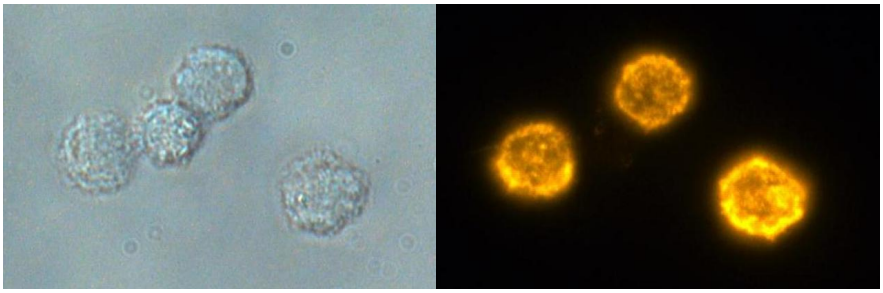
Aus dem Institut für Molekulare Infektionsbiologie
der Universität Würzburg
Vorstand: Professor Dr. Dr. h. c. mult. Jörg Hacker

The role of dendritic cells in the immunoregulation of leishmaniasis – transfection of dendritic cells with mRNA encoding a molecularly defined parasitic antigen



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Referentin:	Prof. Dr. Heidrun Moll
Koreferent:	Prof. Dr. Ulrich Vogel
Dekan:	Prof. Dr. Matthias Frosch

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Der Promovend ist Arzt.

meinen Eltern gewidmet

A-t-on le droit de dire que la science des microbes a su accomplir la plus merveilleuse des révolutions humaines?

Nous serions tentés de le prétendre si, après avoir contemplé le beau chemin parcouru, nous ne nous retournions pas pour apercevoir immense, illimitée, la rude montagne qui reste à gravir.

Elias Metchnikoff

Contents

1 Introduction

1.1 Function of DC in immunoregulation

1.1.1 The DC system

While dendritic cells (DC) had been described morphologically to reside in the skin by Langerhans in 1868, who believed them, according to their structure, to be cells of the nervous system [129], it was Aschoff who described these cells in the spleen, observed their phagocytic activity and forged the term reticulo-endothelial system for them [8, 9]. However, it was not until 1973 that DC were discovered in peripheral lymphatic organs by Steinman and Cohn who started their characterization in a series of publications [220-222, 224, 225]. This was only the beginning of a more refined way of understanding the immune response; since then, many research groups in basic science and clinical settings have focused on studying DC and accumulated an impressive amount of information.

DC are key players of both the innate and the adaptive immune system: they register the presence of pathogens, capture antigens in peripheral sites of the organism and make this information available to the immune system by migrating to lymph nodes (LN) and presenting the acquired information to T lymphocytes. They are of haematopoietic origin. Their progenitors arise from bone marrow cells positive for the cluster of differentiation molecule 34 (CD34) and colonize most tissues *in vivo* as immature non-dividing cells. Their growth and differentiation is promoted by various cytokines, such as the transmembrane proteins c-Kit ligand and FMS-like tyrosine kinase 3 (Flt-3) ligand [150], granulocyte/macrophage-colony stimulating factor (GM-CSF) and interleukin (IL)-3 [102]. Once they have settled in the peripheral tissues, they are found to have an “immature” phenotype and capture and process antigens in various ways. Upon infection with a pathogen, DC are “activated” and undergo a profound structural and functional transformation [12]: the expression of major histocompatibility complex (MHC) molecules that present pathogen peptides on their surface – forming peptide-MHC complexes – is up-regulated. A “mature” DC phenotype ensues that is characterized by efficient antigen presentation. Subsequently, DC start to migrate to lymphoid organs, the spleen and the draining LN. These surveillance and migratory

properties are indeed unique to DC, and they are not shared by any other antigen-presenting cell type such as B cells or macrophages.

Once they reach the DC-dependent areas of the lymphoid organs, they upregulate co-stimulatory molecules. Naïve T cells pass by, interact with antigen-presenting DC, seeking their cognate peptide-MHC complexes that are recognized by T cell receptors (TCR), and are being activated in an antigen-specific fashion.

1.1.2 DC subsets

There is no DC prototype. Studying DC biology is demanding because the DC population is very diverse, fulfilling many different functions in a large variety of tissues. Possibilities to classify DC are outlined in Fig. 1.

Fluorescence-associated cell sorting (FACS) analysis and detection of secreted cytokines by enzyme-linked immunosorbent assays (ELISA) have permitted to elucidate phenotypical and functional differences. Whether these divisions reflect subset specialization or functional plasticity is still unclear [269]. Moreover, due to intrinsic differences of the immune systems, a transfer of conclusions from animal models such as the mouse to human biology is not always possible.

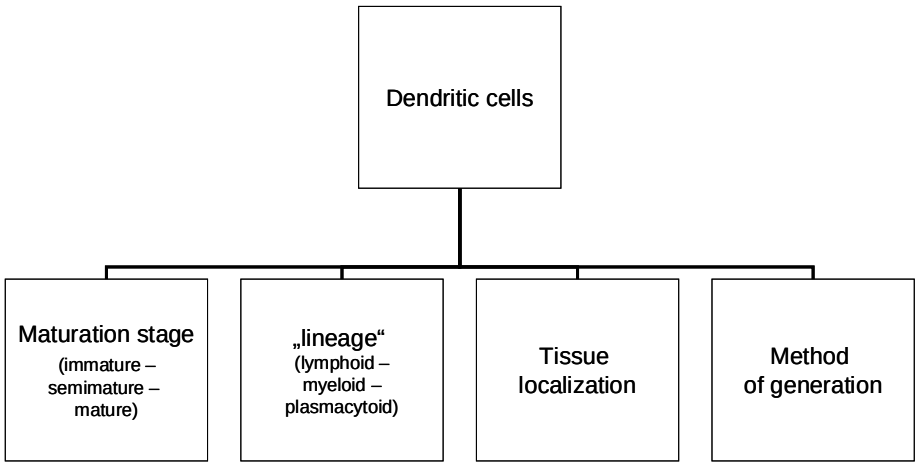

Fig. 1: Possibilities of DC classification. "DC" is a generic term, and their tissue of origin has to be kept in mind when results are interpreted. Attempts to distinguish DC subsets with the consequence to ascribe specific functions are based on different criteria, and these terms are not mutually exclusive. The maturation stage of DC refers to the functional states that DC can acquire when being activated. The assignment to different lineages relates to the progenitor cell population. DC have many different functions in different tissues, which is why their tissue localization is another important distinctive feature. The method of generation is equally important, as the properties of DC extracted from tissues are different from those of DC generated in culture from precursors.

DC spread diffusely over the entire organism; they have different functions in the blood, thymus, the secondary lymphoid organs, the liver and the skin. Specific phenotypic characteristics are associated with their localization. They are closely related, but functionally distinct cell types. Although they are believed to originate from a common progenitor cell in the bone marrow, a lineage-specific marker has not been identified; cells are currently defined by a combination of morphologic and functional properties. Murine DC are usually defined as $CD11b^+$, $CD11c^+$, $MHCII^+$ and $CD205^+$ cells [269].

Nevertheless, one important way to distinguish functionally different DC subsets is their attribution to one of three DC "lineages": Classically, "lymphoid" DC bearing the CD8α molecule were distinguished from CD8α-negative "myeloid" DC. It was shown, however, that CD8α-positive DC can develop from both lymphoid and myeloid precursors [149]. Therefore, some authors suggested the CD8α molecule simply to be a marker of DC maturation [269]. A third variant, the plasmacytoid DC, was first characterized in human blood as interferon (IFN)-γ-secreting "plasmacytoid pre-DC". They were discovered late in the mouse because they bear the B220 molecule on their surface; antibodies against B220 had been used to eliminate B cells from sample cultures [90]. Later, murine plasmacytoid DC were described as $Ly6C^+B220^+CD11c^{lo}CD4^+$ cells that produce IFN-α and IL-12 upon stimulation with viruses and CpG oligodeoxynucleotides (CpG), but not bacteria [10]. In contrast to most other DC types, they enter the LN directly from blood via high endothelial venules, not from peripheral tissues [165, 169].

Another limitation that complicates studying DC subset biology is the collection of a sufficient number of cells for experimental purposes in the mouse model: many experiments were conducted using Langerhans cell preparations from mouse ears, thymi or spleens. In 1999, Lutz *et al.* published a modified protocol for the generation of high numbers of DC at 90 to 95% purity after stimulation of murine bone marrow cultures with GM-CSF. These cells exhibit a myeloid phenotype [144]. Plasmacytoid DC are generated from bone marrow cultures under Flt-3 ligand stimulation. It has to be born in mind that experiments conducted with these cell types do not necessarily reflect "DC" properties *in situ* that can be generalized without caution.

1.1.3 Mechanisms of exogenous antigen capture by DC

DC residing in peripheral tissues, usually under epithelial surfaces and in most solid organs, exhibit an immature phenotype and are very efficient in capturing and uptake of antigens. They share this ability with their close relatives the macrophages. Classically, peptides from exogenous antigens are eventually presented via MHCII molecules. DC have different mechanisms to generate these MHC-peptide complexes that are needed for antigen presentation to T cells. Peptide loading onto MHC molecules can occur on both the intra- and extracellular side of DC.

Extracellular loading of peptides onto MHC molecules does not involve endocytosis and is only based on peptide-MHC affinity. It is a technique being widely used in experimental and clinical settings. It was also shown to be a mechanism of MHC-peptide complex formation *in vitro* and *in vivo* under certain conditions, but the contribution of peptide exchange to MHC loading at the cell surface is not thought to be very prevalent *in vivo* [249].

The primordial mechanism for assembling MHC molecules with peptides derived from exogenous antigens, however, is intracellular peptide loading: Endocytosis is followed by antigen processing, meaning conversion of exogenous antigens into immunogenic peptides that can bind to MHC molecules to be recognized by T cells. To date, three main types of endocytosis have been described in antigen-presenting cells. On top of that, some pathogens (e. g. *Toxoplasma gondii*) help to gain entry to the host cell by forming their own parasitophorous vacuole *de novo* [213].

(1) Phagocytosis is the uptake of pathogen-derived and endogenous particulate antigens. It is partly mediated by receptors, such as Fcγ-receptors, complement receptors and lectins. Immature DC avidly phagocytose apoptotic cells, microbes, inert particles, or liposomes. The ingestion of apoptotic cells is not only believed to play a vital role in sampling self-antigens in the steady state for the maintenance of tolerance [228], but also in sampling pathogens present in the engulfed cells, especially for microbes that may not infect antigen-presenting cells [131]. Yet, DC do not always wait for other cells to die before starting to sample them; in fact, they are able to physically “nibble” pieces of the cell membrane of live neighboring cells [85].

(2) Macropinocytosis represents internalization of soluble antigens from the surrounding fluids, a scenario that is of principal relevance for *in vitro* experimental

settings. It is constitutive in immature DC and macrophages. These cells also use macropinocytosis for antigen uptake after injection of soluble antigens. Its significance *in vivo* is still unclear, as most antigen encounters probably occur in tissues where extracellular fluid is not abundant. Moreover, pathogens only release few soluble antigens; most antigens are found as membrane-integrated proteins that would require uptake as particles via other endocytic mechanisms (e. g. phagocytosis) [249].

(3) Receptor-mediated endocytosis of soluble receptor-ligand complexes was found to be subdivided into at least two major mechanisms: internalization by clathrin-coated vesicles and via caveolin-containing invaginations, termed “caveolae endocytosis”. It has not been confirmed yet if DC exhibit this type of endocytosis, but there is evidence that they may exhibit a related form of endocytosis that involves neither clathrin nor caveolin [249]. Receptors mediating this type of adsorptive endocytosis include scavenger receptors, complement receptors, C-type lectins (e. g., macrophage mannose receptor, DC-specific ICAM3-grabbing nonintegrin (DC-SIGN) and DEC-205), Fcγ and Fcε-receptors. Receptor-mediated endocytosis goes along with two substantial advantages: first, it has a concentrating effect, as DC can present antigen that is found at concentrations lower by several orders of magnitude [130], achieving uptake of antigens in up to nanomolar and picomolar concentrations [202]. This sensitive detection of antigen is not only helpful, but even necessary to prevent antigen escape, as the immune system has to face three other limiting parameters: the low density of peptide-MHC complexes on the surface of tumor and infected cells, the paucity of T cell clones and a low affinity of the TCR [12]. Second, differential expression of receptors or functionally different consequences upon ligand engagement contribute to functional specificity and diversity of DC [147].

DC are not performing all three types of endocytosis constitutively; for instance, after a transient increase, macropinocytosis is down-regulated upon maturation, while clathrin-mediated uptake is still occurring in mature DC [60, 179, 267]. In other words, even though immature DC are specialized on antigen capture and uptake, and mature DC are mainly potent antigen-presenting cells, antigen uptake still continues in the mature stage.

1.1.4 Mechanisms of antigen processing

Generally, exogenous antigens captured and processed by phagocytic cells are eventually presented via MHCII molecules. Some peptides and lipids generated in the endocytic pathway, however, can be bound by MHCI-like molecules that are encoded outside the MHC: the CD1 molecules [124, 180]. Endogenous antigens, on the other hand, are mainly presented in the MHCI context. How DC handle antigens during this process called "antigen processing" is likely to be as important a determinant of immunogenicity and tolerance as is the nature of the antigens themselves [249].

In the classical understanding, the decision whether a peptide generated for presentation will be assembled with MHCI or MHCII molecules depends on its compartment of origin: antigens present in the cytosolic compartment, as it is the case in viral and certain bacterial infections, are degraded in the cytosol, loaded on MHCI molecules and presented to $CD8^+$ T cells. On the other hand, many pathogenic bacteria and some eukaryotic parasites replicate in organelles of the vesicular system where they are degraded; peptides derived from these pathogens are loaded on MHCII molecules and presented to $CD4^+$ T cells.

1.1.4.1 The classical MHCI pathway

Typically, peptides presented by MHCI molecules derive from cytosolic host proteins or from viruses that have taken over the cell's biosynthetic mechanisms to make their own proteins. Other sources are "cryptic" or alternative transcripts (resulting from alternative open reading frames), defective ribosomal products (DRiPs) and secretory and membrane proteins that are retranslocated from the endoplasmic reticulum (ER).

Most proteins are degraded unspecifically by cytosolic peptidases. For the generation of MHCI ligands, proteins need to be conjugated to ubiquitin and are degraded thereafter by a multicatalytic protease complex called the proteasome. Ubiquitination is required for efficient and rapid generation of MHCI peptides by the proteasome [72]. The proteasome is present in virtually all cell types, and its structure and function is highly conserved throughout the eukaryotes. It is interesting to see that evolution has not developed dedicated mechanisms for antigen degradation by immune cells, but chose to avail pre-existing ones [249]. Nevertheless, subunit composition of the proteasome can vary, e. g. in inflammatory conditions when IFN-γ is present, resulting in the production

of a different peptide repertoire and an increase in the production of peptides capable of binding to MHCI molecules [64, 192]. Notably, the proteasome also creates MHCI ligands by splicing peptides that are not contiguous in the original protein sequence [263]. Peptides that are generated by the proteasome are further trimmed by cytosolic peptidases [192], yet they are thought to be protected from exhaustive degradation by cytosolic chaperones [127]. Moreover, under IFN-γ stimulation, the content of certain destructive peptidases is reduced [192].

Loading on MHCI molecules occurs in the ER. MHCI molecules are heterodimers, consisting of the MHCI α chain and β_2-microglobulin. They are pre-assembled in the ER membrane where they form complexes with a variety of chaperone proteins, including calnexin, tapasin, calreticulin and Erp57. Being part of the MHCI loading machinery, they are thereby defined as ER markers. MHCI molecules are not released from the ER for transport to the cell membrane until they bind an appropriate peptide.

Peptide transport from the cytosol into the ER is mediated by heterodimeric proteins called Transporters associated with Antigen Processing (TAP1 and TAP2) that are inducible by interferons. They preferentially bind and translocate peptides with a length suitable for binding to MHC class I molecules, usually eight to nine amino acids [272], although peptides that are considerably longer may also be substrates [120]. Once inside the ER, the translocated peptide binds to an MHCI molecule that is part of a pre-assembled complex and completes its folding. Peptide-MHC complexes are subsequently exported in vesicles to the surface of the DC.

1.1.4.2 The classical MHCII pathway

Conversely, in the MHCII pathway, there are still a number of important questions remaining unanswered. In general, antigens processed in the MHCII pathway are derived from intravesicular pathogens and antigens internalized via endocytosis (see section 1.1.3).

The key question has been: which is the compartment where peptides generated in lysosomal degradation are loaded onto MHCII molecules, and how? Many authors postulated a specialized, MHCII-rich compartment that was termed MIIC [12, 168]. Yet, it has become apparent that DC rather use otherwise conventional organelles (late endosomes and lysosomes) by modifying their features – they were simply found to be

the sites to which the bulk of MHCII molecules accumulate [116].

How does the antigen reach the loading compartment? The organelles that comprise endocytosed antigen are endosomes which become increasingly acidic as they progress to the interior of the cell. They contain proteases that are activated at low pH and are responsible for degradation of intravesicular pathogens and protein antigens, cathepsins S and L being the predominant proteases. Late endosomes gradually undergo a maturation process and become lysosomes, being equipped with typical lysosomal enzymes and performing degradative functions [232]; they also have the capacity to fuse with pre-existing lysosomes [62]. In the immature stage, lysosomes are poorly degradative, due to the still high pH and the accumulation of protease inhibitors like cystatin C [249]. DC maturation lowers the lysosomal pH and thus enhances protein degradation [248].

How do the MHCII molecules reach the loading compartment? The distribution of MHCII molecules in mature and immature DC is distinct: in the immature stage, newly synthesized MHCII molecules are transported to late endosomes/lysosomes where they accumulate, whereas they are transferred to the cell membrane via non-lysosomal vesicles once the DC undergoes maturation. In confocal microscopy imaging, this property confers two impressively distinct morphologies [178].

Immediately after biosynthesis by the ribosomal apparatus, newly synthesized MHCII molecules are transferred into the ER where peptides derived from the host cell and peptides that have been actively transported into the ER are present. To prevent indiscriminate binding of these peptides, MHCII molecules are instantly assembled with a protein called the MHC class II-associated invariant chain (Ii). A part of the Ii peptide chain blocks the peptide-binding groove of MHCII molecules; its cytoplasmic domain contains a signal that diverts Ii:MHCII complexes from the secretory pathway leading to the surface and targets them to the endocytic pathway. Once MHCII and Ii are assembled in nonameric complexes, they are exported from the trans-Golgi network to acidified endocytic vesicles.

The substantial difference of the MHCII distribution in differently mature DC is probably owed to this targeting signal: upon maturation, the cytoplasmic Ii domain is degraded more efficiently due to an increase in lysosome protease activity in general [248]. So-called tubular extensions are formed from late endocytic compartments, the

"multivesicular bodies", which carry MHCII molecules to the cell surface [115].

How is the invariant chain processed and replaced with antigenic peptides? Replacement of Ii takes place in three steps, of which the first two are widely accepted: (1) In endosomes, an intravesicular portion of Ii is cleaved by an asparagine-specific endopeptidase (AEP) and other proteases [148], still leaving the cytoplasmic domain and the part binding to the MHCII binding groove intact. This fragment remains as a trimer. (2) The second step was shown to involve mainly the protease cathepsin S, but also cathepsin B and L: subsequent cleavage of Ii leaves only a short peptide still bound by the MHCII molecule, called "CLIP" (for class II-associated invariant chain peptide). (3) The exchange mechanism of CLIP against the antigenic peptide is still subject to debate. In parallel to the TAP molecules that facilitate peptide binding to MHCI molecules, an MHCII-like chaperone molecule called H-2M in mice (HLA-DM in humans) stabilizes empty MHCII molecules that would otherwise aggregate [125]. Furthermore, it catalyzes the release of CLIP and the binding of antigenic peptides, and it removes unstably bound peptides from MHCII molecules – which happens to ensure a minimum peptide-MHCII complex stability that is necessary for efficient antigen presentation [110]. Different aspects of peptide-MHCII complex formation, such as peptide binding, dissociation of Ii and enzymatic disulfide reduction by the gamma-interferon-inducible lysosomal thiol reductase (GILT) are facilitated by acidic pH [109, 176, 217].

It could not be elucidated so far whether during the process, the MHCII molecule binds directly to an appropriate antigenic peptide (the "peptide capture hypothesis") or whether the peptide is generated by cleavage after binding of the MHCII molecule to an intact antigen (the "epitope capture hypothesis"). There is evidence for both models, and they are thought not to be mutually exclusive [249].

1.1.4.3 Elusive pathways

The classical separation between the two pathways, defining distinct subcellular compartments and molecules to be responsible for the presentation of endogenous or exogenous antigens bestowed a certain beauty upon the concepts of the initiation of the immune response. This paradigm was, however, challenged: It was not able to explain experiments starting in the 1970s which showed that transplantation of allogeneic tissue

as a source of exogenous antigens resulted in a cytotoxic T cell response that was mediated by $CD8^+$ T lymphocytes recognizing peptides bound to MHCI molecules [20, 29, 70, 71]. It is now known that boundaries between both pathways can be crossed.

The presentation of exogenous antigens by MHCI is referred to as "cross-presentation", the elicitation of a $CD8^+$ T cell response towards exogenous antigen is called "cross-priming". While presentation of self-peptides on MHCI molecules is achieved by virtually all nucleated cells, cross-presentation and the capacity to elicit a cytotoxic T cell response is limited to endocytically active cells, specifically to $CD8^+$ DC [1, 47]. This difference is critical; otherwise, healthy cells would put themselves at risk to become targets for killing by $CD8^+$ T cells [1].

To explain the process of cross-presentation, the cell obviously has to offer a mechanism for egress of the endocytosed antigen into the cytosol to achieve its degradation and generation of appropriate MHCI-binding peptides. Indeed, a phagosome-to-cytosol pathway was shown to exist [122]. The generated peptides would, subsequently, have to be loaded on MHCI molecules before they could be exported to the cell surface. For a long time a hypothesis claiming that loading of MHCI molecules with peptides derived from exogenous antigens might take place in the ER after peptide translocation was prevalent. However, researchers were facing technical limitations to prove this.

In 2002 and 2003, evidence was found in macrophages [59] and DC [78, 95] that early phagosomes could be the compartments responsible for cross-presentation. The results suggested that during phagocytosis, the ER membrane fuses with the plasma membrane to form the phagocytic cup and initial phagosome; the process was hence termed "ER-mediated phagocytosis" [78]. Moreover, phagosomes were shown to contain virtually the entire MHCI loading machinery, including TAP1/2 transport across their membranes. The retrotranslocation from the phagosomal lumen to the cytosol might occur via the Sec61 pore, an otherwise ER-resident protein [95]. Interestingly, proteasomes were able to associate with the cytosolic side of phagosomes, notably upon treatment with IFN-γ [95], while ribosomes were never found on phagosome membranes [75]. These findings point to the fact that early phagosomes may hold an MHCI loading machinery similar to that of the ER, with the difference that in phagosomes it only serves for assembly of MHCI molecules with peptides generated

from exogenous antigens, not with peptides derived from newly synthesized cellular proteins. Phagosomes were thus considered as organelles that are self-sufficient for cross-presentation of exogenous antigen. The evidence, however, is contested with arguments derived from theoretical calculations and experimental observations [247].

The pathway operating in the opposite direction, describing the phenomenon of presentation of endogenous antigens on MHCII molecules, is referred to as "autophagy". Two subforms of autophagy, macroautophagy and chaperone-mediated autophagy have been characterized in mammalian cells. In macroautophagy, a cup shape isolation membrane forms from membranes of yet unknown origin [160]. The isolation membrane engulfs cytoplasmic content and upon closure forms autophagosomes with intravesicular membranes. These autophagosomes fuse with lysosomes and late endosomes [141] where they gain access to the MHCII loading apparatus. In chaperone-mediated autophagy, cytosolic proteins are delivered directly into lysosomes via the protein transporter lysosome-associated membrane protein (LAMP)-2a with the assistance of cytosolic and lysosomal chaperone proteins [2]. Notabene, up to 20% of peptides eluted from affinity-purified MHCII molecules were found to originate from cytosolic sources [160].

These current models are based on the understanding that every DC is a single actor in the immune response. Their quality as a "system", as proposed by Aschoff, is appreciated by recent findings pointing to additional pathways for MHCI loading: adjacent DC were shown to form gap junctions, consisting of the broadly expressed protein connexin 43 that is expressed under the influence of inflammatory cytokines or stimulators of the innate immune system [55]. Formation of gap junctions resulted in electrical coupling, and DC were able to exchange peptides with a relative molecular weight of up to 1,800 and eventually present them to $CD8^+$ T cells [166]. This mechanism, termed as "gap junction-mediated immunological coupling" (GMIC), provides neighboring DC with, e. g. virus-derived, antigenic peptides, thus arming them with a tool to elicit cytotoxic T cells before they are infected themselves. Although this happens at the cost of some cells that might otherwise not have been infected, GMIC is thought to help prevent the spread of infection by forming a *cordon sanitaire* surrounding the infected cell [166].

Furthermore, tunnelling nanotubules (TNT), originally described in *Drosophila*

melanogaster and named cytonemes [121], were recently also shown to establish functional continuity of plasma membranes between two adjacent DC [266]. If signaling via TNT could be relevant for the presentation of antigen to T lymphocytes will yet have to be investigated.

New challenges came up when, for experimental purposes, alternative ways of antigen administration, e. g. transfection with nucleic acids, were introduced. These will be discussed further down.

1.1.5 The process of DC maturation as the link to adaptive immunity

One of the most intriguing questions is: How does the immune system differentiate between "dangerous (or infectious) non-self" and "non-dangerous (or non-infectious) self"? In mammals, evolution has brought forth two systems that act in cooperation to achieve this differentiation: an adaptive system that is based on gene rearrangement and clonal expansion upon detection of specific antigens of an invading pathogen, and a phylogenetically older innate nonclonal system that promptly recognizes conserved molecular patterns of pathogenic structures.

The presence of pathogens is signalled by such conserved patterns of molecular structures, often on the surface of microorganisms and mostly non-protein structures like oligosaccharide or lipid residues, termed "pathogen-associated molecular patterns" (PAMP). Recognition by the host organism is mediated by germline-encoded, non-clonotypic "pattern recognition receptors" (PRR) that exist as soluble molecules or molecules bound to intra- or extracellular membranes of phagocytic cells. In the latter case, they can trigger phagocytosis or transmit signals that result in triggering responses of innate immunity or responses that eventually lead to the induction of adaptive immunity [106].

A family of receptors that appears to function exclusively as signaling receptors is evolutionarily conserved and was termed, due to the homology to Toll molecules in the fruit fly *Drosophila melanogaster*, "Toll-like receptors" (TLR). In general, signaling through these receptors results in the activation of two pathways: first, activation of the transcription factor NF-κB occurs, which acts as a master switch for inflammation, regulating the transcription of many genes that encode proteins involved in immunity and inflammation. For most TLR, this pathway is dependent on the myeloid

differentiation factor (MyD) 88 adaptor protein [113]. Secondly, mitogen-activated protein (MAP) kinases are being activated which also participate in increased transcription and regulate the stability of certain mRNAs [171].

Until now, eleven different TLR have been identified [171]. Originally, it was thought that the TLR recognition repertoire was limited to microbial homopolymers. For instance, lipoarabinomannan, peptidoglycan and lipoproteins were found to bind to TLR2 [238, 256, 274]; double-stranded viral RNA is recognized by the intracellular TLR3 [5]; lipopolysaccharide (LPS) from gram-negative bacterial cell walls, probably the best studied TLR ligand, is recognized by TLR4 [94]; unmethylated bacterial CpG DNA was shown to bind to TLR9 [89]. Some microbial structures are recognized by heterodimers consisting of TLR2 and other TLR: LPS molecules from different gram-negative bacterial species will use either TLR2 or TLR4 for signaling [184].

Later, this paradigm was challenged when it was demonstrated that certain proteins, such as the *Trypanosoma cruzi* Tc52-released protein or bacterial flagellin are recognized by receptors of the TLR family as well (TLR 2 and 5, respectively) [87, 173]. It is still subject to debate if host heat shock proteins also act as TLR ligands and initiate inflammatory responses, as a good part of the results was shown to suffer from technical limitations [251].

Upon TLR signaling, the secretion of a number of cytokines, especially IL-12 [191], but also IL-6, IL-10, tumor necrosis factor (TNF) α [128] and chemokines [203], is initiated, depending on the TLR ligand. At the same time, nuclear factor-κB (NF-κB) activation induces the expression of co-stimulatory molecules such as CD80 and CD86 [104] and upregulation of MHC expression [103] to various extents. Hence, even though there are TLR ligand-dependent differences in the ultimate gene expression profile that result in different cytokine secretion patterns [171], these properties serve as appropriate markers for DC maturation in experimental settings.

Classically, T cells need two signals before they can proliferate and act as effector T cells: being part of the adaptive immune system, they (i) have to recognize peptides presented on MHC complexes on the surface of antigen-presenting cells, like DC, by their membrane-bound TCR. Additionally, as an unspecific signal, (ii) interaction of the T cell surface molecule CD28 with the B7 co-stimulatory molecules CD80 or CD86 is required, while absence of co-stimulatory molecules leads to T cell anergy or tolerance

in many settings [74]. DC are by far the most potent activators of T cells: only small numbers and low levels of antigen are needed to induce strong T cell responses [12]. The (iii) secretion of cytokines is critical for the polarization of the T cell response, thus providing a third signal. Only recently has it become apparent that the induction of specific cytokine profiles is actually dependent on the TLR that is involved in signaling. In other words, the type of TLR ligand is critical for the polarization of T cell differentiation [185].

This "polarization" of the T cell response is a differentiation process of $CD4^+$ T cells into two types of T helper (TH) cells, termed TH1 and TH2 cells. TH1 cells are mainly involved in the establishment of cell-mediated immunity. Their development is marked by the production of IFN-γ that enhances the microbicidal properties of macrophages, but also by the induction of B lymphocytes to produce IgG antibodies that have a role in the opsonization of pathogens for uptake by phagocytes. IFN-γ is the cytokine dominating TH1 responses. TH2 cells, on the other hand, are more involved in humoral immunity: they activate naïve antigen-specific B cells to produce IgM antibodies and are subsequently able to induce an isotype switch to IgA or IgE responses. TH2-related cytokines are IL-4, IL-5 and IL-10. Ultimately, the response represents a bias towards one end of the TH1/TH2 spectrum, rather than a canonical TH1 or TH2 response [48]. Infections by intracellular pathogens are predominantly related to TH1 responses, while TH2 responses are more common in diseases caused by extracellular pathogens, parasitic infections and hypersensitivity reactions. Generally, TLR3, TLR4, TLR5 and TLR9, by the release of IL-12, are known as TH1-inducers, while TLR2, via IL-12 suppression and IL-10 release, induces a skewing towards TH2 development [167].

The discrimination between dangerous non-self and non-dangerous self is thus mainly achieved by TLR in phagocytic cells like DC; they register the presence of microbial pathogens. DC subsequently undergo a process called maturation and are able to process and present foreign antigen to specific T cells. By secretion of cytokines, they polarize T helper cells that proliferate and propagate an inflammatory response.

1.1.6 Immunity vs. tolerance

The immune system is a balanced entity. Its principal function is to avoid both self-reactivity and pathogen escape, i. e. leave the body's own tissues unharmed and try to eliminate aggressors. One of the indispensable requirements to achieve this is the generation of a lymphocyte reservoir that has been "educated" to recognize what is "self" and what is "non-self".

Although all lymphocytes are derived from bone marrow precursors, the location of lymphopoiesis, the development of new lymphocytes, varies: B cells develop in the bone marrow, while T cells are generated in the thymus. These cell types relate to different functions of the immune system. Humoral immunity is the aspect of immunity that is mediated by secreted antibodies, produced by cells of the B cell lineage after their differentiation into plasma cells. T cells, on the other hand, are key actors in cell-mediated immunity. In leishmaniasis, their response to leishmanial antigens presented by DC – which has been studied in the present work – is critical for the outcome of the infection. Therefore, this section focuses on the role of T cells in the immune response.

T cell lymphopoiesis results in a state of immunological tolerance to ubiquitous self antigens. This is accomplished in two steps: Precursors with randomly arranged TCR first receive a survival signal from thymic epithelial cells (positive selection) when they are able to recognize the body's own MHC molecules – cells that are unable to recognize self-MHC do not survive. In a second step, lymphocytes that bind strongly to self antigens on self MHC molecules receive a signal that leads to their death; they might initiate detrimental autoimmune reactions and are hence removed from the repertoire ("negative selection"). By this mechanism, non-reactivity to self-antigens is established; this state was termed "central tolerance".

Once mature T lymphocytes that survived in the thymus have started to circulate in the body, they are in a continuous dialogue with antigen-presenting cells. The nature of the dialogue determines the outcome of the encounter; it involves both specific ligand and co-stimulatory signals. If an MHC-peptide complex is not recognized by the T cell, nothing happens; if it is, there are two possible consequences – activation, proliferation and differentiation vs. anergy or induction of tolerance.

Co-stimulation was found to be the culprit: as was discussed above, DC are only activated to express the co-stimulatory B7 molecules (CD80 and CD86) on detecting

the presence of infection through receptors of the innate immune system, e. g. TLR. Expression of B7 molecules by the same DC that presents the antigen is required for clonal T cell expansion. Binding of B7 to CD28 on the surface of T cells leads to IL-2 production and expression of a high affinity IL-2 receptor [7]. Upon T cell activation, other surface molecules including CD40-ligand, CTLA-4 and ICOS are expressed by the T cell and modify the T cell response [91]. After 4-5 days of proliferation under IL-2 influence, T cells differentiate into "armed effector T cells" that have specialized functions as cytotoxic or helper T cells. Once they have reached this stage, they do not require co-stimulation any more whenever they encounter their specific antigen [107]. A model for T cell activation is depicted in Fig. 2.

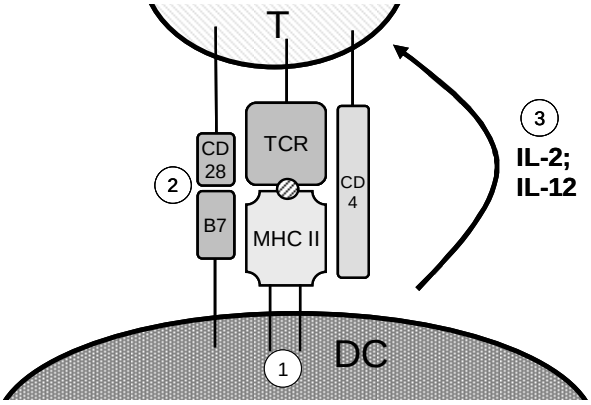

Fig. 2: T cell activation. Three signals are required before T cells are activated to proliferate: 1) antigen presentation, 2) co-stimulation, 3) cytokines that determine the polarization of the T cell response.

Thus, the activation of naïve T cells – the immune system's ambition to establish immunity – is dependent on co-stimulation. This is important, as during lymphopoiesis in the thymus, not all potentially self-reactive T cells are eliminated; especially those with a lower affinity for self antigens escape deletion [25]. Self-tolerance could thus be broken if antigen presentation was the only prerequisite for activation.

This mechanism is of use in vaccination: most antigens administered as vaccines do not by themselves possess the ability to confer a signal that encourages the immune system to establish a protective immune response. If a stimulus for DC maturation is coadministered, upregulation of co-stimulatory molecules can be achieved in the presence of the antigen, importantly: by the same cells that present the antigen. Maturation stimuli may thus serve as adjuvants, and the individual is more prone to develop immunity against the antigen. Indeed, most agents used as adjuvants have TLR

stimulating activities [167].

Central tolerance may be efficient, but it is also incomplete. Another mechanism has evolved that helps to ensure the tolerance of T cells to self tissue antigens in the periphery; it is referred to as “peripheral tolerance”. When T cells encounter their specific MHC-peptide complex in the absence of co-stimulation, they not only fail to be activated: they become refractory, incapable to be activated when specific antigen is subsequently presented to them by a professional antigen-presenting cell. This results in a state of anergy which is thought to be a reversible form of tolerance [223].

