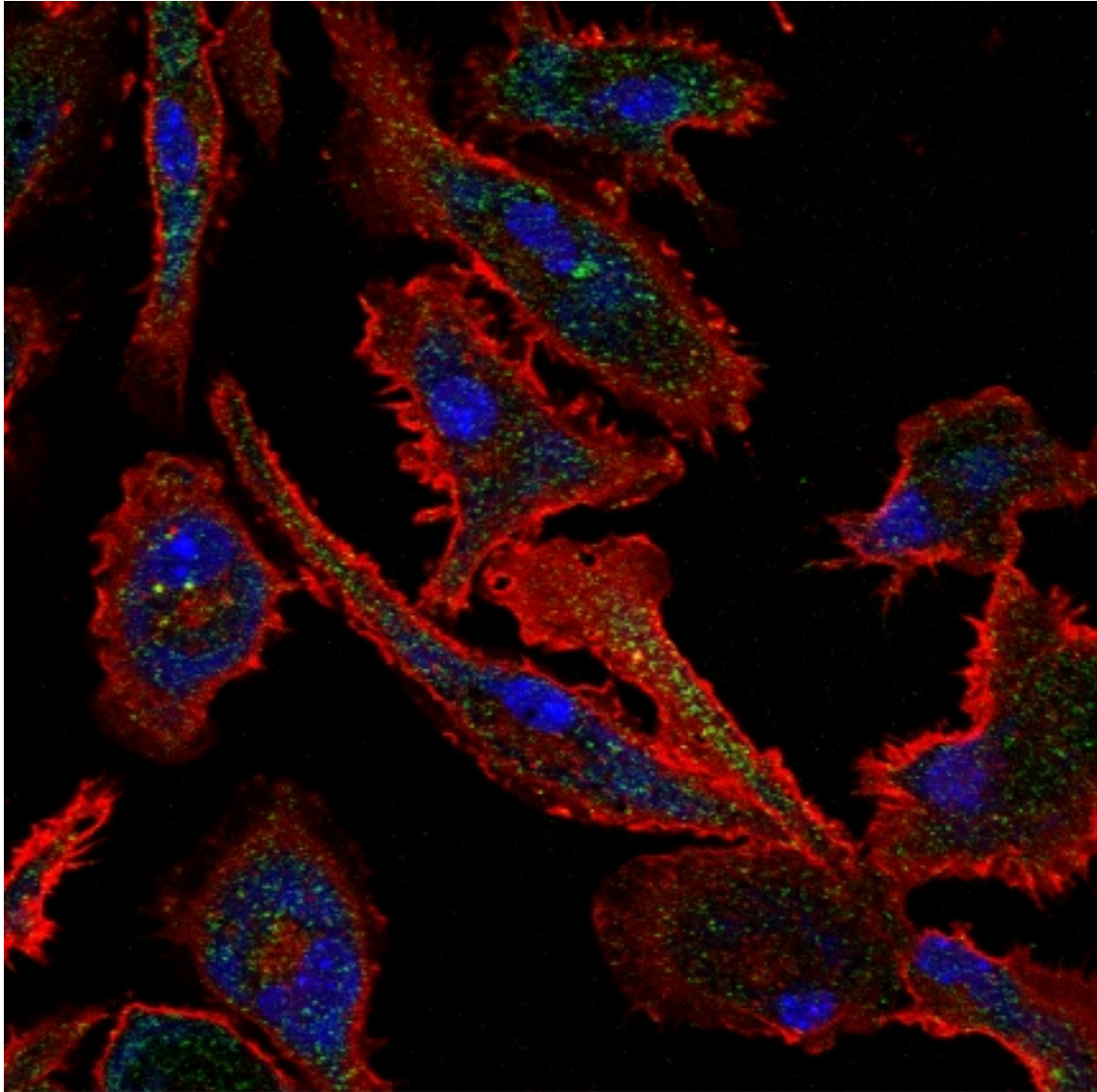


**Dendritic Cell mediated immune responses are shaped by
Interleukin-15 and Interleukin -21**



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Cover: Dendritic cells, stained for membrane (red), IL-15 (green) and the nucleus (blue).

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Abstract

The immune system contains a distinct group of antigen presenting cells, called dendritic cells (DCs), that are specialized to capture antigens and initiate T cell immunity. The complex processes involved are critically balanced by soluble factors, such as cytokines, produced by DCs itself or released by neighboring cells.

The goal of this study was to examine how differentiation, maturation and function of DCs are modulated by two particular cytokines, namely IL-15 and IL-21, which possess regulatory activities on immune responses. Here it is shown that IL-15 has stimulatory effects on DC activation and on T cell priming. Thus IL-15 evoke highly mature DCs whereas in contrast IL-21 mediates inhibitory effects resulting in phenotypic and functional immature DCs with decreased T cell stimulatory capacities *in vitro* and *in vivo*.

To understand by which means DC activation is modulated by IL-15 and consequently which T cell subsets are influenced by this cytokine, several *in vivo* models were investigated. On the one hand we used genetic deficient mice for IL-15 (IL-15^{-/-}) and its high affinity receptor (IL-15R α ^{-/-}) and on the other hand we treated wild type mice with an IL-15 antagonist, a soluble IL-15R α , to block the activity of the cytokine without affecting DC differentiation.

The studies revealed that IL-15 and IL-21, modulate DC function. DC-derived IL-15 is essential for the initiation of Th1 type immune responses. Despite sharing a common receptor subunit, IL-15 and IL-21 exhibit completely opposite effects on DC biology.

The dichotomous effects of IL-15 and IL-21 on DC mediated T cell activation shed new light on the understanding of immune modulatory mechanisms. Thus, the application of agonists or antagonists of these cytokines can be conceived as therapeutical tools to control immune responses.

Zusammenfassung

Das Immunsystem beinhaltet eine besondere Gruppe von Antigen-präsentierenden Zellen, die sogenannten Dendritischen Zellen (DZ), die darauf spezialisiert sind, Antigene aufzunehmen und eine T Zell Immunantwort einzuleiten. Die dabei beteiligten, hoch komplexen Prozesse werden entscheidend durch lösliche Faktoren, bezeichnet als Zytokine, beeinflusst, die von DZ selbst oder von benachbarten Zellen sezerniert werden.

