with LPS, anti-CD40 Ab or TNF-α, they were similarly ineffective [Fig. 9A]. The combination of TNF-α and

anti-CD40 Ab in addition to antigen pulsing was also ineffective [Fig. 9B]. However, when mice were vaccinated with BMDC that were antigen-pulsed and additionally treated with TNF-α, anti-CD40 Ab and CpG ODN, a highly efficient protection against *L. major* challenge was observed [Fig. 9B]. BMDC that were further treated with IL-12 in addition to the other activators were also protective. Clinical protection was striking as indicated by the complete absence of lesions and by the observation that footpad swelling was even lower than that observed in LC-vaccinated mice [see Figs. 7A and 9B].

3.2 CpG-matured antigen-pulsed BMDC protect BALB/c mice from cutaneous leishmaniasis

The experiment shown in Figure 9B indicates that the presence of CpG ODN during pulsing was a prerequisite to confer a protective phenotype to BMDC. However, whether the presence of TNF-α and anti-CD40 Ab is also required or whether CpG ODN treatment is sufficient cannot be inferred from those results. To clarify this, a repeat experiment where CpG was the only BMDC activator added in culture was carried out. To rule out an effect of the DNA backbone in CpG ODN, a non-CpG ODN was also evaluated as control. CpG-treated unpulsed BMDC were also used for treatment of a group of mice to rule out a possible antigen-independent activation of the immune system. The results are shown in Figure 10. Again, both non-activated LmAg-pulsed BMDC as well as DC that were activated with LPS, TNF-α or anti-CD40 Ab were ineffective in protecting against murine leishmaniasis. Interestingly, activation of LmAg-pulsed BMDC with CpG ODN is sufficient to confer protection against subsequent infection with *L. major* in susceptible BALB/c mice [Fig. 10A]. All mice vaccinated with those cells developed a very small footpad swelling [less than 1 mm], which peaked at week 3 post infection, and were completely cured after 6 weeks. None of the mice of this group showed any sign of ulceration, in contrast to the control and other experimental groups, where chronic inflammation, ulceration and necrosis were evident [Fig. 10B]. Control groups with unpulsed CpG-treated BMDC or LmAg-pulsed BMDC treated with a non-CpG AT-rich ODN showed a course of lesion development comparable to the control group [Fig. 10A]. Protected mice were followed up for more than 4 months and no clinical manifestations of disease were observed. Control and other non-protected groups, in contrast, had to be killed after 12-14 weeks post infection due to the presence of large necrotic lesions in

the footpads and the severe loss of weight most likely due to parasite dissemination [not shown]. I have vaccinated more than 50 mice in at least 10 different trials and reproducibly observed a very strong protective effect. These findings demonstrate that a single i.v. injection of CpG-matured LmAg-pulsed BMDC mediates complete protection against murine leishmaniasis and that CpG activation is necessary and sufficient to activate this protective phenotype in BMDC.

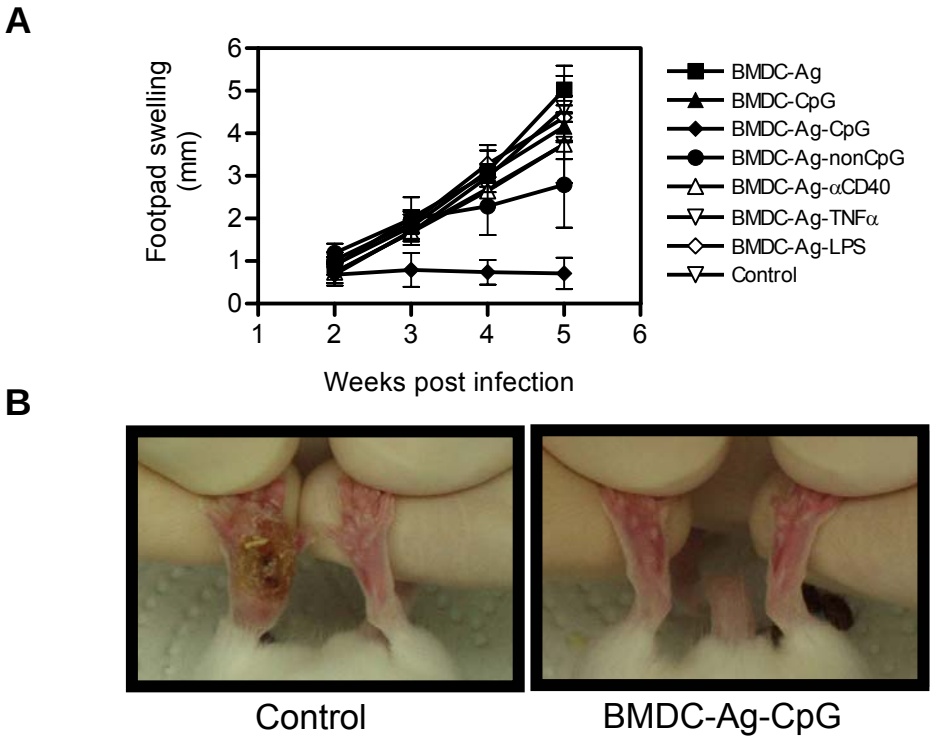

Fig. 10. Antigen-pulsed BMDC activated with CpG protect mice from *L. major* infection. A: BMDC were prepared, incubated overnight with the indicated treatment and used in a vaccination trial. B: the infected and uninfected foot from one representative animal belonging to the indicated group is shown. Ag: antigen, αCD40: anti-CD40 Ab, BMDC-Ag-CpG: CpG-matured antigen-pulsed BMDC.

3.3 Clinical protection induced by CpG-activated BMDC correlates with a significant reduction in the parasite burden at the site of infection

It was very important to know whether the clinical protection observed in mice vaccinated with CpG-matured LmAg-pulsed BMDC is due to the control of parasite replication at the site of infection. Therefore, mice from the most relevant groups of the experiment shown in Figure 10A were sacrificed, the infected feet removed and the number of parasites quantified by a limiting dilution procedure as described in Material and Methods. Figure 11A shows the parasite burden in mice analyzed individually. Only mice vaccinated with CpG-matured LmAg-pulsed BMDC were significantly less parasitized than control mice. On average, there was a more than

10^5-fold reduction in the number of parasites per footpad [2.63 x 10^{10} and 9.07 x 10^4 for control and protected groups, respectively].

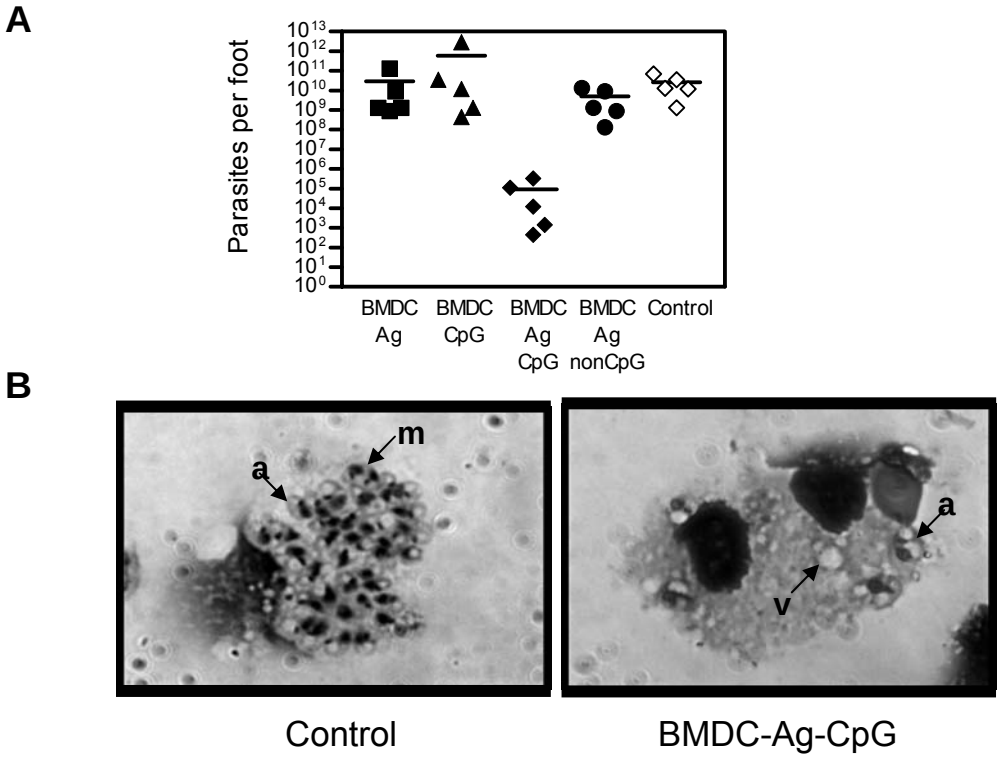

Fig. 11. The protection induced by CpG-matured antigen-pulsed BMDC is due to an effective control of parasite replication. A: animals from some of the experimental groups shown in Figure 10 were individually analyzed to quantify the parasite load in the footpads. B: a representative field of "Diff-Quik"-stained samples from animals belonging to the indicated groups is shown. Ag: antigen, BMDC-Ag-CpG: CpG-matured antigen-pulsed BMDC, a: amastigote, m: mitotic figure, v: empty vacuole.

When smears from the control footpads were stained and analyzed microscopically, an uncountably high amount of parasites was seen and, as shown in Figure 11B, macrophages were typically full of intracellular amastigotes. Mitotic figures were frequently observed in the preparation obtained from those mice indicating active replication of the parasite within macrophages. In contrast, in samples obtained from the footpads of protected mice, parasites could hardly be detected and the typical observation was no or very few intracellular amastigotes. In these samples, empty vacuoles in the cytoplasm of macrophages were frequently observed, a picture strongly suggestive of effective intracellular elimination of *Leishmania* parasites [Fig. 11B]. These results indicate that the clinical resistance induced by immunization with CpG-matured LmAg-pulsed BMDC in susceptible mice is due to the acquisition of the ability to efficiently activate anti-*Leishmania* effector mechanisms at the site of

inoculation. Interestingly, when the parasite burden in draining LN was analyzed, no significant reduction in the frequency of infected cells from protected mice in comparison to the control group was observed [not shown].

3.4 BALB/c mice vaccinated with CpG-matured antigen-pulsed BMDC develop a Th1-like immune response after infection with *L. major* promastigotes

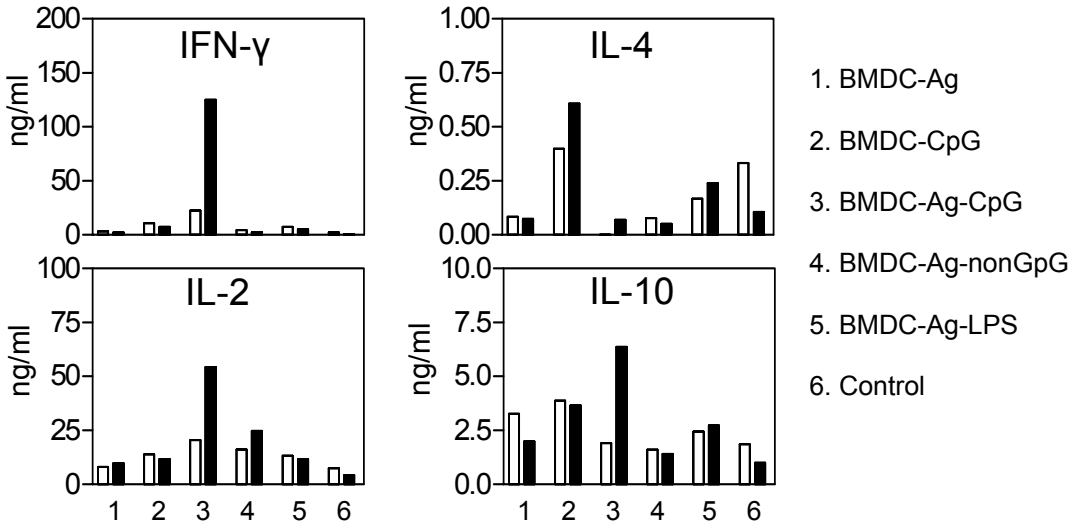

Fig. 12. Mice vaccinated with CpG-matured antigen-pulsed BMDC develop a Th1-like response after *L. major* infection. Animals from some of the experimental groups shown in Figure 10 were sacrificed and the production of cytokines by pooled draining LN cells were analyzed in the absence [open bars] or presence [solid bars] of antigen. Ag: antigen.

In order to determine whether the protection induced by BMDC is associated with a different profile in cytokine expression, the secretion of IL-2, IFN-γ, IL-4 and IL-10 by LN cells was assessed. Mice from the most relevant experimental groups shown in Figure 10A were sacrificed 5 weeks after infection, and total LN cells were cultured in the absence or presence of LmAg. After 72 hours of incubation, the concentration of those cytokines in the supernatants was measured by ELISA. The levels of IL-2 in the absence of LmAg ranged between 7.6 and 20.7 ng/ml with the maximal level exhibited by the protected group that had been vaccinated with CpG-matured LmAg-pulsed BMDC [Fig. 12]. However, this difference was increased strikingly when LmAg was added to the culture. A 13-fold higher level of this cytokine was observed in the protected group compared with the control group. An even more pronounced difference was observed when IFN-γ levels were determined. In the absence of

LmAg, a 10-fold increase was observed when the protected group was compared with the control group and 2- to 7-fold when compared with other groups. When LmAg was present in the cultures, 151-fold and 16- to 60-fold higher levels of IFN-γ were observed when the protected group was compared with the control and the other groups, respectively. In contrast to IL-2 and IFN-γ, LN cells from mice vaccinated with CpG-matured LmAg-pulsed BMDC secreted no detectable, or very low, levels of IL-4 in the absence or presence of LmAg, respectively [Fig. 12]. Finally, more than the double amount of *L. major*-specific IL-10 production was observed in protected mice when compared to the control group. Thus, only in those mice that were vaccinated with CpG-matured LmAg-pulsed BMDC, the cytokine profile induced in LN cells after *L. major* challenge was strongly shifted towards a Th1-like immune response.

3.5 The pattern of *Leishmania*-specific IgG Ab correlates with the induction of a Th1 immune response in mice vaccinated with CpG-matured antigen-pulsed BMDC

In spite of the fact that the Ab response does not play a critical role in protection against leishmaniasis, it is known that different IgG subclass profiles correlate with Th1 or Th2 immune responses. The presence of high levels of IgG1 and low titers of IgG2a anti-*Leishmania* Ab is associated with a Th2 response and the opposite distribution with a Th1 response. Thus, it was investigated whether the pattern of IgG subclass production was shifted towards a Th1-type of response in the protected group. Mice from the most relevant experimental groups shown in Figure 10A were sacrificed 5 weeks post infection, and the serum levels of total IgG, IgG1 and IgG2a *Leishmania*-specific Ab were determined by ELISA. As shown in Figure 13A, the levels of *Leishmania*–specific total IgG Ab were variable but significant in all the groups. When the IgG subclass distribution was determined, a clear tendency to produce low IgG1 and high IgG2a levels was observed in the serum of protected mice which had been vaccinated with CpG-matured LmAg-pulsed BMDC [Fig. 13A]. Some groups showed low levels of IgG1 and some others high levels of IgG2a, but only mice vaccinated with CpG-activated BMDC had the combination of both. A simpler parameter to see shifting towards a Th1-like pattern seems to be the relative ratio of IgG2a/IgG1, with higher values indicating Th1 induction. As shown in Figure 13A, the protected group treated with CpG-matured LmAg-pulsed BMDC exhibited

the highest IgG2a/IgG1 average ratio which was 4 times higher than the ratio of the control group [1.4992 and 0.3661, respectively].

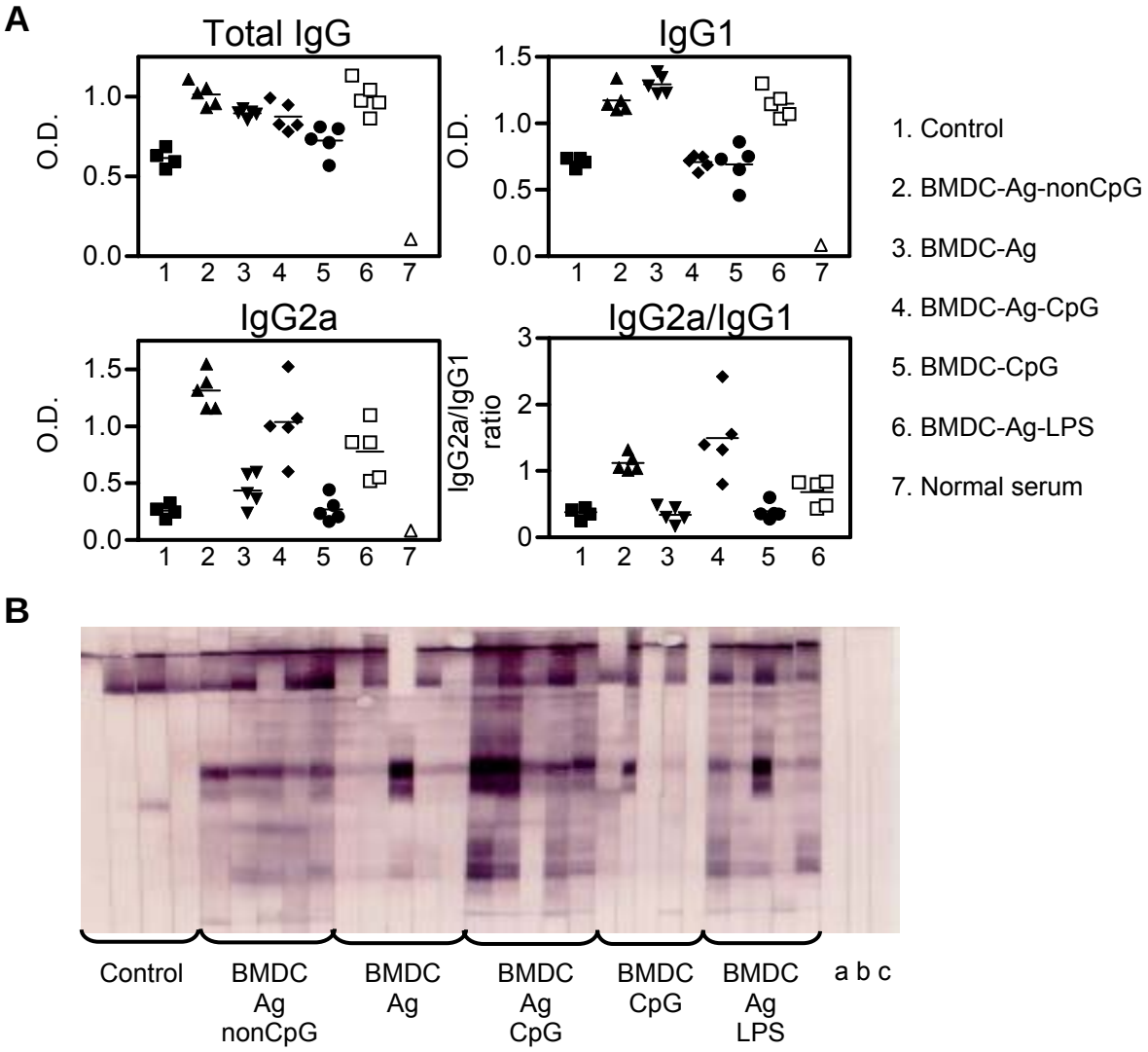

Fig. 13. *L. major*-specific IgG subclasses correlate with the induction of a Th1 response in mice vaccinated with CpG-matured antigen-pulsed BMDC. Animals from some of the experimental groups shown in Figure 10 were sacrificed and the IgG distribution of anti-*L. major* Ab analyzed by ELISA [A]. The pattern of reactivity of anti-*L. major* IgG2a Ab was also assessed by immunoblot [B]. Ag: antigen, a: normal serum control, b: no serum control, c: no serum and no conjugate control.