In experimental settings, tolerance could be induced in a number of different ways. It was shown that it is not merely the presence or absence of typical maturation stimuli that determines if the DC is tolerogenic or immunogenic. Antigens that were targeted in low doses to the DC surface receptor DEC-205 by conjugation to or engineering into an anti-DEC-205 antibody induced tolerance [23, 86]. Likewise, in the steady state, DC are able to acquire antigen from different types of apoptotic somatic cells, e. g. pancreatic islet β cell antigens or the proton pump ATPase of gastric parietal cells [100, 208]. This mechanism may explain the immune system’s tolerance to tumors: with DC being unable to sample antigens from tumors that fail to undergo apoptosis, a tumor is recognized as self, induction of tolerance ensues and the tumor is left unharmed by T lymphocytes.

If DC have both the capacity to take up self antigen from apoptotic cells and are also able to induce immunity upon encounter of a foreign pathogen with the help of MHCII, co-stimulatory molecules and cytokine production, one central question arises: should DC that receive a maturation stimulus not be expected to upregulate presentation of self antigens they have sampled before a subsequent infection, and thereby cause autoimmunity on a regular basis? What is done to prevent this? It is postulated that, here, the induction of peripheral tolerance is of crucial importance: immature DC are thought to induce antigen-specific peripheral tolerance in the steady state, before maturation during infection or under inflammatory conditions. This way, self-reactive T cells are silenced or deleted and are prevented from elicitation of detrimental immune reactions during infections [226].

1.1.7 Immature, semi-mature and mature DC

Certain aspects of the tolerance phenomenon cannot be fully explained with the classical model introduced above. The tolerogenic DC population seemed to comprise a second – so to say intermediate – subtype that was termed "semi-mature", also referred to as "partially mature" DC. They were found to be the "missing link" between the immature, antigen-sampling DC that reside in peripheral tissues, and the potent antigen-presenting DC that were found in T cell-dependent areas of lymphatic tissues.

DC migration does not only occur in inflammatory conditions, but also in the "steady state"; there is a homeostatic turnover of DC in the skin and in secondary lymphoid organs [135, 199]. These migratory DC are found in lymphatic vessels and represent a cell population that seems to be derived from immature DC. They contain apoptotic material and are loaded with antigens from their sentinel tissue. This uptake of dying cells is remarkably selective for the $CD8^+$ subset of splenic DC [105]. Morphologically and phenotypically, they exhibit dramatic differences in comparison to tissue-resident DC: anchor receptors (e. g. E-cadherin) are down-regulated, whereas expression of chemokine receptors (e. g. CCR7) and matrix metalloproteinases is induced. Thereby, a phenotype that is predestined for migratory functions is established [188].

Antigen processing and tolerogenic cross-presentation of apoptotic material by DC was shown to require at least some degree of maturation. These tolerogenic DC, however, still differ from mature DC, notably by their lack of cytokine producing ability: secretion of IL-12 or other proinflammatory cytokines cannot be detected. The maturation process of these cells seems to have arrested at a "semi-mature" stage [145]. Production of IL-2 by DC was found to be necessary to induce T cell priming [73]. The ability of DC to produce cytokines might thus represent a switch from tolerance to immunity.

The phenotype of these tolerogenic semi-mature DC was found to be $MHCII^{high}$, co-$stimulation^{high}$, $cytokines^{low}$. Taking into consideration that they exhibit both MHCII and co-stimulatory molecules at a level that has been thought to be sufficient to initiate primary immune responses and lead to immunity, these cells questioned the established models on T cell activation. Under the influence of TNF-α, semi-mature DC were shown to stimulate IL-10-producing $CD4^+$ regulatory T cells [4, 152, 153]. Upon further stimulation with maturation inducers, e. g. LPS, they are still able to reach the state of

terminal differentiation as fully mature DC [145].

It has even been shown recently that the same semi-mature DC type is able to assume both tolerizing and immunogenic properties at the same time, depending on the MHC context in which peptides are presented [117]. Thus, semi-mature DC can assume an important role in the regulation of the immune response towards self and non-self antigens.

These concepts led researchers to postulate a new three stage maturation model for the role of DC in the control of immunity, introducing semi-mature DC, a tolerogenic cell population with migratory function in the steady state, as the link between immature and mature DC.

1.2 DC as adjuvants for vaccination

Since the revolutionary trials on smallpox by Jenner in the 1790s and on rabies, anthrax and chicken cholera by Pasteur in the 1880s, vaccination has become an extremely powerful medical tool, saving the lives of many million people annually. For the best of the 20th century, scientists have attempted to understand the underlying causes of infectious diseases and improve vaccination techniques.

As it was demonstrated, DC represent a key pressed very early on the keyboard of the immune response. Their pivotal role in immunoregulation is appreciated in the term “nature’s adjuvants” which was forged by Steinman [12]. Many of the classical vaccination techniques using live attenuated organisms, including measles, mumps and Sabin polio vaccines, may have unintentionally taken advantage of this adjuvant function. The critical property that is exploited in vaccination is the capacity of DC to elicit strong primary T cell responses, which is necessary to establish an immunological memory by the formation of memory T cells and antibody-producing B cells.

Not every pathogen can be attenuated and used as an effective and safe vaccine. With the knowledge emerging from basic immunological research, acellular vaccines could be developed (e. g. against Hepatitis B infection). In some diseases, it is not primarily the interaction between the pathogen and the host organism, but the action of a toxin that determines the clinical outcome, as in infections caused by *Clostridium tetani*, *Bordetella pertussis* and *Corynebacterium diphtheriae*. Attenuated variants of these toxins, called toxoids, are usually not immunogenic on their own. In the absence of an

adjuvant that would be able to act on DC, the administration of a toxoid does therefore not elicit a sufficient level of immunity. To solve this problem, an interesting approach was undertaken for the development of a vaccine against tetanus and diphtheria: tetanus and diphtheria toxoids were mixed with pertussis toxin which has adjuvant properties in its own right. Thus, pertussis toxin not only vaccinates against whooping cough, but also acts as an adjuvant for the other two toxoids [79].

DC cannot not only be targeted indirectly by application of adjuvants, they can also be isolated directly from animals and humans, loaded with antigens *ex vivo* and used to elicit protective or therapeutic effects in several malignant and infectious diseases. Malignancies for which adjuvant immunotherapeutic function of DC was assessed include breast, renal cell and prostate carcinoma, melanoma and certain lymphoproliferative diseases. Some studies have advanced to phase III clinical trials [52, 205].

So far, immune interventions utilizing DC in clinical settings are confined to the field of tumor therapy. The role of DC as anti-infective vaccines is a relatively new field of research, but has yielded important insights in the mechanisms of immunoregulation.

1.2.1 Targeting of DC *in vivo*

As DC are spread over the entire organism, even if their immunostimulatory action is mainly carried out in the lymphatic tissues, it is difficult to target them with respect to their localization. Therefore, some vaccines have been designed to exhibit a specific property that can be recognized selectively by DC – to prevent “misuse” by other cell types. Furthermore, despite the fact that DC are the main cell population that is responsible for antigen uptake after injection of antigen, efficiency of antigen delivery and the quality of antigen processing are important parameters that may be influenced favorably by appropriate targeting [227].

Based on these prerequisites, experiments were conducted using antigens targeted to endocytic receptors that are specifically expressed on DC, e. g. DEC-205, either by conjugation to anti-DEC-205 antibodies or by specifically engineered antibodies [23, 147]. As another novel approach, transcriptional targeting was introduced: high expression levels of the actin-bundling protein fascin and its selective expression in mature DC had been observed. This encouraged researchers to construct a DNA expression vector containing the sequence of the enhanced green fluorescent protein

(EGFP) under the control of the fascin promoter. DNA vaccinations of mice using a gene gun consistently resulted in selective EGFP expression by Langerhans cells [198]. DC targeting *in vivo*, however, has not yet advanced into clinical settings.

1.2.2 Antigen loading of DC *ex vivo* and reinjection

The direct application of DC, taking advantage of their adjuvant effect in vaccination, has been studied more extensively in the recent past and, at least in tumor vaccination, reached the stage of clinical trials. The protocols are simple: isolating and enriching DC cultures *ex vivo* is followed by antigen administration, with various possible modes of administration. Eventually, the DC are reinjected into the donor (in humans) or into a syngeneic organism (in mice). Again, the desired effect is the elicitation of a T cell response. This approach has become even more appealing when methods for generation of larger amounts of DC from bone marrow or blood precursors became available.

1.2.2.1 DC stimulated by exogenous pulsing

With respect to the fact that for antitumor immunity, a cytotoxic $CD8^+$ T cell response is required, and the mechanisms of cross-presentation were largely unknown until the recent past, exogenous antigen delivery for DC manipulation – resulting in predominant MHCII presentation – played a limited role in tumor models. Still, loading of immunodominant antigen-derived or synthetic peptides on MHCI molecules of DC is feasible, and DC pulsed this way were indeed used for vaccinations against malignant diseases. Later, dead allogeneic tumor cells or exosomes were utilized as sources of antigens [11].

At the same time, DC were shown to confer protective immunity against pathogen infections in murine models when pulsed with attenuated or nonviable microorganisms, such as against *Mycobacterium tuberculosis* [46] and *Chlamydia trachomatis* [234] infections. Other approaches favored loading of DC with peptides from defined antigens; e. g., a gp63-derived peptide was able to confer protection against cutaneous leishmaniasis [250]. In a study involving cutaneous Langerhans cells as vaccine carriers it was shown that for *ex vivo* pulsing, DC do not need to engulf an intact organism; they are rather able to utilize an antigen cocktail derived from lysed *Leishmania major* promastigotes to confer protective immunity against *L. major* infection [58].

The development of subunit vaccines, for reasons of safety, efficacy and easy applicability, has always been desirable. In systemic application, recombinant antigens have been proven protective in various infectious diseases [33, 268]. While for systemic application, subunit- and multi-subunit vaccines were shown to confer at least partial protection against *L. major* infection [39, 45, 214], one study revealed that this effect was also attained when Langerhans cells were isolated and pulsed with single, molecularly defined recombinant antigens [17]. Among other *Leishmania* antigens, LeIF (*Leishmania* homologue of eukaryotic ribosomal initiation factor 4a) was shown to be particularly apt, owing to its property to bridge the innate and adaptive immune system by both acting as an adjuvant – inducing IL-12 secretion – and delivering peptide epitopes to generate LeIF-specific TH1 cells [17].

1.2.2.2 Transfection of DC

Exogenous antigen pulsing does not account for certain vaccination requirements: Peptide loading is dependent on the individual's human leukocyte antigen (HLA) haplotype; protein purifications may be costly and time-consuming; finally, due to the lack of knowledge about the mechanisms of cross-presentation, the question of how $CD8^+$ T cell responses to exogenously obtained antigen could be elicited reliably was not solved for a long time. A technique overcoming these limitations would have to gain access to the MHCI presentation pathway, either by endogenous synthesis or direct introduction of the antigen into the cytosol.

An appealing approach to address this problem is by viral transduction. Besides retroviral transduction that was described first [16, 189], other viruses including vaccinia virus, adenovirus, herpes virus, lentivirus and influenza virus were manipulated to encode antigen sequences [reviewed in 108]. Although the transduction rate was remarkably high in some cases, certain disadvantages have to be kept in mind: the virus-host interaction sometimes may lead to the inhibition of maturation, and some viruses down-regulate the level of co-stimulatory molecules in DC, both of which, while being a central mechanism to escape the host immune response, reduce their immunostimulatory capacity. Furthermore, some viruses have a cytopathic effect on their host cell which causes a decreased viability of DC. While initial results were promising, many viral immunomodulatory effects on DC have been elucidated from

these studies which were soon deemed to limit the use of viruses for DC-based immune intervention against tumors and most infections [101, 239]. However, many data still suggest that virally transduced DC may be useful as vaccines in viral infections [275], and some authors still postulate a role for certain viruses, e. g. lentivirus, in cancer therapy [51].

Another strategy to manipulate DC genetically is to inject nucleic acids into the organism. In the first nucleic acid-based vaccine studies in the early 1990s, plasmid DNA encoding antigen was injected intramuscularly. The studies were based on two assumptions: first, the plasmid would be delivered to local muscle cells which would express and release the encoded protein. After uptake and presentation of the protein via MHCII by antigen-presenting cells, an immune response was expected to be elicited. Second, the plasmid would be taken up by antigen-presenting cells that would express and present it directly via MHCI [240, 255]. It was shown that the immunogenicity of a DNA vaccine correlated with the number of DC containing the plasmid; this number was typically very low (< 1%). Indeed, immune responses of both $CD4^+$ and $CD8^+$ T cells were reported [32, 40].

Direct transfection of DC had been limited for a long time by their scarcity in the organism and the lack of an efficient method for expansion. In the late 1990s, studies evaluating systematically different gene transfer methods for manipulation of human DC – that could be generated more easily in sufficient numbers than murine DC – were published. First results did not favor physical methods in general [6], but delivery of plasmid DNA by electroporation was soon shown be superior to other techniques using liposomes, non-liposomal reagents or calcium phosphate [260].

The efficiency of DNA transfection, however, is not overwhelming. It is reasoned that there are too many steps involved before the encoded protein reaches the cytosol: DNA needs to penetrate the cell membrane and the nuclear envelopment until it reaches the transcriptional machinery in the nucleus. The permeability of the nuclear membrane is low in most primary cells, especially postmitotic cells, and therefore represents a fundamental limitation to gene expression [28]. Subsequently, the resultant RNA has to be transported into the cytoplasm in a form that can be translated [27]. As it was shown in cell line studies, this latter step also represents a limiting parameter for the efficiency of the technique [252].

Furthermore, DNA transfection of DC may lead to unspecific (i.e. antigen-independent) stimulation of cytotoxic T cells which, especially for vaccination, constitutes an unacceptable adverse immunological property. This effect was attributed to possible DC activation by CpG DNA motifs [259].

Another elegant way to target antigen to the cytosolic pathway is to deliver RNA to DC. While a number of trials focused on RNA application *in vivo*, on self-replicating RNA vaccines and on targeting RNA to DC via certain receptors [reviewed in 27], a novel technique – delivering RNA to DC *ex vivo* by electroporation – was pioneered by Boczkowski *et al.* in 1996 and has since greatly influenced the field of DC research [21]. It is based on the assumption that RNA is directly transferred to the cytosol where it is able to bind to ribosomes and initiate translation of the encoded protein. Once the RNA-encoded protein is available intracellularly, it is subjected to the same processing and presentation mechanisms as is cell-derived protein.

Indeed, both the efficiency of RNA transfection [259] and the capacity of RNA-transfected DC to stimulate cytotoxic T cells (CTL) [233] are superior compared to DNA transfection. Unanimously, electroporation of DC has been shown to be the more efficient method of RNA delivery, compared to lipofection [112, 258].

Electroporation of DC with RNA is a very attractive technique and has been favored recently as a preferable method of antigen delivery, not only because it circumvents the limitations imposed by peptide loading (MHC restriction) or by the complicated laboratory infrastructure needed for viral transduction. Furthermore, there is no unspecific stimulation of CTL as observed in DNA transfection. Most importantly, RNA transfection of DC is a very safe technique, as RNA, unlike DNA, cannot be integrated into the host cell's DNA – whereby many adverse effects, from gene dysregulation to malignant degeneration, can be avoided.

It has to be kept in mind that stable transfection in terms of maximal duration of antigen expression is not the goal of nucleic acid-based DC transfection. The intention is to generate a potent T cell response. To that end, transient intracellular expression is sufficient to generate MHC-peptide complexes that are stable and that can recirculate from the interior of the cell to the surface.

Electroporation of mammalian cells is not restricted to nucleic acids; cell membranes can be permeabilized for other macromolecules like proteins as well: it was shown in

several studies that soluble proteins can access the MHCI presentation pathway when delivered to the interior of the cell via electroporation [37, 84]. One group was able to demonstrate that delivery to the MHCII pathway occurs as well [140].

1.3 A note on electroporation

Electroporation is a term used for transient permeabilization of the cell membrane that allows exogenous molecules to enter the cell. It requires the application of an external electric field on a cell suspension.

While transfer of proteins and nucleic acids by electroporation is a technique widely used in molecular biology, on one hand for generation of transgenic microorganisms, on the other hand in the field of genetic manipulation of mammalian cells or tissues *in vitro* or *in vivo*, even in clinical settings, its molecular mechanisms remain poorly understood. What is certain is that molecules do not gain access to the cytosol through a simple "hole" in the cell membrane that is formed by a sliding door-like closed-open-closed mechanism. Electroporation has rather challenged the classical models of membrane biology. First, it has to be kept in mind that the effects of electroporation are not confined to the short duration of the electrical pulse. Electroporation is rather a procedure that subjects the cells to a sudden stress situation with profound effects on the membrane structure which is followed by complex reorganization.

Electroinduced permeabilization of the cell membrane only occurs in cell membrane regions where the transmembrane electric potential difference exceeds a threshold value of about 200 mV [243]. The potential difference induced by an external electric field in a cell at a given point M of the cell membrane is dependent on the membrane conductance factor $g(\lambda)$, the shape factor f (a cell being not an ideal sphere, but a spheroid), the cell radius r, the field intensity E and the angle θ between the electric field vector and the membrane normal vector at the point M, described by the equation [242]:

$$\Delta V_M = f\, g(\lambda)\, r\, E \cos\theta$$

Five steps have been postulated to describe electroporation:

(1) Induction or trigger step: Once the increase in the potential difference reaches the critical value of 200 mV, membrane leakage can be detected within less than a

microsecond after the onset of the pulse. During this phase, a mechanical stress is exerted on the cell membrane.

(2) Expansion step: As long as the electric field is maintained at an overcritical value, the size of the cell membrane's permeabilized part does not change, but the density of defects increases.

(3) Stabilization: As soon as the external field is below the critical permeabilizing threshold, there is a fast decrease of conductance in the permeabilized part of the cell surface within milliseconds; the membrane organization recovers dramatically. Still, the cell membrane remains slightly leaky to polar compounds, albeit their flow also decreases strongly.

(4) Resealing: Annihilation of the membrane permeability takes place within a time scale of seconds to minutes. Resealing kinetics is dependent on physical strains like hydraulic stress [276] and an intact organization of the cytoskeleton [195]; moreover, at low temperatures, the permeabilized state can be maintained for several hours [143]. Thus, a cellular response is thought to influence the quality and the kinetics of resealing.

(5) Memory: Although cell viability may not be strongly affected, electroporation still induces certain cellular alterations from which cells need to recover on a time scale of several hours: asymmetrical phospholipid distribution in the plasma membrane [82] and the establishment of macropinocytosis of otherwise non-endocytic cells can be observed [194].

In general, the cell membrane is permeable for exogenous molecules during the phases 1 through 3, while the membrane potential difference is beyond the threshold of 200 mV. Depending on the specific experimental conditions and the type of molecule, a slightly permeable state may be maintained in the aftermath during phase 4. Afterwards, macropinocytosis may be induced.

Molecular descriptions of membrane electropermeabilization still remain highly speculative to date. Presumably, membrane disruption during electropulsation does not cause the formation of a toroidal pore as suggested originally, but rather a structureless defect. Theoretical models account for field-induced alterations of membrane lipids and proteins, postulating three different protein and four different lipid states, but these models still await experimental validation [242].

These mechanisms hold only true for small molecules of up to 4 kD. The transfer of macromolecules to the interior of the cell follows a slightly different mechanism. Currently, the largest quantity of information is available on DNA transfection, and it remains to be seen if the conclusions can be extended to other macromolecules such as RNA or proteins. As the mechanisms are different from "electropermeabilization", DNA electroporation is referred to as "electrotransfection".

Results from systematic studies suggested that DNA electroporation occurs in a multi-step process: During the pulse, (1) cells are electropermeabilized. (2) The polyanion DNA is electrophoretically attracted to the positive pole and driven into contact with the cell surface, whereupon (3) a metastable DNA-membrane complex is formed. The pulse duration needs to be at least 1 ms to allow plasmid-cell complex formation. (4) After the pulse, plasmids leave the complex and are released into the cytoplasm, while this step involves active participation by the cell, indicated by its ATP dependence. (5) A small fraction of DNA molecules crosses the nuclear envelope to be expressed [66].

While small molecules diffuse into the cytoplasm over the permeabilized area of the cell membrane, uptake of DNA is not a result of diffusion [65]. Its exact nature has not yet been elucidated, but it is dependent on electrophoretic forces [235]. Moreover, presence of DNA even facilitates pore formation through direct interaction with the membrane [218].

Importantly, macromolecules like DNA do not simply cross the cell membrane during electropulsation. This was concluded from experiments demonstrating that hyperosmolarity of post-pulse medium [65], low temperature of post-pulse incubation [193] and the presence of DNAseI (which is known not to cross the membrane) in the medium up to 60 s after the pulse [56] reduced transfection efficiency.

Detailed studies showed that the formation of the DNA-membrane complex is the critical step in electrotransfection: although DNA does not diffuse through the pores, it needs to be "trapped" in the permeabilized part of the membrane; moreover, the complex has a certain stability [66]. The crossing and release to the cytosol happens within the minute following the electropulsation.

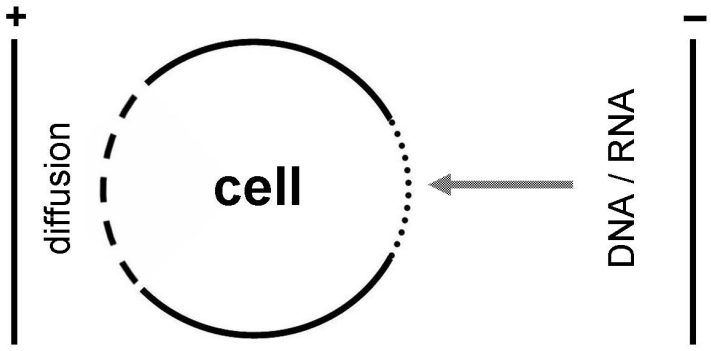

Fig. 3: Electroporation and macromolecule transfer. Initially, permeabilization will happen at the cell pole facing the positive electrode where the membrane capacitance is first exceeded when an external field is applied. Subsequently, permeabilization of the cell pole facing the negative electrode occurs. While the membrane area that is permeabilized is larger on the cell pole facing the positive electrode, the degree of permeabilization is greater on the opposite side facing the negative electrode. This has consequences for the flux of molecules over the cell membrane: diffusion of smaller molecules is predominant by the positive cell pole; the entry of larger molecules occurs preferentially at the pole facing the negative electrode [244]. Macromolecules like DNA (and presumably also RNA), being polyanions, are driven by electrophoretic forces to travel towards the positive pole, and indeed enter the cell via the pole facing the negative electrode, as was shown by fluorescence studies [67].

When DNA is added after the pulse, no plasmids cross the membrane. Electric field parameters critical for membrane permeabilization are the field intensity, the number and the duration of pulses: the area that is (prone to be) permeabilized increases with the field intensity, while with a constant electric field intensity value, but increasing values of pulse numbers and/or pulse duration, the density of transient permeated structures (permeabilization) in that area increases [196].

A model for the mechanisms of macromolecule transfer into mammalian cells is depicted in Fig. 3. Electroporation experiments with recombinant RNA in the present study were carried out at 300 V and 150 µF which had been shown to result in efficient transfection of DC before [258, 259].

1.4 Leishmaniasis – a parasitic infection

1.4.1 The disease

Leishmaniasis is a collective term that is used to describe a range of closely related diseases caused by parasites of the genus *Leishmania*; they occur in humans and different rodent species and are transmitted by adult female phlebotomine sandflies.

Leishmaniases are divided into three main clinical forms: cutaneous, mucocutaneous and visceral leishmaniasis. Some forms have been reported since antiquity, e. g. on pre-

Inca pottery from Ecuador and Peru and in Egyptian and Assyrian papyri. The first relevant clinical description of cutaneous leishmaniasis was given in 1756 by the British physician Alexander Russell who travelled to Syria and termed the disease "Aleppo boil". From the late 1880s, microscopic observations of biopsies taken from skin lesions of patients suffering from the "oriental sore" were made, and the causative agent was thought to be an intracellular parasite. In 1898, the Russian physician Borovsky was the first to recognize correctly that it was a protozoon. In 1900, Sir William Leishman examined spleen biopsies from an Irish soldier suffering from "Dum-Dum fever" and found intracellular corpuscles he thought to be *Trypanosoma*; similar corpuscles were described by Charles Donovan in 1903. Both observations were published in 1903 [49, 136]. The new species, originally referred to as "Leishman-Donovan bodies", was later termed *Leishmania donovani*. The taxonomic classification is depicted in Fig. 4.

Scientific classification	
Kingdom	Protista
Subkingdom	Protozoa
Phylum	Sarcomastigophora
Class	Zoomastigophorea
Order	Kinetoplastida
Family	Trypanosomatidae
Genus	*Leishmania*

Fig. 4: Scientific classification of *Leishmania* [43].

Leishmania parasites develop along a life cycle. They are prevalent in two groups of hosts: in mammals, including humans and other hosts (e. g., dogs, rodents, sloths etc.), where they develop from the promastigote to the amastigote form, and in the intestine of phlebotomine sandflies where they develop from the amastigote to the promastigote form. The only insect vectors for *Leishmania* parasites are *Phlebotomus* spp. – which occur only in the Old World – and *Lutzomyia* spp. – which are found only in the New World, especially in the forested areas of Central and South America.

After an infective blood meal, the parasites reside and multiply in the intestine of

sandflies. They develop a flagellum while being attached to the gut wall. Once they have developed into the metacyclic stage (with a size of 10 – 25 µm), which happens within about 4 to 12 days after the uptake, they are found in the anterior mouth parts. From here, they are regurgitated and inoculated into a new host during another blood meal.

Once they have entered the host organism, they primarily infect cells of the mononuclear phagocytic system like macrophages and Langerhans cells; rarely, fibroblasts are infected. Here, they transform into the amastigote form and multiply within vacuoles (phagolysosomes) by continuous dichotomy. Eventually, the host cell is destroyed; the amastigotes are released and can infect neighboring cells.

Cutaneous leishmaniasis (CL) of the Old World is caused by *L. tropica*, *L. major* and *L. aethiopica*; here, only the skin is affected. Within weeks to months, papules, nodules and ulcerations develop next to the puncture. These lesions usually resolve spontaneously and are associated with subsequent lifelong immunity. In contrast, CL of the New World is caused by parasites from the *L. mexicana* and *L. braziliensis* complexes. Lesions caused by *L. mexicana* are similar to CL of the Old World. Conversely, *L. braziliensis* disseminates into body areas distant from the puncture and may also affect the mucosa of e. g. the nasopharyngeal space; these lesions resolve less easily and may cause considerable disfiguration.

Visceral leishmaniasis (VL) is the disease that was also called “Dum-Dum fever” by Charles Donovan, named after a town near Calcutta where the disease was particularly endemic, or kala-azar (a Hindi expression meaning “black fever”, relating to the typical black skin pigmentation of infected patients). It is caused by *L. donovani* and *L. infantum* in the Old World, while *L. chagasi* is the prevalent subspecies in Central and South America. The parasites are not confined to macrophages at the site of infection, but disseminate to visceral organs (especially spleen, liver and also bone marrow) and cause a systemic infection of the entire reticulo-endothelial system.

The majority of infections are asymptomatic. Clinically, after an incubation time of weeks, or even months to years after infection, there is a sudden onset of fever (39 – 40°C) within 24 hours. Patients will present with indolent hepatosplenomegaly, anaemia and, in advanced stages, with ascites and black skin pigmentation. Untreated, the lethality is up to 90%. Current therapy regimens include treatment with pentavalent

antimony, amphotericin B, pentamidine, paromomycin and recently miltefosine [18, 163].

To date, even though leishmaniases are not perceived as a critical health issue in Europe, *Leishmania* infections represent a worldwide threat: according to a current WHO report, they are endemic in 88 countries and account for 1.5 million new cases and 59,000 deaths annually; worldwide, 12 million people are affected with a total of 350 million at risk. The WHO has declared leishmaniasis a category 1 disease: emerging and uncontrolled. Evolving problem are the spread of vectors to formerly non-endemic regions and an increasing number of *Leishmania*/HIV coinfections.

1.4.2 Immunobiology

Murine leishmaniasis has not only proven to be a useful model to study specifically the immunology of *Leishmania* infections – it was also the disease in which the regulatory importance of T helper cell subsets, the TH1/TH2 balance, was first described [211]. Typically, murine leishmaniasis is established by needle inoculation of a high number of metacyclic promastigotes into subcutaneous sites, such as the base of the tail or the right hind footpad.

Initially, it was shown that different inbred mouse strains react differently to subcutaneous infection with *L. major* [83, 96-98, 114]; e. g., BALB/c mice develop progressive lesions and fail to control the disease, while C57BL/6 mice only develop small lesions that eventually resolve. Later, this difference was shown to be paired with different T helper cell polarizations [211]. Today, the general view is accepted that the difference in clinical outcome is associated with an IL-12-driven, IFN-γ-dominated TH1 response in resistant mouse strains and an IL-4-driven TH2 response in susceptible strains [200]. It is believed that the healing forms of murine leishmaniasis are useful models for CL in humans, while disease exacerbation in *L. major*-susceptible mouse strains mimics non-healing forms of the human disease such as VL. Murine leishmaniasis is also considered a model disease for infections with intracellular pathogens.

The relationship between genetic, molecular, pathologic and immunologic bases of the mechanisms leading to either resistance or susceptibility to *L. major* infection has so far not been elucidated satisfactorily to provide a consistent explanation about how the

decision is being made and which keys must be pressed to redirect it. So far, immunologic models have provided the most comprehensive approach. As has been indicated, *L. major* parasites primarily infect and persist in macrophages. The key event in antileishmanial host defence is IFN-γ-mediated activation of parasite-killing mechanisms in macrophages: upregulation of expression of inducible nitric oxide synthethase is followed by production of reactive nitrogen intermediates (RNI) that have potent activity to destroy intracellular pathogens; apoptosis plays a role, too [99].

As sources of IFN-γ, natural killer (NK) cells [207], $CD8^+$ T cells [15] and, most importantly, $CD4^+$ T helper cells [36, 53] – the principal actors in TH1 responses – have been identified. In contrast, TH2 responses are dominated by IL-4, IL-10 and IL-13 – cytokines that inhibit RNI production by macrophages.

Susceptibility to *L. major* can be explained best by the immune system's failure to generate IFN-γ-producing T helper cells. Recent immunologic research has refined the simplified view about the TH1/TH2 concept in leishmaniasis immunology and indicates that there are at least two distinct phases of the immune response, the early phase and the late phase.

Interestingly, the early events, probably until about two weeks post infection [88], are similar in resistant and susceptible mice: an IL-4-producing, oligoclonal population of $CD4^+$ T cells whose TCR recognizes the *Leishmania* antigen LACK (*Leishmania* homologue of receptors for activated C kinase) dominates the early immune response in either case [230]. Indeed, even in resistant mice that eventually develop an IL-12-driven TH1 response, IL-12 production might be absent initially, and some authors conjecture that the early TH2 response could be explained by the inability of *L. major* parasites to induce early IL-12 production in both types of mouse strains [200]. This property is subspecies-specific, as other *Leishmania* strains, e. g. *L. donovani*, cause a rapid, transient IL-12 burst conferred by splenic DC in BALB/c mice immediately after infection [69].

In contrast to the early phase, the late phases in susceptible and resistant mouse strains differ. In the current models, IL-4 plays the central role in promoting disease development during the late phase in *L. major*-susceptible mice. It is believed that, in these mouse strains, the inability of *Leishmania* antigens to activate DC to produce IL-12 results in default differentiation of naïve T cells into IL-4-secreting TH2 cells.

IL-4 is derived from activated DC and binds to IL-4 receptors on naïve T cells, thus activating the STAT (signal transducer and activator of transcription) 6 pathway with subsequent binding of the transcription factor GATA 3 and secretion of typical TH2 cytokines (IL-4, IL-5, IL-13 and others). IL-10, which is also secreted in the late phase, has similar functions. The crucial role of these cytokines in directing the immune response was revealed by demonstrating that even resistant mice that were engineered genetically to express IL-4 or IL-10 constitutively did not exhibit a reduced number of IFN-γ-producing TH1 cells. They were nevertheless unable to control the lesions [54, 76].

IL-4 production and -signaling, however, is not the only, and under certain conditions not even a necessary, influencing factor for TH2 development in *L. major*-susceptible mice. Recently, a more flexible model that takes into account immunological differences observed in infections with different *L. major* subspecies and that postulates relative contributions of different cytokines and cytokine signaling pathways for disease development has been favored [200].

Besides the instructional role of IL-4 and IL-10, several other factors have been proposed to be determinants of TH2 polarization in susceptible strains. (1) The cellular composition of the tissue environment, e. g. a high proportion of neutrophils in the inflammatory infiltrate of infected BALB/c mice, in contrast to an only transient presence of neutrophils in lesions of C57BL/6 mice, contributes to the sustained induction of a TH2 response [237]. Moreover, (2) dissemination of parasites is rapid in susceptible mice, while resistant mice manage to contain them at the infectious site and the draining LN [132]. (3) The site of infection is another important predictor; e. g., normally resistant mice develop progressive lesions associated with a TH2 response when infected intranasally [164]. (4) Increased or decreased expression of certain chemokines [77, 219] and interaction between co-stimulatory molecules or their receptors [42] can also have crucial roles in the development of a TH2 response and point to the possibility that differential expression of these molecules might define functionally different antigen-presenting cells in different tissues that guide the outcome of the disease. (5) Instable expression of the IL-12R (IL-12 receptor) β2-chain on activated $CD4^+$ T cells has also been suggested as a disease-promoting factor [93].

While TH2 dominance with IL-4 production persists in mice susceptible to *L. major*, the

late phase in resistant mice is marked by the onset of IL-12 secretion that promotes redirection towards a TH1 response [236]. It is dependent on initial IL-12/IL-12R signaling, although alternative factors, e. g. IL-18 signaling, might cooperate. In resistant mice, the failure to produce IL-12 during the early phase is likely related to the fact that macrophages, being the primary targets for *L. major* parasites, are unable to produce IL-12 upon parasite ingestion: Their – otherwise pronounced – ability to secrete IL-12 upon pulsing with strong proinflammatory stimuli is selectively impaired by the ingested parasite [190].

Production of IL-12 and the induction of a specific immune response are finally achieved by epidermal Langerhans cells that take up parasites, mature thereupon and migrate to the draining LN [158]. TLR may be involved in the signaling pathway leading to IL-12 production, as it could be shown that MyD88-deficient C57BL/6 mice, which is required for signaling by all TLR, develop a high susceptibility to *L. major* infection that is associated with a polarized TH2 response [161].

Importantly, IL-12 not only initiates a TH1 response, but is also required for maintaining immunity to *L. major* [174]. Another requirement for long term immunity is parasite persistence in the host – a latent infection needs to be established, leading to concomitant immunity [14]. It is, however, a double-edged sword: with an intact immune system, the host is resistant to reinfection, but in conditions of immunosuppression, disease exacerbation is bound to occur – as it is the case in *Leishmania*/HIV coinfection.

In IL-10-deficient mice, the parasite is cleared entirely and sterile immunity is established, but the host is rendered susceptible to re-infection [14]. IL-10-producing cells that are required for maintaining a latent infection were shown to belong to a population of $CD4^+$ $CD25^+$ regulatory T cells [14].

Above and beyond detailed immunological research, the phenomenon of susceptibility vs. resistance was also addressed in genetic studies. They revealed gene loci that are associated with susceptibility to *L. major* [156]. Certain susceptibility loci were shown to be related to characteristic immunological reactions or pathological symptoms [142]. However, correlation between development of disease and TH2-type immune responses in recombinant congenic mice used in these experiments was absent or limited to specific genotypes, which suggests that in addition to the postulated antagonistic

TH1/TH2 effects, other genetically programmed mechanisms have an impact on the development of leishmaniasis. Thus, the host's genotype affects disease development in a more complex way than had been thought [142].