Das Ziel dieser Arbeit war, zu untersuchen, wie die Differenzierung, Reifung und Funktion von DZ durch IL-15 und IL-21, zwei Zytokine mit Immun-regulatorischen Eigenschaften, modulieren werden können. Dabei zeigte sich, dass IL-15 eine steigernde Wirkung auf die DZ Aktivierung und die darauffolgende T Zell Differenzierung hatte. Demzufolge, unterstützte IL-15 die DZ bei der Reifung, wohingegen IL-21 hemmende Effekte vermittelte, was zur Ausbildung von phänotypisch und funktional unreifen DZ führte, die eine geringe Kapazität zur T Zell Stimulation *in vitro* und *in vivo* hatten.

Um besser zu verstehen, wie die Aktivierung von DZ von IL-15 moduliert wird und infolgedessen, welche T Zell Subklassen davon beeinflusst werden, haben wir uns verschiedener *in vivo* Mausmodelle bedient. Wir verwendeten zum einen Gen-defiziente Mäuse für IL-15 (IL-15^{-/-}) und zum anderen für dessen hoch affinen Rezeptor IL-15R α (IL-15R α ^{-/-}). Zusätzlich haben wir die Funktion von IL-15 in Wildtyp Mäusen durch die Gabe eines antagonistischen sIL-15R α geblockt ohne die DZ Differenzierung zu beeinflussen.

Diese Untersuchungen geben neue Einsichten, wie die Zytokine IL-15 und IL-21 die Funktion von DZ modulieren. Es wurde deutlich, dass von DZ sezerniertes IL-15 essentiell ist für die Induktion von Th1 Immunantworten. Obwohl IL-15 und IL-21 eine gemeinsame Rezeptorkette zur Signaltransduktion nutzen, zeigen sie konträre Effekte auf die Funktionen von DZ.

Diese Befunde werfen ein neues Licht auf das Verständnis von Immun-modulatorischen Mechanismen. Daher ist es vorstellbar, dass Antagonisten oder Agonisten dieser Zytokine als therapeutische Instrumente angewandt werden, um unerwünschte Immunantworten zu kontrollieren.

Introduction

Dendritic Cells and their central role in T cell mediated immunity

Host defense relies on a concerted action of both Ag nonspecific innate immunity and Ag-specific adaptive immunity (Fearon 1996). Key features of the mammalian innate immune system include the ability to rapidly recognize pathogen and/or tissue injury and the ability to signal the presence of “danger” to cells of the adaptive immune system (Matzinger 1994). The innate immune system includes phagocytic cells, natural killer cells, the complement system and interferons. Evolutionary pressure has led to development of adaptive immunity, the key features of which are the ability to rearrange genes of the immunoglobulin family, permitting creation of a large diversity of Ag specific clones and immunological memory. Yet this highly sophisticated and potent system needs to be instructed and regulated by antigen presenting cells (APCs).

Dendritic cells (DCs) are unique APCs because they are the only ones that are able to induce primary immune responses, thus permitting establishment of immunological memory (Banchereau 1998). DC progenitors in the bone marrow give rise to circulating precursors that home to tissues, where they reside as immature cells with high phagocytic capacity. Following tissue damage, immature DCs capture Ag and subsequently migrate to the lymphoid organs. There, DCs present Ag, bound on major histocompatibility complex (MHC) and select rare Ag specific T cells which recognize this complex via a specific T cell receptor. In addition, DCs express different costimulatory molecules, like CD80 and CD86 that bind to receptors on T cells and are a prerequisite that they become activated and differentiate into effector and memory T cells. A central aspect of the immune system is the generation of “memory cells” after the first antigen contact, residing for many years in different locations of the lymphoid system. These memory T cells become rapidly activated after repetitive contact to the same antigen, resulting in clonal expansion, thereby mediating a highly efficient secondary immune response (Janeway 2002).

This “defense system” is critically modulated by soluble factors, called cytokines, produced by DCs itself or released by neighboring cells and providing cellular cross-talk. Cytokines bind to specific receptors on the membrane of target cells, triggering signal transduction pathways that ultimately alter gene expression of target cells. With this, cytokines are able to regulate the intensity and duration of an immune response by stimulating or inhibiting

activation, proliferation, and/or differentiation of various cells and by controlling the secretion of antibodies and other cytokines. As described in detail later, cytokines often use multipart receptors, composed of different binding and signaling chains, some of them shared with other cytokines. Specificity is guaranteed by unique, private high affinity receptor components.

DC maturation is a continuous process initiated upon Ag encounter and/ or simulation with inflammatory cytokines and is completed during DC-T cell interaction. While presenting Ag to T cells, DCs pass through different functional states and are induced to enter maturation (Banchereau 2000). Numerous factors induce and/ or regulate DC maturation, including pathogen-related molecules like lipopolysaccharide (LPS, Rescigno 1999), bacterial DNA (Akbari 1999; Hartmann 1999), and double stranded RNA (Cella 1999). The balance between pro- and anti-inflammatory signals in the local microenvironment further shapes the outcome of DC activation. The course of maturation is associated with several coordinated events (**Fig. I**) such as loss of endocytic and phagocytic receptors and activity, up-regulation of co-stimulatory molecules and MHC class II, change in morphology and cytoskeleton reorganization, and alteration of adhesive structures to acquire high cellular motility (Winzler 1997).