I also wanted to know whether the increase in the production of IgG2a anti-*Leishmania* Ab observed in the protected group was accompanied by qualitative changes in the recognition of parasite antigens. To this end, total LmAg was separated by gel electrophoresis, transferred to a nitrocellulose membrane and the pattern of IgG2a reactivity was individually determined by Western blot as described in Material and Methods. As shown in Figure 13B, the intensity of the bands recognized by sera from the different groups of mice correlated with the quantitative results

obtained by ELISA. The order in the strength of reactivity was CpG-matured LmAg-pulsed BMDC > nonCpG-matured LmAg-pulsed BMDC >> LPS-matured LmAg-pulsed BMDC >> LmAg-pulsed BMDC > CpG-treated BMDC >> PBS. Interestingly, the pattern of antigens recognition by those IgG2a Ab was generally conserved among most of the groups indicating that differences in Ab production observed by ELISA were rather quantitative than qualitative. When blots were developed for IgG1 Ab no significant quantitative or qualitative differences in the pattern of reactivity were observed [not shown]. Taken together, these results indicate that only mice vaccinated with CpG-matured LmAg-pulsed BMDC produce a pattern of anti-*Leishmania* IgG Ab that correlates with the induction of a strong Th1 immune response after infection with *L. major*.

3.6 The protective effect of CpG-matured antigen-pulsed BMDC is also observed in the resistant strain of C57BL/6 mice

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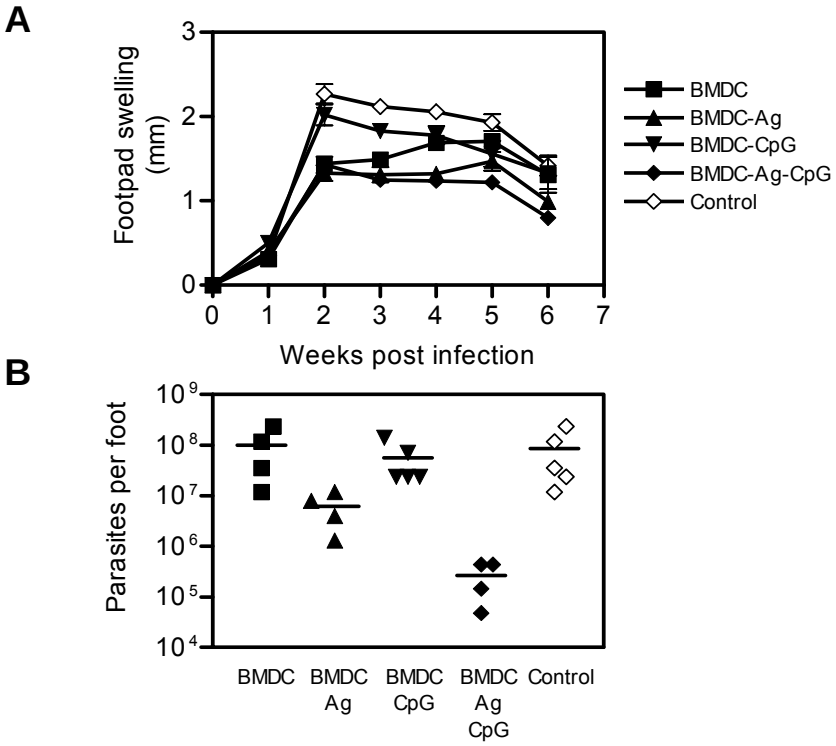

Fig. 14. Antigen-pulsed BMDC activated with CpG also protect C57BL/6 mice from *L. major* infection. A: BMDC were prepared, incubated overnight with the indicated treatment and used in a vaccination trial. B: animals were sacrificed 6 weeks after infection and the parasite burden in footpads determined. Ag: antigen.

It was also investigated whether this approach is applicable to a different strain of mice that is resistant to *L. major* infection. C57BL/6 mice develop a limited

inflammation in the footpad after parasite challenge, which usually peaks 2-4 weeks post infection, followed by a progressive reduction in the swelling and complete cure. However, when these mice were vaccinated with CpG-matured LmAg-pulsed BMDC one week before the infection, a significant reduction in the footpad swelling was observed, with a lower maximal peak and faster healing [Fig. 14A]. When these mice were vaccinated with BMDC alone, an initial unspecific effect was observed. However, these mice reached a footpad swelling comparable to the control group after 4-5 weeks of infection. As expected, vaccination with CpG-treated unpulsed BMDC showed no effect. However, in contrast to BALB/c, C57BL/6 mice vaccinated with BMDC pulsed with LmAg in the absence of CpG treatment also showed a significant reduction in lesion development, when compared to non-vaccinated mice, but this effect was less pronounced than that induced by BMDC additionally activated with CpG [Fig. 14A].

When the parasite load of the different experimental groups was analyzed, a striking correlation with the clinical outcome was observed. The parasite numbers of mice with non-protective treatments were similar to those of the control group [Fig. 14B]. Mice vaccinated with LmAg-pulsed BMDC showed a 10-fold reduction in parasite load and, most notably, those mice vaccinated with CpG-matured LmAg-pulsed BMDC had approximately 100-fold less parasites in the footpads. These results demonstrate that not only in BALB/c, but also in C57BL/6 mice, the vaccination with CpG-matured LmAg-pulsed BMDC induces clinical protection against leishmaniasis that is associated with a significant reduction in the parasite load at the site of infection.

3.7 Resistance induced by immunization with CpG-matured antigen-pulsed BMDC is solid and protects against re-infection

Because of the exceptional efficacy of CpG-matured LmAg-pulsed BMDC in mediating protection against leishmaniasis [total cure of 100% of the mice, Fig. 10A], it was very important to know whether mice that resolved the primary infection are also able to resist a second challenge with *Leishmania* parasites. To this end, mice that completely cured from a primary infection were re-challenged with 500,000 metacyclic parasites [2.5-fold more than the primary infective dose] 10 weeks after the first challenge. The results in Figure 15A show that a solid immunity was

established by immunization with these BMDC, since the swelling after secondary infection was even lower than that observed after primary challenge. Re-challenged mice showed an almost undetectable footpad swelling [less than 0.5 mm] and most of them completely cured after 3 weeks of secondary infection. When the parasite load in the footpads of those mice was measured, a significant reduction in the numbers of parasites was again observed, compared with a newly infected group of naive mice used as control [Fig. 15A]. Animals protected from re-infection were followed up for more than 20 weeks after secondary challenge and no sign of disease was observed.

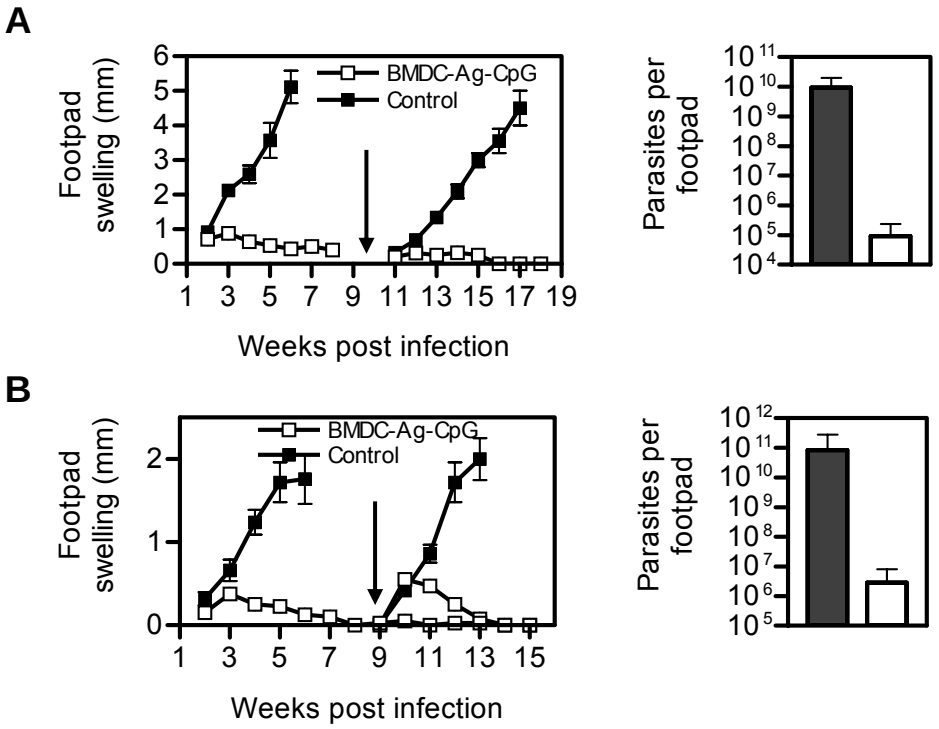

Fig. 15. Mice vaccinated with CpG-matured antigen-pulsed BMDC are also resistant to re-infection. Mice recovered from primary infection were re-challenged with higher parasite doses at the indicated time [arrows]. Swelling was monitored weekly [left] and the parasite load in footpads determined [right]. Re-challenge doses were 5 x 10^5 [A] or 8 x 10^5 parasites [B]. Ag: antigen.

In a repeat experiment, mice that had cured from the primary infection were re-challenged with an inoculum that was 4-fold higher than the primary infection [800,000 *L. major* promastigotes] and lesion development as well as parasite burden were analyzed. As shown in Figure 15B, re-challenged mice developed a swelling that was larger than that observed after the primary infection but it lasted shorter, as they were completely cured after 4-5 weeks of infection. Again, those animals had almost 10^6-fold less parasites in the footpads than a group of naive mice infected with the same inoculum [Fig. 15B]. Together, these results demonstrate that vaccination with

CpG-matured LmAg-pulsed BMDC induces a level of resistance against *L. major* challenge that is solid enough to protect not only from a primary infection, but also against a stronger secondary challenge.

3.8 Protection induced by CpG-matured antigen-pulsed BMDC is long-lasting

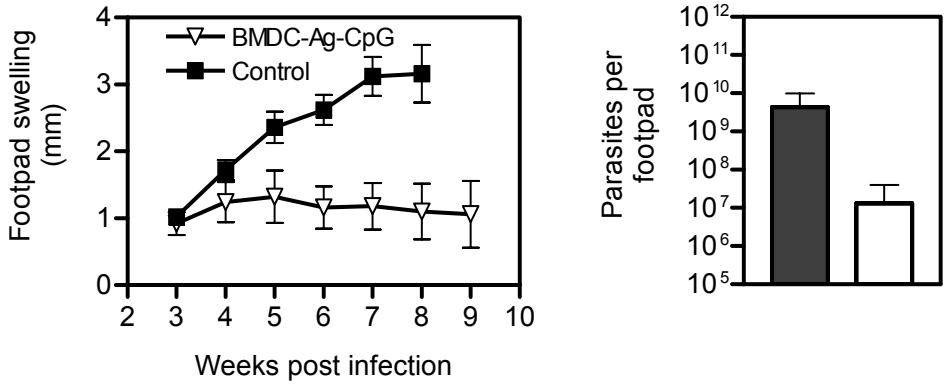

Fig. 16. Protection induced by CpG-matured antigen-pulsed BMDC is long-lasting. Naïve mice were vaccinated as indicated and 16 weeks later were infected with *L. major*. Swelling was monitored weekly [left] and the parasite load in footpads was determined 9 weeks after infection [right]. Ag: antigen.

A more striking result in terms of the potency and durability of the protection induced with BMDC is shown in Figure 16. Mice were vaccinated with CpG-activated and LmAg-pulsed BMDC and were infected with *L. major* parasites 16 weeks after immunization. The protection was evaluated clinically and parasitologically. Whereas PBS-injected mice developed progressive lesions, mice vaccinated with BMDC were significantly protected [Fig. 16]. From the 5 BMDC-vaccinated animals, 3 showed a level of protection that was comparable to that observed in the short-term challenge model [infection at one week after immunization]. These three mice were almost cured at the time of sacrifice [9 weeks post infection] and the parasite load in footpads was 10^4- to 10^5-fold lower than the mean of the control group. The fourth mouse showed a moderate swelling and the number of parasites was 10^2-fold reduced compared to the control group, whereas the remaining animal was clinically not protected but showed a more than 10-fold reduction in parasite burden. When the means of both groups were compared, an almost 10^3-fold reduction in the parasite burden was observed [Fig. 16]. Thus, the level of clinical and parasitological protection achieved by CpG-matured LmAg-pulsed BMDC after long-term infection in BALB/c mice is highly significant.

4. Standardizing parameters and testing new applications

4.1 Determination of the amount of donor BMDC required for the induction of protection

An outstanding observation in the present report is the high level of protection achieved with a single injection of CpG-activated and LmAg-pulsed BMDC. A clinical cure as well as a dramatic reduction in the parasite burden in the footpads was consistently observed. This protective effect is even more pronounced, both clinically and parasitologically, than the one observed with LmAg-pulsed LC and therefore can potentially be exploited in clinical settings. For this reason, it was important to determine the minimal amount of BMDC required for inducing protection in susceptible BALB/c mice. CpG-matured LmAg-pulsed BMDC were therefore prepared from BALB/c mice and titrated amounts were injected i.v. in a total volume of 100 µl into naive mice one week before *L. major* challenge.

Figure 17 shows the results of two independent experiments. A complete protection of 100% of mice was achieved when 500,000 BMDC or more were used as vaccine carriers. When mice were vaccinated with 100,000 BMDC, a significant reduction in the footpad swelling could still be observed when compared to the control group, but only one mouse from this group cured after 6 weeks of infection. The remaining mice presented chronic lesions that did not resolve and reached a plateau at 6 weeks post infection. Mice vaccinated with 10,000 BMDC [Fig. 17A] or less [not shown] were not protected.

In a second experiment BALB/c mice were treated with 100,000, 300,000 or 500,000 CpG-matured LmAg-pulsed BMDC and protection was analyzed. Again, 500,000 BMDC were sufficient to induce total protection in 100% of mice [Fig. 17B]. Mice immunized with 100,000 BMDC showed partial protection, since a reduction in the footpad swelling was observed in only one mouse whereas the course of disease in the remaining mice was comparable to the unprotected control. When mice were inoculated with 300,000 cells, a significant reduction in the footpad swelling was observed. Four out of 5 mice were clinically cured at 7 weeks post infection, and inflammation persisted only in one mouse [Fig. 17B]. These results lead to the

conclusion that a protective effect can be observed when more than 100,000 BMDC are injected, but optimal protection with complete cure in all of the vaccinated mice is observed when 500,000 or more cells are used. The use of the double amount of the optimal inoculum, or more cells, does not deteriorate the effect [Fig. 17A and not shown].