1.4.3 Strategies for intervention

Endeavors for prevention of leishmaniasis are based on two pillars: vector control on one hand, vaccination against the parasite on the other hand [163]. Despite all drawbacks, the WHO is aiming to eliminate certain subforms of leishmaniasis that are restricted to particular areas, such as VL [175]. To ensure an efficient disease control, not only scientists will have to enforce the search for an effective vaccine – public health programs are likewise needed to implement prevention strategies.

Physical protection from sandflies by bed nets leads to a substantial reduction of sandfly bites. Insecticides are used for indoor spraying and impregnation of beds. Recently, in areas where leishmaniasis is primarily zoonotic, targeting the animal reservoir was shown to be an effective way to reduce incidence of human disease: Dipping dogs in insecticides, or providing them with impregnated collars provided protective effects. Even vaccinating dogs was shown to be feasible [139, 187, 201].

Vaccination of humans against *Leishmania* parasites has proved difficult so far, as classical requirements for an appropriate vaccine – low costs, easy applicability, high level of protection and high safety – could not be met. While considerable knowledge about leishmaniasis immunology has been accumulated in murine models, less is known about its immunology in human disease.

For successful vaccine development, the knowledge on the immunology of leishmaniasis needs to be implemented. The principles that appear be responsible for the establishment of resistance have to be taken into account. This suggests that for a future vaccine, three criteria will have to be fulfilled: (1) ablation of the TH2 cytokines after infection, (2) establishment of an IL-12-driven TH1 response, possibly with a TH1-promoting adjuvant, and (3) elicitation of both $CD4^+$ and $CD8^+$ T cell responses.

2 Aims of the study

DC have not only been shown to be key actors in the regulation of immune responses, but they were also demonstrated to be promising vaccine candidates against malignant and infectious diseases. Trials in murine leishmaniasis by our own group revealed their efficacy as vaccines against *L. major* infection [17, 58, 186].

Several leishmanial antigens have been proposed to be useful for a potential DC-based vaccine. From our own experiments with Langerhans cells, LeIF emerged as a promising candidate. LeIF is an abundant intracellular leishmanial protein involved in the parasitic translation machinery. It unites the properties of a protein antigen and an IL-12-inducing adjuvant and was shown to elicit protective, antileishmanial TH1 type immune responses. By virtue to its capacity to stimulate DC and macrophages to secrete IL-12 and IL-18 and to activate NK cells, LeIF was suggested to be an atypical PAMP [24, 215, 216]. Its immunologically active part was shown to be tied to its 226 aminoterminal amino acids.

There are different ways to administer antigens to DC. Besides classical pulsing by co-incubation of DC with antigen, methods to manipulate DC genetically to express the antigen of interest have evolved. One of them is based on delivery of *in vitro* transcribed RNA to DC by electroporation.

So far, RNA transfection of DC has mainly been performed with tumor antigens that, obviously, are of mammalian origin. LeIF, however, is a microbial antigen and a potent stimulator of the innate immune system. It is not known what effect transfection with *in vitro* generated RNA encoding for such a protein molecule would have on DC. This study was conducted to determine the immunological characteristics of murine BMDC transfected with *in vitro* transcribed RNA of the leishmanial antigen LeIF, and to see if and how these characteristics differ from LeIF-pulsed BMDC. As nothing was known about how the properties of a recombinant protein relate to its properties after RNA transfection, two variants of LeIF-RNA were constructed: LeIF(fl)-RNA, encoding the complete LeIF sequence, and LeIF(226)-RNA, encoding only the aminoterminal half of the LeIF sequence (226 amino acids), the immunogenic part of LeIF.

However, a reporter assay was needed first that would provide information on how

efficiently DC could be transfected at all; for this purpose, EGFP which is easily detectable by flow cytometry was chosen as a reporter antigen.

The present study adressed these central questions:

1. What is the kinetics of transfection efficiency and antigen expression after transfection of DC with EGFP-RNA, and is it influenced by DC maturation?
2. Can LeIF be detected intracellularly after transfection of DC with LeIF-RNA?
3. Do LeIF-transfected and LeIF-pulsed BMDC stimulate T cells in an antigen-specific fashion?
4. Does transfection with LeIF-RNA induce the maturation of DC?
5. Does the duration of BMDC generation have an influence on the strength of the immune response?
6. Is intracellular LeIF immunologically equivalent to extracellular LeIF, and is the capacity of LeIF to stimulate cells of the innate immune system such as DC also conferred by transfection with LeIF-RNA?

3 Material

3.1 Technical equipment

Product	Brand
agarose electrophoresis gel chambers	Peqlab m
cell culture centrifuge	Heraeus [Langenselbold, Germany]
cell culture incubator	WTC Binder [Tuttlingen, Germany]
cuvettes	Peqlab
electrophoresis power supply	BioRad [München, Germany]
electroporator	BioRad
ELISA reader	Dynatech Laboratories [Stuttgart, Germany]
FACScalibur	Becton-Dickinson [Heidelberg, Germany]
fluorescence microscope	Zeiss [Oberkochen, Germany]
heater	Axon lab AG [Baden-Dättwil, Switzerland]
light microscope	Zeiss
magnetic stirrer	Heidolph [Kehlheim, Germany]
photometer	Eppendorf [Hamburg, Germany]
pH-meter	Inolab/WTW [Weilheim, Germany]
protein electrophoresis chamber (Miniprotean II unit)	BioRad
scale	Sartorius [Göttingen, Germany]
shaker	Eppendorf
sterile bench	Nuaire [Plymouth, MN, USA]
table centrifuge	Eppendorf
Thermocycler	Eppendorf
UV wave transilluminator	Hartenstein [Würzburg, Germany]
vortexer	Heidolph
Western Blot transfer chamber	BioRad

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

3.2 Culture Media

Complete medium for DC cultures was prepared with RPMI medium [Biochrom, Berlin, Germany], adding 10% heat-inactivated fetal calf serum [FCS; PAA Laboratories, Linz, Austria], 2 mM L-glutamine [Biochrom], 10 mM HEPES buffer

[Biochrom], 0.05 mM 2-mercaptoethanol [Sigma, Taufkirchen, Germany], 20 µg/ml gentamicin [Sigma] and 60 µg/ml penicillin [Sigma].

Cell counting was performed with 1:10 dilutions in 0.4% Trypan Blue [Invitrogen, Karlsruhe, Germany]. Plastic tubes (15 and 50 ml) were from Sarstedt [Nürnberg, Germany]. Petri dishes were purchased from Greiner [Kremsmünster, Austria]. 6-, 12-, 24- and 96-well culture plates were from Nunc [Wiesbaden, Germany]. Cell scrapers were obtained from BD Biosciences PharMingen [Heidelberg, Germany]. Plastic caps (0.5 and 1.5 ml) and pipette tips were purchased from Eppendorf.

For bacterial culture, lysogeny broth (LB) medium was prepared from 25 g LB powder [Roth, Karlsruhe, Germany] in 1000 ml aqua dest.

3.3 Solutions

For preparation of phosphate-buffered saline containing magnesium and calcium [PBS (Mg^{2+}, Ca^{2+})], 100 ml Ca^{2+}-/Mg^{2+}-free PBS [Invitrogen] was mixed with 50 µl of a 0.5 M magnesium chloride solution (end concentration: 0.25 mM) and 50 µl of a 0.7 M calcium chloride solution (end concentration: 0.35 mM).

For a final volume of 100 ml 4% paraformaldehyde (PFA), 4 g PFA was mixed with about 85 ml aqua dest. to which 10 ml 10x PBS (Mg^{2+}, Ca^{2+}) was added. While stirring constantly, 1 M sodium hydroxid was added drop-wise until the solution was clear. Using diluted hydrochloric acid, the pH was lowered to about 7.2, the solution was filled up with aqua dest. to a total volume of 100 ml and passed through a 0.2 µm filter. The solution was stored at 4°C.

Blocking solution contained 5% fat free milk powder in PBS and 0.05% Tween20.

3.4 Buffers

For 1x TE Buffer, 10 mM Tris-buffer (pH 8) and 0.1 mM ethylene diamine tetraacetic acid (EDTA; pH 8) were filled up to a volume of 50 ml with sterile water [Ampuva; Fresenius, Bad Homburg, Germany]. For preparation of TAE buffer, 242 g Tris, 57.1 ml acetic acid and 100 ml EDTA were added to 1000 ml aqua dest. and adjusted to pH 8.0.

6x sample buffer for DNA agarose gels consisted of 1 mM EDTA (pH 8), 50% glycerol, 0.25% bromophenol blue, and 0.25% xylene cyanol. MOPS buffer contained 0.2 M MOPS (pH 7.0), 50 mM sodium acetate and 5 mM EDTA (pH 8.0). The sample buffer

for RNA agarose gels contained 10.0 ml deionized formamide, 3.5 ml 37% formaldehyde, 2.0 ml MOPS buffer. It was dispensed into single use aliquots in 500 µl tubes and stored at -20°C.

Loading buffer for RNA agarose gels contained 50% glycerol, 1 mM EDTA, 0.4% bromophenol blue. For the RNA loading buffer, high grade glycerol was used. The buffer was dispensed into single use aliquots and stored at -20°C.

Radioimmunoprecipitation assay (RIPA) buffer for eukaryotic cell lysates consisted of the following liquids: 25 ml 10% Triton X-100 (1%), 12.5 ml 1 M HEPES pH 7.4 (50 mM), 7.5 ml 5 M sodium chloride (150 mM), 25 ml 100% glycerol (10%), 2.5 ml 100 mM EGTA (1 mM), 375 µl 1 M magnesium chloride (1.5 mM), 10 ml 250 mM sodium pyrophosphate (10 mM), 50 ml 0.5 M sodium fluoride (100 mM) and 1.25 ml 20% sodium dodecyl sulfate (SDS; 0.1%) that were slowly added. This solution was filled up to 240 ml with bidest water. 2.5 g dry deoxycholic acid (1%) was slowly added and dissolved while stirring slightly until the solution was clear. Subsequently, 2.5 ml 100 mM sodium orthovanadate (1 mM) was added dropwise. The solution was filtered and filled up to a total volume of 250 ml. Subsequently, 250 µl of 5 mg/ml leupeptin (5 µg/ml), 250 µl 10 mg/ml aprotonin (10 µg/ml), 250 µl 10 mg/ml Pefabloc (10 µg/ml), 250 µl 5 mg/ml pepstatin (in methanol; 5 µg/ml), 250 µl 10 mM benzamidin (in ethanol; 10 µM) were added. The solution was stored at 4°C.

The labeling buffer for MACS cell isolation [MACS kit by Miltenyi Biotec, Bergisch Gladbach, Germany] contained PBS (pH 7.2), 0.5% FCS and 2 mM EDTA. The buffer was degassed by applying a vacuum pump overnight and kept cold at 4°C.

FACS buffer was made of 980 ml PBS, 20 ml FCS and 200 µl from a 10% sodium azide dilution (0.02%).

Intracellular staining buffer ("saponin buffer") was composed of 0.1 g saponin, 5 ml inactivated serum (e. g. goat serum, depending on the antibodies used) and 45 ml PBS (Mg^{2+}, Ca^{2+}). If stainings were performed with rabbit antibodies, anti-CD16/CD32 antibody in a 1:50 PBS (Mg^{2+}, Ca^{2+}) dilution was used instead.

Extracellular staining buffer for fluorescence microscopy consisted of 45 ml PBS (Mg^{2+}, Ca^{2+}) and 5 ml serum (or anti-CD16/CD32 antibody in 1:50 PBS (Mg^{2+}, Ca^{2+}) dilution).

For 6x sample buffer for SDS gels, 1 ml 0.5 M Tris (pH 6,8), 0.8 ml glycerol, 1.6 ml

10% SDS, 0.4 ml β-mercaptoethanol and 0.4 ml bromophenol blue were added to 4 ml water. 10x running buffer for SDS gels contained 0.25 M Tris, 2 M Glycin and 1% SDS. 1x transfer buffer for Western Blot contained 25 mM Tris/HCl, 192 mM Glycin and 20% methanol that were filled up with water to 1 l.

3.5 Plasmids

Plasmids used for experiments in this study are listed in Table 2. The pEGFP-C1 plasmid which was purchased from Clontech [Heidelberg, Germany] was used as a template from which the EGFP sequence could be amplified (see section 4.5). The pSP64 plasmid, purchased from Promega [Mannheim, Germany], is a vector allowing *in vitro* transcription of RNA under control of the SP6 promotor.

name of plasmid	origin	description
pEGFP-C1	Clontech	contains the EGFP sequence
pSP64	Promega	*in vitro* transcription vector
pET3-LeIF(fl)	Y. Skeiky, Corixa Corporation, Seattle, WA, USA	encodes the entire 403 amino acids sequence of *Leishmania* homologue of eukaryotic initiation factor 4A (LeIF)
pET3-LeIF(226)	Y. Skeiky, Corixa Corp.	encodes the truncated, N-terminal 226 amino acids sequence of LeIF
pGEM-3'UT-5'UT-A64	K. Thielemans, Vrije Universiteit Brussel, Belgium	*in vitro* transcription vector
pGEM-3'UT-EGFP-5'UT-A64	K. Thielemans, Vrije Universiteit Brussel	*in vitro* transcription vector containing the EGFP sequence
pTRI-Xef-1	Ambion [Austin, TX, USA]	control plasmid for *in vitro* transcription

Table 2: Plasmids.

The pET3-LeIF(fl) plasmid contains the full nucleotide sequence of the LeIF molecule (1209 bp), while the pET3-LeIF(226) plasmids contains only the first 678 nucleic acids of the LeIF molecule, thus encoding for LeIF(226), an N-terminally truncated LeIF molecule which was shown to be the immunologically active part of the LeIF molecule [216]. The empty pGEM-3'UT-5'UT-A64 plasmid, an *in vitro* transcription vector that

features a *Bgl*II/*Eco*RI polycloning site, was obtained from Dr. Thielemans' group in Brussels. The pGEM-3'UT-EGFP-5'UT-A64 plasmid was obtained from Dr. Thielemans as well. The pTRI-Xef-1 plasmid (encoding for *Xenopus* elongation factor 1A) was provided by Ambion as a control plasmid.

Table 3 shows the plasmids that were constructed for this work according to the molecular cloning techniques described in the methods section (sections 4.5 to 4.7). The EGFP sequence was amplified and cloned into the pSP63 vector; the LeIF(fl) and LeIF(226) sequences were amplified and cloned into the pGEM-3'UT-5'UT-A64 vector.

name of plasmid	description
pSP64-EGFP	*in vitro* transcription vector containing the EGFP sequence
pGEM-3'UT-LeIF(fl)-5'UT-A64	*in vitro* transcription vector containing the LeIF(fl) sequence
pGEM-3'UT-LeIF(226)-5'UT-A64	*in vitro* transcription vector containing the LeIF(226) sequence

Table 3: Constructed plasmids.

4 Methods

4.1 Competent cells

From an *Escherichia coli* XL-1 Blue strain glycerol stock stored at -80°C, some colonies were picked with a tooth pick, transferred into 10 to 20 ml LB medium prepared in a sterile 250 ml Erlenmeyer flask and grown overnight in a 37°C shaker. From this overnight culture, 1 ml was transferred into a sterile 1 l Erlenmeyer flask containing 50 ml LB medium and grown at 37°C and about 300 shaking cycles per minute. The optical density at 600 nm (OD_{600}) was determined after approximately 3 hours; once the bacterial culture had reached an OD_{600} of 0.4, which reflects a stage in the bacterial cell cycle when they are most apt to take up foreign DNA, it was transferred into 50 ml Sarstedt tubes and stored on ice for 10 minutes. The tube was centrifuged for 10 minutes at 4°C at 4,000 rpm, the supernatant was decanted, and the pellet was suspended in 10 ml ice-cold 0.1 M calcium chloride. After resuspension, the cells were stored on ice for another 10 minutes, centrifuged for 10 minutes at 4°C and 4,000 rpm and suspended in 2 ml ice-cold calcium chloride. This suspension was dispensed in aliquots of 100 µl into 1.5 ml Eppendorf caps and frozen at -80°C.

4.2 Transformation of competent cells

E. coli XL-1 Blue strain cultures (see section 4.1) were thawed from -80°C storage for 5 minutes, 0.5 µg plasmid DNA were added, and the cap was placed on ice for 10 to 20 minutes. Subsequently, the cap was placed in a heater at 42°C for 90 seconds and then cooled on ice for about 5 minutes. The bacteria were then suspended in 1 ml LB medium and allowed to grow for 1 hour at 37°C. The suspension was centrifuged at 13.000 rpm for 2 minutes, the supernatant was poured out, sparing about 100 µl, and the pellet was resuspended in the minimal amount of LB medium remaining in the cap. The bacteria suspension was then spread on an LB agar plate containing the correct antibiotic and allowed to grow in an incubator overnight at 37°C.

4.3 Overnight Cultures

Sterile glass tubes for bacterial cultures were sterilely filled with 3 ml LB medium. For selection of bacterial strains that contained a specific plasmid which also conferred resistance against a specific antibiotic, the respective antibiotic was added to the culture medium. The final antibiotic concentration was 100 µl/ml. Using sterile toothpicks, one colony from an LB agar plate was picked and transferred into the medium. The tube was incubated overnight at 37°C.

4.4 Plasmid DNA Preparation

For plasmid purification from transformed *E. coli* XL-1 Blue strains, columns from Qiagen [Hilden, Germany] were used, following the plasmid mini preparation protocol for small amounts, the midi preparation protocol for larger amounts of DNA. In brief, 1.5 ml of overnight culture volume was centrifuged at 13.000 rpm for 2 min. The pellet was resuspended in different buffers, finally applied to the Qiagen column, washed and eluted. DNA obtained from this procedure was precipitated with isopropanol, washed with 70% ethanol and taken up in 25 µl (Mini preparation) or 250 µl (Midi preparation) of autoclaved TE buffer. After linearization with the restriction enzyme *Eco*RI, purification and subsequent quality control by gel electrophoresis (1% agarose), the concentration was determined by ultraviolet (UV) photospectrometry.

4.5 Polymerase Chain Reaction for cloning

For cloning the antigen sequences into the pGEM-3'UT-5'UT-A64 vector, they were amplified by polymerase chain reaction (PCR) using specific primers.

The sequence "GCC A/GCC *AUG* G" flanking the AUG start codon has been shown to maximize the efficiency of translation of eukaryotic mRNA (Kozak consensus sequence) [123]. To enable efficient translation, this Kozak sequence was used for primer design.

For selection of the appropriate restriction site, vector and inserts were analyzed with a computer program [Clone Manager 3.0, Scientific & Educational Software Inc., State Line, PA, USA] in order to verify if they contained any of the restriction sequences available for cloning. The pGEM-3'UT-5'UT-A64 plasmid contains a small polylinker site between the 5' and 3' untranslated regions (UTR), providing a *Bgl*II site at the 5'

end and an *Eco*RI site at the 3' end. As both the LeIF(fl) and LeIF(226) sequences contain a *Bgl*II site within the encoding sequence, *Bgl*II could not be used for cloning. However, *Bgl*II and *Bam*HI both leave sticky GATC ends at their cleavage sites which are able to recombine, and the LeIF(fl) and LeIF(226) sequences do not contain intrinsic *Bam*HI sites. Therefore, a *Bam*HI site was cloned at the 5' end of all LeIF inserts. After ligation, the site is not a restriction site any more ("lost in cloning").

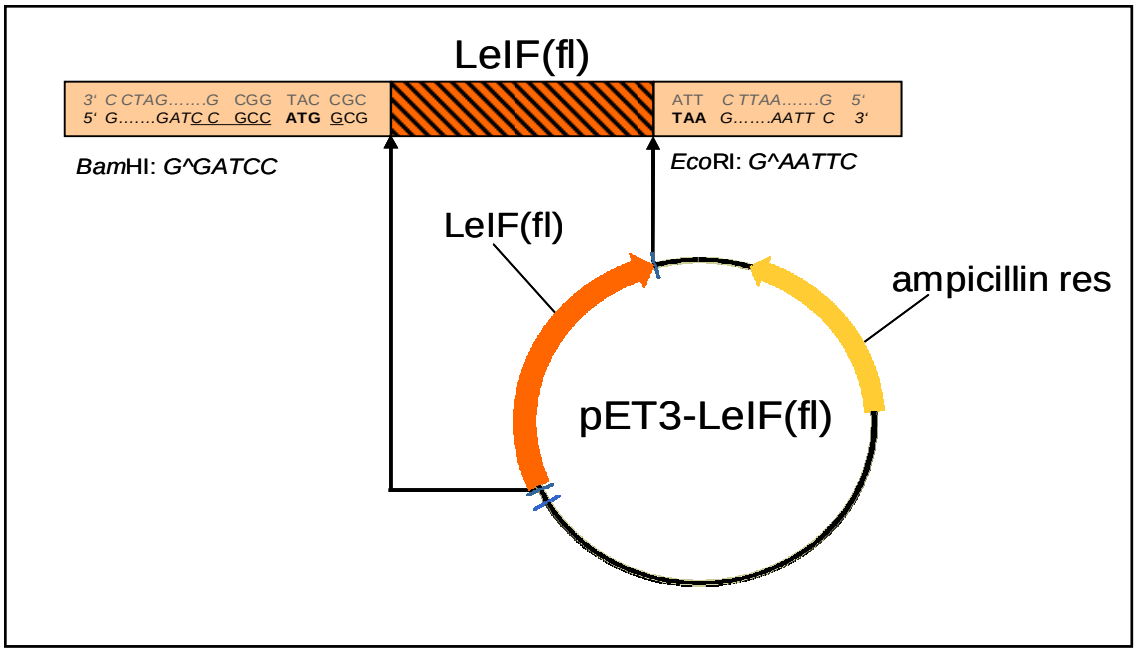

Fig. 5: Amplification of LeIF(fl) sequence. The pET3-LeIF(fl) plasmid was used as a template for amplification of the LeIF(fl) sequence. For primer design, a *Bam*HI restriction site is added on the 5' end, an EcoRI site is added on the 3' end (in italics). The start and stop codons are bold, the Kozak consensus sequence is underlined. The dotted lines indicate the restriction cleavage sites.

The primers were thus designed by inserting a *Bam*HI restriction site at the 5' end and an *Eco*RI restriction site at the 3' end for correct orientation; a part of the Kozak sequence (CC as part of the *Bam*HI restriction site, GCC and G after the ATG start codon) was inserted around the start codon ATG (see Fig. 5). For exact sequence reference, data were cleared with Dr. Skeiky's lab [Corixa Corp.] according to his publications [215, 216]. The primer length was chosen with the help of a computer program [Vector NTI Advance 10, Invitrogen, Carlsbad, CA, USA] in a way for all primers to have a similar annealing temperature. Another computer analysis [Clone Manager 3.0] was carried out to ensure that the chosen restriction sites were indeed unique within the amplified DNA inserts. The primer design and PCR conditions are outlined in Table 4.

target sequence	sense antisense	size (bp)	annealing temp.	number of cycles
LeIF(fl)	5' – GGG *GGA TCC* GCC **ATG** GCG CAG AAT GAT AAG ATC GCC 3' – GGG *GAA TTC* **TTA** CTC GCC AAG GTA GGC AGC	36 30	65°C	30
LeIF(226)	5' – GGG *GGA TCC* GCC **ATG** GCG CAG AAT GAT AAG ATC GCC 3' – GGG *GAA TTC* **TTA** GTC GCG CAT GAA CTT CTT CGT CAG	36 36	62°C	30

Table 4: Primer design. Start/stop codons in bold, restriction sites in italics, Kozak sequence underlined.

For amplification of protein or antigen sequences from purified plasmids, PCR reactions were performed using a high fidelity DNA polymerase (VENT polymerase) according to the protocol outlined in Table 5.

reagent	brand	volume
DNA, different molarities	–	3 µl
desoxynucleotides, 2.5 mM	New England Biolabs, Frankfurt, Germany	4 µl
primer 1, 25 pmol/µl (sense)	Operon, Köln, Germany	1 µl
primer 2, 25 pmol/µl (antisense)	Operon	1 µl
10x buffer	New England Biolabs	5 µl
magnesium sulfate, 2 mM	New England Biolabs	4 µl
VENT polymerase	New England Biolabs	0,25 µl
sterile water, filled up to a volume of	New England Biolabs	50 µl

Table 5: PCR amplification of inserts.

The reaction samples were set up in a 50 µl reaction tube [Eppendorf]; the PCR reactions were run in a thermocycler [Eppendorf] using the following cycle profile:

1. 2 minutes, 94°C
2. 1 minute, 94°C (denaturation)
3. 1 minute, see temperatures above (annealing)
4. 1 minute, 72°C (polymerization)
5. 10 minutes, 72°C (extension)

Steps 2 to 4 were repeated 30 times. The PCR was first run at different temperatures between 60 and 75°C to determine a temperature that would allow both primers to bind adequately to the template and to receive a clean reaction product, if necessary, by using the gradient function of the PCR machine. A good result was achieved at 65 and 62°C respectively, and the assays were repeated at a larger scale of 8 samples per reaction to receive a sufficient amount of DNA for restriction digestion, purification and ligation. The reaction product was purified using the Qiagen plasmid purification kit and taken up in 100 µl nuclease-free water.

4.6 Restriction digestion for cloning

Plasmid digestions were performed according to the protocols in Table 6. The digest was run at 37°C for 2 hours. For removal of 5' phosphate residues, samples were treated with 2 µl of calf intestinal phosphatase (CIP) during the last 30 minutes to prevent unspecific self-ligation [204].

reaction	reagent	brand	volume
1. pGEM: *Bgl*II	Plasmidal DNA (1 µg/ml)	–	16.5 µl
	10x buffer D	Promega	6.0 µl
	Bovine serum albumine (BSA)	Promega	0.6 µl
	*Bgl*II	Promega	1.5 µl
	Nuclease-free water	Promega	35.4 µl
	Total volume		60.0 µl
2. pGEM: *Bgl*II / *Eco*RI	Plasmidal DNA (1 µg/ml)	–	33.0 µl
	10x buffer D	Promega	12.0 µl
	BSA	Promega	1.2 µl
	*Bgl*II	Promega	3.0 µl
	*Eco*RI	Promega	3.0 µl
	Nuclease-free water	Promega	67.8 µl
	Total volume		120.0 µl

Table 6: Restriction digestion reactions of vectors for subsequent cloning.

Subsequently, a 0.7% TAE agarose gel was run overnight at 30 V to allow exact separation of reaction products. The bands representing the digested plasmids of the expected size were excised from the gel slice with a scalpel under a UV transilluminator. The Qiagen gel extraction kit was used to extract the DNA from the

gel slice. The DNA was then taken up in 30 and 60 µl of nuclease-free water, respectively, and the concentration was determined by UV spectrometry. Another agarose gel was run to check DNA size and purity.

The PCR products from eight 50 µl PCR reactions were purified using the QIAquick PCR purification kit protocol [Qiagen] which purifies DNA fragments from primers, nucleotides, polymerases and salts, and were taken up in 100 µl nuclease-free water. The DNA (LeIF(fl) and LeIF(226)) obtained from PCR amplification was digested at its added cloning sites with *Bam*HI and *Eco*RI in a restriction digestion reaction in order to obtain inserts with "sticky ends", thus suitable for the subsequent ligation reaction into a predigested vector. The protocol is shown in Table 7.

reagent	brand	volume
Insert DNA from PCR (0.13 µg/µl)	–	20 µl
10x buffer E	Promega	4.0 µl
BSA	Promega	0.4 µl
*Bam*HI	Promega	1.0 µl
*Eco*RI	Promega	1.0 µl
Nuclease-free water	Promega	13.6 µl
Total volume		40.0 µl

Table 7: Restriction digestion reactions of inserts for subsequent cloning.

Incubation was allowed at 37°C for 1 hour. The reaction was purified using the Qiagen PCR purification kit, and the DNA was taken up in 30 µl of nuclease-free water.

Restriction digestions for qualitative control were similarly assembled in 20 µl reactions.

4.7 Vector-insert ligation reaction

For ligation reactions involving cleaved plasmids and DNA inserts, different vector-insert-ratios are recommendable. A dependency of the number of colonies from the amount of insert DNA used for the reaction, compared with a control experiment checking for religation of the empty vector, allows an early assessment of the effectiveness and the specificity of the ligation. Table 8 shows the protocol for vector-insert ligation reactions.

reagent	brand	volume
Insert DNA (LeIF(226) or LeIF(fl), 0.13 µg/µl) – cut : *Bam*HI, *Eco*RI) or negative control (nuclease-free water)	–	1.0 µl
Vector DNA (pGEM) – cut: *Bgl*II, *Eco*RI (0.06 µg/µl and dilutions 1:5, 1:10, 1:20)	–	3.0 µl
10x buffer E	Promega	1.1 µl
T4 DNA ligase	Promega	1.0 µl
Nuclease-free water	Promega	6.0 µl
Total volume		12.1 µl

Table 8: vector-insert ligation reaction.

The reaction was allowed to incubate at 4°C for 14 hours. The reaction product was used for transformation of competent cells (see section 4.2). Samples were spread on agar plates that contained ampicillin (8 samples per agar plate).

4.8 PCR detection of positive bacteria clones

To check if the fragment DNA had indeed been inserted correctly into the plasmid – as far as its orientation, number and length are concerned – a PCR, following the protocol in Table 9, was run.

reagent	brand	volume
Water	–	34.0 µl
Magnesium sulfate, 2 mM	New England Biolabs	2.0 µl
10x buffer	New England Biolabs	5.0 µl
Desoxynucleotides, 2.5 mM	New England Biolabs	4.0 µl
Primer 1, 25 pmol/µl (sense)	Operon	1.0 µl
Primer 2, 25 pmol/µl (antisense)	Operon	1.0 µl
Bacterial suspension / template	–	3.0 µl
VENT polymerase	New England Biolabs	0.25 µl
Total volume		50.25 µl

Table 9: PCR for detection of positive bacterial clones.

First, a sterile yellow pipette tip was used to transfer some bacterial colonies of each sample from the agar plate into 10 µl of water in an Eppendorf cap. This bacterial suspension was heated to 100°C for 5 minutes to provoke breakdown of the bacterial

cell wall and to release plasmid DNA. The annealing temperatures were as indicated before (see Table 4). Elongation was allowed for 90 seconds.

4.9 *In vitro* transcription of mRNA

1. *Spe*I was used for linearization, according to the plasmid map. The reaction was assembled according to the protocol in Table 10. The reaction was incubated at 37°C overnight. A 1% agarose gel was run to check if the plasmid digestion had been completed, i. e. if the gel showed a single sharp band. If this was not the case, another 5 µl of enzyme was added and incubated for several hours. If the digestion was complete, 10 µl proteinase K [Qiagen] was added and incubated at 50°C for 30 minutes.

reagent	brand	volume/amount
plasmid DNA	–	50 µg
*Spe*I	Promega	5.0 µl
reaction buffer	Promega	25.0 µl
BSA	Promega	2.5 µl
Nuclease-free water	Promega	variable
Total volume		250 µl

Table 10: Linearization of plasmids for *in vitro* transcription.

Subsequently, the DNA was extracted from the reaction by adding an equivalent volume of TE-saturated (pH 4.5) phenol:chloroform:isoamyl alcohol (25:24:1), vortexed for 60 seconds and centrifuged for 2 minutes at 13,000 rpm and 4°C. The upper, aqueous phase was transferred into a new cap, an equivalent volume of chloroform:isoamyl alcohol (24:1) was added, the cap was vortexed again for 60 seconds and centrifuged for 2 minutes at 13,000 rpm and 4°C. To precipitate the DNA, 0,1 vol. 3 M sodium acetate (pH 5.2) and 1 vol. isopropanol were added, mixed and incubated on ice for 2 – 5 minutes, then centrifuged at 13,000 rpm and 4°C. The supernatant was removed carefully, and the pellet was washed with 1 ml 70% ethanol and centrifuged at 13,000 rpm and 4°C for 5 minutes. The pellet was then dried and suspended in 50 µl nuclease-free water. The concentration was determined by UV photospectrometry using a 1:100 dilution. A 1% agarose gel was run to document the plasmid size. Aliquots of 5 µg DNA were distributed in Eppendorf caps and frozen at -20°C.

2. For RNA synthesis, the Ambion *in vitro* transcription mMessage mMachine T7 Ultra Kit and protocol [Ambion] were used. It generates mRNA from a bacterial plasmid under control of the T7 promotor. Additionally, RNA transcripts are capped with an anti-reverse cap analogon (ARCA) which is shown in Fig. 6; ARCA capping in reverse orientation renders RNA a lot more stable than uncapped RNA and has a strong stimulatory effect on subsequent translation [229].

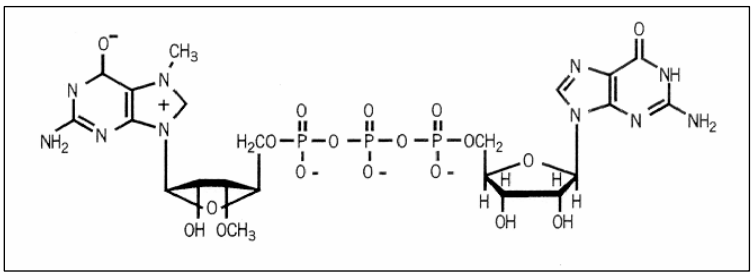

Fig. 6: Schematic of the ARCA molecule [Ambion]. Using the 3'-O-methyl-m7(5')Gppp5'G molecule (ARCA) for RNA capping prevents reverse incorporation of the cap that hampers correct recognition by eIF4E [229]. Translation of RNA capped with ARCA is more efficient than translation of conventionally capped RNA [157].

Importantly, all materials used were RNAse-free or treated with RNA-removing reagents. Briefly, the reaction was assembled in a 1.5 ml Eppendorf cap according to the protocol in Table 11.

T7 NTP/ARCA	50 µl
10 x reaction buffer	10 µl
Linear template DNA	5 µg
T7 enzyme mix	10 µl
Nuclease-free water up to a volume of	100 µl

Table 11: *In vitro* transcription.

The reaction was incubated at 37°C. After 3 to 4 hours, 5 µl DNAse I was added, and the reaction was incubated for another 15 minutes at 37°C. The reaction was stopped and the DNA was precipitated by adding 50 µl lithium chloride precipitation solution. The cap was frozen at -20°C. For pelletation of the RNA, the cap was then centrifuged at 13,000 rpm and 4°C for 15 minutes. The supernatant was removed; the pellet was washed with 1 ml 70% ethanol and recentrifuged for 5 minutes. The ethanol was carefully removed pouring the cap; the remainders were allowed to evaporate for 20 –

30 minutes. The pellet was suspended in nuclease-free water and frozen at -80°C. Analysis on a 1% agarose gel was performed to check the size of the transcript; RNA concentration was determined using UV photospectrometry.

4.10 Agarose gels

For detection of DNA, 0.8 - 1.5% agarose gels were cast. For a 1% agarose gel, 1 g agarose was weighed out and sprinkled into a flask with 100 ml 1x TAE buffer. The flask was heated in a microwave and swirled in between to facilitate dissolution of the agarose. When bubbling was seen, the flask was taken out and allowed to cool until the gel temperature was about 60°C. Afterwards the gel was poured into a gel chamber up to a height of 0.5 cm, and the well-forming comb was inserted into the mounting. When the gel had cooled down completely, 500 ml 1x TAE buffer was poured into the electrophoresis cell, covering the gel completely with buffer. DNA samples were diluted with 6x sample buffer, vortexed, spun down with a centrifuge and transferred into the wells. Gels were run at a current of 100 mA and a voltage of 70 - 100 V. When the blue dye had reached about the middle of the gel, the gel slice was taken out, bathed in an ethidium bromide solution (0.5 µg/ml) for 20 minutes and viewed under a UV wave transilluminator.