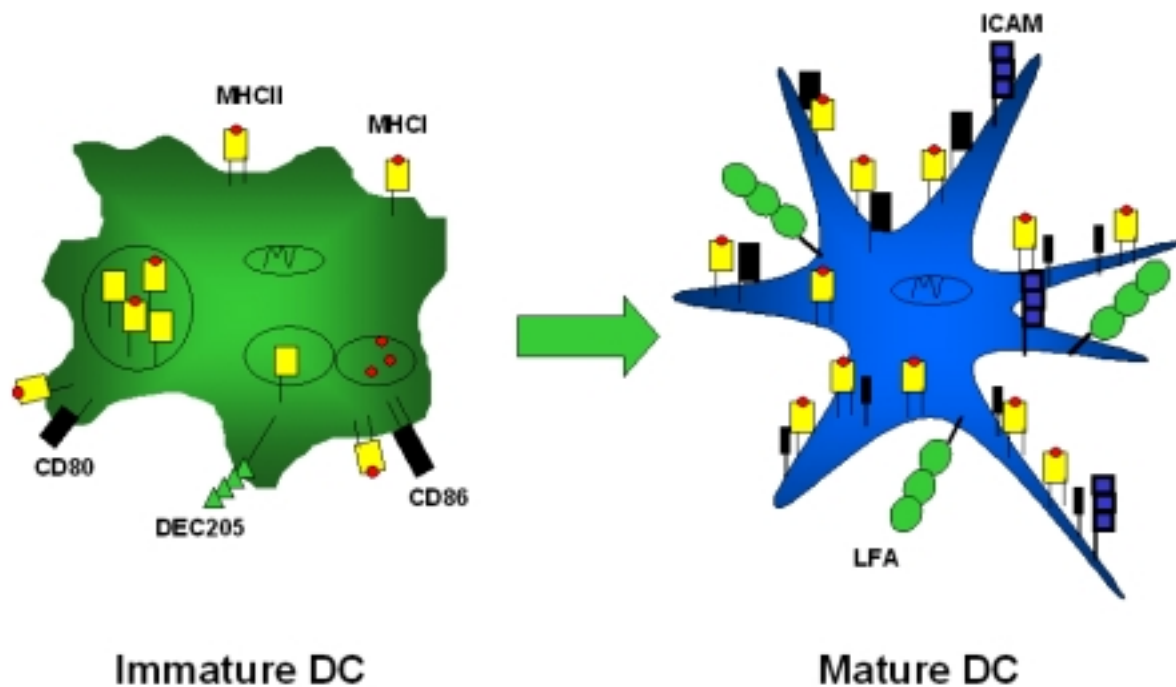


Fig. I. DCs mature through at least two definable stages to become potent antigen-presenting cells in lymphoid tissue. DCs arise from bone marrow progenitors and migrate

via the blood to peripheral tissues and organs, where they are highly phagocytic via receptors such as DEC 205 and express only few co-stimulatory molecules but store much intracellular MHC molecules in large vacuoles (left). When they pick up Ag in the peripheral tissues, they migrate via the afferent lymphatic vessels into the regional lymph node. Here they express high levels of T cell activating potential such as CD80 and CD86, MHC and high levels of adhesion molecules DC-SIGN, ICAM-1, ICAM-2, LFA-1 and LFA-3 but are no longer phagocytic (right). In addition, the phenotype changes from a relatively round cell morphology into mature DCs with prominent protrusions.

Mature DCs exhibit aside elevated surface expression of MHCII and co-stimulatory molecules, high T cell stimulatory capacity and thereby convert from a high efficient antigen-capture and uptake to an antigen presenting state. Therefore DC maturation is a key checkpoint in the initiation of immunity and is important for the overall quality of an immune response (Liu 2001b).

The paucity and difficulties of isolating primary DC populations *in vivo* has limited their study. This was circumvented by generating large amounts of these cells *in vitro* making cell biologic and molecular approaches more feasible. A common progenitor for granulocytes, macrophages, and DCs had been identified in mouse bone marrow (BM) (Inaba 1993) as a MHC class II negative cell that can develop under the aegis of the growth factor GMCSF (granulocytes- macrophage colony stimulating factor). Expansion of *in vitro* generated BM-derived DCs was shown to be promoted by IL-4, (Sallusto 1994). However, under some culture conditions GMCSF and IL-4 are not able to drive the full maturation of DCs, in that the final steps are mediated by T cells itself after Ag presentation and the influence of pro-inflammatory cytokines.

Taken together, DCs comprise a unique system of APCs designed to capture Ag at the side of entry and then to identify cognate T cells to prime a specific immune response. Modulators of this critical defense system would be powerful tools to better control pathogenic DC mediated processes.

Interleukin-15 and its modulatory effects in immunobiology

IL-15 is a pleiotropic cytokine that functions in development and/ or survival of NK-, NKT cells respectively homeostasis and development of lymphocytes. This results in an immunoregulatory cross-talk between natural and specific immune cells which bridges innate and adaptive immunity as described in **Fig. II**.