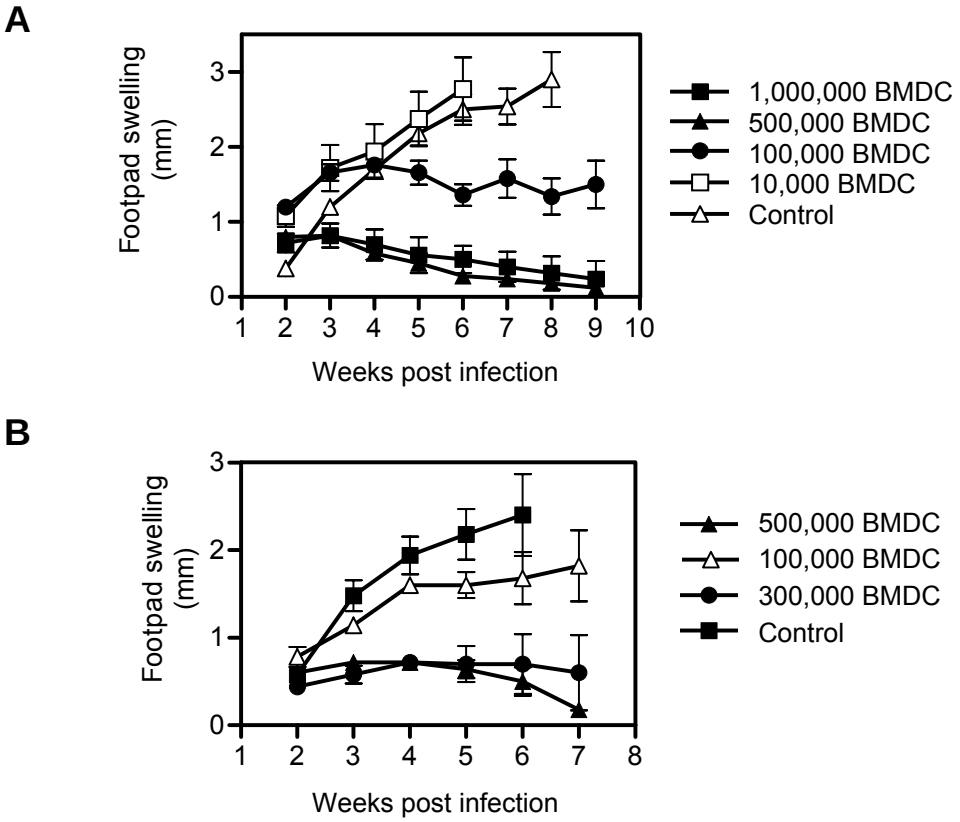

Fig. 17. Titration of the amount of BMDC required for the induction of protection. Naïve mice were vaccinated with the indicated amounts of CpG-activated antigen-pulsed BMDC and infected with *L. major* parasites one week later. Footpad swelling was monitored weekly. A and B show two independent experiments.

4.2 The route of BMDC administration is critical for complete protection

Antigen-pulsed LC are protective against murine leishmaniasis when injected i.v. but not s.c. or i.p. [300]. It was therefore relevant to find out whether this is also true for BMDC-based vaccination. Naive mice were immunized with 500,000 CpG-matured LmAg-pulsed BMDC s.c., i.p. or i.v. and subsequently infected with *L. major* parasites. A group of mice were treated i.v. with PBS as a control. As expected, mice treated i.v. developed a very limited swelling after challenge, and only one out of 5 mice showed a very low inflammation after 8 weeks of infection [Fig. 18]. Mice vaccinated s.c. were heterogeneous in the development of lesions: one mouse showed

a clear reduction in the footpad swelling [0.9 mm after 8 weeks of infection] and another showed intermediate inflammation [1.5 mm]. However, the remaining mice were comparable to those of the non-vaccinated group with regard to the size of the lesions. Although mice immunized with BMDC i.p. showed a significant reduction in the footpad swelling, there was also variability within this group. Whereas two mice showed very low footpad swelling [0.5 mm] with a clear tendency to resolve the lesion, the remaining three mice had lesions of 1.2-1.5 mm in size that tended to remain on a plateau [Fig. 18]. These observations indicate that, when a single injection of CpG-matured LmAg-pulsed BMDC is given, the route of administration is critical and only i.v. injection can induce complete protection in 100% of vaccinated mice. Although s.c. and, even more, i.p. injections were partially protective, a single inoculum with these modes of administration is not sufficient to completely protect BALB/c mice against leishmaniasis.

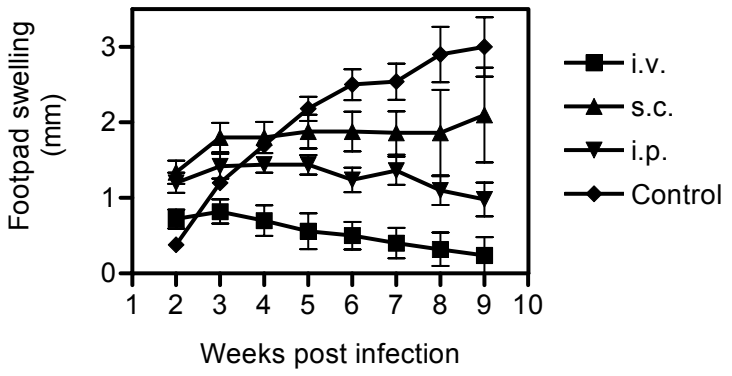

Fig. 18. The induction of protection by BMDC is dependent on the route of administration. CpG-activated antigen-pulsed BMDC were injected into naïve mice as indicated, and protection against *L. major* challenge was determined by measuring the footpad swelling.

4.3 BMDC pulsed with some molecularly defined antigens are not protective

Since DC pulsed with single antigens have been used for prophylaxis and therapy in tumor and infectious disease models [259, 308], it was important to test whether BMDC pulsed with molecularly defined antigens are also able to prevent murine leishmaniasis. As already mentioned, the LeIF protein was reported as a prominent leishmanial antigen that may induce partial protection [303], and very recently, it was shown that LeIF-pulsed LC were comparable to LmAg-pulsed LC in inducing protective immunity against leishmaniasis [308]. As shown in Figure 19A, whereas a group of mice vaccinated with LmAg-pulsed BMDC were typically protected from

leishmaniasis, BMDC that were pulsed with LeIF did not protect and mice showed a swelling comparable to PBS-treated mice.

Fehler! **A**

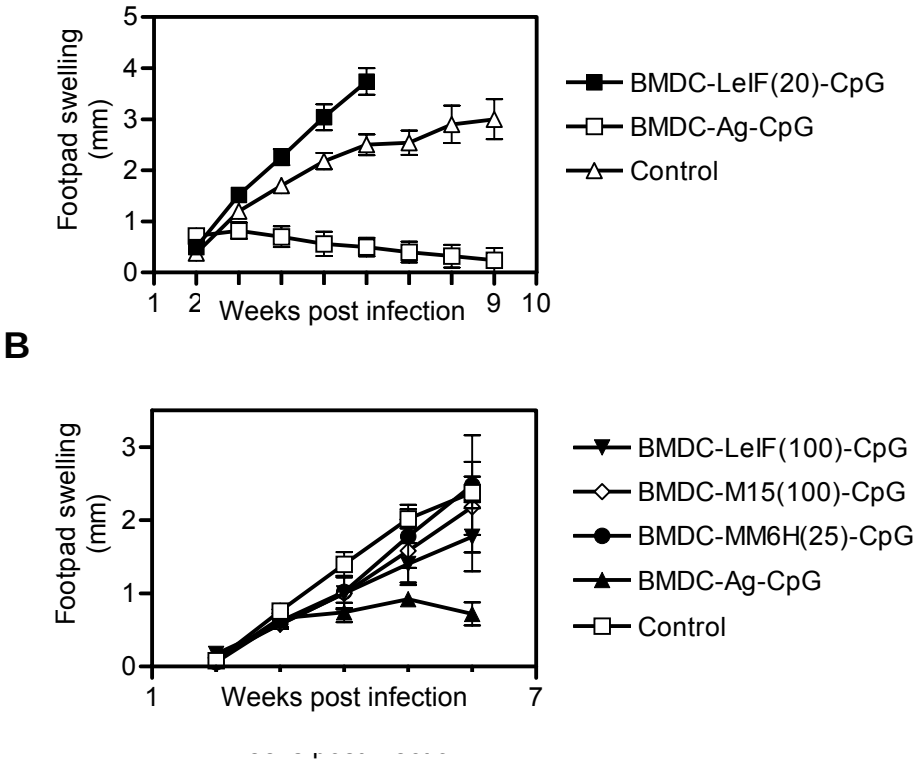

Fig. 19. BMDC are protective when pulsed with LmAg but not with molecularly defined antigens. BMDC were pulsed with single antigens at the concentrations indicated in parenthesis [in µg/ml] and with CpG overnight, and used in a vaccination trial. Protection against *L. major* challenge was determined by measuring the footpad swelling. Mice injected with CpG-matured LmAg-pulsed BMDC or PBS were used as positive and negative controls, respectively. A and B show two independent experiments.

A repeat experiment, in which the concentration of LeIF during BMDC pulsing was increased to 100 µg/ml, showed similar results [Fig. 19B]. A second immunodominant antigen called M15 [Y. Skeiky, personal communication] was also tested and again, no protection was observed. Moreover, a trimeric protein [MM6H] that consists of a linear arrangement of three major leishmanial antigens was tested in this system. As shown in Figure 19B, MM6H-pulsed CpG-matured BMDC did not protect against *L. major* infection. Thus, in contrast to LC, BMDC could not induce protection when pulsed with the molecularly defined antigens LeIF, M15 or the multi-component hybrid vaccine MM6H.

4.4 Once *L. major* infection is established in susceptible mice, the treatment with BMDC does not improve the clinical outcome

Given the unusual potency of these cells in the induction of a long-lasting protective Th1 immune response, the next question to address was whether it is possible to cure an already established *Leishmania* infection in BALB/c mice. For this purpose, a series of experiments was designed in which naive mice were infected with *L. major* promastigotes and subsequently treated with CpG-matured LmAg-pulsed BMDC at different time points.

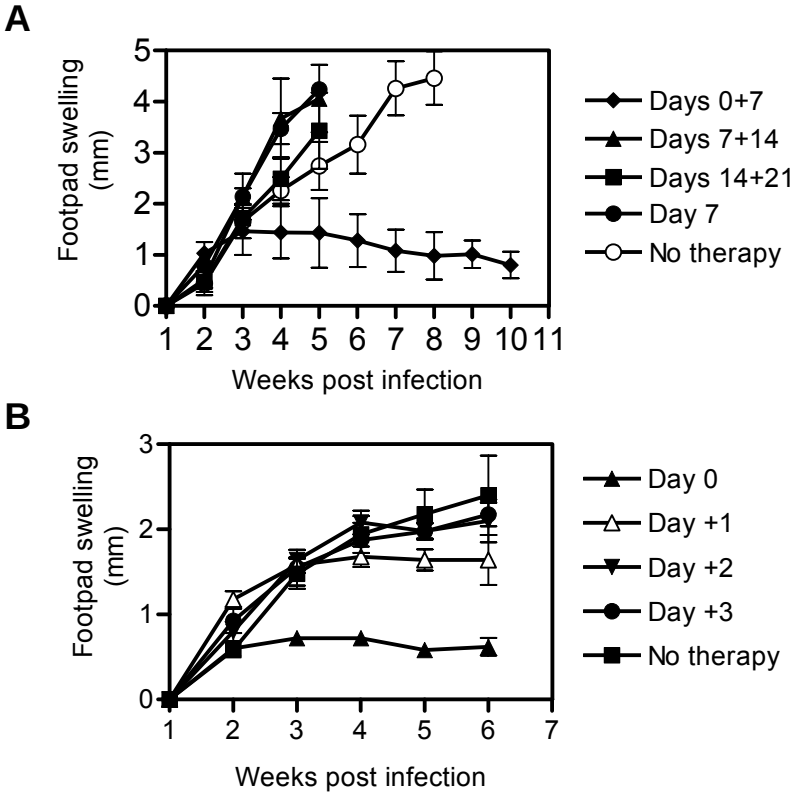

Fig. 20. BMDC can prevent leishmaniasis but can not cure an established infection. Naive mice were infected with *L. major* and subsequently treated with BMDC at the indicated time points. The therapeutic effect was monitored by measuring the footpad swelling. A and B show two independent experiments.

When mice were treated on day 0 [1 hour post infection] and one week after infection, a clear therapeutic effect was observed [Fig. 20A]. When therapy was given one and two weeks after infection the effect was reversed, since the treatment seemed to be exacerbating. A similar curve of disease progression was observed when a single therapeutic dose was injected one week after infection, indicating that the therapeutic efficacy exerted by the schedule 0 plus 7 days post infection was more dependent on the first dose than the second. Finally, two therapeutic doses on days 14 and 21 post

infection did not show any effect as evidenced by a kinetic in disease progression comparable to that of the control group [Fig. 20A]. These results suggest that once a Th2 immune response is established in susceptible BALB/c mice after *L. major* infection, a possible redirection towards a protective Th1 response by CpG-matured LmAg-pulsed BMDC is restricted to a very narrow time window. It was therefore very important to determine how long after challenge BMDC can still be protective. Figure 20B shows the results of a second therapeutic experiment where mice were infected and treated with BMDC at days 0, 1, 2 and 3 after challenge. Again, mice that were treated 1 hour [day 0] after infection were protected from leishmaniasis. However, no therapeutic effect was observed when mice were treated at days 1, 2 or 3 after infection. These results lead to the conclusion that CpG-matured LmAg-pulsed BMDC have the potential to initiate a protective Th1 immune response in BALB/c mice when administrated before or during initial contact with the parasite. But once a disease-promoting Th2 response is initiated, BMDC are not only unable to change the course of disease but can even accelerate the development of lesions.

5. In vitro approaches to determine the mechanism of protection

5.1 Pulsing and maturation of BMDC modulates their cytokine expression

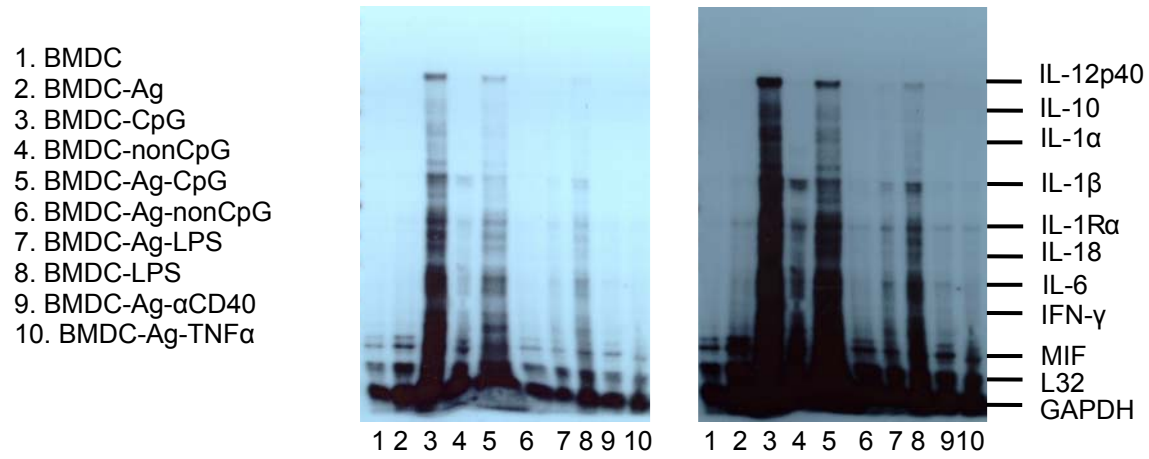

Fig. 21. Antigen pulsing and maturation of BMDC modulate cytokine production. BMDC were treated overnight as indicated and the expression of cytokine mRNA was assessed by RPA. Two different times of film exposure [24 and 72 hours] are shown. Ag: antigen, αCD40: anti-CD40 Ab.

As an initial attempt to explore the mechanisms enabling BMDC to induce a protective Th1 immune response in vivo, we wanted to know whether pulsing and maturation of BMDC modulates the expression of DC-related cytokines. Particularly, since IL-12 is a major DC product driving naive T cells to the Th1 phenotype, it was of interest to analyze the level of expression of this cytokine after pulsing and maturation with the different inducers. Therefore, BMDC were treated with LmAg and/or the different maturation inducers and total RNA was isolated to be used in RPA assays as described in Material and Methods. From the molecules evaluated in this set, only the chemokine macrophage migration inhibitory factor [MIF] is expressed at low levels in untreated BMDC [Fig. 21]. Pulsing of BMDC with LmAg up-regulated the expression of MIF and induced a low level of expression of the α-chain of the IL-1 receptor [IL-1Rα]. Treatment of BMDC with CpG ODN induced a vigorous expression of the mRNA for almost all the molecules evaluated. Most notably, IL-12p40 expression was readily induced in CpG-treated BMDC. LPS induced a similar pattern of cytokine production although at a lower level. Non CpG ODN significantly induced the expression of IL-1β, IL-1Rα and IL-6, up-regulated the MIF expression but, interestingly, did not induce IL-12 expression. BMDC that were treated with CpG ODN, LPS and non-CpG ODN in addition to LmAg expressed lower levels of cytokines when compared with unpulsed cells. However, even in the presence of LmAg, the induction of IL-12, IL-1β and IL-6 by CpG was still significant. Finally, BMDC that were treated with TNF-α or anti-CD40 Ab in addition to LmAg exhibited very low levels of IL-1β, IL-1Rα and IL-6.

In summary, from the different treatments analyzed, CpG showed a potent pro-inflammatory effect with a remarkable potential to induce IL-12. LPS was less potent than CpG in inducing IL-12 expression. Although some cytokines are produced by BMDC that were treated with LmAg plus maturation inducer, IL-12p40 is only produced when CpG is the activating signal. Thus, an unique expression pattern of cytokines is observed in the protective CpG-treated LmAg-pulsed BMDC [Fig. 21].

5.2 Pulsing and maturation modulate chemokine and chemokine receptor expression by BMDC

Homing to tissues and subsequent migration to secondary lymphoid organs are crucial for DC function. The guided movement of DC through their life cycle critically

depends on the modulation of the expression of chemokine receptors by DC. On the other hand, DC can also produce chemokines that are chemotactic for them or for other types of cells of the immune system. In addition, the expression of chemokines and chemokine receptors by DC is known to be highly regulated during the process of maturation [193]. I therefore wanted to know whether pulsing with LmAg and maturation by treatment with additional stimuli can modulate the expression of some chemokine receptors that are known to be critically required for DC migration and also whether the expression of some DC-related chemokines is regulated. For this, BMDC were treated with LmAg and/or activated with maturation inducers as described in the section Material and Methods. After total RNA preparation, the levels of mRNA for a set of chemokines and chemokine receptors were determined by RPA.