For detection of RNA from *in vitro* transcription reactions, the electrophoresis cell and other accessories were cleaned with ethanol and RNase Away [Invitrogen]; RNase-free pipette tips were used. A 1.5% agarose gel containing 0.5 µg/ml ethidium bromide in TAE buffer (prepared with diethylpyrocarbonate (DEPC)-treated water) was cast. 1 to 2 µl of RNA from the reaction were mixed with 18 µl sample buffer and 2 µl loading buffer and subsequently heated at 65°C for 5 to 10 minutes. The gel was run at 100 mA and 80 V until the blue dye had reached the middle of the gel and afterwards immediately viewed under a UV wave transilluminator where a photo was taken.

4.11 *In vitro* translation of RNA transcripts (Rabbit reticulocyte lysate system)

To check the translatability of RNA molecules generated by *in vitro* transcription, RNA transcripts were translated in a rabbit reticulocyte lysate system [Promega] that contains all necessary components to initiate protein synthesis from RNA; it is a sensitive system to check if *in vitro* transcribed RNA is indeed recognizable by a mammalian protein

biosynthesis apparatus before it is used for transfection into mammalian cells.

All reagents were stored at -80°C and were slowly allowed to thaw on ice. A standard reaction was assembled in a 0.5 ml polypropylene microcentrifuge tube according to the protocol in Table 12.

The reaction tube was spun down in a centrifuge to return the sample to the bottom of the tube; it was thereafter incubated immediately at 30°C for 90 minutes. For further analysis, a protein gel and subsequent Western Blot were performed to detect the translated protein.

reagent	brand	volume
Rabbit reticulocyte lysate	Promega	35.0 µl
Amino acid mixture, complete	Promega	1.0 µl
RNasin Ribonuclease Inhibitor (40 units/µl)	Promega	1 µl
RNA substrate in water (1 µg/µl)	Promega	2 µl
Nuclease-free water to a final volume of		50 µl

Table 12: *In vitro* translation (Rabbit reticulocyte lysate system).

4.12 Mice

Female BALB/c mice were purchased from Charles River Breeding Laboratories [Sulzfeld, Germany]. The animals were kept in a conventional animal facility. They were 6 to 8 weeks old at the onset of experiments.

4.13 Generation of DC from bone marrow

The generation of bone marrow-derived DC (BMDC) was performed according to the protocol by Lutz *et al.* [144]. Briefly, both femurs and tibias were dissected from BALB/c mice; all muscle tissue was removed by rubbing it off manually with an unsterile Kleenex tissue. The intact bones were left for 2 – 3 minutes in 70% ethanol for disinfection and afterwards washed with sterile PBS. Both ends of each bone were cut with sterile scissors. The marrow was rinsed with sterile complete medium using a syringe (0.5 mm needle diameter) until the bone was completely white. Marrow clumps were disintegrated by pipetting up and down with a 5 ml serological pipette. The cell suspension was then centrifuged for 10 minutes at room temperature and 1,600 rpm. The cell pellet was washed with complete medium. Cells were counted using a 1:10

dilution with bromothymol blue. $2x10^6$ cells were suspended in 10 ml complete medium in a 10 cm bacterial Petri dish to which 200 U/ml recombinant mouse GM-CSF [rmGM-CSF; Peprotech, London, Great Britain] were added. On day 3 and 6 after preparation, 5 ml fresh culture medium containing 200 U/ml rmGM-CSF was added.

4.14 Generation of LeIF-primed lymph node single cell suspensions

A method to expand antigen-specific T cells for experimental purposes has been described before [216]. Briefly, 70 µg of recombinant LeIF (rLeIF) was dissolved in PBS in a final volume of 200 µl. Depending on the experiment, 5 to 10 BALB/c mice [Charles River] were shaved at their flanks and anesthetized in an Erlenmeyer flask containing 100 µl halothane [Hoechst, Frankfurt, Germany]. They received 70 µg rLeIF injected subcutaneously in PBS distributed over three sites of their shaved flanks. After 7 days, an immunological boost was performed injecting 35 µg rLeIF. On day 10, the mice were sacrificed; well visible bilateral inguinal, brachial and axillary LN that drained the injection sites were removed with a forceps, passed through a sterile 70 µm strainer [BD Falcon, Heidelberg, Germany] with a syringe stamp which was rinsed with PBS several times. For generation of naïve LN cells serving as a negative control, LN from the popliteal, brachial, inguinal, axillary and paraaortic regions of non-treated mice were removed and processed in the same manner. The cell suspensions were washed with PBS twice and either immediately used for experiments or for further purification.

4.15 Purification of T cells

T cells were purified from LN suspensions using the MACS Pan T cell isolation kit [Miltenyi Biotec], following the company's protocol. The MACS technology is based on a negative selection assay in which non-T cells (i. e. B cells, NK cells, DC, macrophages, granulocytes and erythroid cells) are depleted by indirect magnetic labeling, using a cocktail of biotin-conjugated antibodies and magnetic anti-biotin MicroBeads. Labeled cells are retained in a column that is suspended in a magnetic field, and T cells are eluted from the column.

Briefly, cells were counted when still in the PBS suspension. After centrifugation at 4°C and 1,600 rpm, 40 µl of labeling buffer per 10^7 total cells was used for resuspension. 10 µl of biotin antibody cocktail per 10^7 total cells was added to label cells. The suspension

was mixed well and incubated in a refrigerator at 4°C. After 10 minutes, 30 µl of labeling buffer and 20 µl of anti-biotin MicroBeads were added per 10^7 total cells. After an incubation time of 15 minutes at 4°C, the cells were washed with buffer adding 10 – 20x labeling volume. After centrifugation, the pellet was resuspended in 500 µl of buffer per 10^8 total cells, but no less than 500 µl. For up to 10^7 total cells, one medium size (MS) MACS column was used and placed into the magnetic field of a MACS separator. The column was prepared by rinsing three times with 500 µl of buffer, without having the column run dry. The effluent was discarded. Thereafter, the suspension containing the labeled cells was applied to the column, and the column was rinsed three times with 500 µl of buffer. The effluent, containing the enriched T cell fraction, was collected, and the column was discarded. This procedure was carried out several times until the entire volume was purified. The cells were kept on ice until they were centrifuged and taken up in fresh complete medium.

4.16 Lymphocyte cultures for cytokine analysis

Lymphocyte cultures were set up to study the effect of LeIF RNA-transfected and rLeIF-pulsed BMDC on naïve and LeIF-primed T cells by cytokine analysis. A DC to T cell ratio of 1:2 was chosen.

First, BMDC were collected from cell culture 24 hours after RNA transfection. The 6-well plates were placed on ice for 5 minutes, the supernatant was removed using a 5 ml serological pipette, and the wells were rinsed with PBS. Previous studies showed that cells collected from the supernatant have the same properties as cells adhering to the surface of the culture plate [V. Fuss, personal communication; 144]. The cells were washed in a suitable volume of complete medium, counted, centrifuged and taken up in fresh complete medium at a concentration of $2x10^6$ cells/ml.

BMDC were either cultivated with LN cells or with purified T cells. Cells from a LN single cell suspension were taken up in fresh complete medium at a concentration of $8x10^6$/ml; purified T cells were adjusted to a concentration of $4x10^6$/ml. Cell cultures were assembled in 96-well plates in triplicates. Using a multipette, 50 µl from BMDC suspensions and 50 µl from T cell or LN cell suspensions were transferred into every well, so that a final concentration of $1x10^6$ cells/ml for BMDC, $2x10^6$ cells/ml for T cells and $4x10^6$ cells/ml for LN cells was reached. If a sample only contained one cell

type, the remaining volume was filled up with fresh complete medium. The cell cultures were incubated at 37°C for 72 hours. Culture plates were then centrifuged and the supernatant collected for ELISA analyses.

4.17 FSDC cell line

A fetal skin-derived DC (FSDC) line culture [63] was split after reaching a confluence of about 80% under the inverted microscope. The cell culture medium was removed from the flask using a pipette, and 4 ml cold, calcium- and magnesium-free PBS was added to the cell culture with the intention to enhance cell detachment. After 5 minutes, the cells were removed from the surface using a cell scraper, and 6 ml PBS was added. The cells were resuspended carefully 5 to 10 times. Depending on the number of cells needed for the experiment, 0.5 to 2.0 ml of the cell suspension was transferred into a culture flask containing 35 to 45 ml RPMI medium and cultured at 37°C.

4.18 RNA transfection/protein electroporation of DC

BMDC were used for electroporation on day 7 to 9 of cell culture, FSDC were used after reaching a confluence of 70 to 90% under the inverted microscope.

FSDC were removed from culture flasks using 5 ml of cold PBS to facilitate detachment. BMDC were used on day 7 of culture; they were resuspended 5 to 10 times with a 10 ml serological pipette, and the Petri dish was rinsed thoroughly. Cells were pooled in 50 ml Sarstedt tubes and centrifuged for 10 minutes at 4°C and 1,600 rpm. The supernatant was removed.

All cells were washed twice in 10 ml PBS (to ensure RNAse-free conditions that are otherwise mainly impaired by the use of FCS) and counted in a Neubauer counting chamber. They were taken up in an appropriate volume of warm Opti-MEM medium [Gibco BRL, Eggenstein, Germany] (37°C), counted and adjusted to a final concentration of $20x10^6$ cells/ml. Of this suspension, 200 µl was transferred into a sterile 4 mm electroporation cuvette [Peqlab] and, using RNAse-free pipette tips, mixed with 10 to 40 µg RNA or 10 to 40 µg of recombinant antigen that was carefully resuspended. The cuvette was placed into the electroporator, and a pulse of 150 µF, 300 mV and 6 ms was carried out. The cells were transferred immediately into 3.8 ml fresh complete medium to reach a final concentration of $1x10^6$ cells/ml. Depending on what

experiment was to be performed, the cell suspension was then transferred into 6-well or 12-well culture plates and stored in an incubator at 37°C and 5% CO_2.

4.19 Antigen pulsing of BMDC

Day 7 to 9 BMDC were collected, washed in PBS and taken up in fresh complete medium at a concentration of $1x10^6$ cells/ml and transferred to 6- or 12-well plates [Nunc]. Recombinant antigen was added as indicated (10 to 40 µg) and mixed thoroughly by resuspension; plates were stored thereafter in an incubator at 37°C and 5% CO_2 for 24 hours.

4.20 Preparation for analysis by FACS and extracellular staining

Flow cytometry or FACS is a technique that allows quantitative analyses of cell surface markers and intracellular proteins. During the staining procedure, cells are exposed to specific monoclonal antibodies that bind to their target structures. Antibodies are either labeled with a fluorescent dye, or are detected by a labeled secondary antibody. Within the flow cytometer, the cell suspension is forced through a small capillary. Here, laser beams of appropriate wavelengths are directed onto the suspension. The fluorescent dyes are excited and emit light at a lower frequency which is picked up by detectors. Usually, 10,000 cells were measured; the results can be visualized as dot plots, coordinate systems that visualize distinct cell populations.

To check EGFP transfection of DC by measuring fluorescence in a FACS machine [FACScalibur, Becton-Dickinson], 300 – 500 µl of the cell culture was removed after 3 to 96 hours, transferred into FACS staining tubes, washed twice with PBS and eventually taken up in 500 µl 1% PFA in PBS. The samples were vortexed afterwards to prevent cell clumping.

For extracellular staining against the surface markers MHCII, CD4, CD8, CD11c, CD40 and CD86, 0.5 to $1x10^6$ cells of every sample were transferred into a FACS tube, washed with 2 ml PBS and centrifuged for 10 minutes at 4°C and 1,500 rpm. The supernatant was removed; the pellet was washed with 2 ml FACS buffer, recentrifuged and the supernatant again removed. 100 µl of a 1:50 dilution of anti-Fc receptor antibody [purified rat anti-mouse CD16/CD32, BD Biosciences PharMingen] in PBS were added to the pellet. After short vortexing and an incubation time of 10 minutes at

4°C, 1 µl of a specific monoclonal antibody labeled with a fluorescent dye (phycoerythrin (PE) or fluorescein isothiocyanate (FITC)) was added. For each analysis, a staining with an isotype-matched control antibody was performed. After incubation for another 20 minutes at 4°C, cells were washed with 2 ml of FACS buffer.

If a directly labeled antibody was not available, 1 µl of an unlabeled or biotinylated primary antibody directed against the structure in question was used instead. After washing, 2 µl of a PE- or FITC-labeled secondary anti-immunoglobulin antibody with specificity against the unlabeled primary one was added and allowed to incubate for 30 minutes at 4°C. Equally, if the primary antibody was biotinylated, 2 µl of PE- or FITC-labeled streptavidine was added. The pellet was subsequently washed with FACS buffer as described. To maintain the cell structures for appropriate analysis, 0.5 ml of 1% PFA in PBS was added; the sample was vortexed thereafter.

4.21 Intracellular staining for analysis by FACS

Intracellular staining against LeIF was performed not until extracellular staining was completed. The last step of the extracellular staining procedure was omitted, and 2 ml of intracellular staining buffer ("saponin buffer") was added and incubated for 30 minutes at room temperature in the dark. The suspension was centrifuged, and the supernatant was decanted. 100 µl of a 1:50 dilution of the anti-CD16/CD32 antibody was added and incubated for 30 minutes in the dark, at room temperature. The suspension was washed with 1 ml saponin buffer and centrifuged with subsequent removal of the supernatant. The primary anti-LeIF antibody was added in a 1:100 dilution in saponin buffer and incubated for 30 minutes as described above. The sample was washed as indicated, and the secondary anti-rabbit antibody was added in a 1:100 dilution with saponin buffer in a total volume of 100 µl and incubated for 30 minutes as described. Again, the sample was washed with 1 ml saponin buffer, and the cells were resuspended in 0.5 ml FACS buffer or fixated with 1% PFA in PBS.

4.22 Preparation of adherent cells for fluorescence microscopy

The day before the experiment, at least 3 hours before staining, the cells to be analyzed by fluorescence microscopy were transferred in a volume of 400 µl of a $1x10^6$ cell suspension into 8-well chamber slides [Nunc] to allow their adherence to the glass surface. Every well was washed 3 times with 400 µl of warm PBS, then once with 400 µl PBS (Mg^{2+}, Ca^{2+}) – see section 2.3.

The supernatant was removed by a vacuum pump. The cells were fixed by adding 200 µl 4% PBS (Mg^{2+}, Ca^{2+}), allowing an incubation time of 30 minutes. Two washing steps with PBS (Mg^{2+}, Ca^{2+}) followed. For blocking unbound surface area, 200 µl extracellular staining buffer ("staining buffer", see section 3.4) was added and incubated for 5 minutes at room temperature, then washed. Subsequently, the first antibody was added using the appropriate dilution with staining buffer in a total volume of 100 µl. Incubation was performed for 45 minutes at room temperature in a dark, humid environment on a shaker: the chamber slides were covered with aluminum foil and placed on a humidified tissue. The wells were washed twice with PBS (Mg^{2+}, Ca^{2+}). 200 µl extracellular staining buffer was added and incubated for 5 minutes. The second antibody was added using an appropriate dilution with staining buffer in a total volume of 100 µl. Incubation was performed as described. Again, the wells were washed twice with PBS (Mg^{2+}, Ca^{2+}).

The well mounting was removed with a forceps as were the glue remainders. Remaining PBS was allowed to drop off. 15 to 20 µl mounting solution [DAKO Diagnostika, Hamburg, Germany] was added to the slide surface evenly, and the coverslip was dropped gently while avoiding gas bubbles. Excessive solution was removed with a tissue. Nail polish was used to seal the slide and the coverslip to avoid running dry. The slides were stored at 4°C until used for fluorescence microscopy.

4.23 Preparation of DC lysates for protein analysis

The entire procedure was performed at 4°C. The supernatant was removed from the cell cultures using a Pasteur pipette, and the cells were washed with 1x PBS. Subsequently 0.6 to 1 ml RIPA buffer (see section 3.4) was added to the cell culture. The cells were removed from the dish surface using a cell scraper, transferred into an Eppendorf cap and resuspended 5 to 10 times in order to shear the DNA. 100 µl microbeads [Miltenyi

Biotec] was added, and the suspension was incubated for 5 minutes in a rotor. After centrifuging the samples for 20 minutes at 13,000 rpm, the supernatant containing cell proteins was removed and immediately frozen at -80°C.

4.24 Protein Gel

The glass plates were cleaned with ethanol and assembled in a clamping fixture. A 10% separation gel was prepared from 2 ml aqua dest., 1.25 ml 1.5 M Tris/HCl (pH 8.8) and 100 µl 10% SDS. Under a fume hood, 1.65 ml acryl amide, 50 µl 10% ammonium persulfate (APS) and 10 µl tetramethylethylene diamine (Temed) were added. The separation gel was poured into the gel chamber up to the first marker line and covered carefully with water. After 10 to 20 minutes, allowing the gel to polymerize, the water was removed as completely as possible with a pipette. Filter paper was used to dry the area above the gel.

The 4% stacking gel was prepared from 1.5 ml aqua dest., 0.625 ml 0.5 M Tris (pH 6.8) and 100 µl 10% SDS. Under the fume hood, 0.325 ml acryl amide, 50 µl 10% APS and 10 µl Temed were added. The stacking gel was poured onto the polymerized separation gel up to the upper edge of the chamber, and a well-forming comb was placed in position, avoiding air bubbles getting trapped between the teeth. The stacking gel was then allowed to polymerize for 15 to 60 minutes. After disassembling the clamping fixture and removing the comb, the wells were rinsed with water several times, and excessive gel in the upper part of the chamber was removed, using a small syringe. The glass plates were inserted into the electrophoresis cell [Miniprotean II unit, BioRad]; the reservoirs were filled with running buffer. Samples were mixed with 6x sample buffer for protein gels, heated to 95°C for 5 minutes, centrifuged and then loaded into the wells in the stacking gel. The first well was loaded with a 10 kD marker [Benchmark, Invitrogen]. Electrophoresis was started at a low voltage of 80 V for the stacking gel and was continued at a voltage of 100 to 150 V after the sample had entered the separation gel. It was continued until the blue dye had reached the bottom of the separation gel. After running the electrophoresis, the glass plates were taken out and carefully separated with a dispenser to enable careful removal of the gel.

4.25 Western Blot

Blotting the protein gel onto a nitrocellulose membrane allowed detection and visualization of transfected proteins.

A piece of nitrocellulose membrane [0.45 µm, BioRad] and 4 Whatman filters were cut to fit to the size of the protein gel and soaked in transfer buffer. While assembling the different components of the transfer unit in the correct order, attention was paid to avoid gas bubbles. The mounting was then compressed and inserted into the transfer chamber [Mini Trans Blot cell, BioRad]. An ice block was equally fixed and the chamber filled to the rim with transfer buffer. Transfer was performed at 250 mA for 90 minutes.

To verify the effectiveness of the Western Blot, the transferred proteins were visualized with a Ponceau staining (2 minutes incubation, then rinsing with water until the background was clear). Overnight, the membrane was stored in a flat Petri dish covered with blocking solution at 4°C.

The following day, the membrane, still in a Petri dish, was washed three times with sufficiently PBS + 0.05% Tween20, each time for 5 minutes on a shaker. Thereafter, it was incubated with the primary antibody (monoclonal anti-EGFP [Clontech] or rabbit anti-LeIF [kindly provided by Dr. Skeiky, Corixa Corp.]) in a 1:2000 dilution in blocking solution for 2 hours while shaking. After 3 washing steps in PBS + 0.05% Tween20 (as described), each for 5 minutes, the membrane was incubated with the alkaline phosphatase-conjugated secondary antibody in a 1:5000 dilution in blocking solution. After 3 more washing steps in PBS + 0.05% Tween20, 10 ml of WesternBlue [Promega] was added to visualize the transferred protein. The reaction was halted and the membrane washed with PBS + 0.05% Tween20 when the expected bands were well recognizable and the background was still clear. The marker was transferred from the protein gel immediately and could be seen without further staining.

4.26 Cytokine detection by ELISA

For detection of cytokines in the supernatants of lymphocyte cultures, sandwich ELISAs were performed. On day 1, the 96-well ELISA plates were coated with the primary antibody. The purified capture anti-cytokine antibodies were diluted in carbonate coating buffer, 50 µl of this solution was dispensed into every well, and the plates were incubated in a refrigerator at 4°C overnight. Monoclonal capture antibodies

were used in the following concentrations: rat anti-mouse IFN-γ, 1 µg/ml; rat anti-mouse IL-2, 2.5 µg/ml; rat anti-mouse IL-4, 1 µg/ml; rat anti-mouse IL-6, 4 µg/ml; rat anti-mouse IL-10, 5 µg/ml; rat anti-mouse IL-12p70, 2.5 µg/ml [BD Biosciences PharMingen]; rat anti-mouse IL-1β, 4 µg/ml [R & D Systems, Wiesbaden, Germany].

On day 2, the plates were rinsed three times with PBS/Tween20. To block unspecific binding, 200 µl of a 10% BSA in PBS/Tween20 blocking solution was transferred into every well and incubated at 4°C for 4 hours. After 3 washing steps with PBS/Tween20, a 100 µl standard sample of the cytokine in question, having a defined concentration, was added to the plate, and a serial 1:2 dilution in 1% BSA/PBS/Tween20 was set up for a standard curve. The samples to be measured were added to the plate at a volume of 50 µl per well; incubation was at 4°C overnight.

On day 3, the plates were again washed three times with PBS/Tween20. To detect antibody-cytokine complexes, a biotinylated detection antibody directed against the cytokine was diluted 1:500 in 1% BSA/PBS/Tween20, and 50 µl of this solution was transferred into each well. The antibody was incubated for 1 hour at room temperature and washed with PBS/Tween20. End concentrations of detection antibodies used were: biotin-conjugated rat anti-mouse IFN-γ, 1 µg/ml; biotin-conjugated rat anti-mouse IL-2, 1 µg/ml; biotin-conjugated rat anti-mouse IL-4, 1 µg/ml; biotin-conjugated rat anti-mouse IL-6, 2 µg/ml; biotin-conjugated rat anti-mouse IL-10, 1 µg/ml; biotin-conjugated rat anti-mouse IL-12p70, 2.5 µg/ml; [BD Biosciences PharMingen]; biotin-conjugated rat anti-mouse IL-1β, 0.1 µg/ml [R & D Systems].

A streptavidine-alkaline phosphatase complex [DAKO Diagnostika] was then prepared as a 1:1000 dilution in PBS and transferred at a volume of 50 µl per well. For visualization, 100 µl of p-nitrophenyl phosphate [1 mg/ml, Sigma] dissolved in diethanolamine buffer was pipetted into every well and incubated at room temperature under an aluminum foil cover. After 1 hour, the first determination of the OD at 490 and 405 nm was performed using an ELISA reader [MRX, Dynatech Laboratories, Chantilly, USA]. The measurement was repeated later several times, and the one revealing the most accurate standard values was used for further analysis. The cytokine concentrations were calculated by comparing the absorbance values of the samples to the standard curve obtained by linear regression.

Detection thresholds were 0.012 ng/ml for IL-1β; 0.488 ng/ml for IL-2; 6.1 pg/ml for

IL-4, IL-6 and IL-10; 0.157 ng/ml for IL-12p70; 0.098 ng/ml for IFN-γ.

For statistical analysis, unpaired Student's *t* tests were performed. If statistical significance was observed, bars in the graphs are marked as follows: One asterisk denotes $p < 0.05$, two asterisks denote $p < 0.01$, three asterisks denote $p < 0.001$.

5 Results

5.1 Generation of translatable RNA

Initial experiments of this study were conducted with the intention to clone the EGFP sequence into the commercially available *in vitro* expression vector pSP64 [Promega]. This vector provides a polycloning site within a very short distance of only a few base pairs of the SP6 promotor and a poly(A) region. EGFP was cloned successfully into this vector. The RiboMAX Large Scale RNA production system [Promega] was used for *in vitro* transcription, utilizing the Ribo m^7G Cap Analog for enhanced stability [270].
EGFP-RNA generated by this method was well translatable in a cell-free system (Rabbit Reticulocyte Lysate System [Promega]), as could be shown by its detection in a Western Blot using an EGFP-specific monoclonal antibody [data not shown]. However, when used for transfection, DC receiving up to 20 µg RNA would show a maximum transfection efficiency of 5% [data not shown] which, for the elicitation of an immune response, seemed by far too low.

5.1.1 Successful ligation of inserts into the pGEM-5'UT-3'UT-A64 vector

The molar vector-to-insert ratio was varied from 1:1 to 1:20 to minimize the risk of inadequate ligations. After the ligation reaction, ligated plasmids were transformed into competent cells and spread on agar plates.

insert	vector dilution		
	pGEM 1:1	pGEM 1:5	pGEM 1:20
no insert	0	2	8
LeIF(226)	2	4	13
no insert	4	1	0
LeIF(fl)	95	10	5

Table 13: Numbers of clones after transformation of competent cells. Predigested LeIF(226) and LeIF(fl) inserts were ligated into pGEM vectors in different molar ratios, or pGEM vectors were religated in the absence of inserts. Upon ligation, competent *E. coli* XL-1 Blue were transformed with the reaction products. The numbers represent the number of clones per agar plate. The number of clones in samples containing inserts is higher than in samples containing no inserts. There is also a dependency between vector molarity and the number of clones.

Table 13 shows the dependency of the number of bacterial colonies on the molar ratio. The higher number of clones in insert-containing samples suggested that the ligation was successful.

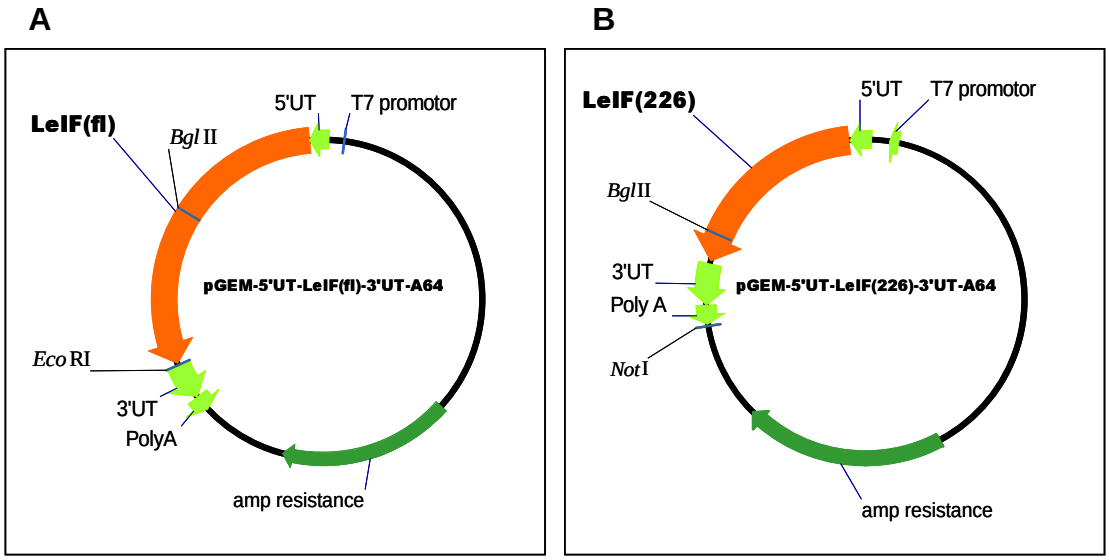

Fig. 7: Schematics of pGEM-LeIF(fl) [A] and pGEM-LeIF(226) [B]. The sequences of LeIF(fl) and LeIF(226) were cloned into the *in vitro* transcription vector pGEM-5'UT-3'UT-A64 kindly provided by Dr. Kris Thielemans, Brussels. To control successful cloning, plasmids obtained from cloning were digested with single cutters of whom one would cut within the vector, the other one within the insert (*Bgl*II and *Eco*RI for pGEM-LeIF(fl); *Bgl*II and *Not*I for pGEM-LeIF(226)). The predicted fragment sizes are 321 bp (pGEM-LeIF(fl)) and 632 bp (pGEM-LeIF(226)).

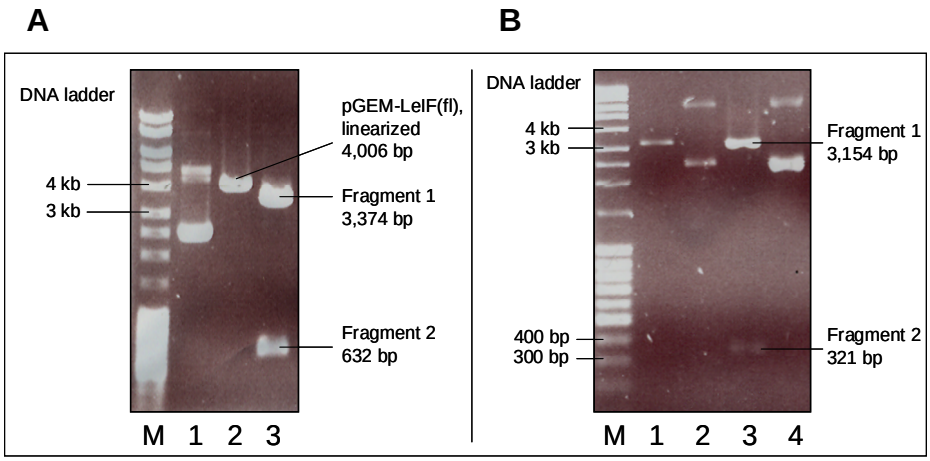

Fig. 8: Differential digestions of pGEM-LeIF(fl) [A] and pGEM-LeIF(226) [B]. After digestion, the reaction products were visualized on an agarose gel. [A] shows one representative clone that was digested with *Bgl*II and *Eco*RI: the undigested pGEM-LeIF(fl) plasmid in lane 1, the linearized plasmid in lane 2 and the double digest in lane 3. The fragments were of the expected sizes. [B] shows two clones obtained from pGEM-LeIF(226) cloning that were digested with *Bgl*II and *Not*I: the digested plasmids in lanes 1 and 3, the undigested plasmids in lanes 2 and 4. A fragment of the expected size is visible in lane 3; the clone in lanes 1/2 is negative, the clone in lanes 3/4 represents the expected plasmid.

In order to check for the correct orientation of the insert, 12 (pGEM-LeIF(fl)) and 17 (pGEM-LeIF(226)) colonies were picked and spread on agar plates. The DNA was prepared by mini preparation from overnight cultures as described in section 4.4 and digested with single cutting restriction enzymes at two different restriction sites: pGEM-LeIF(fl) was cut with *Bgl*II and *Eco*RI, pGEM-LeIF(226) was cut with *Bgl*II and *Not*I. In both cases, as can be seen in Fig.7, *Bgl*II is supposed to cut within the insert, and *Not*I and *Eco*RI, respectively, are supposed to cut within the vector part of the construct. The two expected bands in the agarose gel, as shown in Fig. 8 A (lane 3) and 8 B (lane 3), would therefore appear only if both parts were present. One clone was chosen for further experiments.

5.1.2 *In vitro* transcription and subsequent *in vitro* translation

Following the Ambion protocol for *in vitro* transcription, capped RNA was generated. The transcripts were run in an agarose gel and had the appropriate sizes, which is shown for LeIF(fl)- and LeIF(226)-RNA in Fig. 9.

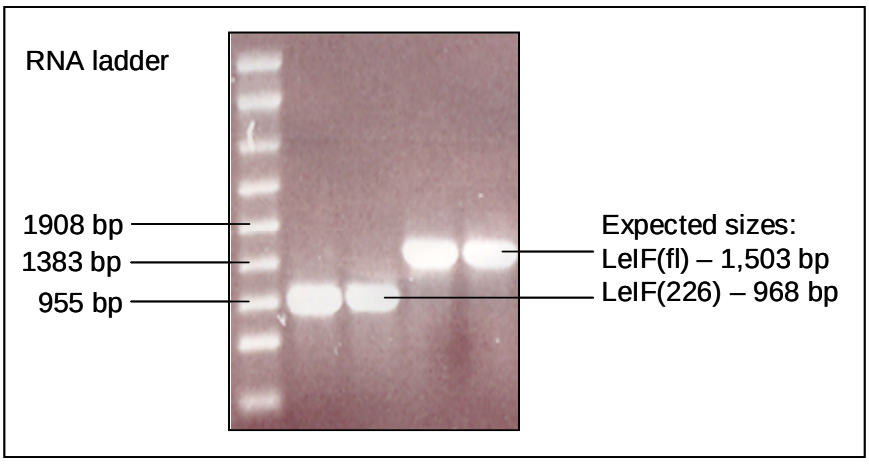

Fig. 9: RNA transcripts. *In vitro* transcripts were visualized on an agarose gel. The left lane shows a standard RNA marker [Promega]. LeIF(fl)- and LeIF(226)-RNA are shown to have the expected sizes.

To ensure that the transcripts were indeed readily recognizable by the ribosomal apparatus, they were translated in a cell-free Rabbit Reticulocyte Lysate System [Promega] according to the protocol. A protein gel was run afterwards, and a Western Blot was performed that showed single protein bands of the expected size.

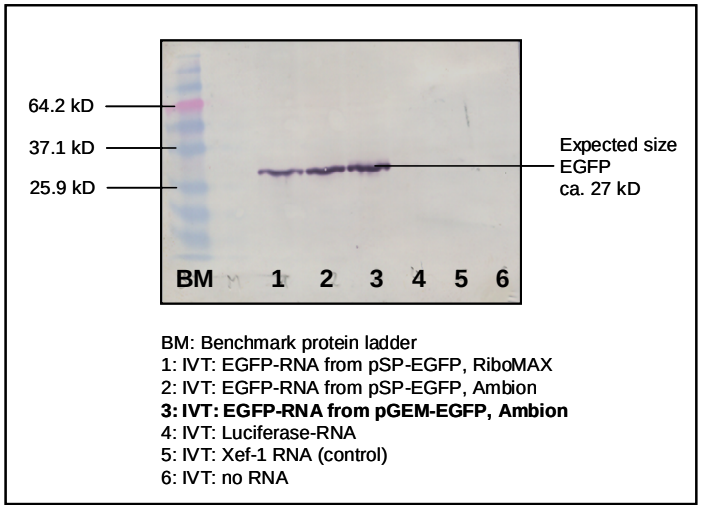

Fig. 10: *In vitro* translation of RNA from different plasmids using different *in vitro* transcription kits. RNA was transcribed *in vitro* in a cell-free system from different linearized plasmids containing the EGFP sequence and from two control plasmids. The reaction product was detected with a monoclonal anti-EGFP [Promega] antibody in a Western Blot.

Fig. 10 gives a semiquantitative impression of translation efficiencies obtained with different RNA encoding for the same protein, but having different primary structures and using different *in vitro* transcription kits. EGFP-RNA transcribed from the pSP64-EGFP vector (lanes 1 and 2) is translatable in the cell free system, and the protein is recognized by the monoclonal anti-EGFP antibody. The band, however, appears weaker than the one resulting from pGEM-EGFP RNA transcription in the Ambion system (lane 3). Reaction products from control RNA (lanes 4 and 5) and from a transcription sample containing no RNA (lane 6) were not detected by the anti-EGFP antibody. Hence, RNA obtained from *in vitro* transcription was translatable into the correct protein.

5.2 Proof of transfection: antigens are expressed intracellularly by DC

5.2.1 Kinetics of transfection efficiency and EGFP expression in FSDC

Before analyzing the effects of RNA transfection on BMDC, it was necessary to develop a reporter assay that would provide detailed and reliable information on how efficiently the antigen is actually translated by BMDC, i. e. what percentage of cells express the antigen and how stably it is being expressed.

To that end, the leishmanial antigen LeIF that was to be studied would have been difficult or at least impractical to detect. However, nucleic acid constructs containing EGFP are a common tool for transfecting various types of cells. The fluorescence of EGFP allows simplified tracking of transfected cells by confocal laser scanning microscopy (CLSM), standard fluorescence microscopy or FACS.