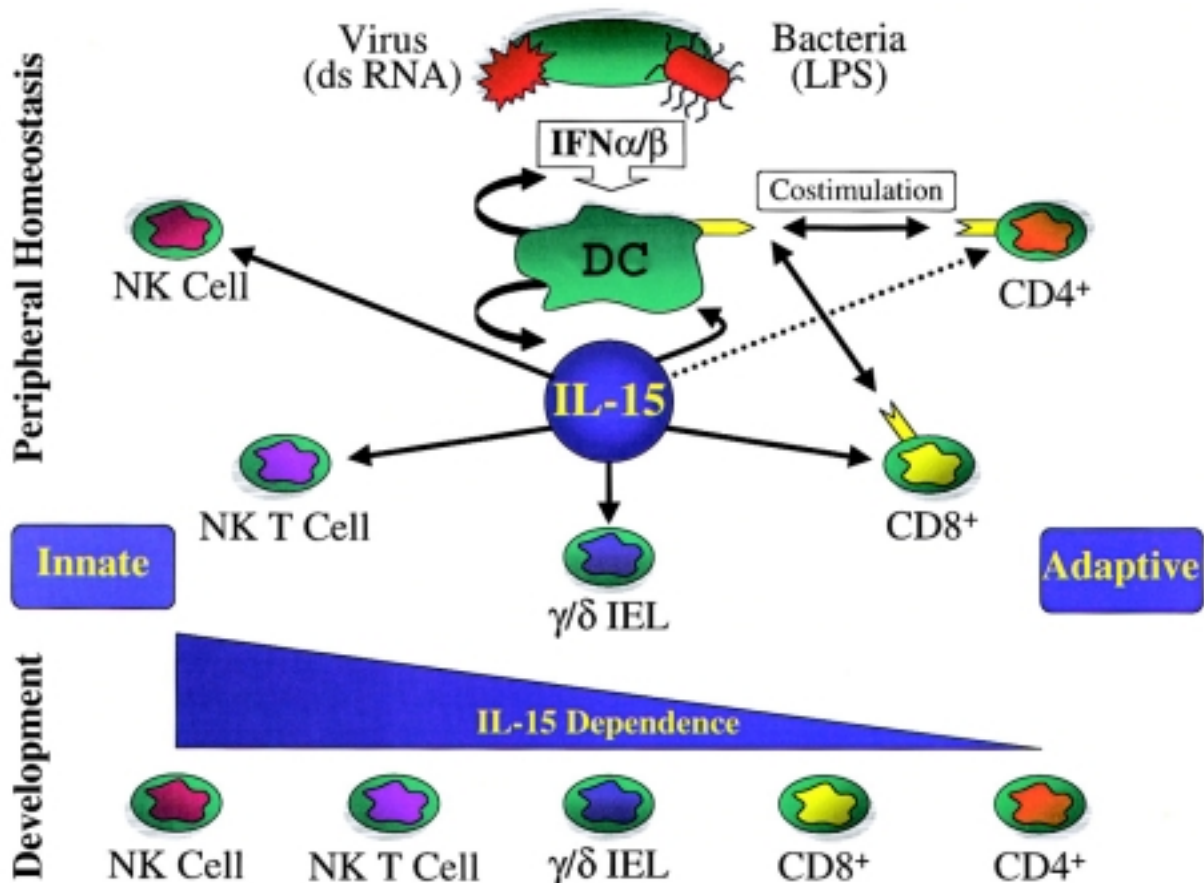


Figure II. Multiple functions of IL-15 in the innate and adaptive immune response. *IL-15 supports innate and adaptive immune cell development and homeostasis. Upon bacterial or viral infection conserved motifs such as double-stranded RNA or LPS result in release of type I interferons (IFN α/β) from infected host cells. Type I interferons activate DCs, stimulating them to produce IL-15 as well as to induce the up-regulation of co-stimulatory molecules. IL-15 selectively activates NK-, NKT-, and several T cell subclasses including CD8⁺ T cells (modified after Ma 2000).*

Although IL-15 was initially identified through its ability to mimic IL-2 induced T cell proliferation, both cytokines exert specific functions provided by binding to unique private α -chains that complement the IL-15R $\alpha\beta\gamma$ and IL-2R $\alpha\beta\gamma$ hetero-trimeric high affinity receptor complexes (Bulfone-Paus 1997a; Bamford 1994; Wilkinson 1995). Dramatic differences exist between these two cytokines in terms of their specific expression and the control of their synthesis and secretion (Doherty 1996; Krause 1996; Onu 1997). For example, most tissues express IL-15 and IL-15R α in response to inflammatory stimuli (Ma 2000; Fehninger 2000) including dendritic cells (DCs) (Mattei 2001), macrophages (Doherty 1996) and epithelial cells (Rückert 2000), whereas IL-2 and IL-2R α are expressed primarily by activated T cells (Tagaya 1996; Grabstein 1994).

The unique roles of IL-15 became clearer with the generation of knock out mice for IL-15 (Kennedy 2000) and the IL-15R α (Lodolce 1998), giving further evidence that IL-2 and IL-15 mediate very different functions *in vivo*: While IL-2 $^{-/-}$ and IL-2R $\alpha^{-/-}$ mice spontaneously accumulate activated T and B cells and die prematurely from autoimmune disease, IL-15 $^{-/-}$ and IL-15R $\alpha^{-/-}$ mice are generally healthy and specifically lack NK, NK T cells and activated CD8 $^{+}$ T cells (Ku 2000; Dai 2000). The loss of these cells demonstrates that IL-15 signals via IL-15R α are critical for lymphoid development and/or maintenance. The underlying mechanisms by which IL-15 supports homeostasis of memory T cells (Zhang 1998, Yajima 2002; Sprent 2001) are not yet understood. Neither is it known which cells serve as the major source of IL-15 for T cells which express the IL-15R α (Bulfone-Paus 1997a), nor when exactly during the process of T cell activation IL-15 is required *in vivo*.

Nevertheless, it has been reported that the capacity of mature DCs to prime naïve T cells and promote their differentiation is influenced to a high degree by the secretion of T cell activating cytokines (reviewed by Guermonprez 2002 and Moser 2000), notably IL-15 (Li 2001). Indeed DCs produce a large array of these soluble tools used by immune cells to modulate informative cues (Banchereau 1998). Focusing the fundamental role of IL-15 in establishing T cell memory and activation it was conceptually attractive that DCs would produce this cytokine to directly shape the outcome of a T cell mediated immune response.

Vice versa IL-15 acts back on DC differentiation and maturation (Mohamadzadeh 2001; Saikh 2001) as becomes evident e.g. by the secretion of pro-inflammatory cytokines (Mattei 2001; Ohteki 1999) and increased expression of co-stimulatory molecules (Agostini 1999).

Therefore IL-15 may be a very important candidate for modulating DC-mediated immune responses.

Interleukin-21: a new player in the cytokine network of the immune system

IL-21, a recently described cytokine belonging to the family of 4 α -helix bundle cytokines, like IL-4 and IL-15, displays similar organization and very high primary sequence and structural homology to IL-15 (Parrish-Novak 2000). Like this cytokine, IL-21 recruits the common γ -chain as an indispensable subunit for its heterodimeric receptor complex with its own private IL-21R chain (Parrish-Novak 2000; Ozaki 2000).

The family of cytokines that uses the common γ -chain like IL-2, 4, 7, 9, 15 (Sugamura 1996) plays a preeminent role in immunology (**Fig. III**). These proteins regulate lymphocyte development and control a broad spectrum of activities that shape innate an acquired immunity. The discovery of IL-21 and its receptor had added an exciting new player with modulatory potential on immune functions.