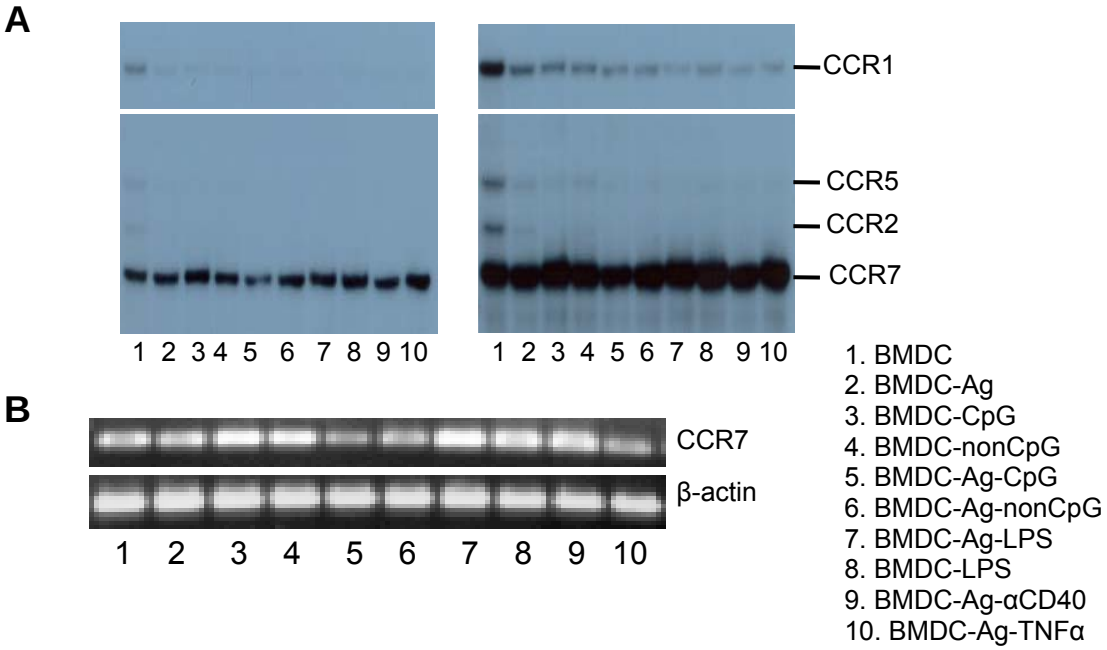

Fig. 22. Antigen pulsing and maturation modulate the chemokine receptor expression in BMDC. A: BMDC were treated overnight as indicated and the expression of mRNA for chemokine receptors was assessed by RPA. Two different times of film exposure [24 and 96 hours] are shown. B: CCR7 expression was determined by RT-PCR and the amplification of β-actin mRNA was used as control. Ag: antigen, αCD40: anti-CD40 Ab.

As shown in Figure 22A, the chemokine receptor expression in untreated BMDC had the typical pattern reported for immature DC. This included strong expression of CCR1 and detectable levels of CCR2 and CCR5. Significant expression of CCR7, a marker associated with mature DC, was also observed in non-treated cultures and can be explained by the presence of spontaneously matured cells in 10 days-old cultures. In comparison to untreated cells, LmAg-pulsed BMDC expressed lower levels of

CCR1, CCR2 and CCR5 and higher levels of CCR7 mRNA. Additional treatment of cells with maturation inducers like CpG, LPS, CD40 ligation and TNF-α also down-regulated the CCR1 expression and virtually abrogated the CCR2 and CCR5 expression by DC. CCR7 expression was also up-regulated by those activators, remarkably by CpG and LPS [Fig. 22A]. Interestingly, non-CpG ODN-treated DC showed a pattern of chemokine receptor expression similar to those stimulated by CpG ODN. When the expression of CCR7 was analyzed in the different experimental groups by RT-PCR, similar results were observed [Fig. 22B]. Thus, both RPA and RT-PCR assays indicate that pulsing and specially maturation of BMDC induce the down-regulation of CCR1, CCR2 and CCR5 and the up-regulation of CCR7.

Chemokine expression by BMDC was also modulated in response to pulsing and maturation [Fig. 23]. Non-treated DC express high levels of macrophage inflammatory protein [MIP]-2 and RANTES, and low but detectable levels of MIP-1α and MIP-1β. LmAg-pulsing slightly up-regulated the expression of MIP-3β, MIP-2 and MIP-1α, and the expression of those chemokines as well as MIP-1β was readily up-regulated by CpG, LPS and TNF-α treatment. Again, non-CpG ODN were also able to up-regulate, although to a lower extent, the chemokines messages induced by CpG ODN. These results indicate that inflammatory chemokines are produced by BMDC and that they are up-regulated by LmAg pulsing and maturation.

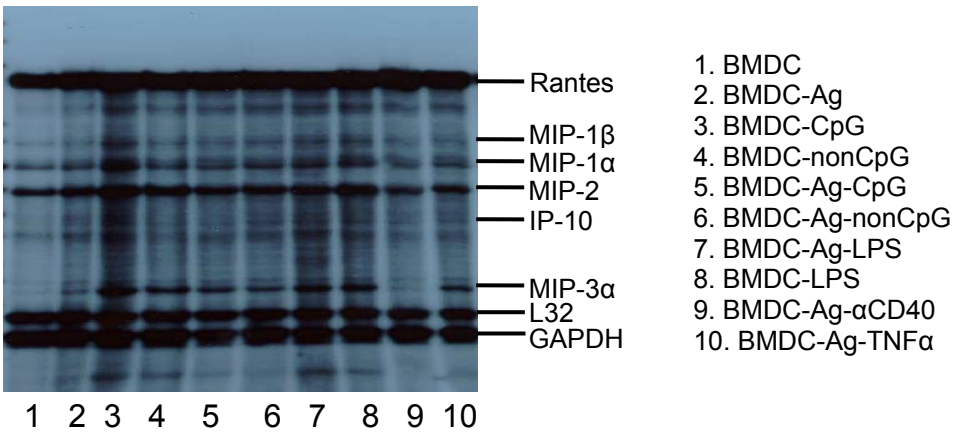

Fig. 23. Antigen pulsing and maturation modulate the chemokine expression in BMDC. BMDC were treated overnight as indicated and the expression of mRNA for chemokine genes was assessed by RPA. Ag: antigen, αCD40: anti-CD40 Ab.

Taken together, these results indicate that untreated BMDC express a pattern of chemokines and chemokine receptors that resembles immature DC and that this pattern is modulated by treatment with LmAg and maturation inducers towards a mature DC-like type. From the stimuli analyzed here, CpG and LPS seem to be the most potent in the modulation of chemokine and chemokine receptor expression.

5.3 CpG-activated BMDC express maximal amounts of IL-12

To explore the mechanisms involved in the activation of the protective Th1-like immune response observed in mice vaccinated with CpG-matured and LmAg-pulsed BMDC, we analyzed the expression of IL-12. This cytokine is formed by the subunits p40 and p35 and is known to play a key role in the development of Th1 cells. For this purpose, BMDC were treated as described in Material and Methods, and after 36 hours total RNA was isolated and the levels of p35 and p40 mRNA were determined by RT-PCR. Supernatants of the same cultures were also collected and the bioactive IL-12p70 form of the protein was measured by ELISA.

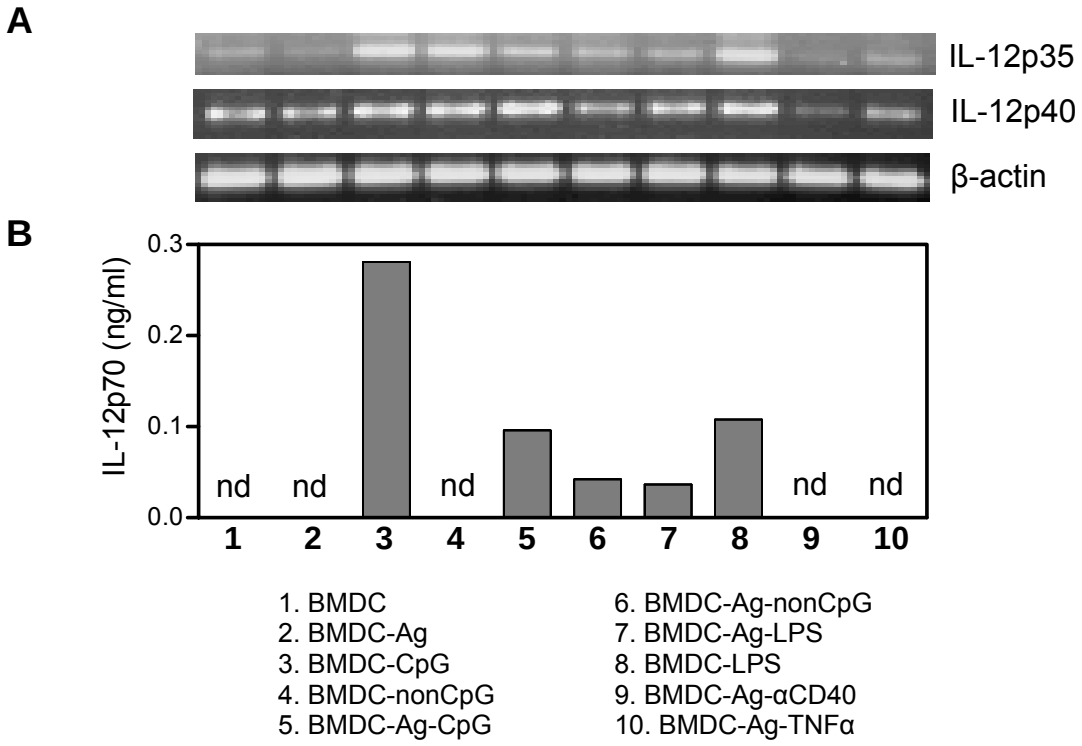

Fig. 24. CpG ODN induce maximal production of IL-12 in BMDC. BMDC were treated overnight as indicated and the expression IL-12 was determined. A: the mRNA for IL-12p35 and IL-12p40 subunits were determined by RT-PCR. B: The levels of bioactive IL-12p70 were determined in supernatants by ELISA. nd: not detected, Ag: antigen, αCD40: anti-CD40 Ab.

As shown in Figure 24A, p40 and p35 mRNA were differentially regulated by pulsing and maturation stimuli in BMDC. CpG and non-CpG ODN as well as LPS induced a very strong upregulation of p35 mRNA. The presence of LmAg in those activator-treated cultures down-regulated the induced expression of p35 mRNA. Among the groups treated with LmAg and maturation stimuli, CpG ODN induced the maximal p35 mRNA level. In contrast to IL-12p35, basal levels of the p40 mRNA were apparently unchanged by only pulsing. However, with the exception of CpG ODN treatment, LmAg-pulsing down-regulated the activator-induced expression of IL-12p40 mRNA [see LPS and non-CpG alone versus Ag-LPS and Ag-nonCpG, respectively, Figure 24A]. Again, CpG ODN stimulated the maximal IL-12p40 mRNA level among the groups that were treated with LmAg and maturation stimuli. Bioactive IL-12p70 protein levels in supernatants were also dependent on DC pulsing and maturation, as shown in Figure 24B. Maximal levels were induced when BMDC were treated with CpG ODN. No IL-12p70 was detected in untreated, LmAg-treated and LmAg-anti-CD40 Ab- or LmAg-TNF-α-treated BMDC cultures. LmAg pulsing also down-regulated the LPS- and CpG-induced IL-12p70 production, but once more, the treatment of BMDC with CpG ODN induced the maximal level of bioactive IL-12p70 among the LmAg-pulsed and activator-treated cultures. These results, together with those shown in Figure 21, reveal an important link between the ability of BMDC to produce high levels of IL-12 and the potential of inducing protection against *L. major*.

6. In vivo approaches to determine the mechanism of protection

6.1 IL-12p70 expression by donor BMDC is not required for the induction of protection in BALB/c mice

Many reports have demonstrated that IL-12 expression is critically required to mount and maintain a protective Th1 immune response against *L. major* infection in mice [95]. Additionally, IL-12 production by DC during their initial interaction with naive T cells is proposed as a major determinant factor leading to the development of Th1 cells. The results shown in Figures 21 and 24 indicate that only the protective CpG-activated BMDC produce prominent amounts of p40 and p35 IL-12 subunits and

secrete high levels of bioactive IL-12p70. These observations strongly suggest that the ability to produce high amounts of IL-12 is the factor rendering BMDC protective.

To formally test this hypothesis, BMDC from IL-12p35-deficient BALB/c mice were generated, pulsed with LmAg, activated with CpG ODN and used in a vaccination trial as described in Material and Methods. Additional groups of mice treated with wild-type BMDC as a protective control and either LmAg-pulsed BMDC or PBS as negative controls were also included.

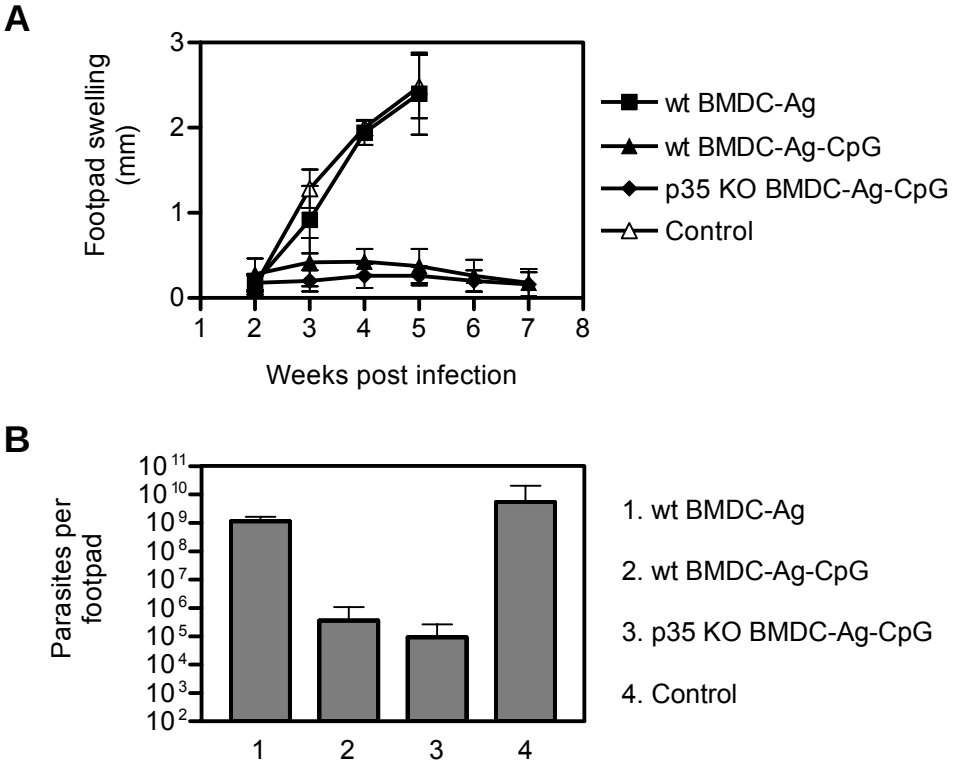

Fig. 25. IL-12 expression by donor BMDC is not required for protection. BMDC were prepared from wild type [wt] and IL-12p35 knock-out [KO] mice and used in a vaccination trial. Protection was determined by measuring the footpad swelling [A] and quantifying the parasite burden at the site of infection [B]. Ag: antigen.

As expected, mice vaccinated with LmAg-pulsed BMDC develop footpad lesions that were comparable in size to those observed in the PBS-treated group, whereas mice treated with CpG-matured LmAg-pulsed BMDC were potently protected [Fig. 25A]. Surprisingly, when naive mice were vaccinated with IL-12p35-deficient BMDC and subsequently challenged with *L. major* parasites, a protective effect was also observed. The level of protection was comparable to that induced by wild-type BMDC in both magnitude and kinetics. Additional evidence was provided when animals were killed and the parasite burden in the footpads analyzed by limiting

dilution assay. The results shown in Figure 25B confirm that, similar to wild-type BMDC, IL-12-deficient BMDC protect mice from clinical leishmaniasis through the activation of anti-parasite mechanisms. Thus, the protection observed in mice treated with CpG-matured LmAg-pulsed BMDC is independent of the production of IL-12 by the vaccinating BMDC.

6.2 Clinical and parasitological protection induced in BALB/c mice by BMDC is independent of the expression of IL-12 or IL-23 by donor BMDC

The unique recognized role of IL-12 in Th1 priming and the unexpected observation that protection against leishmaniasis conferred by CpG-activated and LmAg-pulsed BMDC is independent of donor-derived IL-12 prompted us to analyze in more detail the role of other cytokines that signal through the IL-12 receptor. IL-12 is the prototype member of a family of heterodimeric cytokines recently described. Two of them bind and signal through IL-12 receptor β1 [IL-12Rβ1]. The IL-12p40 subunit described above can associate not only with p35 to form the bioactive IL-12p70 but may also associate with the p19 molecule to form the newly described IL-23. IL-23 induces signal transduction pathways that are similar to those triggered by IL-12 and, therefore, IL-12 and IL-23 have overlapping biological functions like the induction of pro-inflammation and Th1 development [311]. For this reason, it is possible that p35 knock-out donor BMDC are still protective against leishmaniasis because they still produce IL-23 that may be able to signal naive T cell through the IL-12Rβ1/IL-23 receptor and therefore induces the activation of Th1 cells. To test this hypothesis, BMDC were prepared from wild-type, p35- or p40-deficient BALB/c mice, incubated with LmAg and CpG ODN as described in Material and Methods, and used in a vaccination trial.

As shown in Figure 26A and expected, PBS-treated animals developed a progressive lesion in the footpad whereas mice immunized with BMDC from wild-type animals were protected. Again, p35 knock-out BMDC were similarly competent to induce protection against *L. major* challenge. Analysis of the parasite load in the footpad again indicated that clinical cure in those groups was associated with the control of parasite replication [Fig. 26B]. But, surprisingly, BMDC that were generated from p40-deficient BALB/c mice were also able to induce protection. And, more strikingly, parasitic load measurements completely fitted with the clinical outcome since mice

vaccinated with p40 knock-out BMDC had a significant reduction [about 10^5-fold] in the number of parasites per foot [which was similar to that observed in mice vaccinated with wild-type BMDC][Fig. 26].