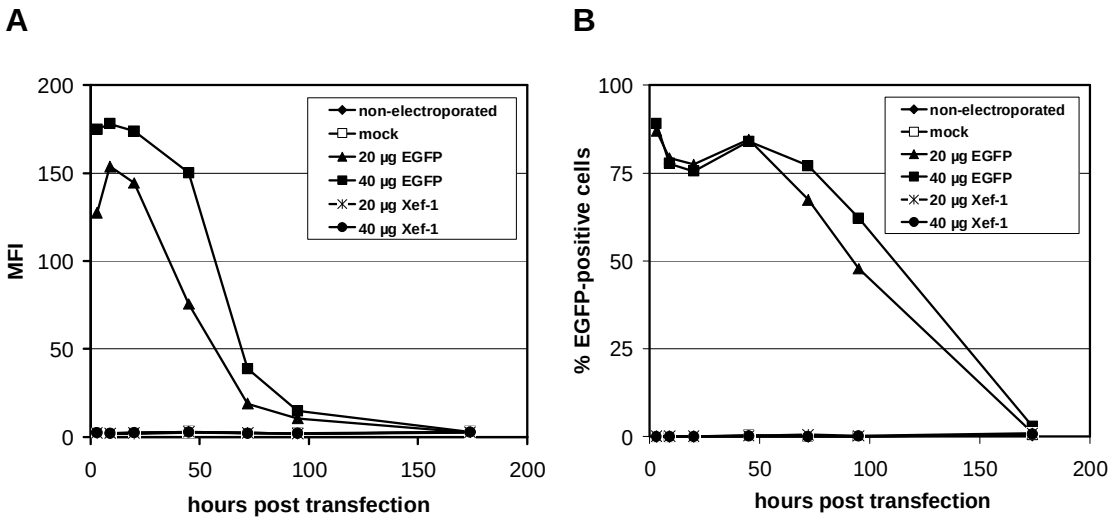

Fig 11: Kinetics of antigen expression and transfection efficiency in FSDC. FSDC were harvested from cell cultures and transfected with the indicated amount of RNA, electroporated without RNA in solution [mock] or not treated. Cells were resuspended in medium, cultured and analyzed for fluorescence by FACS at the indicated time points. The mean fluorescence intensity (MFI) served as a marker for antigen expression [A]; the percentage of EGFP-positive cells indicates the transfection efficiency [B].

For the establishment of a protocol, the FSDC line, first described by Girolomoni *et al*. [63], was used instead of primary cells for easier manageability, to ensure that the electrical parameters reported by van Meirvenne *et al*. for BMDC transfection (capacitance of 150 µF and voltage of 300 V; [258]) would indeed yield the best results. In the presented data, the term “transfection efficiency” refers to the percentage of cells

with fluorescence higher than the gate selected with reference to a mock-transfected cell population (electroporated BMDC without RNA).

Initial experiments utilizing RNA transcribed from the pSP64-EGFP vector showed only very low yields with low antigen expression and a maximum transfection efficiency of 5% [data not shown]. Using EGFP-RNA transcribed from the pGEM-EGFP vector, however, resulted in a maximum efficiency of 86.9% (20 µg) and 89% (40 µg) 3 hours after transfection. Fig. 11 B shows that the transfection efficiencies in FSDC transfected with different amounts of RNA are similar. After the early maximum, the percentage of EGFP-positive cells decreases transiently and reincreases to a second, slightly lower maximum of 84.5% (20 µg) and 84% (40 µg) at 48 hours post transfection. From this second peak, the values continuously decrease to zero after 7 days.

As a marker for the level of EGFP expression, the mean fluorescence intensity (MFI), measured by FACS as the geometrical mean of the (green) fluorescence of the entire cell population, was determined. In contrast to the kinetics of transfection efficiency, its kinetics shows only one peak after 9 hours at an MFI of 153.7 (20 µg) and 177.9 (40 µg), respectively. The MFI was constantly higher in cells receiving 40 µg EGFP-RNA, compared to cells receiving only 20 µg (Fig. 11 A). The MFI values decreased more rapidly than the percentage of EGFP-expressing cells.

5.2.2 Kinetics of transfection efficiency and EGFP expression in BMDC

It was important to know if similar results could be obtained in BMDC, cells that might be used *in vivo*. One critical parameter was the day of culture the cells were to be used for transfection. A study similar to this one has been done by van Meirvenne *et al.* [258] who showed that the highest transfection efficiency is attainable if BMDC are transfected on day 7 of culture, when they are still in an immature stage.

Another study with human monocyte-derived DC had revealed that transfection of immature DC results in superior antigen presentation capacity when compared to transfection of DC that were matured prior to the experiment [259]. This was the reason why transfection was performed on day 7 of cultures in most of the experiments described here.

Fig. 12 A shows how the amount of RNA used for transfection influences the

fluorescence, i. e. the EGFP expression, of the cell population. The more RNA is used, the more the cell population shifts to the right. Fig. 12 B illustrates that there is a proportional dependency of the MFI on the amount of RNA.

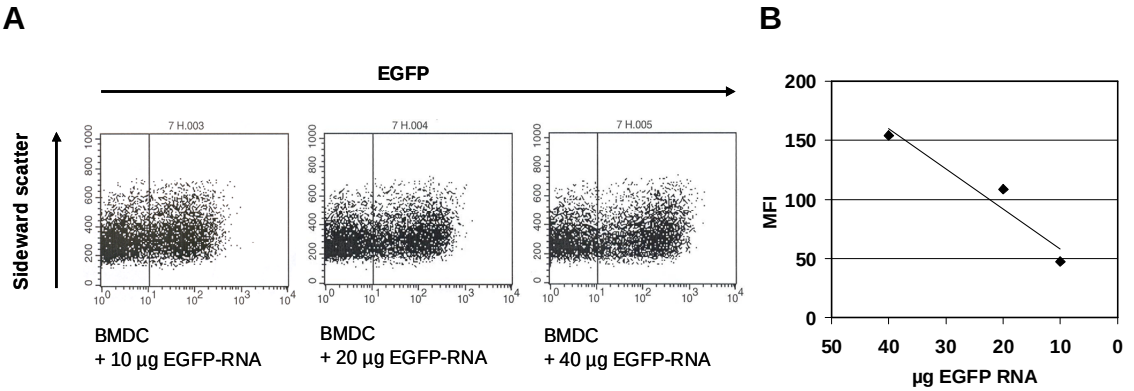

Fig. 12: Antigen expression is directly proportional to the amount of RNA used for transfection. BMDC were harvested at day 7 of cell culture and transfected with the indicated amount of EGFP-RNA. [A] Dot plots show FACS analyses 7 hours after transfection. [B] Dependency of MFI on the amount of RNA 24 hours after transfection.

Fig. 13 shows the kinetics of transfection efficiency and EGFP expression. Both transfection efficiency and maximum EGFP expression in BMDC were shown to be lower than in FSDC. Maximal transfection efficiency (74.2%) was reached with 20 µg EGFP-RNA after 48 hours (Fig. 13 C). On the other hand, Fig. 13 B visualizes the antigen expression, measured as MFI. Even when doubling the amount of RNA from 20 µg to 40 µg, the antigen expression is further increased, up to a maximum of 154.2 after 24 hours. There is a clear dose-dependent effect on the antigen expression that is still obvious after 4 days. Even if the graphs give the impression that after 4 days the level of antigen expression is fairly low, Fig. 13 A shows that a considerable part of the population is still beyond the gate, i. e. expresses EGFP. For future experiments with leishmanial antigens, based on these data, it was decided to use 20 µg of RNA.

Furthermore, it was important to know if the amount of RNA was the only determinant for transfection efficiency and antigen expression. It is known that e. g. LPS from gram-negative bacterial cell walls induces BMDC maturation via binding to TLR4 [113, 258] and enhances antigen presentation and T cell activation [144]. To test if it would also have an impact on expression of proteins encoded by transfected RNA, transfected BMDC were pulsed with LPS (1 µg/ml) for 24 hours.

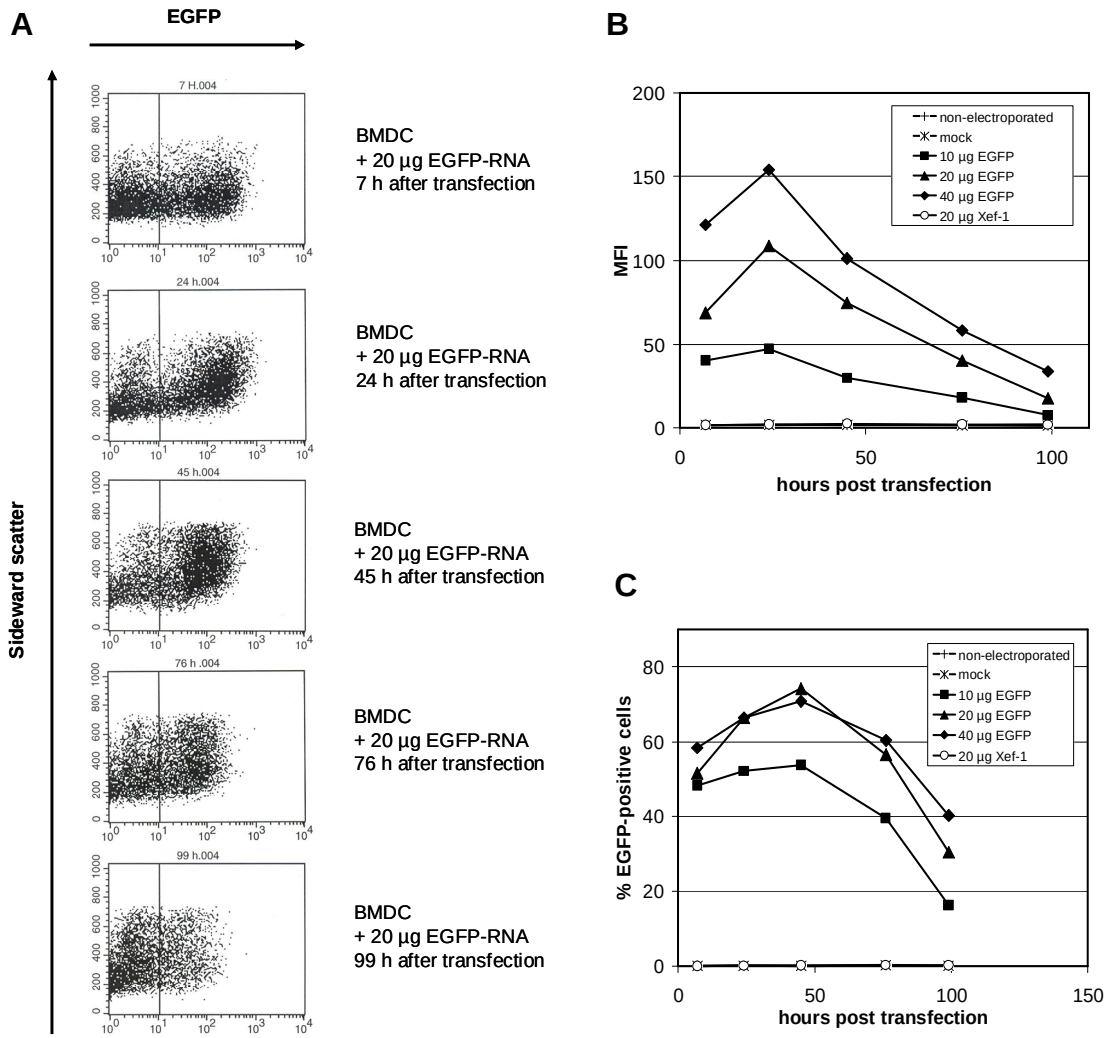

Fig. 13: Kinetics of transfection efficiency and EGFP expression in BMDC is determined by the amount of RNA used. BMDC were transfected at day 7 of culture with the indicated amount of RNA, mock-electroporated or not treated. [A] Dot plots of BMDC transfected with 20 µg of EGFP-RNA; the level of EGFP expression in transfected BMDC [B] and the percentage of EGFP-positive cells [C] are dependent on the amount of RNA used.

As shown in Fig. 14 A, the effect on protein expression (MFI) is not unanimous: LPS treatment of BMDC receiving 10 µg RNA does not cause a higher expression of EGFP. The MFI of cells receiving 20 µg RNA, however, can be further increased with LPS treatment within the first 24 hours. Afterwards, the decline of EGFP expression is similar to if not faster than in BMDC receiving no LPS treatment. Also, LPS treatment does not accelerate the time point of maximum protein expression marked by the MFI.

Also, a higher percentage of cells express EGFP after LPS treatment, as shown in Fig. 14 B. The transfection efficiency of LPS-treated EGFP-transfected BMDC is highest within the first 24 hours (7 hours for 10 µg, 24 hours for 20 µg RNA) and decreases

subsequently, whereas in non-treated BMDC, it reaches its maximum 48 hours post transfection.

While doubling the amount of RNA from 20 to 40 µg does not effectuate an increase of the percentage of EGFP-expressing cells (Fig. 13 C), LPS treatment does: it causes an earlier and higher peak. In other words, a higher percentage of BMDC receiving 20 µg RNA and LPS stimulation is EGFP-positive than after treatment with 40 µg RNA only. However, LPS-stimulated cells also show a more rapid decline of transfection efficiency.

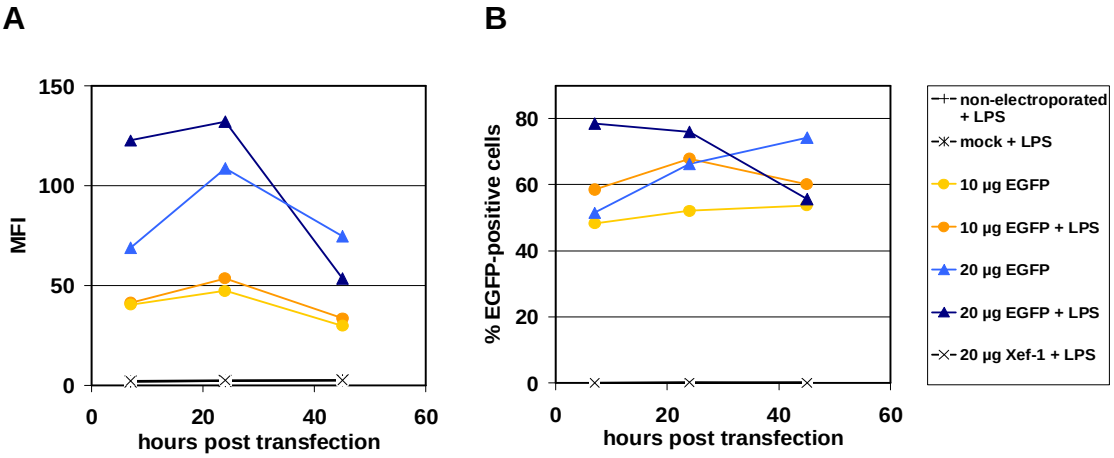

Fig. 14: Upregulation of EGFP expression after LPS treatment of transfected BMDC. BMDC were harvested on day 7 of culture, transfected as indicated and stimulated with LPS (1 µg/ml) for 24 hours. The figures compare the early values of EGFP expression (MFI) [A] and transfection efficiency [B] of LPS-treated and non-treated, EGFP-transfected BMDC.

It was important to know if the cells generated from bone marrow cultures following the protocol described by Lutz *et al.* [144] and expressing EGFP after RNA transfection do indeed exhibit DC properties. Fig. 15 A shows FACS analyses of EGFP-transfected BMDC that were matured with LPS and stained for MHCII expression 24 hours later. Fig. 15 A demonstrates that there is a cell population expressing EGFP without having MHCII markers, and another MHCII-positive population that does not express EGFP. When using 20 µg RNA for transfection, almost 50% of the cells are double-positive for MHCII and EGFP. Hence, the figure shows that a major part of the cells exhibit the desired phenotype. Fig. 15 B illustrates a conventional fluorescence microscope image of EGFP-transfected and MHCII-stained cells. Again, even though there are some cells not expressing EGFP and some MHCII-negative cells, the majority of cells are double positive.

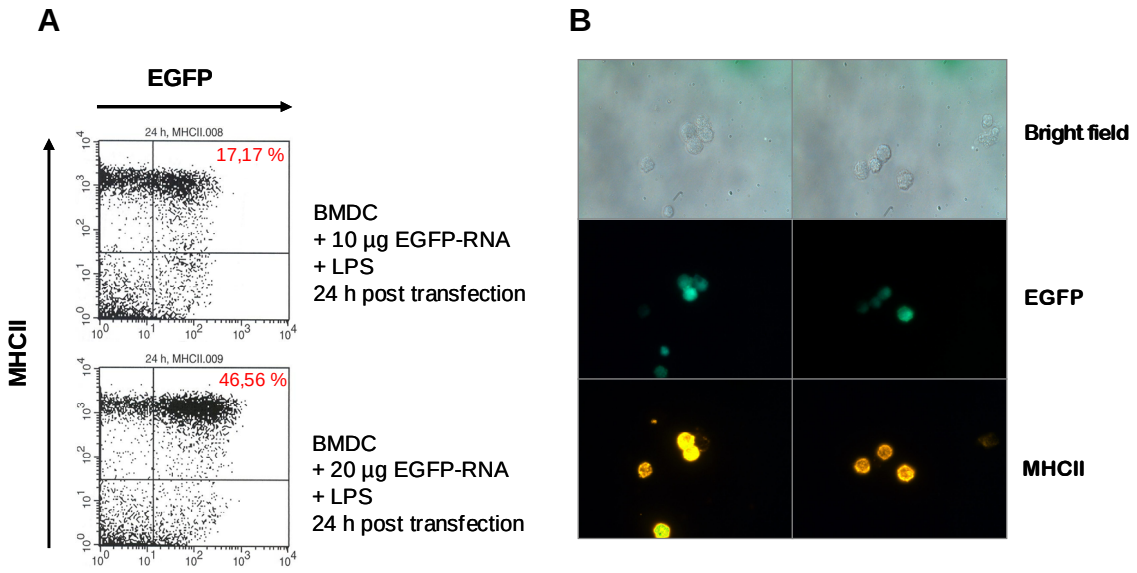

Fig. 15: Transfected cells have characteristics of DC. BMDC were transfected with the indicated amount of RNA on day 7 of culture and stimulated with LPS. After 24 hours, they were collected for staining against MHCII and analyzed by FACS [A], or directly stained in an 8-well chamber slide (see section 4.22) and viewed under a conventional fluorescence microscope [B; two image details].

5.2.3 Viability of BMDC after electroporation

Cells should be alive if they are expected to translate transfected RNA; it would be of great disadvantage if a large number of cells received RNA, but were unable to translate it because the electroporation itself had adverse effects on the viability of BMDC. Fig. 16 shows that BMDC are slightly more resistant to the electric pulse than FSDC. There is no difference in viability between cells receiving only the electric pulse and cells receiving EGFP or irrelevant control RNA; i. e. the (intra- or extracellular) presence of RNA has no additional toxic effect on the cells.

It has been reported in the literature that in certain cell lines, treatment of cells with sodium butyrate at the time of transfection improves transfection rates significantly [241]. This method has been described for certain cell lines and cancer cells, but not for DC. It was reasoned initially that it might also have a positive effect on the transfection efficiency of BMDC; experiments conducted at a sodium butyrate molarity of 1 mM, however, showed a large majority of over 99% dead cells after 24 hours [data not shown]. Owing to this extreme toxicity, further experiments involving sodium butyrate were not performed.

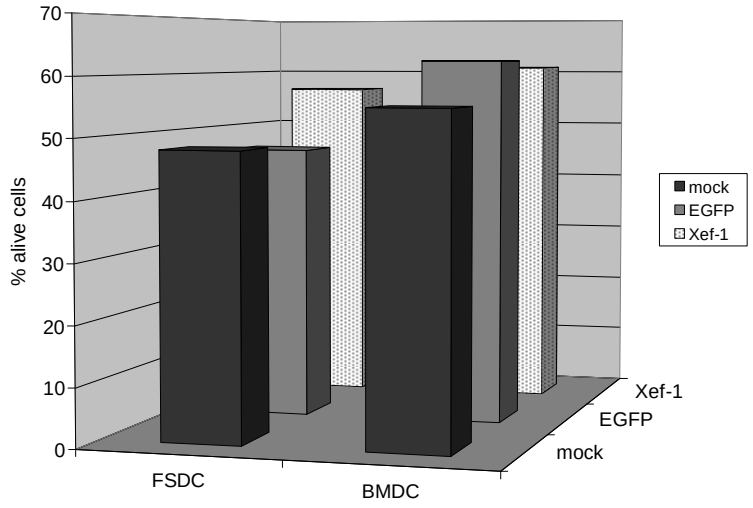

Fig. 16: Viability 24 hours after electroporation. FSDC and BMDC were harvested from cell cultures as described and transfected. After 24 hours, the viability was determined using Trypan Blue staining and counting stained and unstained cells under a conventional microscope.

5.2.4 LeIF(fl), but not LeIF(226) molecules can be detected intracellularly after RNA transfection

In this study, EGFP transfection served mainly as a reporter assay to determine technical parameters for successful transfection. Before conducting experiments with LeIF-RNA for analysis of immunological properties, it was necessary to know that the *in vitro* generated LeIF-RNA was recognized by the mammalian cell and readily translated into the encoded protein.

As has been shown before, the easily detectable EGFP is a very sensitive marker to determine protein expression and transfection efficiency accurately, whereas proof of transfection with non-fluorescent proteins constitutes a somewhat greater challenge, most likely due to the limited sensitivity of the particular assays [258].

Two different methods were used in this study for detecting LeIF in BMDC. First, whole cell lysates from BMDC cultures were prepared 24 hours after transfection as described.

As Fig. 17 shows, no LeIF band is detected in mock-transfected or Xef-1-transfected cells. Furthermore, as expected, transfection with LeIF(fl)-RNA was followed by production of the complete LeIF protein: the polyclonal antibody detected LeIF. Surprisingly, in BMDC that were transfected with LeIF(226)-RNA, the truncated LeIF protein could not be detected.

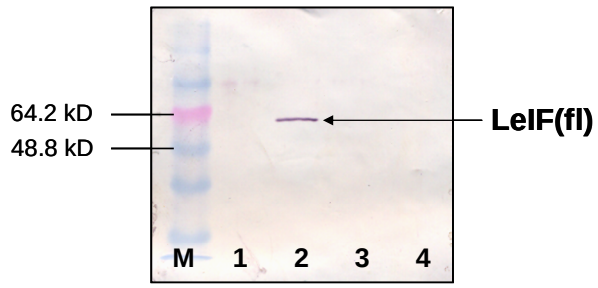

Fig. 17: LeIF(fl), but not LeIF(226) is detectable in DC lysates by Western Blot. BMDC were transfected with 20 µg of the indicated RNA on day 7 of culture; whole cell lysates were prepared as described in section 4.23. The samples were separated in a protein gel, blotted on a nitrocellulose membrane and incubated with a polyclonal rabbit anti-LeIF antiserum. M: marker, 1: BMDC/mock, 2: BMDC/LeIF(fl)-RNA, 3: BMDC/LeIF(226)-RNA, 4: BMDC/ Xef-1-RNA.

The incubation time of the alkaline phosphatase substrate was not the responsible factor, as even after incubation of 30 minutes, there was no band detectable. Furthermore, serial lysates at 2, 6 and 12 hours post transfection were prepared, but the truncated protein was never detected, whereas the LeIF(fl) protein always was [data not shown]. This finding is in distinct contrast with the data obtained from *in vitro* translation in a rabbit reticulocyte lysate system (Fig. 18).

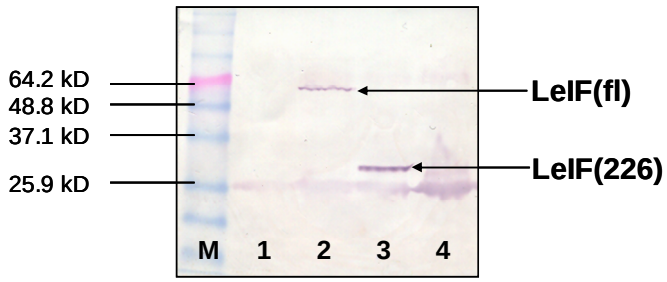

Fig. 18: Western Blot; both LeIF(fl) and LeIF(226)-RNA can be translated *in vitro*. RNA transcribed *in vitro* was used for *in vitro* translation in a rabbit reticulocyte lysate system. The reaction products were run in a protein gel, blotted on nitrocellulose membrane and detected with a polyclonal anti-LeIF antibody. M: marker; 1: no RNA; 2: LeIF(fl)-RNA; 3: LeIF(226)-RNA; 4: Xef-1-RNA.

As the Western Blot shows, LeIF(226)-RNA is well translatable in a cell free system, and the gene product is well recognized by the polyclonal anti-LeIF antibody. These data rule out a potential lack of binding avidity of the antibody towards the C-terminal protein sequence that is missing in the truncated LeIF(226) molecule. They also rule out a frame shift or an inadequate RNA structure that would prevent binding to the ribosome.

As the protein may have been lost during the cell lysis procedure, another approach to detect the LeIF proteins intracellularly was attempted. BMDC, 24 hours after transfection, were permeabilized and stained intracellularly with the anti-LeIF polyclonal antibody; a double stain was performed against MHCII molecules.

While Western Blots of whole cell lysates only allow a qualitative approach, using flow cytometry has the advantage of quantifying results. Fig. 19 A shows that with single intracellular anti-LeIF staining, 11.9% of BMDC transfected with LeIF(fl)-RNA express LeIF, whereas LeIF expression in LeIF(226)-RNA transfected cells is not detectable. Fig. 19 B shows a LeIF/MHCII double stain revealing that 6.3% of LeIF(fl)-transfected cells are double positive for MHCII and LeIF. Fig. 19 B also shows that most cells expressing LeIF are indeed MHCII^{high}.

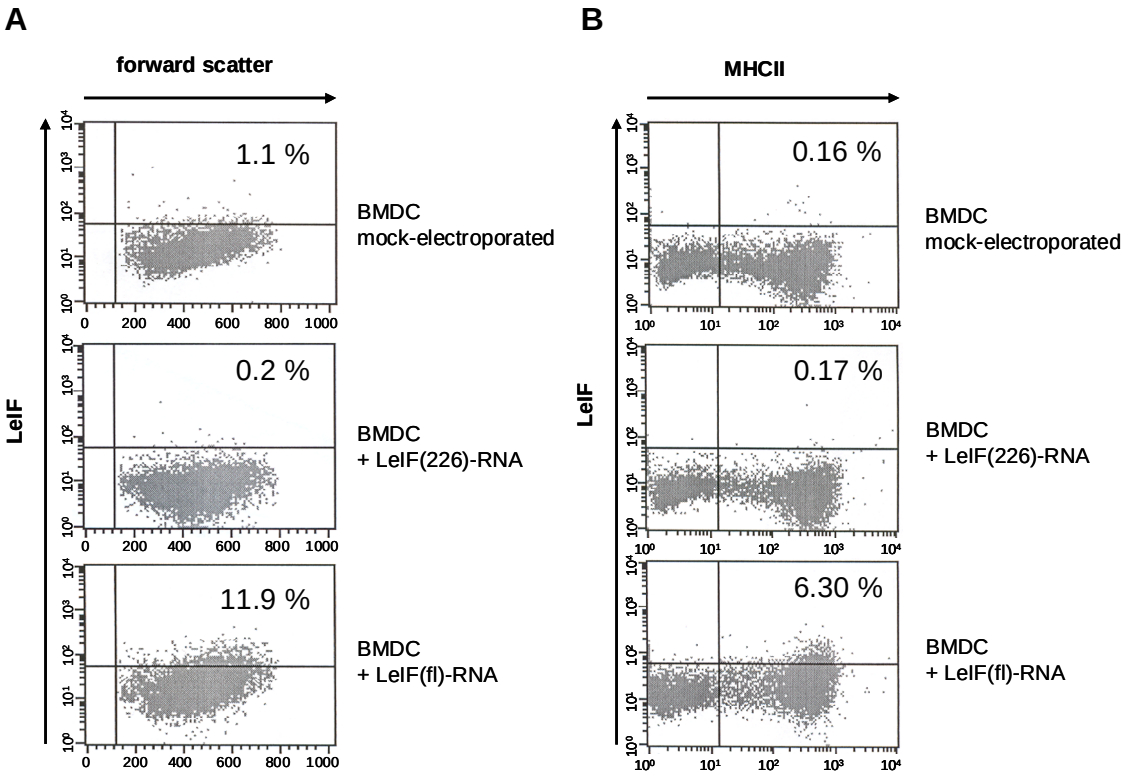

Fig. 19: LeIF(fl), but not LeIF(226) can be detected intracellularly by flow cytometry. BMDC were transfected with 20 µg of the indicated RNA on day 7 of culture. 24 hours later, they were harvested, stained intracellularly with the polyclonal anti-LeIF antibody. [A] shows a single stain; [B] shows a double stain with anti-MHCII antibody; numbers indicate the percentage of positive [A] or double-positive cells [B].

Both Western Blots of BMDC lysates and flow cytometry data illustrate: while LeIF(fl)-transfection of BMDC gives rise to expression of the encoded protein, LeIF(226) is not detectable after transfection with LeIF(226)-RNA. It should be mentioned again that these data contrast with the finding that LeIF(226)-RNA is well translatable in a cell-free system.

5.3 Effects of RNA transfection on the phenotype of DC

5.3.1 Effect on the expression of surface markers of BMDC

There are compelling data that LeIF is able to stimulate the innate immune system: Borges *et al.* showed that recombinant LeIF has a strong potential to induce IFN-γ, IL-12 and IL-18 production by spleen cells and potentiates the cytotoxic activity of NK cells of SCID (severe combined immunodeficiency) mice that completely lack functional B and T lymphocytes. Its ligand has not yet been determined, but TLR4 could be excluded [24].

It was important to know if transfection of BMDC with LeIF, involving introduction of foreign RNA into the cytosol with subsequent intracellular protein expression, would alter the phenotype of the cells.

It has been shown before that electroporation, in the absence or presence of EGFP-RNA, does not inhibit the maturation capacity of BMDC; furthermore, electroporation itself does not induce BMDC maturation [258]. EGFP is a strongly fluorescent protein, so FITC-labeled antibodies could not be used for staining of surface molecules; its strong fluorescence even influences measurements taken at other wavelengths. The flow cytometer software does provide compensation options, but then again, these settings cannot be used for other samples. Therefore, with the intention to avoid this technical problem, Xef-1 RNA that encodes a non-fluorescent protein (as shown above) was used as a control for this experiment.

CD11c expression is not changed after RNA transfection, regardless of the RNA used [data not shown]. Fig. 20 shows that the least alteration in surface expression marker levels with respect to the mock-electroporated control was caused by Xef-1-transfection. The effect on the peak shift of CD40 and CD86 expression by LeIF(fl) and LeIF(226)-RNA is comparable. In both cases, it is more pronounced than the effect caused by Xef-1 RNA. Transfection with LeIF(226)-RNA has the greatest effect on MHCII expression, while the effect of LeIF(fl)-RNA on the MHCII expression level was less obvious, but still more pronounced when compared to the effect of control RNA. The magnitude of the effect of LeIF(226) is dependent on the preexisting MHCII expression level at the time of transfection [data not shown].

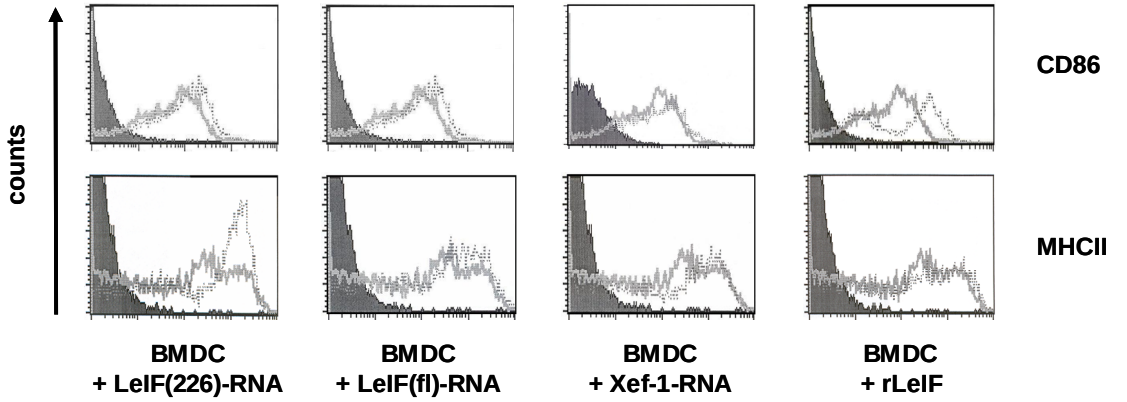

Fig. 20: Effect of RNA electroporation on BMDC phenotype. BMDC were transfected with 20 µg of the indicated RNA on day 7 of culture. 24 hours later, the surface expression levels of MHCII and the co-stimulatory molecule CD86 were analyzed by flow cytometry (dotted lines). Isotype control graphs are filled with dark grey. All values are compared to the surface marker expression levels on mock-electroporated BMDC (thick, light grey lines).

5.3.2 Effect on the cytokine profile of BMDC

DC function is characterized not only by the expression level of surface molecules, but also by their capacity to secrete cytokines. Together with co-stimulatory molecules, the secretion and type of cytokines influence the strength and the polarization of the T cell response.

It has been shown that recombinant LeIF is able to stimulate spleen cells from severely immunodeficient SCID mice that lack functional B and T cells, but have an intact innate immune system, to secrete IL-12 and IL-18 [24]. More specifically, it was also shown to be a potent IL-12 inducer in BMDC [17]. It has not yet been elucidated, however, if stimulation of BMDC with rLeIF also leads to secretion of other inflammatory cytokines. Moreover, it was important to know if the electroporation itself would alter the level of cytokine secretion.

The results depicted in Fig. 21 confirm that mock-electroporated BMDC do not secrete any of the cytokines studied in relevant amounts. Fig. 21 A shows that pulsing of BMDC with different amounts of rLeIF elicits secretion of IL-1β. There was no clear dose-dependency, and there was no difference between the pulsed and electroporated cell populations when only 10 µg of rLeIF were used. When higher amounts were used, the production of IL-1β was generally lower in cells that received an electric pulse. As seen in Fig. 21 B, levels of IL-6 after rLeIF stimulation were comparable in all samples; they were neither dose-dependent nor affected by electroporation.

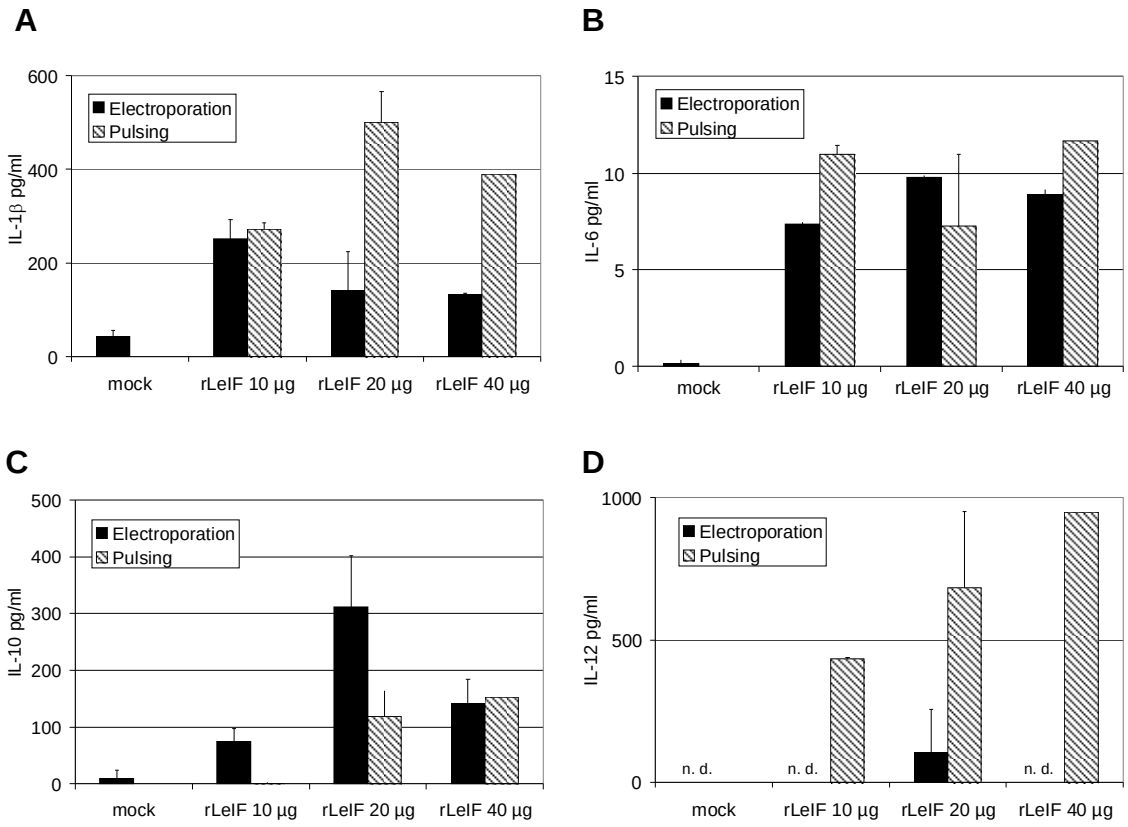

Fig. 21: Cytokine production by BMDC pulsed with rLeIF, with and without electroporation. BMDC were harvested on day 7 of culture; $4x10^6$ cells were either pulsed with the indicated amount of recombinant LeIF in a culture volume of 4 ml for 24 hours (dashed bars), or received the indicated amounts of rLeIF by electroporation with 300 V and 150 µF, followed by 24 hours incubation time (solid bars). Cell culture supernatants were collected for cytokine detection by ELISA. If the concentration was below the detection threshold, bars are labeled "n. d." for "not detectable".