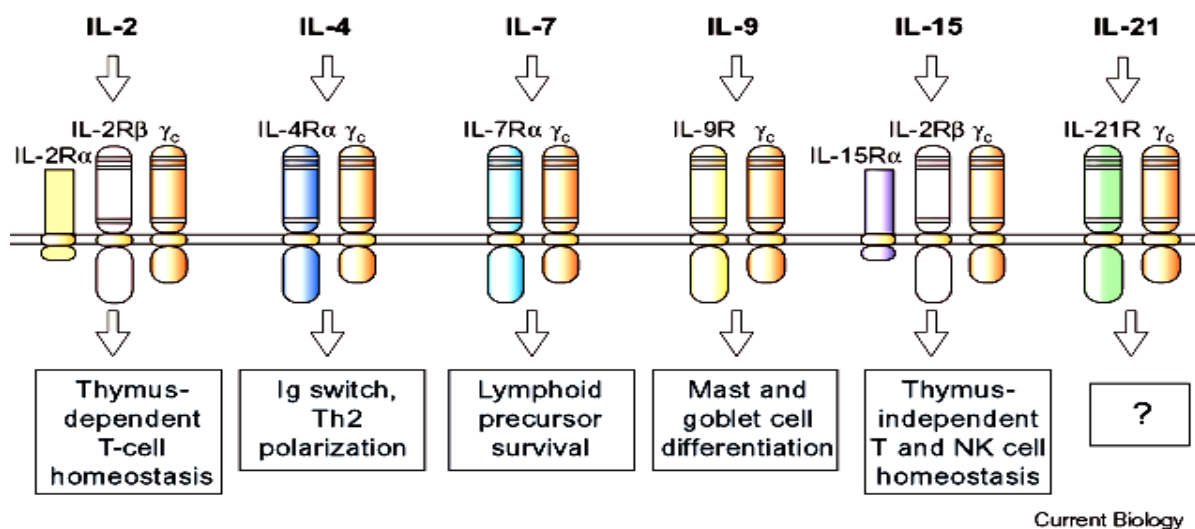


Figure III. The IL-21 and IL-15 receptor share the common- γ -chain. Both cytokines signal through a multipart receptor with a unique, private high affinity chain (IL-21R/IL-15R α) but use (as many other cytokines) the common- γ -chain for signaling. IL-15 in addition shares the IL-2R β -chain with IL-2 (Vossenhenrich 2001).

IL-21 is a product of activated, but not resting, T cells (Parrish-Novak 2000), an expression pattern which parallels that of IL-2, indicating that one of its primary functions may be associated with T cell immune responses. However, it had been shown that IL-21 influences not only the proliferation of T cell but also of B cells and the cytolytic activity of NK and CD8⁺ T cells as well as the differentiation of NK cells out of bone-marrow stem-cells (Parrish-Novak 2000). In addition, IL-21 synergizes with IL-2 and IL-15 in regulating these responses (Parrish-Novak 2002). Notably, IL-21 not only provides “positive” effects on IL-15 induced NK cell expansion or B cell proliferation but mediating the opposite, hence anti-proliferating effects, giving rise to the assumption that IL-21 has the potential not only to support but also to abrogate immune functions at various stages, dependent on co-stimulatory signals provided.

In general, the tissue distribution of receptors offers a strong indication of their potential sites of action. It has been reported that IL-21R is expressed in lymphoid tissues like thymus, lymph node, peripheral blood leukocytes as well as NK-cells (Parrish-Novak 2000), indicating that this receptor/ligand pair could play an important role in immunomodulatory functions. Interestingly, IL-21R expression was also found in bone marrow cells matching the fact that IL-21 promotes the differentiation of lymphoid cells out of BM stem cells. Other γ -chain signaling cytokines, as IL-4 and IL-15 (see above), are important for myeloid cell development like DC generation and their further maturation. However, whether IL-21 has an influence on DC development remains to be elucidated.

***In vivo* models to analyze modulation of DC biology by IL-15 and IL-21**

To study DC biology *in vivo* under the influence of modulatory cytokines like IL-15 and IL-21, two well-defined delayed-type hypersensitivity (DTH) reactions were explored. One of them is a mainly CD8⁺ T cell dependent model of contact hypersensitivity (CHS) (Becker 1993; Hofmann 1998; Grabbe 1998; Rückert 2002) and secondly a classical DTH response to sheep red blood cells (SRBC) was applied. This classical DTH has the advantage to not only consider CD8⁺ T cell mediated effects but also allows to assess the activation of CD4⁺ T cells as well as B cells including their antibody production (Kunzendorf 1996).

The complex process of the CHS response which is a subclass of DTH response and therefore overlapping with mechanisms in DTH reactions can generally be divided in two phases, which are described in the following.

Phase 1: Sensitization

Epidermal immunization, initiated by painting with soluble low molecular weight haptens, serving as model-antigens, induce activation of epidermal Langerhans cells and DCs, which leave the epidermis, enter the local lymph nodes (Kripke 1990, Mehling 2000), and undergo maturation, resulting in down-regulated antigen-uptake but enhanced expression of MHC II, CD80 and CD86 molecules. In the DLNs, where Ag is presented to naïve T cells, specific CHS effector T cells are generated. The passive process of painting an antigen can be mimicked by an active sensitization through direct injection of *ex vivo* antigen-labeled DCs, which circumvents the Ag-uptake in the periphery but induces an equal T cell priming in DLNs (**Fig. IV**).