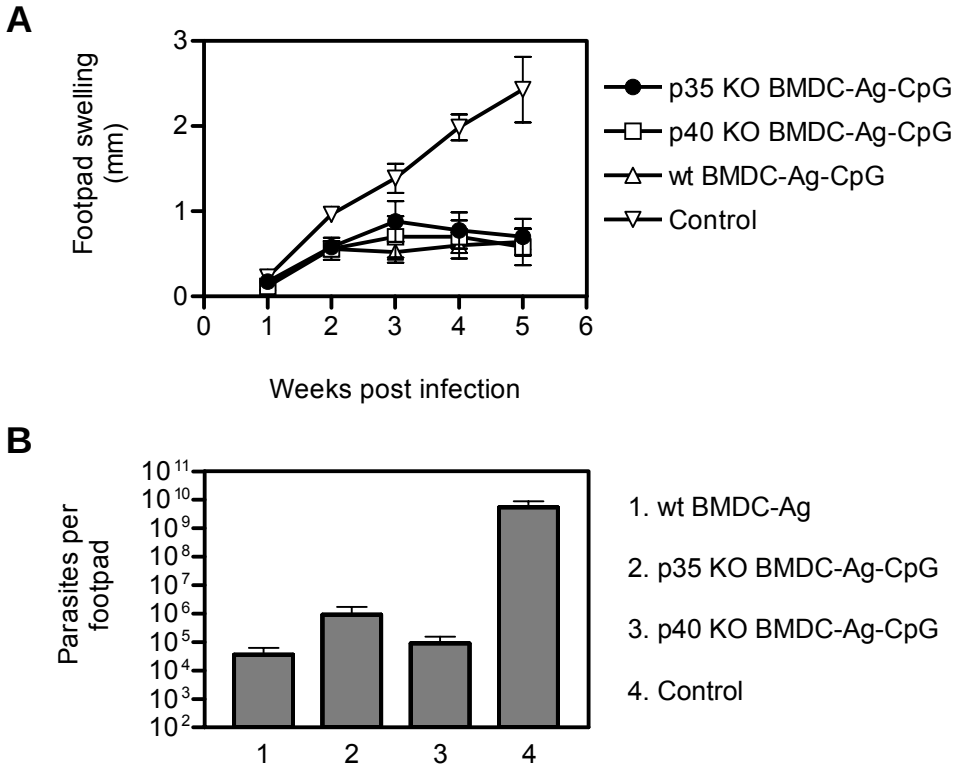

Fig. 26. IL-12 or IL-23 expression by donor BMDC is not required for protection in BALB/c mice. BMDC were prepared from wild type [wt], IL-12p35 and IL-12p40 knock-out [KO] mice and used in a vaccination trial. Protection was determined by measuring the footpad swelling [A] and determining the parasite burden at the site of infection [B]. Ag: antigen.

Those observations clearly show that the clinical and parasitological protection induced by CpG-matured and LmAg-pulsed BMDC in BALB/c mice is completely independent of the capacity of the donor cells to produce IL-12 or IL-23.

6.3 IL-12 expression by donor BMDC is not required for the induction of protection in C57BL/6 mice

To find out whether protection induced by BMDC in resistant mice was also independent of the IL-12 production by donor cells, we set up a similar experiment where naive C57BL/6 mice were vaccinated with both wild-type or IL-12p35-deficient CpG-matured and LmAg-pulsed BMDC and subsequently infected with *L. major* parasites. Appropriate controls that were injected with non-activated LmAg-pulsed BMDC or PBS were also included. As indicated in Figure 27A, C57BL/6 mice

vaccinated with LmAg-pulsed BMDC showed a significant level of clinical protection although in this experiment the parasitic load did not differ with respect to the control group [Fig. 27B]. As expected, mice vaccinated with wild-type CpG-matured and LmAg-pulsed BMDC were potently protected from leishmaniasis and the footpads contained at least 10^4-fold less parasites than control mice.

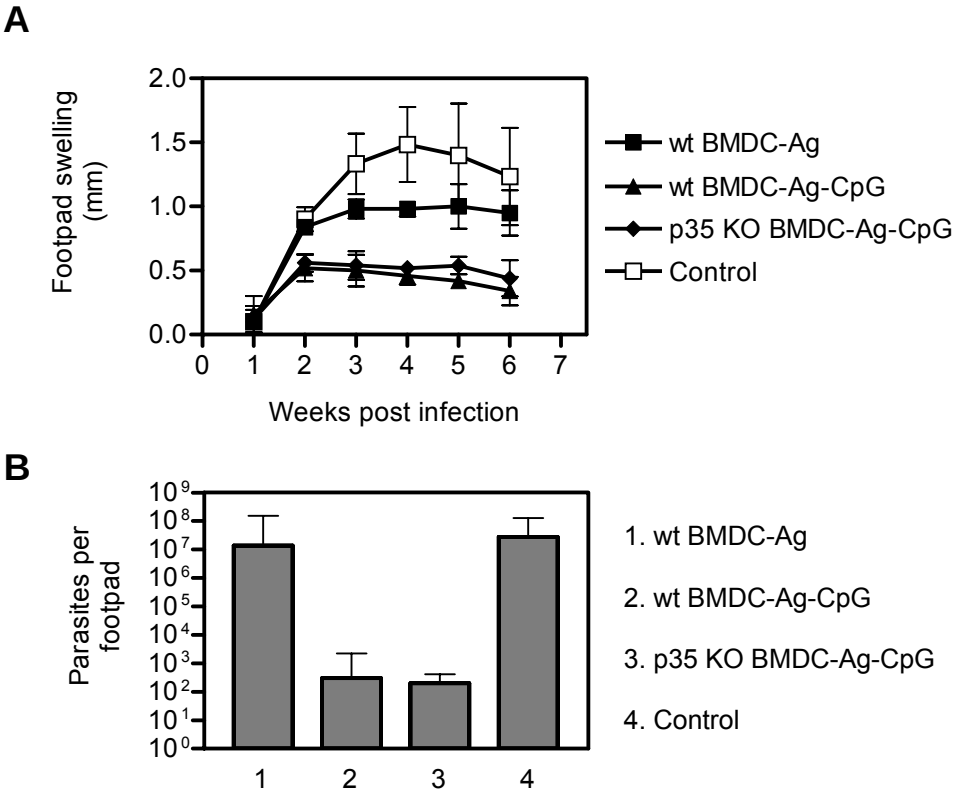

Fig. 27. In C57BL/6 mice, protection is also independent of the IL-12 expression by donor BMDC. BMDC were prepared from wild-type [wt] and IL-12p35 knock-out [KO] C57BL/6 mice and used in a vaccination trial. Protection was assessed by measuring the footpad swelling [A] and determining the parasite burden at the site of infection [B]. Ag: antigen.

Interestingly, mice that had been vaccinated with CpG-matured and LmAg-pulsed BMDC that were deficient in IL-12p35 expression developed a very limited swelling that was comparable to that observed in mice vaccinated with wild-type BMDC. Limiting dilution analysis confirmed that clinical protection induced by IL-12p35-deficient BMDC was also due to the effective control of parasite replication [Fig. 27]. Those results indicate that similar to BALB/c mice, the production of IL-12p70 by donor BMDC is not required to induce protection against leishmaniasis in resistant C57BL/6 mice. Thus, clinical and parasitologic protection induced by CpG-matured LmAg-pulsed BMDC in both susceptible BALB/c and resistant C57BL/6 mice is

independent of the ability of the donor cells to produce IL-12p70 or the functionally related cytokine IL-23.

6.4 IL-12 expression by recipient mice during vaccination is required for the induction of protection in BALB/c mice

The critical role of IL-12 in the initiation of a Th1 immune response protecting against *L. major* infection in mice is well documented. The observation that BMDC induce a protective Th1 response that is independent of the IL-12 expression by donor cells leads to the alternative that cells from recipient mice are the source of this crucial cytokine. Some experiments were therefore set up in order to confirm the hypothesis that production of IL-12 by recipient cells is required for protection. Naïve IL-12p35-deficient BALB/c mice were vaccinated with wild-type BMDC and one week later challenged with *L. major* parasites. Control groups included PBS-treated IL-12p35 knock-out BALB/c mice and wild-type mice treated with either wild-type BMDC or PBS.

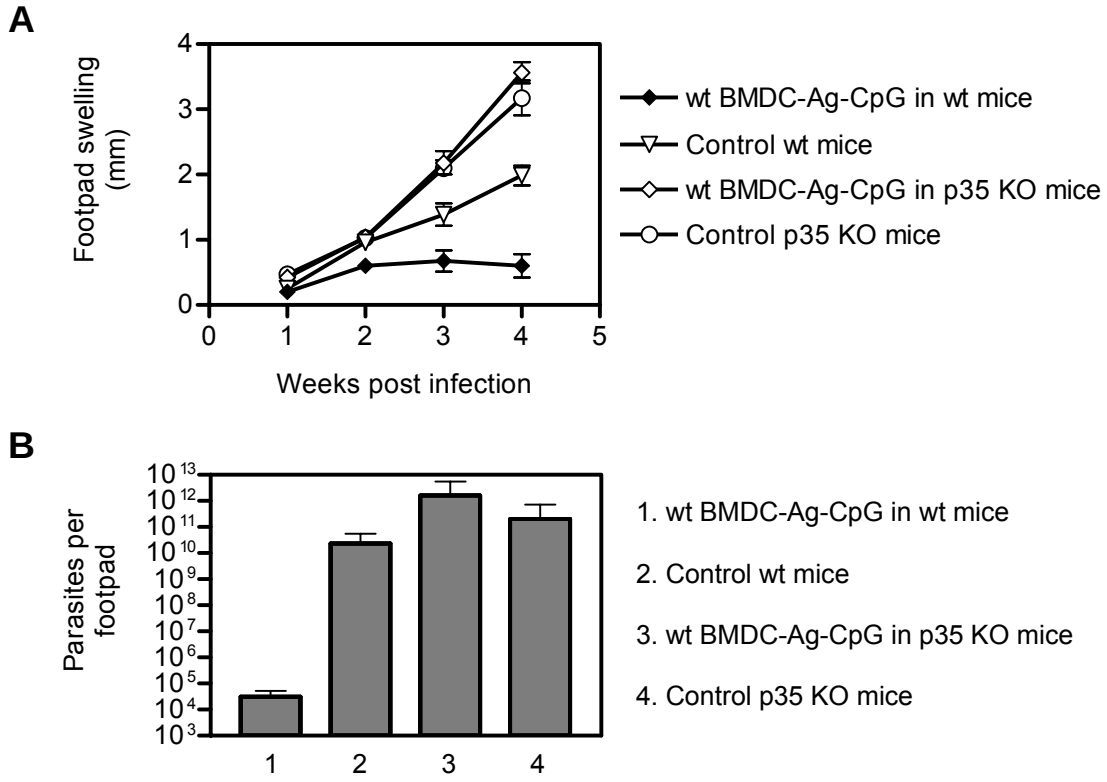

Fig. 28. IL-12 expression by recipient mice is required for the BMDC-mediated protective effect. BMDC were prepared from wild-type [wt] mice and used to vaccinate IL-12p35 knock-out [KO] or wild-type mice. PBS-treated mice were used as negative controls. Protection was assessed by measuring the footpad swelling [A] and determining the parasite burden at the site of infection [B]. Ag: antigen.

As shown in Figure 28A, PBS-injected wild-type mice developed progressive lesions in the footpads whereas BMDC-vaccinated wild-type BALB/c mice are protected against *L. major* challenge. As expected, PBS-treated p35-deficient mice developed lesions in the footpads with a more rapid kinetics than control wild-type mice probably due to a more enhanced Th2 response to parasite challenge. Interestingly, p35-deficient mice that were treated with wild-type BMDC were not protected against infection and showed a kinetic of lesion development comparable to the PBS-treated group. Quantification of the number of parasites in the footpads once more correlated with the clinical outcome [Fig. 28B]. The dramatic reduction in the parasite load observed in wild-type mice vaccinated with wild-type BMDC was completely abrogated when the recipient mice were IL-12-deficient. These results indicate that production of IL-12 by recipient mice is critical to control *L. major* infection in BMDC-vaccinated mice.

Since IL-12 production is known to be required in mice not only to initiate but also to maintain a protective Th1 response against *L. major*, the experiments described above do not answer the question of whether IL-12 production during vaccination is critical for protection in BMDC-vaccinated BALB/c mice. To address this question in vivo, a neutralizing anti-IL-12 mAb was used in BALB/c recipient mice that were vaccinated with BMDC.

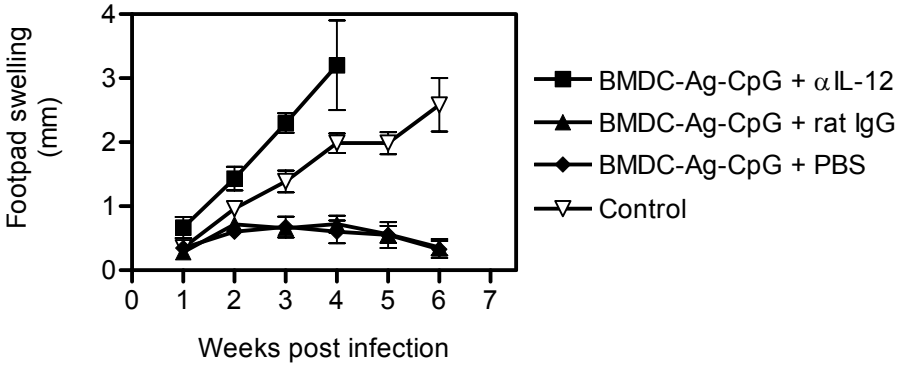

Fig. 29. IL-12 expression by recipient mice at the time of vaccination is required for the BMDC-mediated protective effect. Naive mice were i.p.-treated with anti-IL-12 Ab, rat IgG or PBS at the time of vaccination with BMDC. One week later these animals and a group of PBS-treated mice were infected with *L. major* parasites and protection assessed by measuring the footpad swelling. Ag: antigen.

A group of mice was treated i.p. with 1 mg anti-IL-12p70 mAb one day before the vaccination with BMDC and one week later mice were infected with parasites. A second group of mice received 1 mg of total rat IgG one day before vaccination as isotype control. As additional control, a third group of mice was treated one day before vaccination with PBS. As a non-vaccinated group, some mice were injected i.v. with PBS and similarly infected one week later. The results are shown in Figure 29. Non-vaccinated animals developed progressive lesions in the footpads while BMDC-vaccinated mice were protected. When recipient IL-12 was neutralized during vaccination in BMDC-treated mice the effect was reversed, since all mice developed lesions with a faster kinetics than those from the non-vaccinated control group. Mice that were treated with rat IgG as isotype control were as protected as the PBS-treated group indicating that abrogation of protection in mAb-treated mice was specifically due to the neutralization of the recipient IL-12. Taken together, these results clearly demonstrate that, although donor BMDC-derived IL-12 is not required for protection in BMDC-vaccinated mice, the expression of this cytokine by cells of recipient BALB/c mice at the time of vaccination is absolutely critical.

6.5 IL-2 expression by donor BMDC is not required for the induction of protection in BALB/c and C57BL/6 mice

Although it is widely accepted that the main T-cell growth factor IL-2 is a cytokine produced selectively by activated T cells, recent reports have indicated that DC can also produce this cytokine. Global gene expression analysis unexpectedly showed that IL-2 can be readily expressed by activating DC in vitro and this DC-derived IL-2 could contribute to the initial activation of T cells in vivo [312]. It was therefore interesting to test whether donor BMDC-derived IL-2 is important for the induction of protection in BMDC-vaccinated mice. Naive BALB/c and C57BL/6 mice were thus vaccinated with either IL-2-deficient or wild-type CpG-matured LmAg-pulsed syngeneic BMDC, and the development of lesions was followed up after parasite challenge.

As shown in Figure 30A, BALB/c mice vaccinated with BMDC that are deficient for IL-2 expression exhibited a level of protection against leishmaniasis that was similar to that induced by wild-type BMDC. A similar result was observed in C57BL/6 mice, since the deficiency of IL-2 by donor BMDC did not affect their ability to reduce the

clinical manifestation of leishmaniasis [Fig. 30B]. Thus, protection observed in both susceptible and resistant mice after vaccination with CpG-matured and LmAg-pulsed BMDC is independent of the production of IL-2 by donor cells.

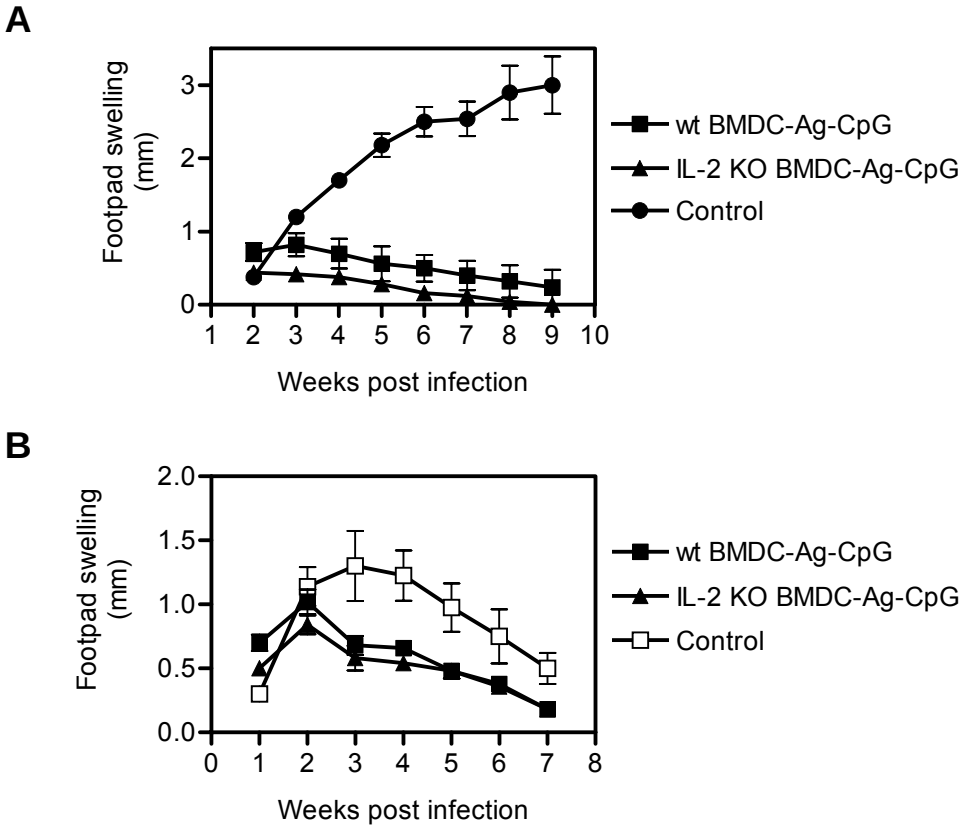

Fig. 30. IL-2 expression by donor BMDC is not required for protection in BALB/c and C57BL/6 mice. Naive mice were injected with wild type [wt] and IL-2 deficient [IL-2 KO] BMDC and used in a vaccination trial. PBS-vaccinated animals were used as controls. Protection was assessed by measuring the footpad swelling. The experiment was performed in BALB/c [A] and C57BL/6 mice [B]. Ag: antigen.