Surprisingly, as shown in Fig. 21 C, IL-10 was secreted more efficiently by BMDC receiving the electric pulse, at least when 10 or 20 µg of rLeIF were used. This difference disappeared when 40 µg of rLeIF were applied. Notably, no IL-10 was secreted by BMDC that were merely allowed to take up 10 µg of rLeIF from the culture medium, whereas BMDC that received 10 µg rLeIF intracellularly by electroporation did secrete IL-10.

Il-12 secretion by BMDC that could take up rLeIF from the extracellular medium was dose-dependent. When the fourfold amount of antigen was used, the IL-12 level doubled. However, IL-12 could only be detected in BMDC cultures that received no electric pulse. Electroporation of rLeIF did not elicit IL-12 production, regardless of the amount of antigen used.

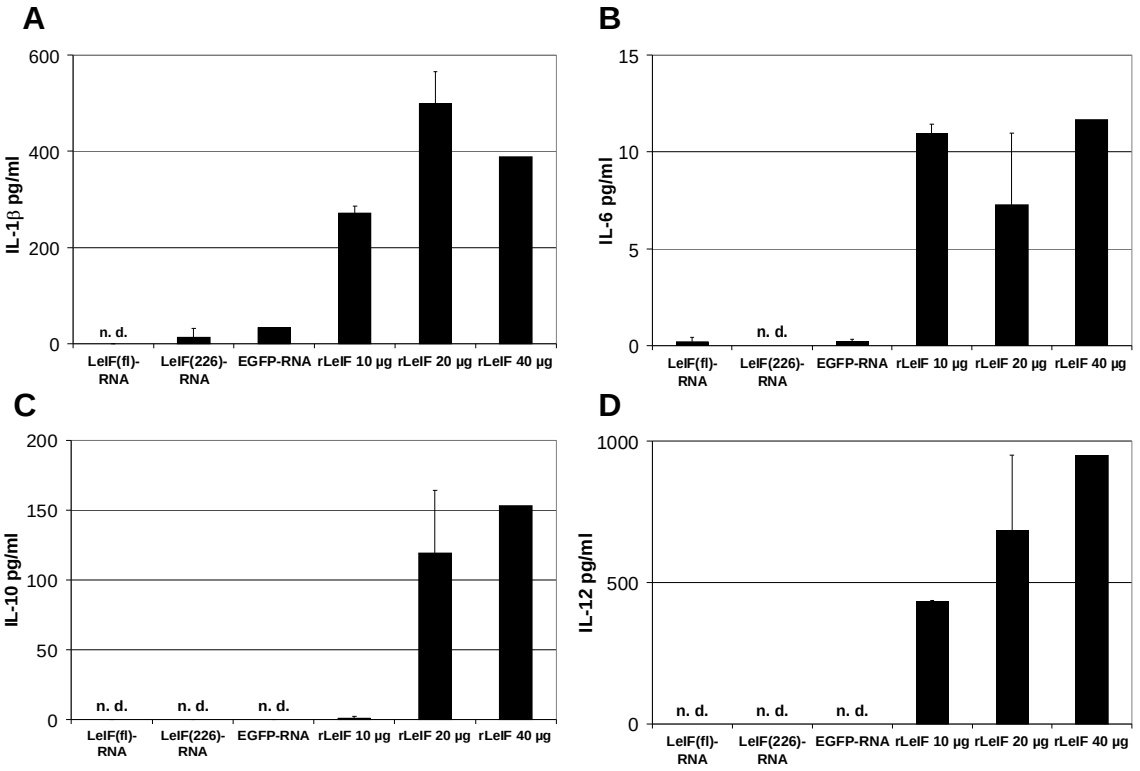

Fig. 22: Cytokine production by LeIF-transfected and rLeIF-pulsed BMDC. BMDC were harvested on day 7 of culture; $4x10^6$ cells were transfected with 20 µg of the indicated RNA or pulsed with the indicated amount of rLeIF in a culture volume of 4 ml. 24 hours later, the culture supernatants were collected and analyzed for cytokine levels by ELISA.

Knowing that recombinant LeIF stimulates day 7 BMDC to secrete different proinflammatory cytokines, the effect of LeIF transfection on BMDC was assessed as well.

Fig. 22 shows that neither EGFP- nor LeIF(226)- or LeIF(fl)-transfected BMDC released any of the cytokines analyzed (IL-1β, IL-6, IL-10 and IL-12), in contrast to BMDC treated with rLeIF. In other words, even though LeIF was detectable as an intact protein intracellularly, at least after LeIF(fl)-transfection as shown above by Western Blot and FACS (Fig. 17 and 19), a stimulatory effect on BMDC in terms of cytokine production could not be seen. This is important, as it might be argued that even if an antigen encoded by transfected RNA does not have the expected effect on transfected cells, these cells would eventually undergo apoptosis, start to leak and release the antigen. Other intact DC would thus be in a position to encounter the released antigen, engulf and present it. At least, if this mechanism exists, its postulated effect is not strong enough to induce cytokine production.

5.4 Immunogenicity of transfected BMDC

5.4.1 Antigen presentation by LeIF-transfected and LeIF-pulsed BMDC and elicitation of a specific T cell response

It was expected that if RNA is delivered to the cytosol, it should be translated by the protein biosynthesis apparatus of the DC; the translational product, being a cytosolic protein and not bearing any specific signal domains, should then be degraded by the proteasome, transferred into the endoplasmic reticulum and associated with MHCI molecules. This in turn was expected to result in presentation of the antigen on the cell surface [27].

Antigen presentation can be proven directly only by detection of peptide-MHC complexes. Reagents that are necessary for this procedure, e. g. antibodies that are specific for such peptide-MHC complexes, are only available for antigens that are more commonly used in antigen presentation studies (like e. g. ovalbumin), and their *de novo* fabrication for an unconventional antigen like LeIF is rather laborious.

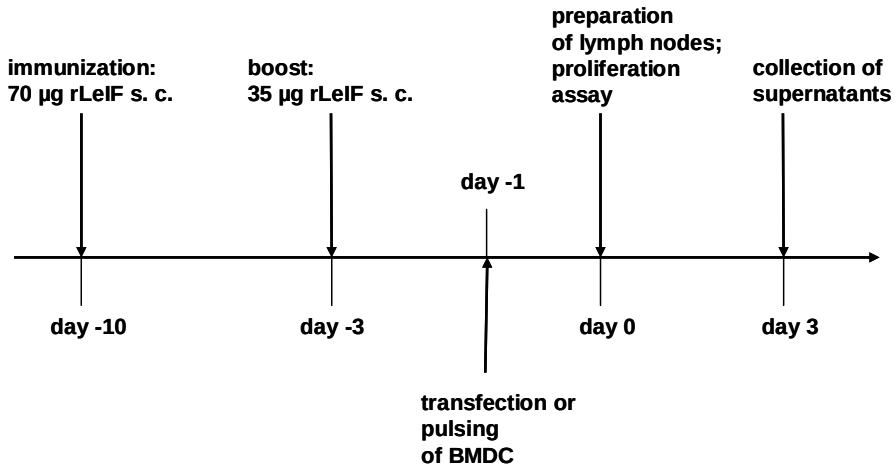

Fig. 23: Enrichment of LeIF-specific T cells and stimulation with transfected or pulsed BMDC. BALB/c mice were injected 70 µg rLeIF in PBS at three sites over the shaved flank; 7 days later, they were boosted with 35 µg rLeIF in PBS. One day before the preparation of LN, day 7 BMDC were transfected with 20 µg RNA or pulsed with 20 µg rLeIF. On day 0, $2x10^5$ T cells or $4x10^5$ LN cells were mixed with $1x10^5$ BMDC in 96-well plates and allowed to proliferate for 72 hours. On day 3, supernatants were collected for cytokine analysis.

However, antigen presentation can be detected indirectly by measuring the response of specific T cells to antigen-presenting cells. Indeed, BMDC from BALB/c mice have been shown to act as professional antigen-presenting cells in a mixed leukocyte reaction [144]. The only *L. major* antigen for which a specific, immortalized BALB/c T cell line, being a tool that greatly facilitates experiments involving antigen presentation and recognition, has been established so far, is the immunodominant antigen LACK [181]. A T cell line for LeIF is not available; its immunodominant epitope has not been described either.

Therefore, a classical immunization assay was set up to obtain a T cell pool with an increased number of LeIF-specific T cells (see Fig. 23): As described by Skeiky *et al.*, 70 µg rLeIF were diluted in 200 µg PBS and injected subcutaneously over three sites on the shaved flank [216]. In the present study, the mice received an additional boost with 35 µg rLeIF after 7 days. On day 0, the draining inguinal, axillary and brachial LN were removed bilaterally for preparation of a single cell suspension as described in section 4.14. LN preparations from naïve mice were used as controls. Production of IFN-γ by LN cells or by purified T cells from these mice served as a read-out for T cell activation. With the appropriate controls, this assay allows to distinguish between unspecific and specific T cell activation upon presentation of LeIF.

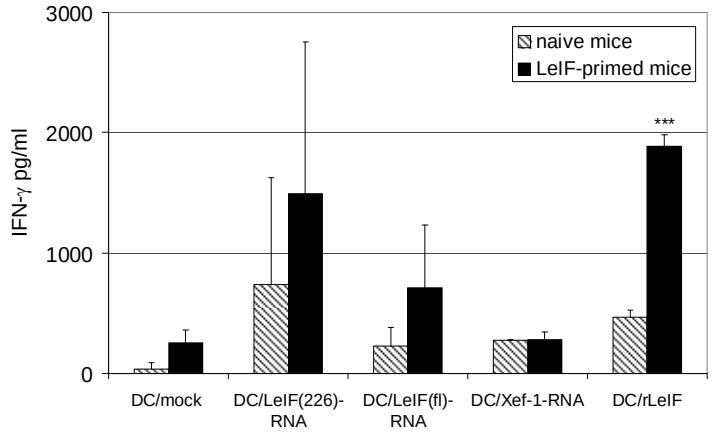

Fig. 24: IFN-γ production by LN cells in response to LeIF-transfected BMDC. BALB/c mice were immunized with rLeIF as described above. Single cell suspensions were generated from LN of naïve (dashed bars) or LeIF-primed mice (solid bars) ten days after the first injection, and stimulated with day 7 BMDC that had been mock-electroporated, transfected with LeIF(226)-, LeIF(fl)- or Xef-1-RNA or pulsed with 20 µg rLeIF. After 72 hours, supernatants were collected for cytokine analysis by ELISA. Three asterisks denote a statistical significance with $p < 0.001$.

As expression of LeIF(226) was detectable neither by Western Blot nor by intracellular FACS, but LeIF(fl) was, it was interesting to know if, likewise, only LeIF(fl)-transfected BMDC would stimulate LeIF-specific T cells to proliferate.

The results of this first experiment are shown in Fig. 24. Mock-electroporated BMDC and BMDC transfected with irrelevant Xef-1-RNA did not elicit production of IFN-γ, neither from naïve nor from LeIF-primed LN cells. It can be seen, however, that LeIF(fl)- and, surprisingly, LeIF(226)-transfected BMDC tend to stimulate LeIF-primed, but not naïve LN cells, to secrete IFN-γ. Remarkably, LeIF(226)-transfected BMDC even caused a higher IFN-γ release than LeIF(fl)-transfected BMDC. This difference was, however, not significant, with p values > 0.05. At the same time, BMDC pulsed with recombinant LeIF were also able to stimulate specifically LeIF-primed LN cells to produce IFN-γ, and here the difference to IFN-γ production by naïve LN cells was indeed statistically significant ($p < 0.001$).

It was reasoned that the relatively high IFN-γ levels in samples with LN cells from naïve mice might reflect the heterogeneity of LN cell suspensions, involving non-T cells like DC or B lymphocytes that might, even in the naïve controls, potentially act as antigen-presenting cells and stimulate IFN-γ production by T cells. A more specific response system, i. e. a cell population consisting only of reactive T cells, not contaminated by other antigen-presenting cells, was therefore desirable.

To achieve this, another experiment was set up involving $CD4^+/CD8^+$ T cells as responders, purified with a Pan T cell purification kit [MACS; Miltenyi Biotec]. The purity of this cell population was verified by FACS [data not shown]. Stimulation of $CD4^+/CD8^+$ T cells with LeIF-transfected BMDC in a lymphocyte culture (Fig. 25 A) confirmed what had been seen with LN cells as responder cells (Fig. 24). Importantly, BMDC transfected with LeIF- or control RNA were no source of IFN-γ [data not shown]. Again, mock-electroporated BMDC did not act as stimulators for neither naïve nor LeIF-primed T cells.

It could be shown that both LeIF(fl)- and, interestingly, LeIF(226)-transfected BMDC are able to stimulate specifically LeIF-primed T cells to secrete IFN-γ, but not naïve T cells. This difference in IFN-γ levels is statistically significant ($p < 0.05$). Notably, stimulation by LeIF(226)-transfected BMDC leads to a higher IFN-γ production of T cells than stimulation by LeIF(fl)-transfected BMDC. This result corroborates the

tendency that had been observed in experiments with LeIF-primed LN cells as responders.

Moreover, although expression of LeIF(226) is not detectable intracellularly (see Fig. 17), LeIF(226)-transfected BMDC are able to stimulate LeIF-primed T cells; the secretion level of IFN-γ is even higher than in samples using LeIF(fl)-transfected BMDC as stimulators, although intracellular expression of LeIF(fl) was detectable. In other words, maintained expression of transfected RNA and sustained cytosolic presence of the antigen is no prerequisite for the induction of an efficient T cell response.

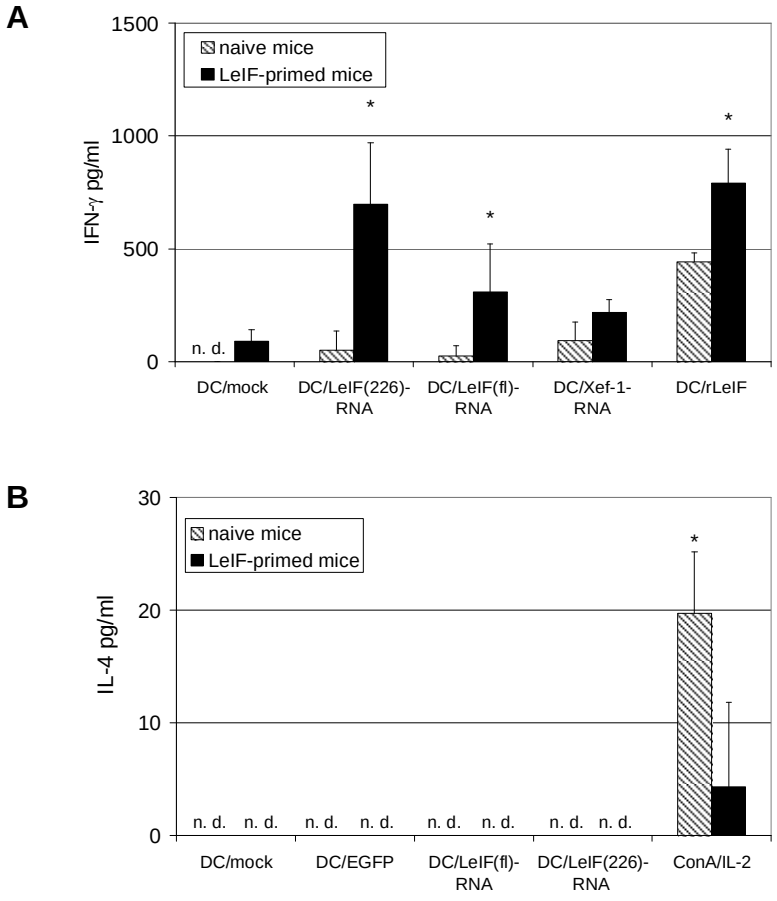

Fig. 25: Cytokine production by T cells in response to LeIF-transfected BMDC. Day 7 BMDC were transfected with 20 µg of the indicated RNA, pulsed with 20 µg rLeIF or mock-electroporated. 24 hours later, they were mixed with purified $CD4^+/CD8^+$ T cells from LeIF-primed (solid bars) or naïve mice (dashed bars). Supernatants were collected after 72 hours for cytokine analysis by ELISA. [A] IFN-γ production; [B] IL-4 production. One asterisk denotes a statistical significance with $p < 0.05$.

BMDC pulsed with rLeIF served as a positive control: IFN-γ production by LeIF-primed T cells stimulated with rLeIF-pulsed BMDC was higher than by naïve T cells (Fig. 25 A). This difference was significant ($p < 0.05$). Still, naïve T cells stimulated with rLeIF-pulsed BMDC produced about 10 times more IFN-γ than naïve T cells stimulated with LeIF-transfected BMDC. This may reflect the adjuvant effect of rLeIF: while LeIF-transfected BMDC are unable to stimulate naïve T cells to secrete IFN-γ, the stimulation of naïve T cells by rLeIF-pulsed BMDC may be related to the capacity of rLeIF to initiate a primary antigen-specific response after eliciting the production of IL-12 and IL-18 [216].

Moreover, LeIF being an inducer of a TH1 cytokine profile, it was important to know if the stimulated T cells would indeed not secrete TH2 cytokines. Therefore, the production of IL-4 was assessed. Fig. 25 B shows the results: Neither mock-electroporated nor RNA-transfected BMDC were able to stimulate naïve or LeIF-primed T cells to secrete IL-4. This goes in hand with reports stating 90% of T cell clones isolated from LeIF-primed mice were TH1, producing only IFN-γ [216].

In order to confirm the principal ability of T cells used in these experiments to secrete IL-4, an unspecific stimulus, containing 20 U/ml IL-2 and 1 µg/ml Concanavalin A (ConA), a mitogen, was used. Naïve T cells, thus stimulated unspecifically, did secrete IL-4. In contrast, T cells from LeIF-primed mice secreted a significantly lower amount of IL-4. This seems surprising, but LeIF has been shown not only to induce a TH1 cytokine profile, but also to down-regulate the production of IL-4 by lymphocytes of *L. major*-infected mice [216] and, when administered as a Langerhans cell-based vaccine, to shift the cytokine profile in *L. major*-infected BALB/c mice towards a TH1 type profile in terms of a down-regulation of IL-4 and an up-regulation of IFN-γ [17]. Here, it could be shown that IL-4 production is also down-regulated in T cells from LeIF-primed mice that are stimulated unspecifically with IL-2 and ConA. In other words, this effect is not confined to restimulation with rLeIF *in vitro*, but rLeIF is able to down-regulate the IL-4 response also when used as a subcutaneous vaccine *in vivo*. This down-regulatory effect is only visible under the vigorous, unspecific stimulation conditions with IL-2 and ConA; stimulation with LeIF-transfected BMDC is not sufficient to cause any IL-4 secretion.

5.4.2 Duration of BMDC generation influences antigen presentation

The experiments on antigen presentation, looking for cytokine production in lymphocyte cultures, were conducted using BMDC on day 7 of culture, because these cells had proven to be transfectable with a maximum efficiency [258]. Even though day 7 BMDC were shown to be immature DC that are more specialized on taking up antigen rather than presenting it [144], day 7 BMDC are indeed able to act as efficient antigen-presenting cells *in vitro* [258]. Furthermore, in human DC, transfected immature DC proved to be more effective T cell stimulators than transfected mature DC [259]. While the present study was conducted, other experiments analyzing the optimal relationship between the time point of RNA transfection and DC maturation status more closely were still under way [206].

It was important to know if the duration of BMDC generation, i. e. the maturation stage, would have any influence on the stimulatory capacity. For this reason, transfection of BMDC on day 7, 8 or 9 of culture was studied.

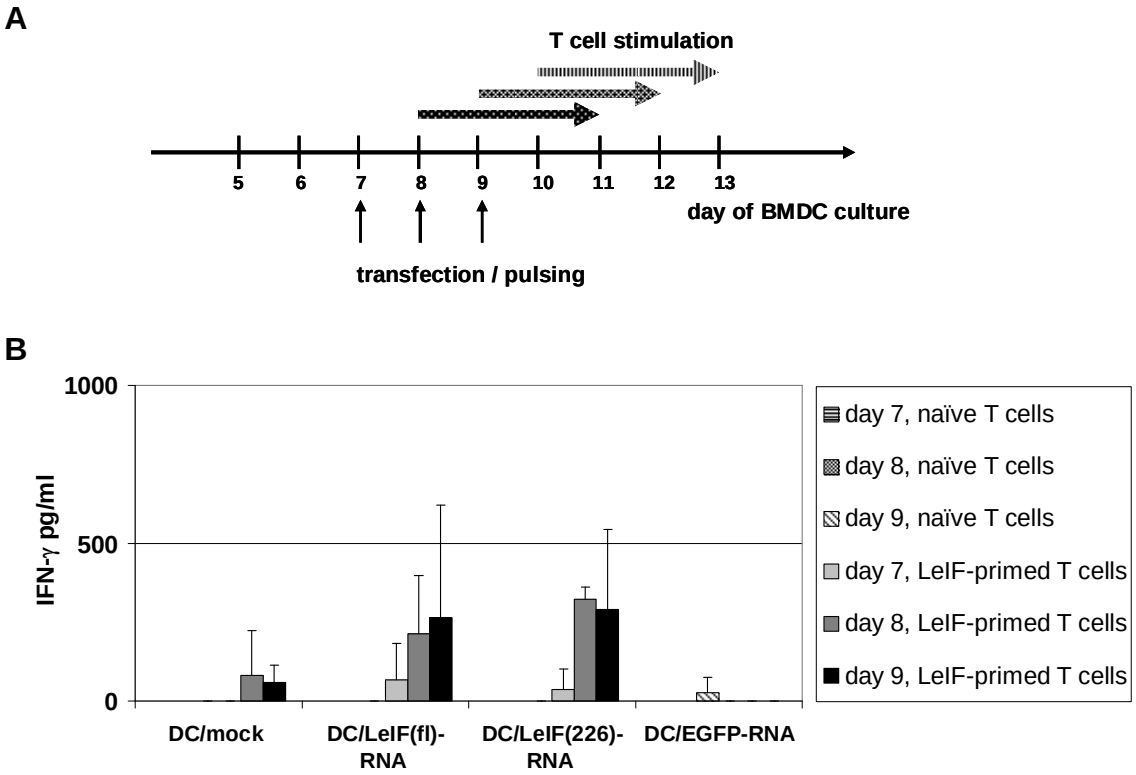

Fig. 26: Influence of BMDC culture duration on stimulatory capacity. BMDC were harvested on day 7, 8 or 9 of culture and transfected with 20 µg of the indicated RNA; 24 hours later, they were mixed with LeIF-primed or naïve purified $CD4^+$/$CD8^+$ T cells as described before. Cell culture supernatants were collected for cytokine analysis by ELISA after 72 hours of incubation. [A] Protocol, [B] IFN-γ levels.

As T cell stimulation in lymphocyte cultures was carried out over 72 hours, the stimulation periods largely overlap as it is shown in Fig. 26 A. Considering the fact that BMDC continue to mature in culture, T cells should be stimulated by antigen-presenting, more mature DC at the end of the 72 hours period even when using day 7 BMDC. It was therefore expected that the date of transfection might only be of minor influence.

Day 7, 8 or 9 BMDC were, as before, transfected with 20 µg RNA. Results are shown in Fig. 26 B. Again, mock-electroporated BMDC stimulated neither LeIF-primed T cells nor naïve T cells. LeIF-transfected day 8 BMDC stimulated LeIF-primed T cells to produce higher levels of IFN-γ than day 7 BMDC, and stimulation with day 9 BMDC was again slightly stronger. This was true for both LeIF(226)- and LeIF(fl) transfections. The differences were, however, not statistically significant.

As seen before, LeIF-transfected and rLeIF-pulsed day 7 BMDC stimulate LeIF-primed T cells to secrete IFN-γ in a similar range (Fig. 25 A). It was important to know if this correlation would be maintained when more mature BMDC were used for antigen presentation. Therefore, an experiment using BMDC pulsed with rLeIF after 7, 8 and 9 days of culture for stimulation was conducted. Its results are depicted in Fig. 27 A.

BMDC pulsed with rLeIF on day 7 of culture stimulated LeIF-primed T cells to produce IFN-γ in amounts comparable to stimulation by LeIF-transfected BMDC, as seen before (Fig. 25 A). The IFN-γ level increased with day 8 BMDC as stimulators; the difference to IFN-γ production by naïve T cells was statistically significant ($p < 0.01$). With day 9 BMDC as stimulators, compared to stimulation with LeIF-transfected BMDC (Fig. 25 A), the IFN-γ values were about 20-fold higher. The difference to naïve T cells was again statistically significant ($p < 0.001$).

Moreover, day 9 antigen-pulsed BMDC stimulated naïve T cells to secrete an amount of IFN-γ that was substantially higher than the amounts secreted by LeIF-primed T cells after stimulation by day 7 or 8 BMDC (both LeIF-transfected and LeIF-pulsed BMDC). This might reflect their capacity to induce primary immune responses.

So far, it was difficult to determine if IFN-γ production by T cells stimulated with antigen-pulsed or transfected BMDC reflected proliferation of T cells, as thymidine incorporation, a classical method to measure proliferation, had not yielded useful information. IL-2 is a T cell-derived cytokine that is responsible for clonal expansion of

activated T cells [209]. Therefore, IL-2 levels in cell culture supernatants are another useful parameter for T cell proliferation.

LeIF-transfected BMDC did not stimulate LeIF-primed T cells to produce IL-2, and the control samples were negative for IL-2 as well [data not shown]. However, as shown in Fig. 27 B, day 7 BMDC pulsed with rLeIF were able to stimulate LeIF-primed T cells to secrete IL-2. In contrast, no IL-2 secretion was detectable from naïve T cells upon stimulation with LeIF-pulsed BMDC ($p < 0.001$). The IL-2 level was maximal when day 8 pulsed BMDC were used as stimulators, being about three times as high as in day 7 BMDC samples. Again, day 8 BMDC specifically stimulated LeIF-primed T cells, not naïve T cells ($p < 0.001$).

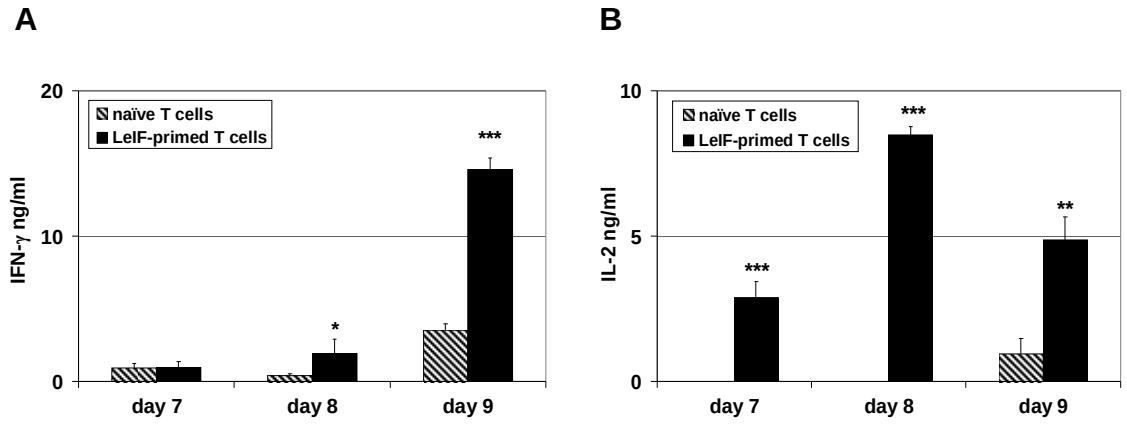

Fig. 27: Influence of BMDC culture duration on stimulatory capacity. BMDC were harvested on day 7, 8 or 9 of culture and pulsed 20 µg rLeIF; 24 hours later, they were mixed with LeIF-primed or naïve purified $CD4^+/CD8^+$ T cells as described before. Cell culture supernatants were collected for cytokine analysis by ELISA after 72 hours of incubation. [A] IFN-γ production, [B] IL-2 production. Statistical significance is marked as follows: one asterisk denotes $p < 0.05$, two asterisks denote $p < 0.01$, three asterisks denote $p < 0.001$.

Conversely, IL-2 production by LeIF-primed T cells decreased again slightly with day 9 pulsed BMDC. Remarkably, day 9 LeIF-pulsed BMDC were also able to stimulate naïve T cells to secrete IL-2. This result correlates with the finding that naïve T cells stimulated by day 9 BMDC secrete more IFN-γ than LeIF-primed T cells stimulated with day 8 BMDC. In other words, while naïve T cells are refractory to stimulation by BMDC pulsed with rLeIF on day 7 or 8 of generation, they do respond with production of IL-2 and IFN-γ to stimulation with BMDC pulsed on day 9. Thus, while LeIF-primed T cells respond to immature rLeIF-pulsed BMDC, antigen-specific activation of naïve T

cells requires BMDC of a more mature state at the time of pulsing (day 9).

This experiment revealed that the BMDC culture duration and the concomitant increase in the number of mature DC have a substantial effect on the immunostimulatory capacity of BMDC only for rLeIF pulsing, not for LeIF transfection [Fig. 27 and data not shown]. Thus, two main conclusions can be drawn: The duration of BMDC generation, i. e. the maturation stage of the DC culture at which the cells first encounter LeIF, is critical for their stimulatory capacity as antigen-presenting cells. Moreover, the way LeIF is administered is decisive: Expression of LeIF by DC after RNA transfection leads to antigen presentation and recognition by LeIF-primed T cells, but apparently the deciding stimulus is only conferred when DC are allowed to take up LeIF by endocytosis from the extracellular medium.

Thus, the combination of maturation stage and type of stimulus (i. e. the route of antigen delivery) is an important parameter for the induction of the immune response.

6 Discussion

The battle against the outbreak of infectious diseases is fought on many grounds. Both therapeutical and preventive strategies play important roles.

Vaccination belongs to the preventive strategies, aiming to confer sustained protective immunity to the host. By exploitation of recent knowledge in molecular biology and immunology, considerable success in vaccination against some bacterial and viral infections has been attained. The development of effective vaccines against parasitic diseases, in contrast, has been more difficult, one group being the leishmaniases, infections caused by intracellular parasites of the genus *Leishmania*.

Cutaneous leishmaniasis caused by *L. major* is well described in mice; its immunology has been investigated thoroughly. Many results suggest that a potent vaccine will have to redirect the TH2 response in susceptible BALB/c mice to a sustained, protective TH1 response – the decisive step to antileishmanial immunity. It has also become apparent that the efficacy of vaccines is dependent on the presence of adjuvants, which thereby represent a critical factor for the development of protective immunity.

Our group has focused on studying the function of *ex vivo*-derived DC as adjuvants in vaccination against leishmaniasis. In a recent study, Langerhans cells were pulsed with recombinant *Leishmania* subunit antigens and used, thereafter, as i. v. vaccines against parasite challenge. Most importantly, LeIF was identified as a single antigen that was able to confer protection when used for pulsing of DC that were subsequently administered i. v. [17]. It appeared to be a protein that is both able to induce an antigen-specific T cell response and mediate an additional adjuvant effect on antigen-presenting cells, as had been suggested before [24, 216].

Pulsing DC with recombinant antigens does not satisfy all requirements that have been postulated for antileishmanial vaccines, e. g. the MHCI pathway with subsequent elicitation of $CD8^+$ T cells is not targeted thereby. Also, protein purification is very costly and time-consuming. An approach involving antigen delivery by nucleic acids, e. g. transfection of DC with DNA or RNA *ex vivo* [259] or targeting genetically encoded antigens specifically to DC for expression [198], seems to be much more appealing in terms of costs and technical feasibility.

Transfection of DC with RNA has emerged from clinical and basic science research as a safe and reliable technique with few side effects. Different methods of transfection have been investigated, and van Tendeloo *et al.* showed that gene delivery into human hematopoietic cells by electroporation of mRNA is far superior to any other means of transfection [259]. Van Meirvenne *et al.* described RNA transfection of murine DC at the time when our first experiments had been accomplished [258].
The present study was set up to investigate the effect of delivering RNA encoding the *L. major* antigen LeIF to murine DC by electroporation in comparison to classical protein pulsing. It was particularly interesting to see if an antigen that is a known maturation inducer retains its innate immunostimulatory properties when administered as an RNA-based vaccine.

Inititally, with RNA transfection not yet being a standard method, a reporter protocol needed to be established; here, EGFP that allows easy detection was used. First, the EGFP sequence was directly cloned into the multiple cloning site of the commercially available pSP64 vector, but results of transfection efficiency and expression levels in BMDC transfected with RNA obtained from this plasmid were disappointing.
Systematic studies revealed that the mere translatability of *in vitro* transcribed RNA in a cell-free system is not a sufficient prerequisite for successful and efficient transfection of DC. In other words, the primary structure, a cap or cap analogon and a polyA tail are by all means necessary, but not yet sufficient components for satisfactory translation of RNA by a mammalian cell. The presence and length of the 3' and especially 5' UTR of RNA as well as the position and the context of the AUG initiator codon were shown to be other critical parameters for translation. For physiological mRNA, they create, depending on their length and sequence, higher secondary structures (with consequences for stability and their property as protein binding sites) that influence translational regulation and may have impact on cell growth, proliferation and even apoptosis [177].
In the study by van Meirvenne *et al.*, RNA containing both a 5' and a 3' UTR flanking the antigen-encoding sequence, thus allowing appropriate folding of the RNA molecule to fit into the ribosomal apparatus, was used for transfection of BMDC. The results showed a much higher transfection efficiency than our initial experiments (over 70%)

and satisfying levels of EGFP expression [258]. The 5' and 3' UTRs thus being indispensable for efficient RNA recognition by the mammalian cell translation machinery, we used a pGEM-4Z plasmid vector containing the 5' and 3' UTRs of *Xenopus laevis* β-globin that was kindly provided by Dr. Thielemans, Brussels, for further cloning procedures. The schematized structure of RNA molecules resulting after *in vitro* transcription from the constructed pGEM-4Z vectors is depicted in Fig. 28.

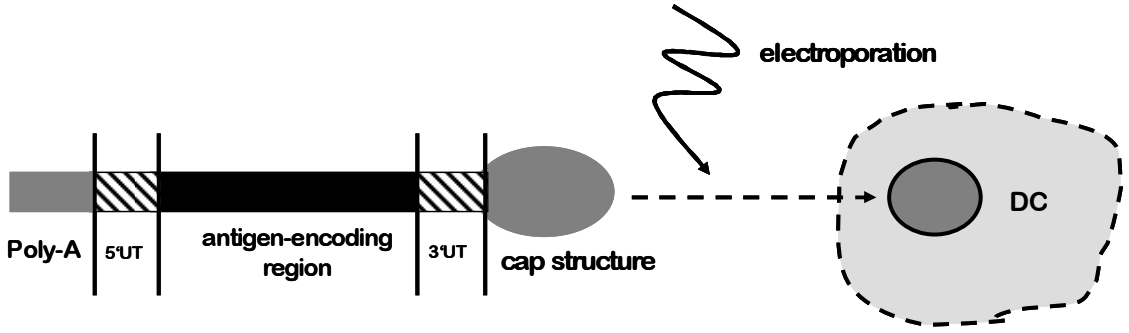

Fig. 28: Principles of RNA electroporation. RNA structure and electropermeabilization.