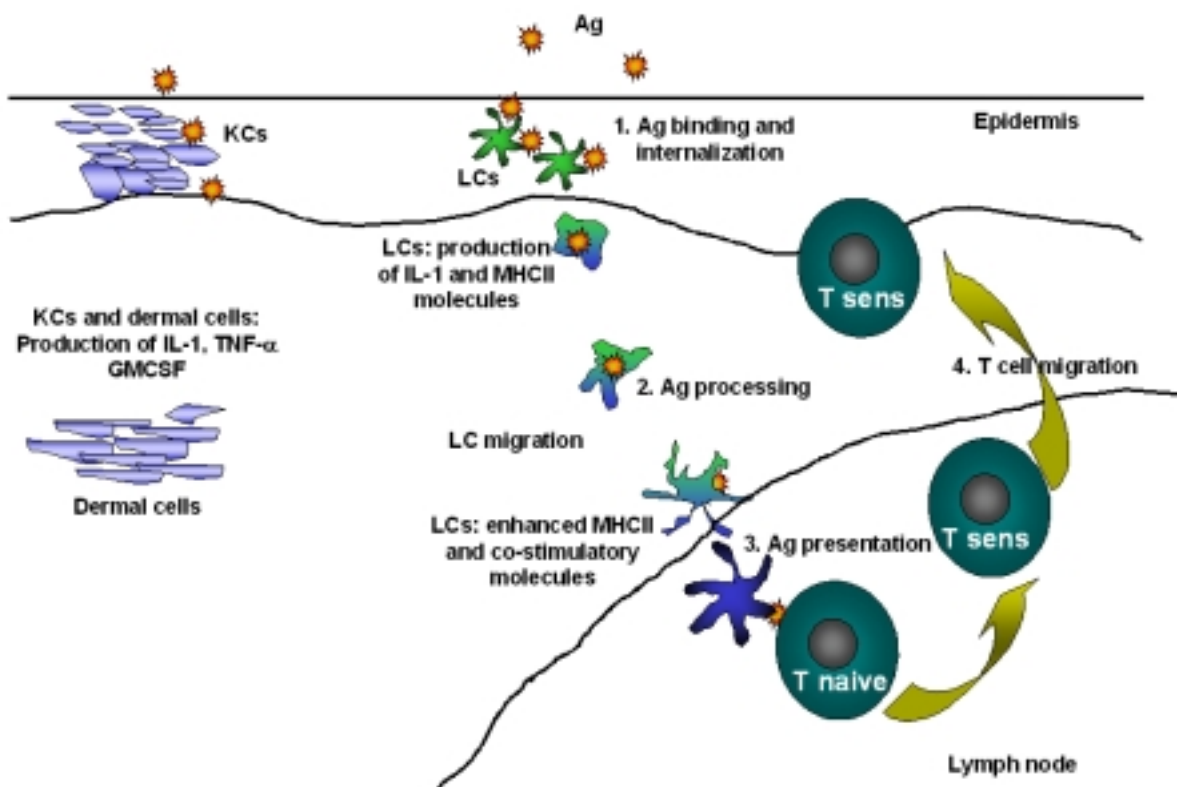


Figure IV. Sensitization phase. DCs – in the skin most notably Langerhans cells (LC) - reside in normal skin in a resting, immature state, characterized by high endocytotic and antigen-processing capacity. Topical hapten application results in cytokine secretion by keratinocytes and DCs and other cells within the skin. This launches DC activation and

migration of Ag-bearing DCs towards regional lymph nodes (Step 1). During this process, DCs process the Ag and acquire a mature, functional state, associated with cytokine secretion, enhanced expression of co-stimulatory molecules and enhanced capacity to prime naïve T cells (step 2). In the lymph node, DCs establish contact with T cells and activate them by sufficient expression of Ag-MHC complexes along with co-stimulatory and adhesion molecules (step 3). As a result of Ag-specific activation, primed T cells alter their migration pathway and begin to recirculate through peripheral tissues (step 4).

Phase 2: Elicitation

After a local antigen challenge on the earlobes of sensitized mice, the elicitation of an ear swelling response depends on the activation of antigen-specific T cells (T_{sens}) and the migration of these specific cells into the skin (Gocinski 1990; Müller 1995, Luger 1996) which is regulated by a complex network of epidermal cytokines. In the ear, at the site of the antigen challenge, an inflammation is induced, mainly mediated by clonal expansion of specific $CD8^+$ effector T cells (Grabbe 1998), resulting in an ear swelling, that correlates with the capacity of the mice or the injected DCs to activate antigen-specific memory T cells. The extend of the ear swelling serves therefore as a readout for the complex cascade of T cell mediated immune responses (**Fig.V**).