6.6 Protection can also be induced by MHC class I- or MHC class II-deficient donor BMDC in C57BL/6 mice

To test whether a cognate interaction between donor BMDC and naive recipient T cells plays a role in the system, BMDC were generated from C57BL/6 mice that were deficient for either MHC class I or class II molecules. Cells were LmAg-pulsed and CpG-treated and used in a vaccination trial. As shown in Figure 31, the expression of MHC class I molecules is not required for the induction of protection in C57BL/6 mice since the ability of BMDC deficient for this molecule to protect mice against leishmaniasis is comparable to that from wild-type BMDC. Surprisingly, BMDC derived from MHC class II-deficient mice were also protective in this model. Thus, the absence of MHC class I or MHC class II molecules in donor BMDC does not

affect their capacity to reduce the clinical manifestations of leishmaniasis in C57BL/6 mice. These findings suggest that the interaction between donor BMDC and recipient $CD4^+$ or $CD8^+$ T cells through MHC molecules is not required in BMDC-vaccinated mice for the induction of protection.

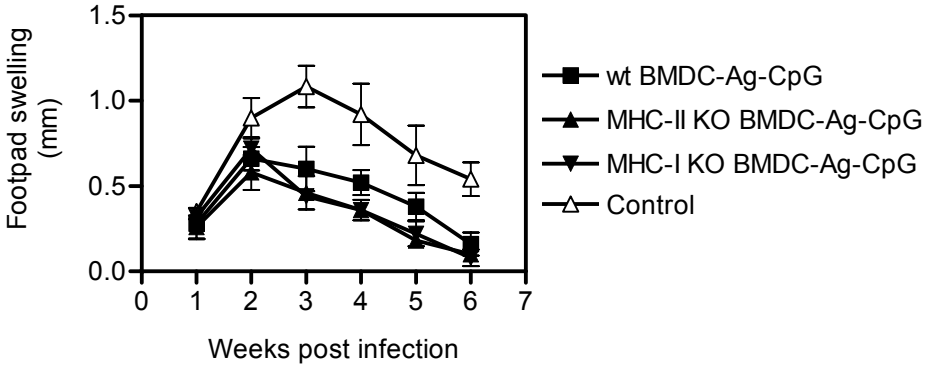

Fig. 31. MHC class I- or class II-deficient BMDC are protective in C57BL/6 mice. BMDC from wild-type [wt] and MHC class I or MHC class II-deficient [MHC-I KO or MHC-II KO] C57BL/6 mice were prepared and used in a vaccination trial. PBS-injected animals were used as controls. Protection was assessed by measuring the footpad swelling. Ag: antigen.

DISCUSSION

Although our understanding of the biology of the interaction between *Leishmania* parasites and their hosts has significantly improved in the last two decades, human leishmaniasis remains one of the most prevalent parasitic diseases worldwide. No effective prophylactic approaches against these diseases are available and therefore, control measures are based only on treatment of the human cases. However, standard chemotherapy with antimonials is expensive, toxic and difficult to administer and its efficacy is dependent on the immune status of the patient. Additionally, the number of patients that are unresponsive to conventional therapy is high and increasing constantly. Therefore, the need of developing new drugs or immunotherapeutic agents against this infectious disease is widely recognized. In particular, there is hope that an effective vaccine against leishmaniasis can be developed. Many vaccine preparations, ranging from live attenuated and killed parasites to molecularly defined antigens delivered with different inert or biologically active adjuvants, have been tested in animal models [313]. In general, the efficacy of vaccines is significant when potent cellular adjuvants in the form of replicating parasites, live bacterial or viral carriers, or IL-12 are administered in the vaccine formulation. Thus, the adjuvant appears to be a critical factor in the development of effective immunoprophylactic and immunotherapeutic strategies against leishmaniasis. In the present thesis, the striking properties of DC as natural adjuvants have been exploited for developing a vaccine formulation that induces an exceptional level of protection against experimental murine leishmaniasis.

A protocol for generating sufficient amounts of DC from bone marrow cultures was established and the typical phenotype and functional characteristics of those DC, such as response to activators and antigen presentation capacity, were confirmed. Interestingly, when antigen presentation functions were tested in a *Leishmania*-specific T cell activation assay, differences between BMDC and LC were found. For instance, when cells were pulsed with total *Leishmania* lysate and therefore antigen processing was required, BMDC but not LC were able to induce IL-2 production by T cells. This could be explained by a continued ability of BMDC to capture antigens during in vitro cultures [307], whereas LC are known to down-regulate *L. major* uptake after overnight culture [314]. Alternatively, since epidermal cell preparations usually contain keratinocytes and other non-DC cell types, the DC:T cell ratio could be actually lower than indicated. However, the lack of IL-2 production even at high

DC:T cell ratios favors the first explanation. Regardless of this observation, the results indicate that BMDC prepared according to the protocol described here have the ability to take up, process and efficiently present antigens to T cells.

In spite of the capacity of the BMDC described here to present atigens in vitro, adoptive transfer of LmAg-pulsed cells to naïve mice failed to confer protection against parasite challenge. This observation was unexpected since our previous reports have indicated that LmAg-pulsed LC protect mice against *L. major* infection and that this protection is associated with the induction of a Th1 immune response [300]. In addition, in another study, BMDC that were pulsed with LmAg induced protection against *L. donovani* infection in mice [301]. For this reason, a number of variables that were thought to influence the in vivo efficiency of the BMDC-based immunization were modified with unsuccessful results. For instance, BMDC that were harvested on different days [after 8, 9, 10 or 11 days of generation], or pulsed with different concentrations of LmAg [1, 3, 10 or 30 μl/ml], or injected in different amounts to the mice [from 3 x 10^5 to 10 x 10^5 BMDC/mouse] were similarly ineffective. A plausible explanation to this finding was suggested by the observation that upon LmAg pulsing, BMDC do not undergo maturation, a process critically required by DC to acquire potent T cell-stimulating capacities [152]. Interestingly, infection with *L. major* induces BMDC maturation, indicating that metabolic activity of intracellular parasites is critical for triggering DC activation. Similar results have been observed with other intracellular parasites [315], suggesting that further in vitro maturation of LmAg-pulsed DC may be required to confer a protective function to DC.

When several representative inducers of DC maturation were tested, namely TLR ligands [LPS and CpG], proinflammatory cytokines [TNF-α] and T cell-derived signals [CD40 ligation], very interesting findings were obtained. Whereas all stimuli induced DC maturation, only CpG ODN provided the appropriate signals to confer a protective phenotype to BMDC. All mice vaccinated with CpG-activated DC were completely protected from disease and harbored a dramatically lower amount of parasites at the site of infection, indicating an effective restriction of parasite multiplication and dissemination. Protection was due to the induction of a Th1 response, as confirmed by the profound changes in the *Leishmania*-specific cytokine

and IgG Ab profiles induced in protected mice after infection, in comparison to the control group. Notably, IFN-γ levels of LN cells were strongly increased, explaining the potent clinical and parasitologic protection achieved. Consequently, serum IgG2a Ab levels and the IgG2a/IgG1 ratio were maximal in those animals. Other Th1 cytokines, like IL-2, were increased and Th2 cytokines, like IL-4, reduced. Interestingly, the *Leishmania*-specific IL-10 production in LN was also increased in protected mice as compared to non-protected groups. Although this cytokine has formerly been linked to a disease-promoting Th2 response, recent findings suggest that IL-10 is also produced in the presence of a strong Th1 response, and represents an immune regulatory loop preventing the development of immunopathology. In addition, IL-10 has been associated with the mechanisms allowing parasite persistence and long-lasting immunity [95, 316]. Whether protected mice harbor persistent parasites and whether this persistence is IL-10-mediated was not determined in the present study. However, protection was solid and long-lasting.

The requirement of DC activation for the acquisition of a protective phenotype reported here contrasts with previous reports. LC were shown to be protective when pulsed in vitro with LmAg in the absence of any exogenous maturation factor [300]. Endogenous mediators released during epidermal cell preparation as well as cytokines produced by keratinocytes and other cells during overnight culture may have induced LC to mature and to acquire a protective phenotype. Indeed, LC undergo maturation in vitro even in the absence of any exogenous antigen [our unpublished observations]. BMDC, in contrast, required additional treatment to reach a fully mature phenotype. Previous studies in our laboratory showed that, although protection mediated by LC is significant, complete regression of lesions is not observed in every experiment and every mouse [300, 308]. In addition, analysis of the parasite load of draining LN in protected mice that were vaccinated with LC showed an effect that was significantly lower than the one observed in the footpads of BMDC-vaccinated animals [5-fold versus 10^5-fold reduction, respectively][308 and this thesis]. This indicates that CpG-activated BMDC are significantly more potent than LC to induce protection and emphasizes the importance of proper activation of the DC for the acquisition of a powerful protective phenotype. It will be interesting to test whether CpG treatment of LC may improve the level of clinical and parasitological protection. An alternative

explanation is that those different levels of protection are intrinsic to the different DC populations.

By infecting BALB/c mice i.v. with *L. donovani*, a model frequently used to mimic human VL, Ahuja and colleagues [301] also reported that antigen-pulsed BMDC protect against leishmaniasis. In this study, the analysis of the parasitic load in spleen and liver indicated that, whereas antigen-pulsed BMDC induced some protection, antigen-pulsed cells that were genetically manipulated by viral transduction to constitutively express IL-12 induced a dramatic reduction [about 10^6-fold] of the parasite burden. Interestingly, in addition to leading to high expression of IL-12, viral infection also induced BMDC maturation. Thus, though those DC cultures were not supplemented with any activator, the genetic manipulation automatically led to maturation. Although caution should be taken when comparing data from those two different settings, these reports are consistent with the conclusion that maturation is critically required by LmAg-loaded BMDC to acquire a protection-promoting phenotype.

Although maturation appears to be a logic requirement for the induction of protection by DC, more intriguing in this work is the observation that only CpG were able to provide the appropriate signals. BMDC activated by TNF-α, CD40 ligation, LPS and CpG have also been tested in experimental tumor models, and the results demonstrated a significant promotion of anti-tumor immunity by all of them when compared to non-activated BMDC [263, 317]. CD40 ligation and TNF-α showed the maximal prophylactic effect in one study, whereas CpG was more potent than TNF-α in the other. The discrepancy of these reports with my results can be explained by the clear differences in the requirements for immunity to tumors and to intracellular parasites like *L. major*. Activation of $CD8^+$ T cells and generation of killer cells are prerequisites for protection against tumors. Indeed, cancer development is usually linked to a failure in the activation of an anti-tumor response, and, therefore, it is conceivable that any DC maturation inducer will improve the tumor antigen recognition and T cell response. Murine leishmaniasis, in contrast, results from an aberrant and continued Th2 immune response rather than the absence of any T cell response [95]. Indeed, in BALB/c mice, effective vaccination against fatal infection crucially depends on the induction of a *Leishmania*-specific Th1 response. It is

therefore reasonable that a DC-based prophylactic strategy against *L. major* and other intracellular microbes requires the activation of signals that not only induce immunogenic but also Th1-promoting DC.

Although many pathogens and substances can activate DC, profound qualitative and quantitative differences in the DC response can be observed, depending on the stimulus used [318]. It is currently hypothesized that those subtle differences constitute the codes that DC use to instruct the type of adaptive response required. How membrane-bound and soluble mediators expressed by activated DC dictate the type of T cell response is largely unknown. A notable exception is the cytokine IL-12, whose role in the initiation of a Th1 response has been extensively studied. The presence of IL-12 during the activation of naïve T cells is accepted as the most important force driving the differentiation of Th1 cells [63, 195, 311]. Therefore, DC maturation that leads to IL-12 production will promote Th1 cells. Consistent with those concepts, the protective CpG-activated and LmAg-pulsed BMDC described here produced significant levels of IL-12 and induced a Th1 response upon parasite challenge, whereas non-protective cells did not produce IL-12 and did not induce a Th1 response after infection. In the absence of CpG, the activation of DC by TNF-α or CD40 ligation induced the production of several pro-inflammatory cytokines, but undetectable or very low levels of IL-12 [240, 263, 317, 319 and this thesis]. This correlates with the reports indicating that DC activated with these agents do not induce Th1 cells in vitro [245, 319] and do not protect against *L. major* challenge [this thesis] . In contrast, CpG that represents a type 1 microbial signal is a potent inducer of IL-12 production [242, 263, 320 and this thesis], has a potent Th1-promoting activity in vitro [224, 321] and enables BMDC to induce protection against *L. major* infection [this thesis].

Interestingly, under the conditions established here, CpG- but not LPS-activated BMDC were able to induce a protective Th1 response. As a member of the TLR family ligands, LPS was expected to activate DC and induce a protective response. Indeed, LPS-treated BMDC produce appreciable levels of IL-12 [234, 235, 317, 319 and this tesis] and have been reported to induce Th1 cells in vitro [234, 319]. A number of considerations have to be taken into account to explain these apparently contradictory results. The concentration of LPS used in the present work to activate

BMDC in vitro ranged from 0.1 to 1 μg/ml, corresponding with the doses used in other reports where Th1 induction in vitro was observed. At those doses, the number of fully mature DC in LPS-treated cultures was comparable to that found in CpG-treated cultures [not shown], indicating that quantitative differences in the expression of MHC class II or co-stimulatory molecules are not the explanation for the final outcome. Whether higher or lower concentrations of LPS may be required to activate protective BMDC remains to be determined. Similarly, a report indicating that LPS derived from different bacterial sources may induce different Th responses [233] suggests an alternative explanation. We are currently exploring those possibilities. However, a more tempting alternative is that differences in the quality and intensity of DC activation by CpG and LPS are responsible for their different capacities of promoting protective DC. Several lines of evidence support this hypothesis: first, CpG are more potent inducers of IL-12 production in DC than LPS, and an enhancement of the IL-12 expression by CD40 ligation is observed in CpG- but not LPS-treated cultures [234, 235, 240, 242, 263, 317, 320]. Second, global gene expression analysis of macrophages treated with LPS and CpG showed some qualitative and dramatic quantitative differences [S. Vogel, personal communication, macrophage and DC meeting, Erlangen, April 2002]. Similarly, global gene expression studies of DC infected with closely related pathogens shows dramatic differences in the profile of up- and down-regulated genes [318], indicating that subtle changes in the stimulus may lead to the activation of functionally different DC. Third, although initial reports suggested common signaling pathways among TLR, recent findings indicate that different TLR ligands activate particular pathways. For instance, CpG activation of DC is completely dependent on the adaptor molecule myeloid differentiation factor-88 [MyD88], whereas LPS molecules activate DC through MyD88-dependent and -independent pathways [194]. Thus, these findings together with my observations indicate that related activators may activate DC in a dramatically different way, leading to functionally different DC, and that activation through TLR9 by CpG may represent an important pathway in the development of protective immunity against leishmaniasis. This last conclusion is additionally supported by the observation that CpG-treated mice are protected from fatal leishmaniasis [321] and by the recent report demonstrating that intact MyD88-dependent signaling pathways are essential in C57BL/6 mice to resist *L. major* infection [288].

From the immunobiological point of view, the findings of this study contribute to the understanding of Th1 polarization by DC. It has been suggested that different DC subsets have an intrinsic tendency to promote either a Th1 or a Th2 response [165]. Recently, however, a number of reports indicated that there is a striking plasticity in the ability of a given DC subset to respond to different microbes [234, 235, 239, 245], suggesting that the type of DC stimulus is a critical factor leading to DC-mediated polarization of the Th cell response. A major limitation has been the lack of an in vivo read-out system reflecting the development of Th cells with impact on disease. The criterion usually applied is the in vitro production of different cytokines associated with Th1 or Th2 cells after in vitro (humans, mice) or in vivo (mice) priming, but formal evidence for the generation of Th1 or Th2 cells that are able to mediate effective control of pathogens in vivo has not been provided. In addition, a mixed pattern of Th1 and Th2 cytokines is observed in some studies [165], leading to further ambiguity regarding the relevance of the findings for the situation in vivo. Moreover, a recent study demonstrated that DC pulsed with a microbial antigen stimulated Th1 cells in vitro, but induced a Th2 response after transfer in vivo, as characterized by the cytokine profile and the lack of protection upon challenge with the pathogen [322]. Th1 and Th2 cells are known to coexist after pathogen encounter and, although the preferential expansion of one population will eventually dictate the overall response, predictions of whether an effective humoral or cell-mediated immune response will predominate in vivo can presently not be made on the basis of cytokine distribution. Therefore, the use of in vivo models to assess the induction of physiologically relevant Th1 and Th2 cells by DC is desirable. By using the murine model of leishmaniasis, I report here that an effective pathogen-restricting Th1 response can readily be induced in vivo by CpG-activated murine myeloid DC bearing microbial antigen.