As shown by Western Blot, RNA obtained from the pGEM-EGFP vector was well translatable in a cell-free rabbit reticulocyte lysate system. To demonstrate that translation was also possible in DC, FSDC were used for exploratory transfection experiments. The principle is shown in Fig. 28. The FSDC cell line possesses a macrophage/immature DC-like surface phenotype and priming capacity unless treated with a maturation inducer [63]. For transfection, electric field parameters proposed by van Meirvenne *et al.* (300 V, 150 µF) were used [258].

The percentage of EGFP-positive FSDC decreased slightly after 3 hours, and reincreased after 48 hours post transfection. This first transient decrease in transfection efficiency should be linked to the fact that every DC receives a different amount of RNA: some of them have received only low numbers of RNA molecules, and then may have run out of intact RNA early due to its degradation. The few resulting EGFP molecules may have also been degraded quickly to leave an expression level below the detection threshold with the consequence that a number of cells was not counted as "EGFP-positive" any more. The origin of the second peak in transfection efficiency

after 48 hours is unclear. It might be related to the cell cycle and could reflect the fact that transfected FSDC divide at a higher rate than non-transfected FSDC. This way, a relatively higher number of cells appear to express EGFP at a level above the detection threshold after 2 days, although the absolute level of expression decreases. This could cause a transient increase in the percentage of EGFP-positive FSDC.

EGFP-transfection of FSDC revealed that the level of antigen expression (with the MFI as the corresponding marker) is dependent on the dose of RNA used for transfection. With the high amounts of RNA used in this study (more than 20 µg), a difference in transfection efficiency was not detected. In analogy to experiments in BMDC [258] and human PBMC [206, 254], it can be assumed that lower amounts of RNA would cause lower transfection efficiency rates also in FSDC.

Eventually, both transfection efficiency and antigen expression level decreased to zero. The decrease in EGFP detection is influenced by RNA stability, the relatively long half life of 26 hours of the EGFP protein [41], and of course cell death plays a role. It was found that the kinetics of the transfection efficiency and of the EGFP expression level was different. Already after 3 hours, about 90% of the cells electroporated with 40 µg RNA were EGFP-positive, this being the maximum. Antigen expression, in contrast, took 9 hours to become maximal. In other words, in about 90% of DC, the transfected protein is being expressed at some point, thus available for subsequent peptide generation. The onset of RNA translation occurs immediately after electroporation; within a time frame of only 3 hours, a maximal percentage of FSDC is EGFP-positive. In contrast, it takes longer – 9 hours – until a maximal EGFP expression level is reached. This suggests that within the first 9 hours, translation products accumulate until a maximum is reached; afterwards, the metabolic balance between accumulation and degradation shifts towards degradation. The MFI being a paramater for the level of antigen expression, the amount of antigen expressed by the cells over time can be related to the area under the MFI curve.

Remarkably, antigen expression decreases more quickly than transfection efficiency – while a relatively high percentage of FSDC are still EGFP-positive after 4 days, the expression level has decreased to about a tenth of its maximal value. In other words, small amounts of antigen are still present intracellularly in a comparably large proportion of cells after several days.

The satisfactory results in the FSDC cell line prepared the ground for more detailed analysis in primary cells. As the highest transfection efficiency in BMDC generated according to the protocol developed by Lutz *et al.* [144] was found in immature DC after 7 days of culture [258], transfection was carried out in day 7 BMDC in these experiments. Considering the subsequent proliferation experiments, it was also taken into account that day 8 BMDC were demonstrated to be the best stimulators of allogeneic T cells *in vitro* [144], which is why transfection of BMDC on day 7, allowing one day for translation, seemed the ideal time for transfection.

Similar transfection efficiencies were found in BMDC electroporated with 20 and 40 µg RNA. With 10 µg EGFP RNA, transfection efficiency was lower, yet the curve had a similar shape. The observation from FSDC experiments that transfection with more than 20 µg RNA does not lead to a further increase of the percentage of EGFP-positive cells was thus confirmed in BMDC. A peak in transfection efficiency was reached after 48 hours, compared to 3 hours in FSDC. There was no initial peak immediately after transfection. The first peak in the BMDC transfection efficiency curve after 48 hours corresponded to the second peak observed in FSDC, indicating that an increased cell division rate of the transfected cell fraction is not an entity influencing transfection efficiency in BMDC. The lower transfection efficiency in primary cells (75% vs. 90%) could be linked to the fact that FSDC, which are subjected to uncontrolled cell division after retroviral transduction with an env^{AKR}-myc^{MH2} fusion gene [63], have a higher cell division rate than primary DC and are therefore more susceptible to intrusion of foreign molecules.

The level of antigen expression in transfected BMDC is directly proportional to the dose of RNA used for electroporation. This confirms similar findings by Schaft *et al.* and Tuyaerts *et al.* [206, 254]. Antigen expression peaks after 24 hours, in contrast to peaking after 9 hours in FSDC. Again, this difference may be related to the slower metabolism of primary DC in comparison to the immortalized cell line: It can be assumed that translation of electroporated RNA by ribosomes occurs more slowly in primary cells than in immortalized cells, and RNA should also be degraded less quickly in BMDC than in FSDC. Therefore, RNA turnover by the primary cell is less rapid, leading to delayed accumulation and a deferred maximum of antigen expression.

The translation kinetics of electroporated RNA by BMDC is, as was also shown in this study, sensitive to treatment with maturation inducers; here, the TLR4 ligand LPS was used for stimulation.

The peak level of EGFP expression (measured as MFI) was not influenced by LPS treatment in BMDC receiving only 10 µg RNA, but it was almost doubled in BMDC receiving 20 µg. LPS treatment also had no influence on the time point of the inflection of the MFI curve (the maximum of antigen expression), but the decrease of the curve was faster.

This LPS-induced increase of MFI, i. e. an increased accumulation of the RNA-encoded antigen, might be caused either by a more efficient translation of electroporated RNA (as also suggested by Michiels *et al*. [155]) – that would result in faster intracellular production of EGFP protein – or reflect decreased protein degradation. Furthermore, the more rapid decline of the MFI caused by LPS-induced maturation could be the result of the onset of an increased activity of protein- and/or RNA-degrading enzymes at a later time point. In fact, some authors favor the view that regulation of intracellular protein levels by degradation rivals, and often surpasses in significance, the regulation through transcription and translation [261].

LPS treatment also increased the transfection efficiency, depending on the amount of RNA used, by 15 to 30% at 7 hours post transfection. The observed increase of the absolute percentage of cells expressing EGFP after RNA transfection and LPS treatment is likely to be related to limitations of the sensitivity of FACS analyses: stimulation of RNA translation by LPS treatment in cells receiving only low amounts of RNA might increase EGFP expression from levels that are otherwise still below the “detection” threshold (i. e. the gate chosen for analysis) to then detectable levels (i. e. to values above the gate). At least, as transfection efficiency is influenced by electrical parameters, and membrane transition of macromolecules starts with complex-pore formation at the onset of the pulse and is completed at latest within the minute range [196], it is unlikely that the complex maturation process induced after the pulse should have an impact in real terms on the percentage of cells loaded with RNA molecules.

Protein degradation in the cytosol is mainly carried out by the proteasome: over 80% of cellular proteins are ultimately recycled through the proteasome [92]. The regulation of its activity is therefore critical for the fate of cytosolic proteins. EGFP is devoid of inherent targeting sequences which permitted its successful use as a molecular tag for proteins in a large variety of subcellular compartments [253]. After RNA transfection, EGFP can therefore be expected to be mainly localized in the cytosol and thus be subjected to proteasomal degradation. The proteasome is subjected to two important types of regulations: subunit modification and activation by binding of the activating complex PA28. Inflammatory stimuli alter the proteolytic properties via both mechanisms [118, 172, 231], but only PA28 strongly increases the maximal velocity of the hydrolytic reaction and decreases the concentration of substrate required for cleavage by the 20S proteasome, albeit this applies predominantly for smaller molecules [146]. In that course, the faster decrease of MFI seen in LPS-treated EGFP-transfected BMDC could be related to LPS-induced PA28-dependent activation of the proteasome.

Another possibility to explain the different expression kinetics of LPS-stimulated BMDC is the subcellular reorganisation of DC after activation: upon stimulation with maturation inducers, DC accumulate newly synthesized ubiquitinated proteins in large cytosolic structures, termed “DC aggresome-like induced structures” (DALIS) [138]. Ribosomes are enriched in rings surrounding the aggregates, and sometimes, the ribosomal apparatus is almost completely redistributed in the vicinity of DALIS. These structures begin to form 4 hours and peak around 8 hours after LPS stimulation, while after 24 to 36 h, most of them have disappeared. For formation and maintenance, continuous protein synthesis is required, as it is the case after RNA transfection. The proteasome activity is not affected by the maturation process, but polyubiquitinated proteins and DRiPs within DALIS have nevertheless a considerably extended half-life and seem to be protected from proteasome degradation, even after leaving the DALIS [137, 138]. This increased stability could well explain why the experiments showed an increased EGFP expression level within the first 24 to 36 hours after LPS stimulation. Resolution of DALIS thereafter could explain the decrease of expression levels to normal or even subnormal values.

It was important to know which cell type was predominantly targeted by RNA transfection of BMDC cultures. BMDC used in this study had been generated, according to the protocol by Lutz *et al.* [144], by 7 days *in vitro* culture before transfection. Typically, on day 8 of culture, which was the time for analysis, a large majority of these cells still exhibit a predominantly immature phenotype [144]. Stimulation of DC with LPS, leading to subsequent upregulation of MHCII molecules, permitted to conclude if the transfected cells were indeed DC or if a different cell type would be the primary target of transfection. It could be shown that with an amount of 20 µg RNA – yielding a maximal transfection efficiency – about 50% of cells were double-positive for MHCII and EGFP. This could be confirmed by fluorescence microscopy. Contaminating cells such as lymphocytes are not transfected under the conditions used for DC transfection, possibly because of different properties in the electrical field (size, membrane conductance) [242]. Thus, the RNA-encoded antigen is indeed expressed by DC after RNA transfection of BMDC cultures. Another conclusion that can be drawn from this experiment is that BMDC do not lose their maturation capacity after EGFP transfection.

In this study, the viability of FSDC and BMDC post transfection was determined to rule out possible cytotoxic effects of the electroporated RNA on BMDC, and to ensure that the percentage of dead cells was within the range that has been reported previously. RNA electroporation, when compared to mock electroporation, did not result in decreased viability. However, the viability of EGFP-expressing cells or the percentage of viable cells expressing EGFP cannot be deduced from these experiments. To obtain such results, BMDC need to be stained with ethidium bromide and analyzed by FACS, as reported by van Tendeloo *et al.* in human Mo-DC [259]. In this study, less than 20% dead cells were found, and it was shown that dead cells do not express EGFP after RNA transfection. When gating only on the viable population, the transfection efficiency increased from about 60% (entire cell population) to over 85% (only viable cells) [259]. Based on these experiments, the percentage of EGFP-expressing cells should be expected to be even higher than 70% when considering only viable BMDC.

Detection of intracellular expression of the *L. major* antigen LeIF after transfection of BMDC with LeIF-RNA was not as easy as detection of the highly fluorescent EGFP molecule. Also, nothing was known about the intracellular stability of LeIF. Skeiky *et al.* had shown that the amino-terminal half of LeIF was more immunogenic by itself than the untruncated LeIF molecule [216]. As it was unknown if this difference would be of functional importance also in RNA transfection, experiments were carried out with both LeIF(226)- and LeIF(fl)-RNA constructs. Comparably to EGFP, LeIF is devoid of any specific signaling or transmembrane domains [24] and was therefore expected, after RNA transfection, to exhibit properties of a cytosolic protein.

In the present study, it was demonstrated that translation of LeIF(226)- and LeIF(fl)-RNAs in a cell-free system resulted in reproducible expression of proteins, as shown by Western Blot. When LeIF-RNA was electroporated into BMDC, only the non-truncated molecule LeIF(fl) was detectable in Western Blots of whole cell lysates between 2 hours and 48 hours post transfection; LeIF(226) was never detectable. Similar results were obtained by intracellular FACS staining 24 hours post transfection. This difference in protein stability was unexpected, especially with view to the sustained EGFP expression.

It has to be kept in mind that even under the influence of intracellular proteases, EGFP is a very stable protein, exhibiting a half life of approximately 26 hours [41]. Other proteins are less stable: a report by Schaft *et al.* on transfection of human monocyte-derived DC with RNA encoding tumor-associated antigens (TAA) revealed that in spite of transfection with equivalent amounts of RNA, the detectable expression levels of different antigens may differ by the factor 10. The expression of some antigens starts with low values within the first 3 hours and decreases to background levels after 6 to, at latest, 24 hours post transfection [206]. It is thus well possible that the truncation of the LeIF molecule confers a reduced intracellular stability.

One important factor determining the half life of cytosolic proteins is degradation by the proteasome, leading to generation of antigenic peptides for presentation by MHCI molecules. The degradation rate of cytosolic proteins with subsequent MHCI presentation is influenced by ubiquitination [72]. However, ubiquitination occurs at the N-terminus of a given protein and depends on the N-terminal amino acid residue (the “N-end rule”) [273]. As LeIF(226) is identical with the 226 N-terminal amino acids of

LeIF(fl), it should be unlikely to assume that increased ubiquitination of the N-terminus is the reason for its instability. However, it has been suggested that ubiquitin-conjugating enzymes may be responsible for degradation by recognizing structural features downstream and distinct from the N-terminus [68, 151]. Possibly, differences in secondary or tertiary structure due to the truncation (nb. the C-terminus of LeIF(226) exhibits a different terminal amino acid than LeIF(fl)) are responsible for the different handling by the intracellular degradation machinery. These hypotheses remain, however, speculative and require further ultrastructural and biochemical analyses for validation. In any case, intracellular presence of the intact protein is not required for its degradation to antigenic peptides. Incompletely translated proteins, DRiPs, are in fact even the most important sources of antigenic peptides presented by MHCI in DC and other cells [182, 210].

Also, with respect to a possible future use in vaccination, it has to be taken into consideration that most migrating DC probably die after arrival in lymphoid tissue [12]. This emphasizes that a prolonged intracellular antigen expression would not even be particularly useful; the critical role of DC is rather effective presentation of immunogenic peptide-MHC complexes in the adequate context and thus the initiation of T cell-mediated immunity.

To determine T cell stimulation, lymphocyte cultures were initially set up using LN cells from naïve and from LeIF-primed mice. As described previously by Skeiky *et al.*, LeIF-primed murine LN cells were generated by subcutaneous injection of the recombinant protein and used as as a read-out for antigen presentation by transfected BMDC [216]. In that course, this study showed that although LeIF(226) was not detectable in BMDC after transfection, probably due to its rapid degradation, the cells did not fail to present it.

LeIF-primed LN cells produced more IFN-γ in response to LeIF-transfected and rLeIF-pulsed day 7 BMDC than naïve LN cells, but the differences were not statistically significant. A possible explanation is the lack of purity of these LN suspensions that also contain antigen-presenting cells that might contribute to the activation of naïve T cells.

Therefore, LN suspensions were purified with a Pan T cell isolation kit to obtain a more

homogeneous responder population. Indeed, the difference was more obvious when naïve and LeIF-primed T cells were used as responders: Day 7 BMDC transfected with LeIF(fl)- or LeIF(226)-RNA both elicited a significantly higher secretion of IFN-γ by primed T cells compared to naïve T cells. Notably, the levels were in the range that was elicited by rLeIF-pulsed BMDC. This suggests that antigenic epitopes were not only generated from LeIF(fl), but also from LeIF(226) which was not detectable by Western Blot in BMDC lysates. As co-incubation with T cells started 24 hours post transfection, the results indicate that peptide-MHC complexes must have been stable enough to allow for antigen presentation to T cells. Similar conclusions for the stability of peptide-MHC complexes were obtained in two studies with the TAA MAGE-3: although in MAGE-3-transfected PBMC, the antigen expression had decreased to background levels after 24 hours [206], the cells retained their immunostimulatory capacity for up to 3 days [155]. Peptide presentation via MHCII was, however, clearly more stable than presentation via MHCI [155].

The source of IFN-γ was not determined in this study. Taking into account the classical mechanism of antigen processing of cytosolic proteins, translated LeIF should have been ubiquitinylated, then degraded by the proteasome and eventually presented on MHCI molecules, which should have resulted in activation of $CD8^+$ T cells. However, it was demonstrated that ~90% of the T cell clones isolated from LN of LeIF-primed mice were TH1 cells, the remainder TH0 cells [216]. This suggests that $CD4^+$ TH1 cells might in fact at least have contributed to IFN-γ production.

Cross-talk between presentation pathways in DC is not limited to cross-presentation, a pathway by which access to the MHCI pathway is granted to exogenous antigens. A mechanism explaining the access of endogenous antigens to the MHCII pathway is autophagy: peptides derived from endogenous antigens, e. g. cytosolic self-antigens or viral antigens, can be bound by MHCII molecules and subsequently be presented to $CD4^+$ helper T cells. Autophagy with subsequent lysosomal degradation and peptide loading on MHCII complexes appears to be selective for long-lived proteins, while short-lived proteins are preferentially degraded by the proteasome and are found to give rise to MHCI, not MHCII ligands [160]. More specifically, it was found that overexpression of a non-self protein after transfection is followed by its MHCII

presentation, mediated by autophagy [170]. It can therefore be expected that the long-lived LeIF(fl) may be associated with predominant MHCII presentation, while the shorter-lived LeIF(226) might give rise to an at least higher involvement of presentation via MHCI. Further experiments should address this question. A hypothetical model of the antigen processing mechanisms after RNA transfection is depicted in Fig. 29.

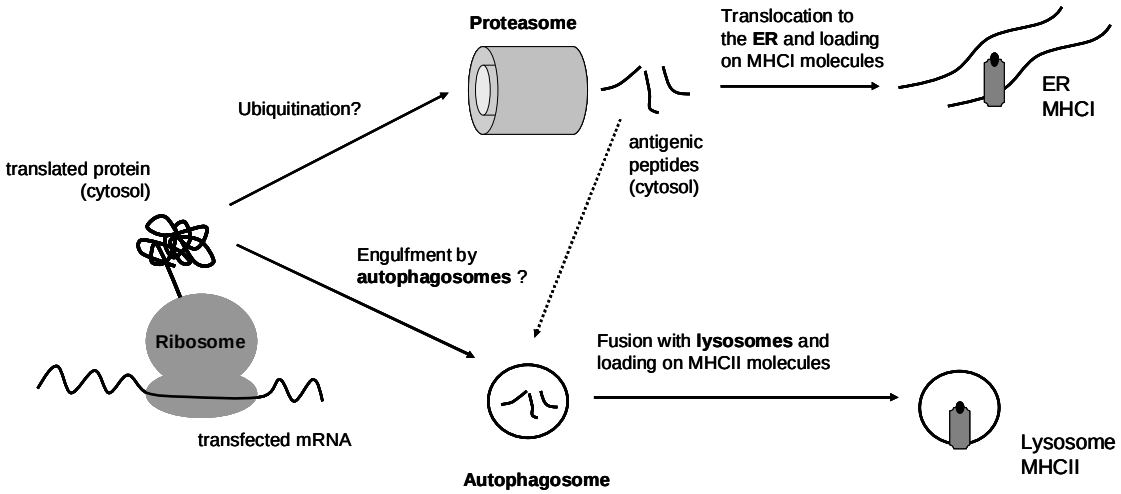

Fig. 29: Hypothetical pathways of antigen processing after RNA transfection. Transfected RNA is translated into the encoded protein by ribosomes and released to the cytosol. Here, it may be conjugated to ubiquitin for proteasomal degradation and subsequent translocation to the ER and MHCI presentation, or be engulfed by autophagosomes and follow the MHCII pathway. It is also possible that peptides generated by the proteasome gain access to the MHCII pathway.

It was also shown in this study that IL-4 was produced neither by naïve T cells nor by LeIF-primed T cells when stimulated with LeIF-transfected BMDC. This is consistent with the fact that in a study by Skeiky *et al.*, no TH2 cell clones could be isolated from rLeIF-primed mice, and an only low proportion of 10% were TH0 cells, producing both IFN-γ and IL-4 [216]. The authors also showed in this study that rLeIF was able to downregulate the production of IL-4 by lymphocytes of *L. major*-infected mice during *in vitro* restimulation [216]. Furthermore, in a study using rLeIF-pulsed Langerhans cells as vaccine against *L. major* infection, the IL-4 production by *L. major* lysate-restimulated LN cells after 11 weeks of infection was reduced significantly in comparison to controls [17].

As shown here, the unspecific stimulation of LeIF-primed T cells with the mitogen ConA and IL-2 also resulted in a significantly lower level of IL-4 production compared

to stimulation of naïve T cells. This could be a result of the substantial (~90%) preponderance of TH1 cells in LeIF-immunized mice with a reduction of the absolute number of IL-4-secreting (TH2) cells. Thus, LeIF is not only able to down-regulate *Leishmania* antigen-specific IL-4 production by LN cells from infected BALB/c mice *in vitro* [216]; the overall IL-4 producing capacity of LN cells from BALB/c mice in response to unspecific stimulators is also down-regulated by immunization with LeIF.

The maturation state of DC is critical for the decision if the cells assume immunostimulatory properties. Van Meirvenne and colleagues showed that electroporation of BMDC itself, in the presence or absence of EGFP-RNA, does not induce maturation [258]. It was, however, not predictable how BMDC would react to RNA transfection with a protozoan antigen: LeIF is a protein that stimulates cells of the innate immune system [24] and BMDC to secrete IL-12 [17]. This effect was proven not to be associated with endotoxin contamination, but linked to its protein nature [183]. It was important to know if LeIF transfection would also result in IL-12 secretion, as the mechanism of how LeIF exerts its potent innate stimulation is not known.
First, the experiments revealed that BMDC receiving rLeIF were able to secrete IL-12 in levels depending on the dose of antigen used. Second, however, no IL-12 secretion could be detected when the antigen delivery was accompanied by electroporation. It is known that exogenous antigens can be delivered to the antigen presentation pathways of antigen-presenting cells by electroporation [140], but so far, no influence on their capacity to secrete cytokines has been described. Moreover, no IL-12 production was elicited by BMDC that were transfected with LeIF(226)- or LeIF(fl)-RNA.
The unability of LeIF-transfected and rLeIF-electroporated BMDC to secrete IL-12 is unlikely to be caused by the same mechanism – it has to be viewed together with the rest of the cytokine profile. While LeIF-transfected cells seem to be more or less unresponsive in general, according to their overall failure to secrete cytokines, IL-12 is the only cytokine whose production by BMDC upon rLeIF treatment is abrogated by electroporation. The production of other cytokines was not abrogated completely. E. g., electroporation did not influence IL-6 production at any point, while IL-1β production was only affected at higher concentrations in the electroporated samples. IL-10 levels were even higher in BMDC samples receiving low amounts of rLeIF (10 and 20 µg) via

electroporation, while there was no difference when they received 40 µg of rLeIF. This could be related to the fact that in samples receiving 40 µg rLeIF, the effect of remaining extracellular rLeIF outweighs the effect of electroporated, intracellular rLeIF.
A decreased BMDC viability is unlikely to be responsible for the complete failure to secrete IL-12, as e. g. IL-6 production was unaffected. Possibly, the IL-12 pathway is interrupted at some point by the electric pulse. Thielemans and colleagues reported that the ability of human PBMC to secrete IL-12p70 upon CD40 ligation was indeed impaired even after mock electroporation, and even more clearly after electroporation with EGFP-RNA. However, the detected amounts were still distinctly above background levels, and IL-12p40 production was only slightly impaired [254].
The complete abrogatory effect of an electroporated protein on IL-12 production by antigen-presenting cells that was seen here has not yet been described in the literature. If it resulted from impaired LeIF recognition early upstream in the signaling cascade, hypothesizing that the LeIF receptor was located exclusively in the cell membrane and taking into account that the process of electroporation can affect the activity of proteins due to an electroconformational change of the protein itself [38], this should have an effect on the secretion of all cytokines. However, not all other cytokines were affected in the same way.
The disturbance could also be located further downstream and primarily affect IL-12 production. The difference might then be linked to the fact that IL-10 is a homodimer, and IL-1β and IL-6 are monomers, but IL-12 is a heterodimer composed of the p35 and the p40 subunit that underlie separate, independent mechanisms of regulation [19] – accounting for the fact that the IL-12p70 heterodimer might actually be a soluble receptor-ligand complex [30]. Indeed, there are different regulating mechanisms that act on both subunits: while the p35 chain level is extensively modified posttranslationally, the p40 chain is mainly regulated transcriptionally and otherwise subjected to only minor modifications during biosynthesis [30]. The intracellular presence of the electroporated LeIF molecule could interfere with the modification of the p35 subunit that is necessary for assembly of the p70 heterodimer, so that IL-12 is not detectable in the supernatant. If so, the impaired mechanism should have a role late in IL-12p70 formation.

Still, inhibition of IL-12 secretion by electroporation of the TH1 adjuvant rLeIF is surprising, especially with respect to the fact that rLeIF was not washed from the samples after electroporation and thus still had the capacity to stimulate BMDC. It cannot be assumed that all protein was electroporated; some rLeIF must have remained in solution and should have elicited IL-12 production. The fact that it failed to do so could speak for the induction of a refractory DC state by intracellular rLeIF. At least, Tuyaerts *et al.* showed that the electroporation process itself only slightly reduces the ability of DC to produce IL-12 [254]. Thus, the effect seen here has to be related to the nature of the electroporated protein, not just the electric pulse.

As for intracellular presence of *Leishmania* parasites in DC, it is well accepted that their uptake results in IL-12 production [264, 265]. In bone marrow-derived macrophages it was shown that *Leishmania* promastigotes evaded IL-12p40 expression in response to other IL-12-inducing stimuli, while the activation pathways for other cytokines like IL-10 remained relatively intact [31], but so far there is not evidence for a similar effect in DC. Possibly, day 7 BMDC represent such an immature state of DC that their response resembles that of macrophages when confronted with leishmanial compounds intracellularly. Also, the secretion of other proinflammatory cytokines is less impaired than IL-12 production in BMDC receiving rLeIF via electroporation, an effect that is comparable to the reaction of macrophages after uptake of promastigotes [31]. It should be mentioned in this context that under certain conditions, immature DC can revert to macrophages [134, 197]. Thus, the IL-12 downregulation upon rLeIF electroporation possibly represents a macrophage-like property. It would be interesting to investigate further if the inhibitory effect of electroporated rLeIF on IL-12 production persists at more mature states, and to see if there is also an effect on the mRNA levels of IL-12p35 and p40.

IL-12 downregulation could also have another correlation on the cellular level: Many parasites aim to evade the host's immune response by targeting intracellular signaling pathways and other mechanisms [reviewed in 44]. IL-12 production is dependent on intracellular signaling, and intracellular rLeIF might have interfered with these pathways. It has been shown that stimulation of DC with TLR ligands is followed by differential modulation of distinct components of the MAP kinase signaling pathway [3, 81, 271]. There are three major groups of MAP kinases: the p38 MAP kinases, the

extracellular signal-regulated kinases (ERK) 1/2 and the c-Jun N-terminal kinases (JNK) 1/2.

It was shown that the stabilized form of the immediate early gene product c-Fos acts as a negative regulator of IL-12p70. The treatment of DC with TH2-promoting stimuli such as SEA (schistosome egg antigens) results in sustained duration and magnitude of ERK 1/2 phosphorylation. Sustained ERK signaling then gives rise to enhanced levels of c-Fos expression in DC [162] and thus leads to suppression of IL-12 production [3]. It is accompanied by enhanced IL-10 production [48].

On the other hand, IL-12p70 production is positively regulated by phosphorylation of p38 and JNK 1/2, promoted by compounds that bias a TH1 response such as *E. coli* LPS and bacterial flagellin [3]. Interestingly, IL-6 production by DC elicited after stimulation with SEA was independent of c-Fos activity and seemed not to be regulated by ERK kinases [3]. This was confirmed in hepatic stellate cells, where IL-6 production appears to be regulated by p38, but is independent of JNK and ERK signaling [245].

The cytokine levels produced by BMDC after electroporation with rLeIF, as seen in this study, had a pattern similar to TH2-skewing stimulants: Comparably to stimulation with SEA, IL-12 production was completely suppressed, while IL-6 production was unaffected. With low and medium amounts of rLeIF, the IL-10 levels were increased. These results suggest that electroporated rLeIF might have interfered with an intracellular MAP kinase pathway, possibly with ERK 1/2. This would be a property shared by other PAMPs: Another conserved leishmanial molecule, lipophosphoglycan, a TLR2 ligand, was shown to stimulate the ERK 1/2 pathway in macrophages, resulting in down-regulation of IL-12 [57]. A hypothetical model of how rLeIF might interact with intracellular MAP kinases is depicted in Fig. 30.

Agrawal *et al.* attributed the different effects after stimulation with different PAMPs to the type of TLR that was involved [3]. The point where rLeIF interferes with the MAP kinase pathways could thus be an intracellular receptor that is different from its receptor involved in extracellular signaling, but it may also immediately interact with other non-receptor components of the MAP signaling cascade further downstream. Other protozoans that can induce a state of “LPS tolerance”, a transient, non-responsive cell state that is marked by inability to produce IL-12, e. g. *Toxoplasma gondii*, were shown to interact with several intracellular targets, namely proximal and distal parts of the TLR

signaling pathway and transcription factors such as STAT 3 [reviewed in 61]. Further research will be required to define the exact point of interaction.

Perhaps leishmanial LeIF that is believed to be relatively abundant within the parasite [24], and although being an intracellular protein, is able to facilitate parasitic survival by inducing IL-12-depressive effects, and thus contributes to parasitic host evasion. It could be indicative of an evasion mechanism that parasitic molecules can have on the host cell even after death of the parasite and release of its compounds into the cytoplasm. The increased IL-10 levels produced by BMDC after rLeIF electroporation equally support this hypothesis: IL-10, biasing a disease-promoting TH2 state, leads to higher parasite persistence and thus survival in normally resistant mouse strains [262]. It was even shown that lack of IL-10 during infection with *L. major* results in total elimination of the parasite [13]; IL-10 is therefore a critical factor for long-term persistence of the parasite within the host.

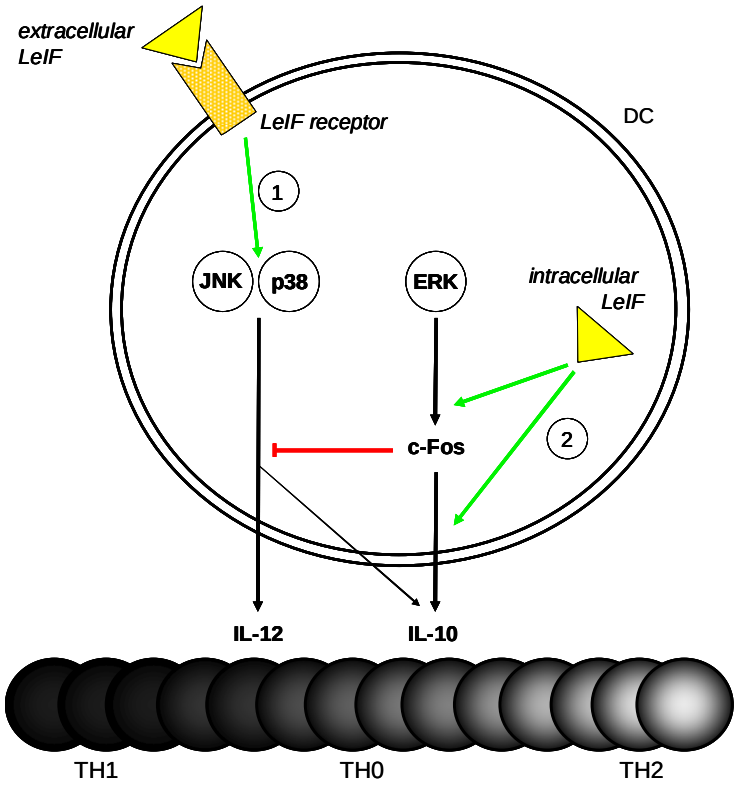

Fig. 30: Hypothetical model of rLeIF interaction with intracellular DC signaling pathways. Experimental evidence suggested a bipartite nature of rLeIF: (1) Extracellular LeIF could cause phosphorylation of JNK or p38 MAP kinases which is critical for the induction of IL-12. IL-10 production is induced to a little extent by activation of JNK and p38. (2) Intracellular LeIF could interfere with different points of the ERK pathway, leading to stabilization of c-Fos with subsequent suppression of IL-12 production and secretion of IL-10. Depending on how strong the activation of the MAP pathways is, the cytokines IL-12 and IL-10 bias the T cell response towards the opposite ends of the TH1/TH2 spectrum.

The altered cytokine profile suggests that LeIF is a protein with a bipartite nature. Its effect on DC might in fact depend on where the cells encounter it: while extracellular pulsing promotes TH1 polarization, intracellular presence favors a TH2 response (Fig. 30).

The fact that no proinflammatory cytokines are secreted upon LeIF transfection suggests that BMDC do not recognize the LeIF molecule they have synthesized themselves as a PAMP. This could be explicable if it was degraded immediately after synthesis, which appears to have happened to the translated LeIF(226) molecule; however, intact LeIF(fl) was detected in whole cell lysates, so DC should have been able to respond to it. According to the absence of major hydrophobic residue clusters and targeting sequences, LeIF is believed to be a cytosolic protein [24], and should thus not be expected to be directed into segregated cellular compartments where it would avoid contact to TLR. The unresponsiveness of DC may be related to a lack of recognition, possibly due to a different tertiary structure of LeIF molecules after translation by the DC protein synthesis machinery, or it may indeed be related to an immunological silencing function mediated by transfection with LeIF-RNA and subsequent overexpression of the encoded protein.

Expression levels of the co-stimulatory molecule CD86 were only slightly elevated in BMDC receiving either LeIF(fl)- or LeIF(226)-RNA via electroporation, compared to BMDC transfected with control RNA. The CD86 expression level of BMDC that were pulsed with rLeIF was not reached. This contrasts with the fact that CD86 expression was found not to be influenced by LeIF in human monocyte-derived DC [183]. It is, however, important to know that LeIF transfection causes a slight, rLeIF-pulsing an even more pronounced up-regulation of CD86, because CD86 was shown to have a role in TH1-priming by DC that have a crucial role in antileishmanial immunity [159].

MHCII expression was slightly enhanced in LeIF(fl)-transfected BMDC, and more pronounced in BMDC receiving LeIF(226)-RNA. Extracellular pulsing only caused a slight upregulation of MHCII expression which contrasts with the clear upregulation of CD86 expression.

What does this imply for the effect of LeIF on the maturation status of DC? The secretion of several proinflammatory cytokines, such as IL-1β, IL-6, IL-10 and IL-12,

as well as the upregulation of CD86 suggests that rLeIF pulsing induces maturation of day 7 BMDC, albeit not strongly. Notably, the expression levels of surface markers are not upregulated unanimously by rLeIF as they are by stronger maturation stimuli such as LPS that causes prominent upregulation of DC surface molecules, e. g. MHCII, CD40, CD80 and CD86 [144]. Conversely, the lack of subsequent cytokine secretion and the failure to up-regulate co-stimulatory molecules more than just slightly suggests that transfection of BMDC with LeIF(226)- or LeIF(fl)-RNA does not induce maturation.