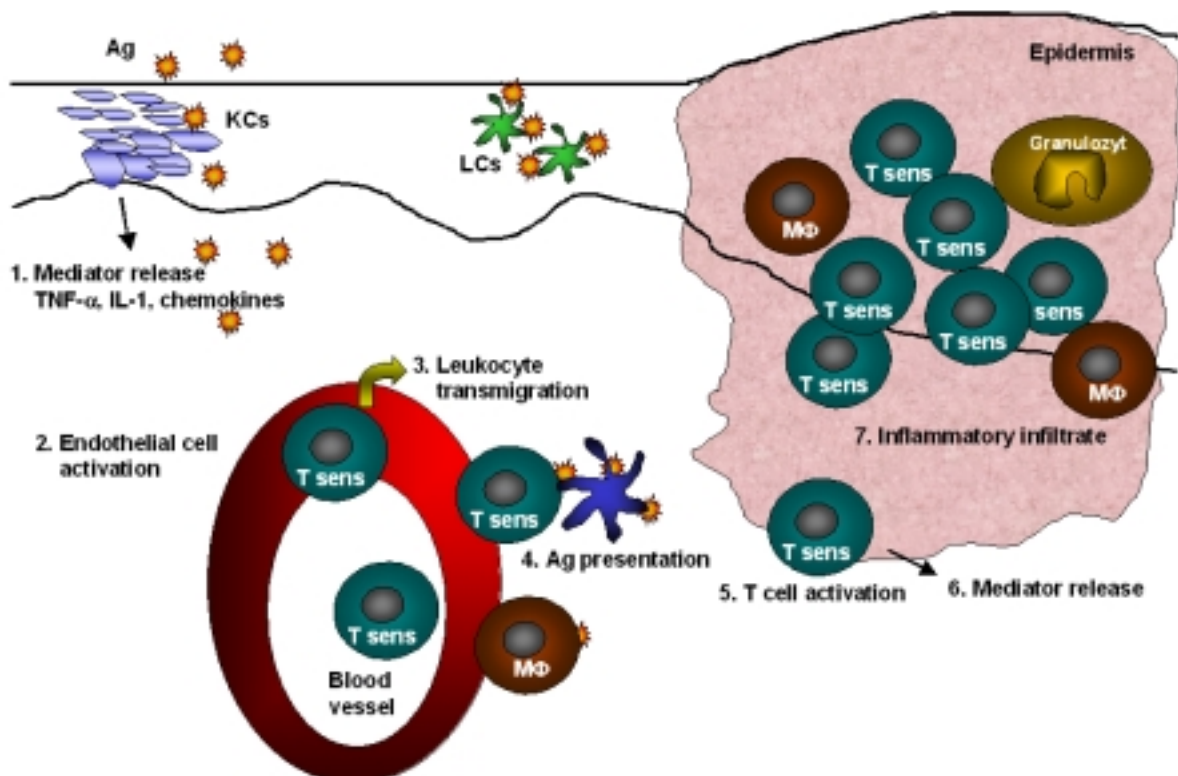


Figure V. A hypothesis to describe the elicitation phase. Secondary Ag application for CHS elicitation has the same primary effects on the skin as primary Ag contact during sensitization (i.e. pro-inflammatory effects, DC activation). Whereas DC activation is the relevant event during sensitization, nonspecific pro-inflammatory effects such as induction of cytokine secretion (step 1), expression of adhesion molecules, and activation of endothelial cells (step 2) trigger elicitation of CHS, probably due to attraction of leukocytes to the site of Ag application (step 3). Among these are primed T cells, which are activated upon Ag-presentation by resident or infiltrating DCs and other APCs (step 4). A specific T cell activation (step 5) induces mediator release (step 6), which amplifies the response by generating an inflammatory process and leads to further accumulation of inflammatory cells (step 7), resulting in clinically manifest contact dermatitis. The time between application of antigen and elicitation of CHS is approximately 24h.

The second model used in this thesis, is the classical DTH response against SRBC. The mechanisms of specific T cell priming by DCs in the classical DTH reaction are comparable to the described events during CHS response. Nevertheless, the sensitization to SRBC affects not only CD8⁺ but also CD4⁺ T cells, which provide co-stimulatory signals to B cells (“B cell help”). Thus specific IgM-, IgG-antibody production is detectable. This gives the great opportunity to analyze in parallel cellular and humoral immunity.

As a very important additional model system to analyze the effects of IL-15 and its high affinity receptor, we also used IL-15- and IL-15R α gene deficient mice. These approaches were further supported by blocking the function of IL-15 in wildtype mice with the help of the sIL-15R α , which constitutes the extracellular part of the high affinity IL-15R α (Fig. IV, Ruchatz 1998).

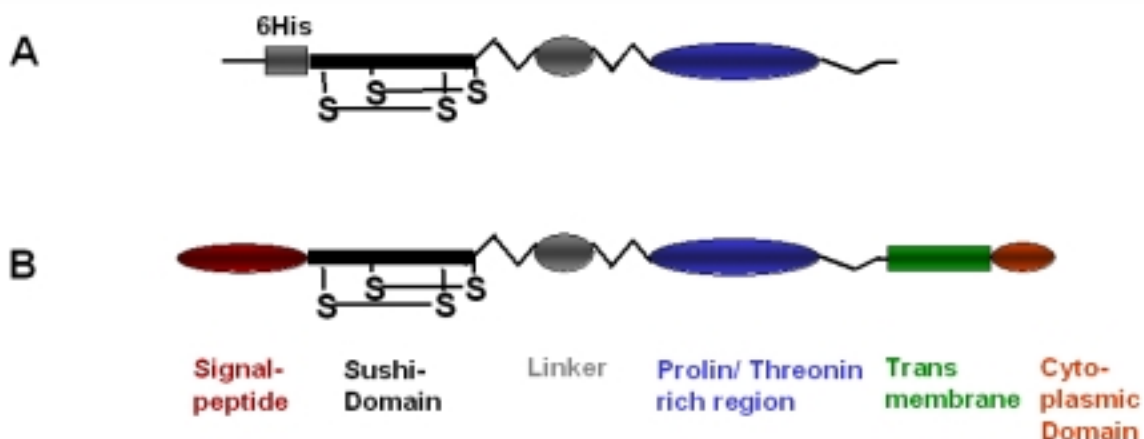


Figure VI. Schematic structure of sIL-15R α (A) and native, full length IL-15R α (B) is shown.

Questions addressed

DCs are professional antigen presenting cells that initiate T cell dependent immune responses, and which pass through different functional states of activation, modulated by cytokines that signal through the common γ -chain as described for IL-4. Given the structural relation between IL-21 and the IL-15/IL-4 cytokines together with their common use of the γ -chain it was reasonable to speculate that they influence DC development in similar ways.

This led us to investigate:

- the ability of IL-15 and IL-21, to influence differentiation, maturation and function of bone marrow derived DCs.
- the role of DC-derived IL-15 in the induction of antigen specific T cell reaction *in vivo and in vitro*.
- the mechanisms by which IL-15 influences DC-mediated T cell priming.

Such investigations will give us new insights in the development of DC mediated T cell immune responses and propose novel strategies to prevent the immune system from overwhelming reactions.

Material and Methods

Mice

C57BL/6 IL-15^{-/-} (Kennedy 2000) and C57BL/6 IL-15R α ^{-/-} (Lodolce 1998) mice were bred at the Research Center Borstel (Germany). As controls served C57BL/6 wildtype (IL-15^{+/+}) mice, which were purchased from Charles River (Sulzfeld, Germany). OTII^{tg} mice, whose transgenic CD4-TCRs specifically recognize the OVA⁽³²³⁻³³⁹⁾ epitope (Robertson 2000) were kindly provided from IFFA Credo (France). OTI^{tg} mice (Rotzschke 1991), which are transgenic for a CD8⁺ T cell restricted TCR, recognizing OVA⁽²⁵⁷⁻²⁶⁴⁾ peptide were kindly provided by Arne von Bonin, Bernhard-Nocht-Institute (Hamburg, Germany). All mice were used between 10-12 weeks age and maintained in specific pathogen-free conditions. All *in vivo* experiments were performed in compliance with national and institutional guidelines.

Culture medium and antibodies

Cells were cultured in RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) from Biochrom, 2mM L-glutamine (Life technologies, Karlsruhe, Germany), 100U/ml penicillin and 100 μ g/ml streptomycin (PAA, Linz, Austria), and 50 μ M β -mercaptoethanol (Life technologies) at 37°C in 5% CO₂.

Unless otherwise specified, all antibodies were from Pharmingen (Heidelberg, Germany). [³H]thymidine was purchased from Amersham Biosciences (Freiburg, Germany).