It has recently been shown that murine myeloid $CD8^-$ DC, previously thought to induce Th2 responses, also promote Th1 responses when stimulated with appropriate microbial signals [235, 241] and that CpG ODN and other bacterial products predispose both $CD8^+$ and $CD8^-$ DC to secrete IL-12 [242]. Both plasmacytoid and myeloid murine DC are able to induce effector Th1 or Th2 cells in vitro depending on the dose of antigen and the presence of microbial stimuli [224]. It has also been

reported that the selective priming of T cells producing IFN-γ or IL-4 in vitro by DC subsets from mouse spleen is primarily determined by the type of microbial signal used for activating the DC and that the intrinsic capacity of different DC subsets to polarize Th cell development is only weak [239]. A similar plasticity is emerging for human DC [245, 246]; human plasmacytoid DC, initially proposed to promote Th2 responses, are now known to drive Th1 differentiation after virus infection [246]. My findings are in agreement with these reports and additionally provide in vivo support to the concept that the nature of the microbial stimulus is the factor primarily determining Th polarization by a given DC subset.

In an attempt to elucidate the mechanisms underlying protection induced by CpG-activated BMDC, a number of in vitro and in vivo experiments were designed. First, the expression of cytokines, chemokines and chemokine receptors at the mRNA level by RPA and RT-PCR was determined in BMDC cultured in the presence of LmAg and/or maturation inducers. As expected, the patterns of chemokine and chemokine receptor expression by DC confirmed the typical modulation during the maturation process. Inflammatory chemokines as well as chemokine receptors associated with homing to lymphoid organs, particularly CCR7, were up-regulated, whereas chemokine receptors associated with homing to peripheral tissues were down-regulated. In general, LmAg pulsing induced minor changes, whereas more pronounced changes were observed when cells were further activated with maturation inducers. CpG and LPS triggered the most pronounced changes confirming their potency as DC activators. However, no qualitative or quantitative differences were observed in the pattern of chemokine and chemokine receptor expression between CpG- and LPS-treated BMDC, suggesting that the modulation of these molecules is not the critical factor rendering a protective phenotype to CpG-matured and LmAg-pulsed BMDC.

More interesting information was obtained when cytokine expression was analyzed. LmAg pulsing induced minor changes in cytokine expression, whereas TNF-α and CD40 ligation induced low levels of pro-inflammatory cytokines such as IL-1β and IL-6. Again, a potent up-regulation of these and other cytokines was observed in CpG- and LPS-treated cultures, but this time with marked differences. First, CpG

induced a clearly stronger up-regulation of cytokines than LPS. Second, IL-12p40 expression was abundant in CpG- but almost un-perceptible in LPS-activated BMDC as shown by RPA assays. And third, although IL-12p40 was down-modulated by LmAg pulsing, considerable levels were still observed in CpG-treated BMDC whereas its expression was abrogated in LPS-activated LmAg-pulsed BMDC. These observations strongly pointed to IL-12 as the molecule playing the critical role in the protective CpG-activated BMDC. The analysis of IL-12p40 and IL-12p35 subunit mRNA by RT-PCR showed similar findings and, more importantly, measurements of the secreted active form of IL-12 in DC cultures confirmed the notion that CpG-activated DC produce maximal levels of IL-12p70.

Thus, since Th1 differentiation induced by DC appears to be related with the amount of IL-12 produced rather than whether IL-12 is produced [195], these findings suggest a mechanism that would operate in CpG- but not LPS-treated BMDC and, therefore, would explain the induction of protection. The hypothesis was that after i.v. injection of CpG-activated LmAg-pulsed BMDC, a cognate interaction between donor DC and recipient *Leishmania*-specific naive T cells takes place in the spleen and other lymphoid organs. Cells would reach lymphoid organs by the blood stream, but would migrate actively to T cell areas due to the high expression of CCR7. These CpG-matured DC, that are potent APC, activate naïve T cells and produce high amounts of IL-12. Activated T cells induce terminal DC maturation and boost IL-12 production, resulting in the establishment of the optimal conditions for Th1 cell commitment and differentiation. A potent *Leishmania*-specific Th1 response is then generated in naïve mice, and, therefore, when parasite challenge comes, the normal Th2 response is shifted towards a protective memory Th1 response. The inability of non-activated or LPS-, TNF-α- and anti-CD40 Ab-activated BMDC to produce sufficient amounts of IL-12 would explain their failure to induce protection.

Experiments designed to formally prove this hypothesis in vivo were carried out and results were completely unexpected. Protection was independent of the expression of IL-12p70 by donor BMDC and a possible role of the related cytokine IL-23 was also ruled out. Protection was monitored at the clinical level and also confirmed by analyisis of the parasite burden at the site of infection, and no differences were observed between wild-type and p35- or p40-deficient BMDC. This observation

contrasts with the recent report showing that the expression of IL-12 is required by LC to protect against *L. major* infection [308]. As discussed before, the fact that LC do not require additional maturation signals to acquire a protective phenotype points to intrinsic differences between these two DC populations in terms of activation requirements. The discrepancy in the IL-12 dependency also suggests fundamental mechanistic differences. This explanation is supported by a recent publication showing that IL-12 production by donor BMDC is not required to induce Th1 response when the levels of IFN-γ in LN were used as read-out [323]. Several lines of evidence, indeed, demonstrate that ex vivo isolated DC differ from in vitro generated DC in some biological properties [162, 224, 324] including the dependency on IL-12 for Th1 priming in vitro [224].

Taking into account the well-documented requirement of IL-12 for the development and maintenance of a Th1 response protecting against *L. major* in mice, it was not surprising that protection was not induced when recipient IL-12 knock-out mice were treated with wild-type BMDC. This demonstrated that the production of IL-12 by cells from recipient mice is critical for the induction of protection in BMDC-immunized animals. This finding also agreed with the report of MacDonald and Pierce that demonstrated that the induction of IFN-γ-producing T cells in mice receiving Th1-promoting BMDC was abrogated when recipient animals were deficient in IL-12 expression [323]. It remains unknown whether IL-12 production by LC-vaccinated mice is required for the development of a response protecting against *L. major*. In order to clarify whether the production of IL-12 by cells of recipient mice at the time of vaccination was required for the initiation of a protective response, an experiment was performed, where IL-12 was neutralized one day before the immunization with BMDC. Whereas mice treated with rat IgG [as an isotype-matched control] were still protected, those from the anti-IL-12-treated group developed progressive lesions. This allowed to conclude that the availability of recipient cell-derived IL-12 at the time of vaccination was a critical requirement. Thus, the protection induced by CpG-activated LmAg-pulsed BMDC in naïve BALB/c mice is an IL-12-dependent process, but the source of this cytokine is not the donor DC, but rather cells of the recipient mice, presumably DC. However, this conclusion again raises the fundamental question: If the levels of IL-12 production by donor DC do not explain protection, what are the exquisite features of CpG activation that make

BMDC protective? The availability of IL-2-deficient mice prompted me to analyze whether the production of this cytokine by BMDC is required for the induction of protection. This experiment was based on a recent report indicating that DC produce IL-2 upon maturation and that this may contribute to T cell activation [312]. However, the results showed that IL-2 production is not necessary for the ability of CpG-activated BMDC to induce protection.

I confirmed the powerful ability of CpG-activated and LmAg-pulsed BMDC to reduce the clinical manifestations of cutaneous leishmaniasis in another strain of mice. C57BL/6 mice are genetically resistant to cutaneous leishmaniasis since they develop a Th1 response after *L. major* infection. After spontaneous cure of the skin lesions within 5 to 6 wk of infection, C57BL/6 mice are completely protected against subsequent challenges. The clinical and immunological features observed in patients with human localized CL appear to be more closely reproduced in C57BL/6 than BALB/c mice [95] and, therefore, *L. major* infection in those mice is accepted as a suitable model for that form of the disease. A single immunization of C57BL/6 mice with CpG-matured antigen-pulsed BMDC induced a significant reduction in the lesion development, with a lower maximal peak and a faster healing, and a significant reduction in the parasite burden in the footpad as compared with the PBS-treated control group. In contrast to BALB/c, C57BL/6 mice immunized with non-activated LmAg-pulsed BMDC showed some reduction in the lesion size and the parasite burden, indicating that the requirements of DC activation may be somewhat different in those different strains. No significant differences in the cytokine profile expressed by LN cells or the IgG subclass distribution in the serum were observed 6 weeks post infection between experimental groups [not shown], consistent with the fact that untreated animals also develop a Th1 response, as expected. We did not analyze the kinetics of the Th1 response but it seems plausible that animals vaccinated with CpG-matured LmAg-pulsed BMDC may have mounted a faster Th1 response than non-protected groups.

Experiments to define the mechanism of protection in C57BL/6 mice revealed striking similarities to BALB/c. Protection in C57BL/6 mice was also independent of the IL-12 expression by donor BMDC and IL-2 was also not required. More intriguingly, when BMDC from C57BL/6 mice that were deficient for MHC class I or

class II were used in a vaccination trial, the protection observed was similar to that observed with wild-type DC. Although this experiment was performed only once and needs to be repeated, statistic significance was observed. However, care has to be taken in the interpretation, since the window of lesion development in these mice is very small, and variation between experiments is frequent. Setting up a similar experiment in BALB/c mice, where a clearer cut between lesion development and cure is observed, would help to confirm that observation. In the case that this observation will be confirmed, two different possibilities have to be considered when discussing the potential mechanisms of protection: first, activation of either $CD4^+$ or $CD8^+$ recipient T cells by donor DC can be sufficient to induce protection, or, second, the induction of protection is independent of the interaction between naïve recipient T cells and donor DC through MHC molecules. The first possibility seems unlikely since $CD4^+$ T cells are known to be required for the induction of Th1 responses and $CD8^+$ T cells are dispensable [95]. However, recent findings indicate that $CD8^+$ T cells are critical for resistance against low doses of *L. major* infection [129] and actively participate in the induction of long-lasting protection induced with DNA vaccines [325]. This hypothesis can be easily proven by using BMDC from double knock-out mice that are deficient for both MHC class I and class II. The second possibility is even more unlikely, but strongly supported by the observed independency of donor DC-derived IL-12 for protection. In this case, LmAg-bearing mature DC are taken up by resident DC in the lymphoid organs, and *Leishmania* antigens are crosspresented to naïve $CD4^+$ and $CD8^+$ T cells in a self MHC-restricted manner. This possibility implies a very exiting possible mechanism for inducing Th1 responses, since it automatically proposes that crosspresented antigens are differentially handled by recipient APC depending on whether they come from a previously activated or not activated DC. Future experiments will help to reveal which of those different alternatives can explain the findings described in this thesis.

From the practical point of view, an outstanding finding of the present study is the high level of protection that can be achieved by this vaccine preparation. A single immunization with CpG-activated and LmAg-pulsed DC completely and consistently protects mice from clinical leishmaniasis by dramatically restricting parasite growth. Moreover, protection is solid, as indicated by the resistance against re-infection even with a stronger parasite challenge, and also long-lasting, since mice that were infected

4 months after vaccination were also protected. This indicates that a stable and protective *Leishmania*-specific memory T cell response is established in vaccinated mice. With the notable exception of DNA vaccination, or vaccine preparations with IL-12 as an adjuvant, such an efficient protection against murine leishmaniasis has not been reported so far [95, 313]. However, in those reports, one or several boosts were applied to obtain such a high level of protection, whereas a single dose of DC was sufficient in this study to induce a strong protective effect.

The exceptional prophylactic effect observed with this BMDC-based vaccine prompted us to perform some standardization experiments that may be important for a potential clinical application. For instance, the minimal amount of DC required for protection was determined and the influence of the route of administration was also studied. An appreciable protective effect could be observed when only 100,000 BMDC were injected and a reproducible and completely protective effect was achieved when 500,000 BMDC per mouse were inoculated. These findings agree with other reports where protection is induced with similar amount of cells [263, 308]. Since more than 200 million BMDC can be readily generated from a mouse, the size of vaccine inoculum does not appear to be a limitation in this approach. This contrasts with LC-based vaccination where about 8-10 mice have to be sacrificed to vaccinate a group of 6 animals. On the other hand, the route of vaccine administration strongly influenced its efficiency. Whereas i.v. inoculation induced complete protection, the administration through i.p., and specially s.c. injection, led to only a partial effect when a single dose of BMDC was used. It will be interesting to test whether i.p. and s.c. injection require a higher inoculum or boosting applications. Our observations agree with previous reports where LC or BMDC-based vaccines against *L. major* and *L. donovani*, respectively, were effective only after i.v. administration [300, 301]. In a more recent report, however, *L. major*-infected FSDC were protective against leishmaniasis when administered intradermally into the ear of BALB/c mice [280]. It remains to be determined whether these different results are related to the different models of infection or rather to functional differences between those DC populations. Also consistent with the present study, s.c. administration of DC has been associated with the induction of a dominant Th2 response whereas i.v. injection induced dominant Th1 responses [301, 326]. In other models of microbial infection and tumors, s.c. application of DC has been shown to be effective [236, 263], indicating

that for achieving protection, the requirements of the homing of injected DC are complex and dependent on the pathogen or disease. This is additionally supported by observations indicating that the route of inoculation of *Leishmania* parasites may strongly influence the outcome of infection [95].

One additional question addressed in this thesis was whether BMDC pulsed with molecularly defined antigens are also able to confer protection. The level of protection induced against murine leishmaniasis by LC pulsed with recombinant LeIF was comparable to that achieved by total LmAg-pulsed LC [308]. However, several attempts to reproduce this protective effect with LeIF-pulsed CpG-activated BMDC were unsuccessful. LeIF-pulsed BMDC not treated with CpG were also ineffective [not shown]. Another antigen that is known to be immunodominant during *Leishmania* infection [M15; Y. Skeiky, personal communication.] was also ineffective. Since the T cell response induced by a single leishmanial antigen is expected to be quantitatively lower than that induced by total parasite lysate, a recombinant trimeric protein spanning the immunodominant regions of three major *Leishmania* antigens was also tested in the BMDC-based vaccine. Again no significant protection was observed. Whether these findings are due to differences in the batch-to-batch production of the recombinant proteins is unknown. The observations that LeIF did not look degraded in SDS-PAGE gels and that a 4-fold higher concentration of the protein during BMDC pulsing was also ineffective argue against this explanation. The reasons for these discrepancies need to be carefully addressed in the future. In agreement with the results reported here, a recent study showed that BMDC pulsed with total microbial antigen, but not with a single recombinant antigen, induced protective immunity in a bacterial model of infection [322], indicating again that functional differences between LC and BMDC may also be reflected in their antigen requirements for the induction of protection.

One exciting possibility that may exploit the ability of CpG-activated and LmAg-pulsed BMDC to induce a Th1 response is their potential use for the treatment of severe forms of leishmaniasis. For instance, patients with VL, in which conventional therapy frequently fails, would be the first candidates for such an approach. The basic question is whether an already established Th2 response can be redirected by CpG-matured and LmAg-pulsed BMDC. Unfortunately, preliminary results indicated that

very soon after infection of BALB/c mice, *L. major* initiate a pathogenic response that can not be redirected by BMDC treatment. CpG-matured LmAg-pulsed BMDC were not only ineffective, but even exacerbating when administrated after parasite challenge. Although BMDC-based therapy alone or in combination with other anti-tumor agents has shown some efficacy in murine tumor models [327], the efficacy of this treatment for microbial infections is poorly documented. In one study, mice treated with *L. donovani* antigen-pulsed BMDC showed a very weak reduction in parasitic load and repetitive injection did not improve the efficacy [301]. The data reported here are consistent with those findings and extend them to the model of *L. major* infection. A very interesting point for the future is to investigate whether combining pharmacologic anti-*Leishmania* treatment with DC immunotherapy has any effect. In addition, it will be interesting to test the therapeutic potential of BMDC in other murine models of leishmaniasis, such as C57BL/6 or C3H mice, which do not show the particularly high level of susceptibility exhibited by BALB/c mice.

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ABSTRACT

The present investigation established a protocol to obtain dendritic cells [DC] that efficiently protect mice against fatal infection with the intracellular parasite *Leishmania major*. DC were generated from bone marrow precursors by culturing them in the presence of granulocyte/macrophage colony-stimulating factor, as previously reported by others, and the properties of the resulting cells were determined. Bone marrow-derived DC [BMDC] exhibited a veiled morphology, expressed CD11c, MHC class II, CD86, CD11b and NLDC-145 on the cell surface, but were devoid of T cell or B cell surface markers. These cells were also potent antigen-presenting cells [APC] for allogeneic T cells in mixed lymphocyte reactions and efficiently presented a *Leishmania*-derived epitope to an antigen-specific T cell hybridoma. BMDC also responded to a set of maturation activators by producing pro-inflammatory cytokines. These properties indicated that BMDC were potent APC that may be used in a DC-based anti-infectious strategy. However, when these cells were pulsed with *L. major* lysate as a source of antigen and used to vaccinate naïve BALB/c mice, no protection against a subsequent parasite challenge was observed. Although some modifications in the protocol of DC generation, the amount of antigen used for pulsing or the number of cells used for the injection were tested, BMDC consistently failed to induce protection.

The observation that antigen pulsing does not induce BMDC maturation suggested that additional activation of the cells in vitro may be required to confer a protective phenotype. Therefore, a number of experiments were performed in order to evaluate the activation signals that enable BMDC to induce a protective T helper [Th] 1 immune response in vivo. BMDC that were pulsed with *L. major* antigen and additionally activated by lipopolysaccharide, tumor necrosis factor-α or CD40 ligation were not capable to protect against leishmaniasis. In contrast, mice vaccinated with antigen-pulsed and CpG oligodeoxynucleotides [CpG-ODN]-activated BMDC were completely protected from leishmaniasis. Protected animals harbored a dramatically lower number of parasites at the inoculation site indicating that the clinical protection was due to an effective control of parasite replication. When the levels of cytokines expressed by lymph node cells draining the site of infection were determined, a significantly higher amount of interferon-γ and interleukin [IL]-2 was shown to be

produced by cells from mice vaccinated with CpG-activated BMDC in comparison to non-vaccinated animals. The determination of the serum levels of anti-*Leishmania* IgG1 and IgG2a antibodies confirmed that only those mice that had been vaccinated with CpG-activated BMDC developed a strong Th1 immune response upon parasite challenge.