However, the maturation state of DC also needs to be judged with view to their ability to stimulate T cells to proliferate. In the experiments presented here, the capacity of BMDC – that were either transfected with LeIF-RNA or pulsed with rLeIF on different days of culture – to elicit production of IFN-γ by naïve or LeIF-primed T cells was assessed. Strikingly, the IFN-γ levels increased substantially when T cells were stimulated with BMDC that were pulsed with rLeIF after one or two more days of culture duration, i. e. on day 8 or 9 of culture; in contrast, they remained within the magnitude elicited by day 7 BMDC when LeIF-transfected cells were used for stimulation.

The interpretation of this result is difficult, as there has not been any report so far describing the effect of electroporated murine DC on primary T cells in standard *in vitro* proliferation assays. Most reports, even on human DC, are based on standard model or tumor antigens for which MHCI- or MHCII-restricted T cell lines are available [50, 155, 257, 258]. It has to be kept in mind that requirements for T cell lines to proliferate are lenient: T cell hybridomas do not require co-stimulation to become activated. This is due to the fact that they are generated from terminally differentiated armed effector T cells that do not require CD28 signaling for activation [107]. In that course, day 7 non-matured OVA-transfected murine BMDC were shown to stimulate an OVA-specific T cell line at a DC:T ratio of merely 1:40 [258]. However, one report demonstrated specific lysis of *ex vivo*-pulsed, adoptively transferred primary CTL by matured, electroporated DC *in vivo* [257]. Thus, if mature electroporated DC are used as stimulators, an activation of primary T cells is basically demonstrable. In this regard, most *in vitro* studies also involved mature DC as stimulators that had been matured

either by cytokine cocktails or by other maturation inducers such as LPS.

It has been shown that the longer BMDC are being cultured according to the protocol by Lutz *et al.*, the higher percentages of cells exhibit a mature phenotype in terms of surface marker expression [144]. However, with respect to the presented results, maturity acquired after longer duration of culture does not seem to be the culprit, as it does not account for the extreme differences in cytokine production in lymphocyte cultures between day 7, 8 and 9 rLeIF-pulsed BMDC as stimulators on the one hand and LeIF-transfected BMDC on the other hand.

The more than sevenfold increase of IFN-γ production from day 8 BMDC to day 9 BMDC is particularly surprising. As stimulation was carried out for 72 hours, T cells in both assays were exposed to DC that were in culture on day 10 and 11 under the same conditions. It seems unlikely that it is merely a temporal difference of stimulation for 24 more hours on day 12 that could explain this enormous increase, especially as IFN-γ production is even higher from naïve T cells stimulated with day 9 BMDC compared to LeIF-primed T cells stimulated with day 8 BMDC (although this possibility could be excluded in a 96 hours lymphocyte culture). In addition, after 12 days of culture BMDC do not mature further unless stimulated with e. g. LPS [144], so there is no reason why during another day the stimulatory capacity should suddenly rise. Here, the high production of IFN-γ in day 9 BMDC samples rather seems to be a result of the "maturation age" of BMDC at the time of pulsing – which is obviously linked to the susceptibility, or "proneness to respond" to a maturation stimulus – and the maturation stimulus itself, rLeIF.

This view is supported by the fact that in samples with naïve T cells as responders, day 9 BMDC, but not day 7 and day 8 BMDC elicited production of IL-2. IL-2 production by LeIF-primed T cells upon stimulation with rLeIF-pulsed BMDC in the adequate context is not as surprising because these T cells have been "educated" to recognize this antigen before, i. e. they have been activated, started to express the high affinity IL-2R [7] and therefore probably do not require such a high level of co-stimulation as naïve T cells. They do not need BMDC-derived IL-2 to become activated and produce IL-2 themselves. Conversely, IL-2 production by naïve T cells is a marker not just for expansion, but for their activation upon the initiation of an immune response by rLeIF-pulsed BMDC. The ability of DC to secrete IL-2 upon innate receptor recognition

marks the state in which they are able to initiate a primary immune response and thus bridge innate and adaptive immunity [73]. This property, in the present study, was acquired only by BMDC that had reached day 9 maturity and that received LeIF as recombinant protein. In contrast, transfection with LeIF-RNA with subsequent LeIF expression did not confer this signal: IFN-γ production remained within the range of day 7 BMDC-stimulated T cells, and the IL-2 level was barely above background. It can be concluded that primed T cells only proliferate when stimulated with rLeIF-pulsed BMDC; they do not proliferate when stimulated with LeIF-transfected BMDC, although they recognize LeIF-derived antigenic peptides, as indicated by IFN-γ production.

How can these differences be explained? It has to be born in mind that several factors influence the magnitude of the T cell response. Classically, presentation of the antigen via MHC molecules has to be paired with recognition by the appropriate TCR; moreover, co-stimulation with CD80 and CD86 is considered as the second signal that is required for T cell activation. Certain cytokines have been suggested to be critical for the polarization of the immune response, such as IL-12 for TH1 and IL-4 and IL-10 for TH2. In addition, recent reports emphasize that DC-derived IL-2 is the first crucial signal for the activation of naïve T cells and might thus represent a key switch from tolerance to immunity as one of the central players on the bridge between innate and adaptive immunity [73].

Another factor that must not be underestimated is the nature of the peptide that is presented via MHC molecules. Different inflammatory or metabolic conditions can lead to the generation of an altered pool of peptides. The proteasome, upon assumption of a different subunit constitution (“immunoproteasome”) or after activation by PA28, can exhibit different cleavage site preferences [118], while the diversity of the peptide repertoire generated by the lysosomal proteolytic degradation machinery can be influenced, upon maturation, by increased protease activity after lysosomal acidification [249]. In addition, under certain conditions, peptide modifications such as post-proteasomal processing [118], peptide splicing [263] or posttranslational modifications [reviewed in 249] may possibly have an influence on the immunogenicity of the peptide. In that course, both the proteasome and the lysosome are thought to contribute to the nature and the immunogenicity of the presented peptides – and thus to the quality

of the T cell response. It is therefore well possible that adequate recognition of rLeIF and subsequent activation of BMDC leads to the generation and presentation of a more immunogenic peptide.

Also, the stability of MHC-peptide complexes is a key parameter for the immunogenicity of an antigen [133], and maturation can, under certain circumstances, increase the stability of both MHCI- [35, 126] and MHCII-peptide complexes [34]. It is thus imaginable that an enhanced stability of MHC-peptide complexes after activation by rLeIF may have contributed to the higher immunogenicity of day 9 rLeIF-pulsed BMDC.

A clue supporting the view that the selection and thus the immunogenicity of presented peptides are influenced by the activation state of the DC is provided by experiments showing that both proteasome inhibition and NF-κB blockade result in decreased T cell responses [26].

In conclusion, for the induction of an immune response, it is not only necessary that DC present an antigen via MHC molecules, but additional factors are also important. Proliferation experiments with transfected DC presented here showed that immature DC were indeed able to present the antigen to primed T cells, but did not elicit prominent proliferation. The culprit could not be the dose of antigen administered: rLeIF-pulsed day 7 BMDC were equally poor stimulators as were LeIF-transfected BMDC, but received the same amount of rLeIF as did day 9 BMDC which elicited a much stronger T cell response.

However, even if rLeIF induced upregulation of co-stimulatory molecules and secretion of proinflammatory cytokines, these factors alone were not sufficient to induce a strong T cell response. The maturation age of DC was important as well, which shows that the induction of maturation (here, with rLeIF) does not always have the same consistent outcome, but that the time of induction is critical too: Only day 9 rLeIF-pulsed BMDC were able to stimulate naïve T cells, albeit at a lower level, and should thus have immunogenic properties, possibly also *in vivo*. If this property related to the maturation age reflects e. g. the differential expression of the receptor for LeIF or an internal functional property of DC that is acquired with time, will have to be studied in the future. Fig. 31 depicts a hypothetical model for the role of components of T cell – DC interaction.

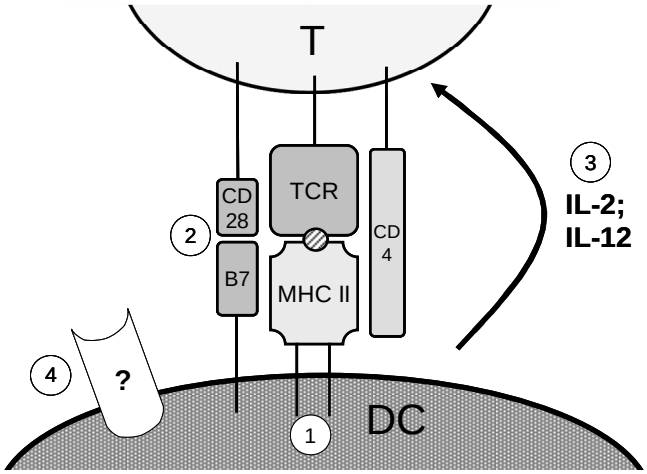

Fig. 31: T cell activation. Before signals 1) to 3) can exert their activating function upon T cells, the BMDC needs to exhibit another property, marked here as a fourth prerequisite – possibly the LeIF recognition receptor or a specific internal function, which is not present before day 9 of culture.

Yet, how can the effects of BMDC transfection be interpreted? The unresponsiveness of primed T cells seems, regardless of all remarks made about co-stimulation, surprising – considering that, as mentioned, antigen-restricted T cell lines proliferated vigorously to transfected BMDC or PBMC in similar experiments. T cell lines are usually expanded from activated T cells, so the persistent unresponsiveness of primed T cells to day 9 BMDC is puzzling.

The phenomenon seen here requires differentiation between anergy and tolerance – a field that is still subject to vibrant discussion. Anergy of T cells, on the one hand, denotes a state of antigen-specific unresponsiveness that prevents further activation. Tolerance, on the other hand, is characterized by failure to respond to an antigen, but it is thought that it is due to the tolerizing capacity of regulatory $CD4^+$ T cells. It has been shown that the molecular bases for these two immunological properties are indeed different: anergic T cells appear to have followed an aborted activation pathway that can be reversed, while regulatory T cells follow a distinct developmental pathway that extinguishes effector functions [119]. Notably, anergic T cells do not secrete cytokines at all, whereas regulatory T cells produce effector cytokines such as IFN-γ or IL-4. It is thus well possible that T cells producing low levels of IFN-γ in the presented experiments are tolerance-mediating regulatory T cells.

In addition, the phenotype of BMDC used here resembled the phenotype of DC that were termed “semi-mature” by Lutz [145]. These semi-mature DC typically induce

regulatory T cells [145]. In conclusion, it is well possible that DC presenting LeIF epitopes after transfection, and also after pulsing with rLeIF in an immature state, act as a tolerogenic, possibly semi-mature, cell population. The production of IL-12, as seen here in experiments using day 7 rLeIF-pulsed BMDC as stimulators, is not a sufficient prerequisite for priming of naïve T lymphocytes. Also, its secretion by BMDC is not necessarily followed by proliferation of primed T cells; therefore, IL-12 does not serve as an appropriate predictor for strong T cell responses. A naïve T cell response, i. e. priming of naïve T lymphocytes, only occurs if full maturation of DC is achieved. Possibly, DC-derived IL-2 production is indeed the critical step [73].

To substantiate this hypothesis, a further characterization of the responding T cell population – if they are primarily $CD8^+$ or $CD4^+$ T cells, if they produce IL-10 as regulatory T cells typically do [4, 111] – and their activation requirements – if the activation is e. g. TNF-α-dependent [153] – will be necessary.

As with LeIF transfection, the necessary signal for DC activation is not conferred, it would be also interesting to see if the administration of another extra- or intracellular maturation stimulus is able to increase the T cell response. In that course, e. g. co-transfection of human PBMC with tumor antigen mRNA and double-stranded viral RNA – poly(I:C), a TLR3 ligand [5] – was observed to cause stronger CTL responses than DC stimulated with a cytokine cocktail [154]. As in leishmaniasis, the TLR9 ligand CpG [89] was shown to contribute to a protective immune response in otherwise susceptible mice [186, 212], CpG might also be an interesting candidate to study activation of LeIF-transfected BMDC.

The results of the present study also suggest that BMDC kept in culture for 9 days at the time of their treatment might serve as more effective stimulators than BMDC cultured for only 7 or 8 days. For further investigation of LeIF transfection of BMDC, emerging results from transfection studies in human PBMC should be considered: The initial pioneer view that electroporation prior to maturation results in a higher level of antigen presentation [259] has been countered recently with systematic studies on the ideal time points of electroporation and onset of maturation. They revealed that RNA electroporation of mature DC – rather than electroporation of immature DC that are subsequently matured – is not only equivalent in terms of transfection efficiency, but even superior in terms of immunogenicity [22, 155, 206].

With respect to the fact that RNA-transfected BMDC might be tested as vaccines against *L. major* infection in mice, several advantages should be pointed out that widen the applicability of the technique. RNA transfection is a method of antigen delivery that is not only independent of the recipient's MHC haplotype, but it also leaves room for application of a variety of different antigens: it was shown that the simultaneous transfection of three different RNAs does not reduce the expression levels of each antigen seperately [206]. It can therefore be imagined that RNA of a multi-subunit polyprotein, e. g. the tandem-linked protein Leish-111f – which also contains the LeIF(226) sequence – that proved to be protective in BALB/c mice when administered as s. c. vaccine, could be tested in RNA transfection [39, 214]. Furthermore, the applicability is not limited by the functional state of DC – the change in endocytic activity during maturation has no impact on the transfection efficiency: it is comparable in DC prior to and after maturation [206].

One limitation pointed out in this study is the possible interference of intracellular parasitic proteins with host cell signaling cascades. Although the phenomenon was not demonstrated in RNA-transfected BMDC, it could be shown that the intracellular presence of a parasitic protein alters the cytokine profile of DC. It has to be born in mind that intracellular parasites such as *L. major* have developed strategies to persist in cells of the phagocytic system. There are means to circumvent this problem: the encoded antigen can be linked to targeting sequences that direct the translated protein to subcellular organelles, thus reducing the exposure to host signaling pathways. Also, processing and presentation via the preferred MHC pathway can be achieved with the help of targeting sequences. Ubiquitin can be added for proteasomal degradation and subsequent presentation via MHCI [246, 258]. For targeting to the MHCII pathway, the antigens can be linked to endosomal sequences such as the invariant chain [258] or to the lysosomal membrane proteins LAMP-1 and DC-LAMP (dendritic cell lysosome-associated membrane protein) [22].

What is the expected role of RNA-transfected BMDC as vaccines against *L. major* infection? As mentioned, the nature of the encoded protein and the expression level can bias the predominance of the processing mechanism, and DC also allow extensive cross-talk between the two classical MHC pathways. Therefore, it cannot be predicted

with certainty which T cell compartment will be activated to what extent. Still, without the introduction of signal sequences, assuming the expected cytosolic expression of the encoded antigen and hypothesizing that presentation via MHCI will occur predominantly, $CD8^+$ T cells should be primarily activated.

Although $CD8^+$ T cells were shown not to be able to compensate for the lack of functional $CD4^+$ T cells in MHCII-deficient C57BL/6 mice with the consequence of disease exacerbation in primary disease [53], they are nevertheless thought to play an immunoregulatory role on IFN-γ-producing $CD4^+$ T cells after vaccination, enabling TH1 differentiation of $CD4^+$ T cells [80]. There is evidence that this effect is IFN-γ-mediated and is dependent on IL-12R expression and IL-12 production *in vivo*. At least for the DNA vaccination model used in this study by Gurunathan *et al.*, the hypothesis was established that a sustained TH1 response requires a bidirectional interaction in which $CD4^+$ T cell activation is required for production of IFN-γ by $CD8^+$ T cells, which in turn helps to maintain the frequency of $CD4^+$ IFN-γ-producing T cells [80].

In the present study, transfection of DC with a protozoan antigen was studied for the first time. BMDC expressed the *L. major* antigen LeIF and presented it specifically to T cells. In comparison to pulsing with recombinant LeIF, profound differences were found with respect to DC function, phenotype and immunogenicity: LeIF did not deliver the same activating signals to DC after transfection as after protein pulsing. This contrasts with the immunostimulatory capacities of DC after RNA transfection in many tumor models.

Nevertheless, the study revealed important differences between antigen delivery to DC via RNA transfection and protein pulsing that will be helpful for further development of vaccination techniques, especially against diseases caused by protozoans. Particularly, it showed that the outcome of RNA transfection of BMDC is not simply transferrable to just any antigen. Still, if the choice of antigens and the activation requirements of DC are carefully taken into consideration, the use of RNA-transfected BMDC as vaccines – a technique that does not underlie the restrictions inherent to classical plasmid DNA vaccination [27] – might eventually be part of the tool box on the search for an antileishmanial vaccine.

7 Summary

Cutaneous leishmaniasis is an infectious disease that is endemic especially in tropical and desert regions with an incidence of 1.5 million cases per year and a prevalence of 12 million people infected worldwide. The infection can be caused by the intracellular parasite *Leishmania major*. The disease has been studied extensively in the murine model. It has become apparent that the induction of a class of interferon (IFN)-γ-producing $CD4^+$ T helper cells (TH1 cells) that activate macrophages to kill the parasites they harbor is desicive for the establishment of immunity. The redirection of the host's immune response towards a protective TH1 phenotype will also be the key to an effective vaccine.

Dendritic cells (DC) loaded with leishmanial antigens *ex vivo* were lately described as vaccines against *L. major* infections. One single recombinant *Leishmania* antigen, LeIF (*Leishmania* homologue of eukaryotic ribosomal initiation factor 4a), which was identified as a protein that stimulates DC to secrete interleukin (IL)-12 and discussed as a pattern-associated molecular pattern (PAMP), was found to mediate a protective TH1-dependent effect when used for pulsing of DC. The application of recombinant proteins is tied to many disadvantages, which is why other methods of antigen administration have been developed. RNA electroporation of DC has recently emerged from tumor research as a safe and versatile method of antigen delivery, by which a large number of RNA molecules encoding a specific antigen gains access to the cytosol of DC by an electrical impulse.

The present study describes, for the first time, transfection of DC with RNA encoding a molecularly defined parasite antigen.

Initially, a standardized protocol for RNA transfection was established, using the enhanced green fluorescent protein (EGFP) as reporter antigen. EGFP-RNA was well translatable in an *in vitro* translation system, and both a DC cell line (fetal skin-derived DC; FSDC) and murine primary bone marrow-derived DC (BMDC) could be transfected efficiently, with a yield of up to 90% and 75%, respectively. In both cell types, maximal transfection efficiency was attained with 20 µg RNA and could not be further increased with larger amounts of RNA. The level of antigen expression, measured as the mean fluorescence intensity (MFI) by flow cytometry, was directly

proportional to the amount of RNA used for transfection. In FSDC, transfection efficiency and MFI were generally higher than in BMDC when the same amounts of RNA were used. Furthermore, the kinetics was shown to be sensitive to treatment with lipopolysaccharide (LPS): the expression peak was higher and was reached sooner, followed by a more rapid decline.

In transfection experiments with LeIF, two variants of LeIF-RNA were used: LeIF(fl)-RNA, encoding the complete LeIF sequence, and LeIF(226)-RNA, encoding only the aminoterminal half of the LeIF sequence (226 amino acids), the immunogenic part of LeIF. Only LeIF(fl) was detectable by Western Blot in whole cell lysates of BMDC after LeIF(fl)-RNA transfection, whereas LeIF(226) could never be detected in LeIF(226)-transfected BMDC. However, as both constructs were well translatable in a cell-free system, the failure to detect LeIF(226) in BMDC lysates did not represent a failure in RNA translation, but rather a rapid antigen degradation.

It was therefore expected that LeIF(226)-transfected BMDC should nevertheless be able to present LeIF(226)-derived antigenic peptides to T cells from BALB/c mice primed with recombinant LeIF (rLeIF). This hypothesis was confirmed by measuring IFN-γ production in BMDC-T cell co-incubation assays, showing that rLeIF-pulsed, LeIF(226)- and LeIF(fl)-transfected day 7 BMDC did indeed activate T cells from LeIF-immunized mice in an antigen-specific manner. In contrast, IL-4 was not produced, which was consistent with the fact that T cells found in lymph nodes from LeIF-primed mice are primarily of the TH1 type.

In the supernatants of LeIF-transfected BMDC cultures, in contrast to rLeIF-pulsed BMDC, the proinflammatory cytokines IL-1β, IL-6, IL-10 and IL-12 were not detected. This effect was not due to the electroporation procedure, as cytokine production by BMDC electroporated with rLeIF was only partially impaired. Also, the expression levels of CD86 were lower upon LeIF transfection than after pulsing with rLeIF. Thus, LeIF transfection did not induce maturation of DC. In conclusion, LeIF-transfected BMDC may have acted as semi-mature antigen-specific tolerance inducers, with regulatory T cells as responders.

The effect of LeIF transfection on the immunostimulatory capacity of BMDC was not significantly increased when day 8 or 9 BMDC were used. However, day 8, and even more day 9 BMDC pulsed with rLeIF mounted a vigorous T cell response. Day 9

BMDC were able to activate naïve T cells.

In conclusion, before a strong T cell response against LeIF can be induced, DC need to – besides presenting antigen and expressing co-stimulatory molecules – exhibit a susceptibility to the innate signaling molecule LeIF which is linked to their maturation age. This third signal is provided by extracellular rLeIF, but it is not conveyed – or is suppressed – by intracellular LeIF after LeIF-RNA transfection.

Furthermore, electroporation of rLeIF abrogated IL-12 production by BMDC completely, the production of IL-1β was reduced with higher antigen doses, and the production of IL-10 was partially increased. The IL-6 production was unaffected.

This altered cytokine profile suggests that LeIF as a PAMP might have a bipartite nature: besides exhibiting the capacity to stimulate IL-12 production upon extracellular presence, thereby enhancing host resistance against *L. major*, LeIF could also contribute to parasitic host evasion mechanisms from intracellular compartments of DC, possibly by interfering with mitogen-activated protein (MAP) kinase signaling pathways. Thus, the adjuvant properties of LeIF depend both on its mode of delivery (transfection with RNA vs. pulsing with the recombinant protein) and the targeted compartment (extra- vs. intracellular).

From this work, it can be summarized that BMDC are well transfectable with a parasite antigen. The antigen is processed and presented, but it is not recognized as a PAMP by DC. Hence, transfection with antigen-encoding mRNA by itself does not convey all necessary signals for the elicitation of a potent immune response.

8 Zusammenfassung

Die kutane Leishmaniose ist eine Infektionskrankheit, die besonders in tropischen und Wüstenregionen endemisch ist, mit einer Inzidenz von 1,5 Millionen Fällen im Jahr und einer Prävalenz von 12 Millionen Infizierten weltweit. Die Infektion kann durch den intrazellulären Parasiten *Leishmania major* hervorgerufen werden. Am Mausmodell ist die Krankheit ausführlich untersucht. Wie dabei deutlich wurde, ist für die Immunität gegen den Erreger die Induktion einer Klasse von Interferon (IFN)-γ-produzierenden $CD4^+$ T-Helfer-Zellen (TH1-Zellen) entscheidend, welche Makrophagen dazu aktivieren, die von ihnen beherbergten Parasiten abzutöten. Die Umlenkung der Immunantwort in Richtung einer schützenden TH1-Antwort wird auch der Schlüssel zu einem effektiven Impfstoff sein.

Ex vivo mit Leishmanienantigenen beladene dendritische Zellen sind vor einiger Zeit als Vakzine gegen *L. major*-Infektionen beschrieben worden. Ein einzelnes rekombinantes Antigen, LeIF (*Leishmania* homologue of eukaryotic ribosomal initiation factor 4a), ein parasitäres Protein, das die IL-12-Produktion durch dendritische Zellen stimuliert und das als mikrobiell konserviertes Strukturmolekül (pattern-associated molecular pattern; PAMP) diskutiert wird, vermittelte dabei, zum Pulsen von dendritischen Zellen verwendet, einen schützenden TH1-abhängigen Effekt. Der Einsatz rekombinanter Proteine ist jedoch mit etlichen Nachteilen verbunden, weshalb andere Methoden zur Verabreichung von Antigenen entwickelt wurden. Aus der Tumorforschung ist unlängst die RNA-Elektroporation dendritischer Zellen als eine sichere und vielseitige Methode hervorgegangen, bei der eine große Anzahl von RNA-Molekülen, die für ein bestimmtes Antigen kodieren, durch einen elektrischen Impuls in das Cytosol dendritischer Zellen gelangt.

Die vorliegende Arbeit beschreibt zum ersten Mal die Transfektion dendritischer Zellen mit RNA eines molekular definierten Parasitenantigens.

Zunächst erfolgte die Etablierung eines standardisierten Protokolls für die RNA-Transfektion mit dem enhanced green fluorescent protein (EGFP) als Reporterantigen. EGFP-RNA war gut translatierbar in einem *In-vitro*-Translationssystem, und es konnten sowohl eine Zellinie (fetal skin-derived dendritic cells; FSDC) als auch primäre, aus Knochenmarkkulturen der Maus gewonnene dendritische Zellen (bone marrow-derived

dendritic cells; BMDC) mit einem Anteil von bis zu 90% bzw. 75% effizient EGFP-transfiziert werden. In beiden Zelltypen wurde die maximale Transfektionseffizienz mit 20 µg RNA erreicht, die mit größeren Mengen an RNA nicht weiter zu steigern war. Die Höhe der Antigenexpression, gemessen als mittlere Fluoreszenzintensität (MFI) in der Durchflußzytometrie, war direkt proportional zur verwendeten RNA-Menge. In FSDC waren die Transfektionseffizienz und die MFI generell höher als in BMDC bei gleicher RNA-Menge. Zudem konnte gezeigt werden, daß eine Behandlung mit LPS die Kinetik beeinflußt: Die maximale Expression war höher und wurde auch eher erreicht, worauf zudem ein schnellerer Abfall folgte.

In den Transfektionsexperimenten mit LeIF wurden zwei Varianten von LeIF-RNA verwendet: eine für die gesamte LeIF-Sequenz kodierende LeIF(fl)-RNA, und eine nur für die aminoterminale Hälfte der LeIF-Sequenz (226 Aminosäuren), dem immunogenen Teil des LeIF-Moleküls, kodierende LeIF(226)-RNA. Im Western Blot von Ganzzellysaten dendritischer Zellen war nur LeIF(fl) nach Transfektion nachzuweisen, wohingegen LeIF(226) in LeIF(226)-transfizierten BMDC nie nachzuweisen war. Da beide Konstrukte aber gut im zellfreien System translatierbar waren, stellte der fehlgeschlagene Nachweis von LeIF(226) kein Fehlschlagen der RNA-Translation, sondern vielmehr einen raschen Antigenabbau dar.

Es bestand daher die Erwartung, daß LeIF(226)-transfizierte BMDC trotzdem in der Lage sein müßten, von LeIF(226) abgeleitete antigene Peptide an T-Zellen von mit rekombinantem LeIF (rLeIF) immunisierten BALB/c-Mäusen zu präsentieren. Diese Vermutung wurde durch Messung von IFN-γ in Stimulationsversuchen mit BMDC und T-Zellen bestätigt, die zeigten, daß am Tag 7 der Kultur mit rLeIF gepulste, LeIF(226)- und LeIF(fl)-transfizierte BMDC in der Tat antigenspezifisch T-Zellen aus LeIF-immunisierten Mäusen aktivierten. IL-4 hingegen wurde nicht produziert, was mit der Tatsache vereinbar ist, daß in Lymphknoten LeIF-vakzinierter Mäusen hauptsächlich T-Zellen vom TH1-Typ zu finden sind.

In den Überständen LeIF-transfizierter BMDC-Kulturen, im Gegensatz zu rLeIF-gepulsten BMDC, waren die proinflammatorischen Zytokine IL-1β, IL-6, IL-10 und IL-12 nicht nachzuweisen. Dieser Effekt lag nicht am Elektroporationsvorgang, da die Zytokinproduktion von mit rekombinantem LeIF elektroporierten BMDC nur teilweise beeinträchtigt war. Die Expression von CD86 war nach LeIF-Transfektion zudem

geringer als nach Pulsen mit rLeIF. LeIF-Transfektion führte mithin nicht zur Reifung dendritischer Zellen. LeIF-transfizierte BMDC könnten im Ergebnis als antigenspezifische Toleranzinduktoren fungiert haben, mit regulatorischen T-Zellen als Respondern.

Der Effekt der Transfektion mit LeIF-RNA auf die immunstimulatorische Wirkung von BMDC war nicht signifikant erhöht, wenn BMDC am Tag 8 oder 9 der Kultur verwendet wurden. BMDC, die am Tag 8, und mehr noch am Tag 9 mit rLeIF gepulst wurden, induzierten hingegen eine energische T-Zell-Antwort. BMDC vom Tag 9 waren sogar in der Lage, naive T-Zellen zu aktivieren.

Bevor eine starke, gegen LeIF gerichtete T-Zell-Antwort eingeleitet werden kann, müssen dendritische Zellen also letztlich – neben Präsentation des Antigens und Expression kostimulatorischer Moleküle – eine gewisse „Empfindlichkeit“ gegenüber dem Strukturmolekül LeIF besitzen, die mit ihrem Reifungsalter in Zusammenhang steht. Dieses dritte Signal wird nicht durch intrazelluläres LeIF nach Transfektion mit LeIF-RNA übermittelt, oder es wird unterdrückt.

Darüber hinaus war nach Elektroporation von rLeIF die IL-12-Produktion von BMDC gänzlich aufgehoben, die Produktion von IL-1β bei höheren Antigendosen reduziert und die Produktion von IL-10 teilweise erhöht. Die Produktion von IL-6 war unbeeinflußt.

Dieses veränderte Zytokinprofil legt eine Doppelnatur von LeIF als PAMP nahe: Neben der bei extrazellulärem Vorliegen von LeIF erwiesenen Eigenschaft, die Produktion von IL-12 zu stimulieren, welches die Resistenz des Wirtes gegen *L. major* steigert, könnte LeIF bei intrazellulärem Vorliegen auch zu Evasionsmechanismen des Parasiten vor dem Immunsystem des Wirtes beitragen, möglicherweise durch Wechselwirkung mit MAP (mitogen-activated protein)-Kinase-Signalwegen. Die Eigenschaften von LeIF als Adjuvans hängen also sowohl von der Verabreichungsmethode (Transfektion mit RNA bzw. Pulsen mit dem rekombinanten Protein) als auch vom Zielkompartiment (extra- bzw. intrazellulär) ab.

Zusammenfassend konnte also in dieser Arbeit gezeigt werden, daß BMDC mit einem Parasitenantigen transfizierbar sind. Das Antigen wird dabei prozessiert und präsentiert, aber von dendritischen Zellen nicht als PAMP erkannt. Durch Transfektion mit antigenkodierender mRNA alleine werden mithin nicht alle notwendigen Signale für die Induktion einer potenten Immunantwort übermittelt.

9 Literature

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10 Abbreviations

AEP	asparagine-specific endopeptidase
APS	ammonium persulfate
ARCA	anti-reverse cap analogon
BMDC	bone marrow-derived dendritic cells
BSA	bovine serum albumine
CIP	calf intestinal phosphatase
CL	cutaneous leishmaniasis
CLIP	class II-associated invariant chain peptide
CLSM	confocal laser scanning microscopy
ConA	concanavalin A
CpG	CpG oligodeoxynucleotides
CTL	cytotoxic T lymphocytes
DALIS	DC aggresome-like induced structures
DC	dendritic cell
DC-LAMP	dendritic cell lysosome-associated membrane protein
DC-SIGN	Dendritic cell-specific ICAM3-grabbing nonintegrin
DEPC	diethylpyrocarbonate
DRiPs	defective ribosomal products
EDTA	ethylene diamine tetraacetic acid
EGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
Flt-3	FMS-like tyrosine kinase 3
FSDC	fetal skin-derived dendritic cells
GILT	gamma-interferon-inducible lysosomal thiol reductase
GMIC	gap junction-mediated immunological coupling
GM-CSF	granulocyte/macrophage-colony stimulating factor

HLA	human leukocyte antigen
IFN	interferon
Ii	MHC class II-associated invariant chain
IL	interleukin
IL-12R	interleukin-12 receptor
JNK	c-Jun N-terminal kinase
LACK	*Leishmania* homologue of receptors for activated C kinase
LAMP	lysosome-associated membrane protein
LB	lysogeny broth
LeIF	*Leishmania* homologue of eukaryotic ribosomal initiation factor 4a
LeIF(fl)	gene or gene product of LeIF relating to the entire LeIF sequence
LeIF(226)	gene or gene product of LeIF relating to the 226 N-terminal amino acids
LN	lymph node
LPS	lipopolysaccharide
MAP kinase	mitogen-activated protein kinase
MFI	mean fluorescence intensity
MHCI/II	major histocompatibility complex class I/class II
MS	medium size
MyD	myeloid differentiation factor
NF-κB	nuclear factor κB
NK cells	natural killer cells
OD	optical density
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PBS (Mg^{2+}, Ca^{2+})	PBS containing magnesium and calcium
PE	phycoerythrin
PFA	paraformaldehyde
PRR	pattern recognition receptor
RIPA	radioimmunoprecipitation assay

rLeIF	recombinant LeIF
rmGM-CSF	recombinant mouse granulocyte/macrophage-colony stimulating factor
RNI	reactive nitrogen intermediates
SCID	severe combined immunodeficiency
SDS	sodium dodecyl sulfate
SEA	schistosome egg antigens
STAT	signal transducer and activator of transcription
TAA	tumor-associated antigen
TAP	transporters associated with antigen processing
TCR	T cell receptor
Temed	tetramethylethylene diamine
TH cells	T helper cells
TLR	toll-like receptor
TNF	tumor necrosis factor
TNT	tunnelling nanotubules
UTR	untranslated region
UV	ultraviolet
VL	visceral leishmaniasis
Xef	*Xenopus* elongation factor

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Curriculum vitae

Persönliche Daten:

Name:	Christian Keller
Geburtsdaten:	22. Mai 1978 in Bonn
Familienstand:	ledig
Nationalität:	deutsch
Adresse:	Buschdorfer Straße 2
Ort:	53117 Bonn
Eltern:	Ulrich Keller, Rechtsanwalt Dr. Marie-Ursel Raether-Keller, Ärztin

Schullaufbahn:

1984 – 1988	Katholische Grundschule St. Augustin
1988 – 1997	Kardinal-Frings-Gymnasium; Abitur Juni 1997

Wehrdienst:

09/1997 – 02/1998	Panzergrenadiertruppe (Rotenburg/Fulda, Koblenz)
03/1998 – 08/1998	Bundeswehrzentralkrankenhaus (BwZK), Koblenz

Studium:

09/1998 – 11/2005	Studium der Medizin, Julius-Maximilians-Universität Würzburg
seit 05/2001	Studium der Rechtswissenschaften, Julius-Maximilians-Universität Würzburg

Examina:

08/2000	Physikum (Ärztliche Vorprüfung)
08/2001	Erstes Staatsexamen
07/2003	Juristische Zwischenprüfung
09/2004	Zweites Staatsexamen
11/2005	Drittes Staatsexamen; Erteilung der Approbation am 06.12.2005, Reg. v. Unterfranken

Promotion:

09/2002 – 10/2003	Experimentelle Doktorarbeit über das Thema "Die Rolle von dendritischen Zellen bei der Immunregulation der Leishmaniasis – Transfektion dendritischer Zellen mit RNA eines molekular definierten Parasitenantigens", Labor Prof. Dr. Heidrun Moll, Institut für Molekulare Infektionsbiologie, Würzburg

Famulaturen:

03/2001	Innere Medizin, Infektionsstation, BwZK Koblenz
03/2002	Praxisfamulatur Urologie, Görlitz
10/2003	Institut für Medizinische Mikrobiologie, Universität Würzburg
02 – 03/2004	Kinderkardiologie, Great Ormond Street Hospital, London, UK

Praktisches Jahr:

10/2004 – 02/2005	Innere Medizin, Missionsärztliche Klinik, Würzburg
02/2005 – 05/2005	Chirurgie, American University Hospital, American University of Beirut, Libanon
06/2005 – 09/2005	Neurologie, West Virginia University Hospital, Morgantown, W. Va., USA

Stipendien:

1998 – 2000	Studienstiftung des Deutschen Volkes
01 – 09/2002	Deutsche Forschungsgemeinschaft, Graduiertenkolleg 520 "Immunmodulation", Universität Würzburg