The protection induced by CpG-activated and *L. major* antigen-pulsed BMDC was solid, as indicated by resistance to re-infection, and long-lasting, because effective protection was also observed when mice were infected 16 weeks after vaccination. This protective effect was not restricted to susceptible BALB/c mice since another strain of mice, C57BL/6, was also efficiently protected against cutaneous leishmaniasis when vaccinated with syngeneic CpG-activated and *L. major* antigen-pulsed BMDC. The route of BMDC administration was shown to be critical since intravenous but not subcutaneous or intraperitoneal injection induced complete protection. Titration experiments demonstrated that complete protection required the injection of 500,000 or more BMDC and that lower numbers induced partial or no protection. When BMDC were used to treat mice that were previously infected with parasites, no therapeutic effect was observed, indicating that once a disease-promoting Th2 immune response is initiated in BALB/c mice by *Leishmania* infection, the injection of BMDC generated according to the present protocol cannot change the outcome of the disease. Similarly, BMDC that were pulsed with the molecularly defined recombinant antigens *Leishmania* homolog of eukaryotic ribosomal elongation and initiation factor 4a [LeIF], the immunodominant antigen M15, or the trimeric protein MM6H were unable to protect BALB/c mice against lethal leishmaniasis.

Experiments conducted to analyze the mechanism of protection indicated that pulsing and maturation of BMDC modulated the expression of several cytokines, chemokines and chemokine receptors. Interestingly, among the different activators used, CpG induced the maximal expression of IL-12 by BMDC, suggesting that this cytokine may play a central role in the induction of protection. However, some experiments designed to test this hypothesis in vivo indicated that the expression of IL-12 or the related cytokine IL-23 by donor BMDC cells was not required for their ability to induce protection in BALB/c and C57BL/6 mice. Conversely, experiments performed

with recipient mice that were deficient for IL-12 expression or that were treated with neutralizing anti-IL-12 antibodies demonstrated that the expression of this cytokine by recipient cells at the time of vaccination was a critical requirement for the induction of protection. The production of IL-2 by donor BMDC was also not required in BMDC-vaccinated BALB/c and C57BL/6 mice. More surprisingly, BMDC that were deficient in the expression of MHC class I or class II molecules were still protective, suggesting an unexpected and complex mechanism.

Taken together, these findings demonstrate that the type of stimulatory signal is critical for activating the potential of BMDC to prime a Th1 response in vivo that confers complete protection against an intracellular pathogen. Furthermore, the mechanism of protection appears to be dependent on the production of IL-12 by recipient cells, but independent of the IL-12 expression by the vaccinating BMDC. Future studies designed to define the particular features of CpG activation that make BMDC protective will help to reveal the mechanism of protection and may provide additional tools for developing new strategies to combat intracellular infections.

ZUSAMMENFASSUNG

In der vorliegenden Arbeit wurde ein Protokoll zur Gewinnung dendritischer Zellen [DC] etabliert, mit deren Hilfe Mäuse wirksam vor einer Infektion mit dem intrazellulären Parasiten *Leishmanior major* geschützt werden können. Die DC wurden aus Knochenmarksstammzellen generiert, indem diese - wie bereits von anderen beschrieben - *in vitro* mit Granulozyten-Makrophagen-Kolonie-stimulierendem Faktor (GM-CSF) inkubiert wurden. Anschließend wurden die Eigenschaften dieser aus dem Knochenmark gewonnenen DC [BMDC] analysiert. Sie wiesen die DC-typische Morphologie auf und exprimierten die Oberflächenmoleküle CD11c, MHC Klasse II, CD86, CD11b und NLDC-145. Für T- oder B-Zellen spezifische Oberflächenmoleküle waren nicht nachweisbar. Die BMDC erwiesen sich als potente Antigen-präsentierende Zellen [APZ] in der Lymphozyten-Mischkultur und zeigten eine hohe Effizienz bei der Präsentation eines Leishmanien-spezifischen Peptids an ein Antigen-spezifisches T-Zellhybridom. Die Stimulation der BMDC mit verschiedenen Reifungsfaktoren bewirkte die Freisetzung von proinflammatorischen Zytokinen. Die genannten Eigenschaften ließen vermuten, dass diese BMDC als ausgezeichnete APZ zur Therapie oder zur Vorbeugung von Infektionskrankheiten eingesetzt werden könnten. Es gelang jedoch nicht, BALB/c-Mäuse durch Verabreichung der mit Leishmanienlysat beladenen BMDC vor einer Infektion mit *L. major* zu schützen. Auch verschiedene Modifikationen des Protokolls zur Gewinnung der Zellen und deren Beladung mit Antigen sowie die Erhöhung der Anzahl der injizierten Zellen befähigten die BMDC nicht zur Vermittlung von Schutz.

Der Befund, dass die Beladung der BMDC mit Antigen alleine nicht ausreichte, um eine vollständige Reifung der Zellen zu erzielen, deutete darauf hin, dass eine zusätzliche Aktivierung der Zellen *in vitro* notwendig sein könnte, um den Zellen eine schützende Wirkung zu verleihen. Deshalb wurden in weiteren Experimenten verschiedene Aktivierungssignale getestet, die BMDC zur Induktion einer protektiven T Helfer [Th] 1-Immunantwort *in vivo* befähigen sollten. Leishmanienantigen-beladene BMDC, die zusätzlich mit Lipopolysaccharid, Tumor-Nekrose-Faktor-α oder Anti-CD40-Antikörpern *in vitro* aktiviert wurden, vermochten aber ebenfalls keinen Schutz zu vermitteln. Im Gegensatz dazu führte die Impfung von Mäusen mit

BMDC, die mit Antigen beladen und zusätzlich mit CpG-Oligodesoxynukleotiden aktiviert worden waren, zu einem vollständigen Schutz vor Leishmaniose. Die geimpften Mäuse zeigten eine deutliche Verringerung der Parasitenlast am Infektionsort; dies ließ auf eine Hemmung der Parasitenvermehrung als Ursache für die verringerte klinische Symptomatik schließen. Die Analyse der Zytokinproduktion in Zellen der ableitenden Lymphknoten ergab bei den geimpften Tieren eine im Vergleich zu den Kontrolltieren signifikant höhere Menge an Interferon-γ und Interleukin [IL]-2. Der Serumspiegel der *Leishmania*-spezifischen IgG1- und IgG2a-Antikörper bestätigte, dass nur die Mäuse, die mit den Antigen-beladenen und CpG-aktivierten BMDC geimpft worden waren, bei Kontakt mit dem Parasiten eine starke Th1-Immunantwort entwickelten.

Der Infektionsschutz, der durch einmalige Gabe der aktivierten BMDC verliehen wurde, erwies sich als stabil und lang anhaltend. Dies konnte aus der Resistenz der geimpften Tiere gegen eine wiederholte Infektion sowie dem Befund, dass der Infektionschutz noch 16 Wochen nach der Impfung vorhanden war, geschlossen werden. Der Effekt einer Impfung mit Antigen-beladenen und CpG-aktivierten BMDC konnte nicht nur bei den für Leishmanieninfektionen anfälligen BALB/c-Mäusen, sondern auch bei den weniger anfälligen C57BL/6-Mäusen beobachtet werden. Als ausschlaggebend erwies sich die Applikationsroute der BMDC, da nur die intravenöse, nicht jedoch die intraperitoneale oder subkutane Verabreichung der Zellen den vollständigen Impfschutz erzeugte. Darüber hinaus musste eine Impfdosis mindestens 500.000 BMDC enthalten, um vollständig vor der Infektion zu schützen. Wurden BALB/c-Mäuse nach der Leishmanieninfektion mit BMDC behandelt, ergab sich keine Verbesserung des Krankheitsverlaufs. Verwendete man zur Beladung der BMDC die molekular definierten, rekombinanten Leishmanien-Antigene „*Leishmania* homolog of eucaryotic ribosomal elongation and initiation factor 4a [LeIF]“, immundominantes Antigen M15 oder das Trimer-Protein MM6H, so erzeugten die Zellen bei BALB/c-Mäusen auch nach Aktivierung mit CpG keinen Schutz vor der Leishmanieninfektion.

Abschließend wurde der Mechanismus, der dem BMDC-vermittelten Schutz vor der Infektion zu Grunde liegt, analysiert. Es zeigte sich, dass die Beladung von BMDC mit Antigen und die Aktivierung der Zellen durch die verschiedenen Stimuli zur

Expression von Zytokinen, Chemokinen und Chemokinrezeptoren in unterschiedlicher Menge und Art führten. Dabei erreichte die Aktivierung von BMDC mit CpG die höchste Produktion von IL-12, womit diesem Zytokin eine zentrale Rolle bei der Induktion von Schutz vor der Infektion zugeschrieben werden kann. Die im Anschluss durchgeführten Experimente zeigten jedoch, dass die Produktion von IL-12 oder dem verwandten Zytokin IL-23 in den zur Impfung eingesetzten BMDC nicht notwendig war, um BALB/c- oder C57BL/6-Mäuse vor der Infektion zu schützen. Im Gegensatz dazu erwies sich die Produktion von IL-12 in den Zellen der Empfängertiere für den Schutz als ausschlaggebend, denn in IL-12-defizienten oder mit neutralisierenden Anti-IL-12-Antikörpern behandelten Tieren ließ sich keine Protektion induzieren. Die Produktion von IL-2 in den Spenderzellen war für den Schutz nicht erforderlich. Überraschend war jedoch die Tatsache, dass BMDC, denen entweder MHC Klasse I- oder MHC Klasse II-Moleküle fehlen, dennoch vor einer Infektion zu schützen vermochten, was auf einen unerwarteten und komplexen immunologischen Mechanismus schließen lässt.

Zusammengefasst zeigen die Ergebnisse, dass die Art des stimulierenden Signals ausschlaggebend ist, um BMDC die Fähigkeit zu verleihen, *in vivo* eine gegen einen intrazellulären Erreger schützende Th1-Immunantwort zu induzieren. Darüber hinaus ist der BMDC-vermittelte Impfschutz von der IL-12-Produktion in Zellen des Impflings abhängig, nicht jedoch von der IL-12-Produktion der zur Impfung eingesetzten BMDC. Die Klärung des Mechanismus der CpG-Aktivierung, die für den von BMDC erzeugten Schutz von entscheidender Bedeutung ist, könnte zur Entwicklung von neuen Strategien der Bekämpfung intrazellulärer Krankheitserreger beitragen.

ABBREVIATIONS

Ab	Antibody
APC	Antigen-presenting cell
BMDC	Bone marrow-derived dendritic cell
BSA	Bovine serum albumin
CD40L	CD40 ligand
CL	Cutaneous leishmaniasis
CLA	Cutaneous leukocyte antigen
CMI	Cell-mediated immunity
CpG ODN	CpG-containing ODN
cpm	counts per minute
CTL	Cytotoxic T lymphocyte
cDNA	copy DNA
DC	Dendritic cell
DC-SIGN	DC-specific ICAM-3-grabbing nonintegrin
DCL	Diffuse cutaneous leishmaniasis
DNA	Deoxyribonucleic acid
DTH	Delayed-type hypersensitivity
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
Flt3	FMS-related tyrosine kinase 3
FSDC	Fetal skin-derived DC
GBP	Gene B protein
GFP	Green fluorescent protein
GM-CSF	Granulocyte/macrophage colony-stimulating factor
HSP	Heat shock protein
ICAM	Intercellular adhesion molecule
i.d.	Intradermal
IFN	Interferon
Ig	Immunoglobulin

IL	Interleukin
iNOS	inducible NOS
i.p.	Intraperitoneal
i.v.	Intravenous
LACK	*Leishmania* homolog of receptors for the activated kinase C
Lag	Langerhans cell-associated antigen
LC	Langerhans cells
LeIF	Leishmania homologue of eukaryotic ribosomal elongation and initiation factor 4a
LmAg	*L. major* antigen
LN	Lymph node
LPG	Lipophosphoglycan
LPS	Lipopolysaccharide
LTE	LACK T cell epitope
mAb	Monoclonal Ab
MCL	Mucocutaneous leishmaniasis
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
MIP	Macrophage inflammatory protein
MLR	Mixed lymphocyte reaction
mRNA	messenger RNA
MyD88	Myeloid differentiation factor-88
NF-κB	Nuclear factor kappa B
NK	Natural killer
NO	Nitric oxide
NOS	NO synthase
OD	Optical density
ODN	Oligodeoxynucleotide
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pDC	Precursor of DC
PE	Phycoerytrin

PKDL	Post kala-azar dermal leishmaniasis
PRR	Pattern recognition receptors
R	Receptor
RNA	Ribonucleic acid
RNI	Reactive nitrogen intermediates
RPA	RNAse protection assay
RT-PCR	Reverse transcriptase-PCR
RT	Room temperature
s.c.	Subcutaneous
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
TAA	Tumor-associated antigen
TCR	T cell receptor
TGF-β	Tumor growth factor-β
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
VL	Visceral leishmaniasis
WHO	World health organization

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PUBLICATIONS AND PRESENTATIONS

Publications [derived from the PhD work]:

Ramirez JR, Gilchrist K, Robledo S, Sepulveda JC, Moll H, Soldati D, Berberich C (2001) *Attenuated Toxoplasma* gondii ts-4 mutants engineered to express the *Leishmania* antigen KMP-11 elicit a specific immune response in BALB/c mice. Vaccine 20, 455-61

Berberich C, Ramirez-Pineda JR, Hambrecht C, Alber G, Skeiky YA, Moll H (2003) Dendritic Cell (DC)-based protection against an intracellular pathogen is dependent upon DC-derived IL-12 and can be induced by molecularly defined antigens. J. Immunol., 170, 3171-3179.

Ramirez-Pineda JR, Berberich C and Moll H (2003) Dendritic cells [DC] activated by CpG DNA ex vivo are potent inducers of host resistance to an intracellular pathogen that is independent of IL-12 derived from the immunizing DC. J. Immunol. [in revision].

Oral presentations, posters and meetings participation

Workshop on *Leishmania* and leishmaniasis, Bernhard Nocht Institute for tropical medicine, Hamburg, November 10-12, 2000. **Poster** presentation: Diagnosis of cutaneous leishmaniasis in Colombia: the sampling site within lesions influences the sensitivity of parasitological diagnosis.

6th Minisymposium "Infection and Immunity", Burg Rothenfels, March 9-11, 2001. **Oral** presentation: Attenuated *Toxoplasma gondii* Ts-4 mutants engineered to express the *Leishmania* antigen KMP-11 elicit a specific immune response in BALB/c mice.

3rd Berlin symposium on Immunology of infection: Dendritic cells and macrophages in infectious diseases, Berlin, May 25-27, 2001.

Meeting of SFB and Graduiertenkollegs "Infektion und Entzündung", Lübeck, June 14-16, 2001. **Poster** presentation: Attenuated *Toxoplasma gondii* Ts-4 mutants engineered to express the *Leishmania* antigen KMP-11 elicit a specific immune response in BALB/c mice.

1st joint retreat of the Graduate colleges 520 and 592 "Immunomodulation and Lymphocyte activation", Schloss Zeilitzheim, Zeilitzheim, July 5-7, 2001. **Oral** presentation: Inducing protective immune response against murine leishmaniasis by using novel antigen delivery systems.

The Cologne Spring meeting "Immunity", Cologne, March 13-15, 2002.

International conference "Immunity to pathogens: receptors, recognition, response", Erlangen, April 4-6, 2002.

6th Elsinore meeting "Induction and maintenance of the immune response to infection", Copenhagen, Denmark, May 17-21, 2002. **Poster** presentation: Avirulent *Toxoplasma gondii* Ts-4 mutants engineered to express the *Leishmania* antigen KMP-11 elicit a specific immune response in BALB/c mice.

2nd joint retreat of the DFG graduate training programs 520 and 592 "Immunomodulation and Lymphocyte activation", Schloss Zeilitzheim, Zeilitzheim, May 9-11, 2002. **Oral** presentation and poster: Dendritic cell-based vaccination against leishmaniasis: mature DC are required for the induction of complete protection.

International symposium in Honor of Prof. Dr. med. Volker ter Meulen, Würzburg, June 6-8, 2002.

7th International symposium on dendritic cells, Bamberg, September 19-24, 2002. **Poster** presentation: Dendritic cell-based vaccination against infection with an intracellular pathogen: mature DC are required for the induction of complete protection.

1st Spring workshop of Immunology of the German Society of Immunology, Leipzig, March 13-15, 2003. **Oral** presentation: CpG-activated Dendritic cells induce a protective Th1 immune response in vivo.

Graduiertenkolleg 592 "lymphocyte activation", University of Erlangen. Invited speaker to the regular seminars, Erlangen, February 13, 2003. **Talk**: Using parasite and host-derived components to induce a protective Th1 immune response against murine leishmaniasis.

34th Annual meeting of the German Society of Immunology [DGfI], FU Berlin, September 24-27, 2003. **Oral** presentation: CpG-activated dendritic cells induce a potent Th1 immune response in vivo: recipient but not donor cells-derived IL-12 is required for protection.

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Publications

Ramirez, JR, Berberich, C, Jaramillo, A, Alonso, C, and Velez, ID (1998) Molecular and antigenic characterization of the *Leishmania (viannia) panamensis* kinetoplastid membrana protein-11. Mem. Inst. Osw. Cruz, 93, 247-254.

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Ramirez-Pineda JR, Berberich C and Moll H (2003) Dendritic cells [DC] activated by CpG DNA ex vivo are potent inducers of host resistance to an intracellular pathogen that is independent of IL-12 derived from the immunizing DC. J. Immunol. [in revision].