DC preparation

Bone marrow-derived DCs (BMDCs) were generated as previously described (Lutz 1999). Briefly, bone marrow cells were flushed from the femurs and tibias of mice and erythrocytes were lysed in 155mM NH₄Cl solution for 15min at room temperature. 2x10⁶ cells were seeded in bacterial grade, non adherent petridishes (Falcon, Becton Dickinson, Heidelberg, Germany) in 10ml medium supplemented with 20ng/ml GMCSF (Tebu, Frankfurt, Germany) or in addition to GMCSF plus 20ng/ml IL-15 (RnD, Wiesbaden, Germany) or 20ng/ml IL-21 which was kindly provided by D. Foster, ZymoGenetics (Seattle, U.S.A.). At day 3, another 10ml medium containing 20ng/ml GMCSF alone or plus IL-15 or IL-21 were added to the plates. At day 6 half of the cell-free culture supernatant was exchanged, and fresh medium containing 10ng/ml GMCSF alone or plus IL-15 or IL-21 was added. At day 8, cells were gently removed from the culture dishes by Accutase treatment (PAA) and washed twice with

medium. Purity was routinely >95% as determined by flow cytometric analysis of CD11c⁺ expression.

Antibodies and flow cytometric analysis

The phenotype of the BMDCs was analyzed using following mAbs: CD11c (FITC or APC labeled), MHC class II (I_A/I_E), CD80, CD25, CD122, γ_c , CD40, CD54, CD11b, CD95, CD95L, OX40L and CD86 (Southern Biotechnology, Birmingham, USA) (all PE conjugated). PE and FITC conjugated anti-CD3, -CD8 α , -CD45/B220, -Gr1, -DX5, -NK1.1 (all from Pharmingen) and -F4/80 (Serotec, Eching, Germany) were used as a control and found negative. We also stained for IL-15 binding on BMDCs with an IL-15-IgG2b fusion protein (Bulfone-Paus 1997b) and secondary with biotinylated goat anti-mouse IgG2b (Southern Biotec, Birmingham, USA) followed by streptavidin conjugated PE (Dianova, Hamburg, Germany). Mouse IgG2b (Sigma, Taufkirchen, Germany) was used as control.

T cells were analyzed with PE and FITC conjugated antibodies specific for CD3 (145-2C11), CD8 α (53-6.7), CD25, CD44, CD62L, CD69, CD122, and CD4-APC (RM4-5). Unless otherwise specified, all antibodies were from Pharmingen (Heidelberg, Germany). Propidium iodide at 2 μ g/ml (Sigma) was added in all probes to exclude dead cells.

For staining procedure 2x10⁵ cells were incubated with 1 μ l mAb for 15min on ice in a total volume of 100 μ l. After incubation cells were washed with PBS/EDTA/BSA. Flow cytometric analysis was performed with a FACSCalibur, equipped with standard filters (Becton Dickinson, Heidelberg, Germany) and CELLQuest software (Becton Dickinson).

Histomorphometry

Harvested BMDCs (5x10⁴) were transferred in 300 μ l fresh medium onto 8 well chamber slides (Nunc, Wiesbaden, Germany) and incubated for 48h at 37°C, 5% CO₂ to allow adhesion and spreading of dendrites. Slides were stained with Pappenheim after drying and pictures were taken with an Olympus Camedia digital camera adapted to an inverse Olympus CK 40 microscope (Olympus, Hamburg, Germany).

RT-PCR

Total RNA was extracted from 5x10⁶ cells after 8 days of culture using RNAzol (Life technologies) following the manufacturer's instructions. cDNA was synthesized using random hexanucleotide primers and the Superscript preamplification system II (Life technologies). Obtained cDNA was amplified in a 20 μ l reaction, using 1U of AmpliTaq DNA polymerase

(Roche, Mannheim, Germany), 250 μ M of each dNTP and 2 μ l 10-fold PCR buffer (Roche). The final concentration of each primer was 0.5 μ M. Thermal cycling conditions were 5min at 94°C and additional 30sec at 94°C. Annealing temperature was 60°C for 30sec, followed by extension at 72°C for 30sec. 30 cycles were performed and ended with final extension at 72°C for 10min. Reaction products were analyzed on a 1.5% agarose gel and visualized by ethidium bromide staining. All primers used were commercially synthesized and obtained from TIB Molbiol (Berlin, Germany) or Metabion (Martinsried, Germany). Primer sequences were as follows: IL-21R sense: 5'-CTC AGC CAG GCA CTT CAT TCA GG-3', IL-21R antisense 5'-ATC ACA GGA AGG GCA TTT AGC-3'; IL-15R α sense: 5'-AAC ATC CAC CCT GAT TGA GTG T-3', IL-15R α antisense 5'-GTT TCC ATG GTT TCC ACC TCA A-3'; IL-15 sense: 5'-GGC ATT AAG TAA TGA AAA TTT TGA-3', IL-15 antisense 5'-CTC GCA TGC AGT CAG GAC GTG TTG-3'; IL-21 sense: CCC TTG TCT GTC TGG TAG TCA TC-3', IL-21 antisense 5'-ATC ACA GGA AGG GCA TTT AGC-3'; IL-2R α sense: 5'-GGA TCC AAG ATG GAG CCA CGC TTG CTG ACG-3'; IL-2R α antisense 5'-AAG CTT TCA ATA CTC CAT AGT GAG CAC AAA TGT CAC C-3'; IL-2R β sense: 5'-GTC GAC GCT CCT CTC AGC TGT GAT GGC TAC CAT A-3', IL-2R β antisense 5'-GGA TCCCAG AAG ACG TCT ACG GGC CTC AAA TTC CAA-3'; γ_c sense: 5'-GTC GAC AGA GCA AGC ACC ATG TTG AAA CTA-3', γ_c antisense 5'-GGA TCC TGG GAT CAC AAG ATT CTG TAG GTT-3'. β -actin sense 5'-GTG GGG CGC CCC AGG CAC CA-3', β -actin antisense 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3'. To exclude contaminations all experiments were run with a mock PCR. β -actin message expression was used to normalize the cDNA amount. As positive controls for IL-21 and its receptor cDNA from CD4⁺ T cells from C57BL/6 mice was isolated (Parrish-Novak 2000). For IL-15 and its receptor we used cDNA from L929 fibroblasts (Bulfone-Paus 1999) and CTLL-2 cells were the positive reference for all IL-2R chains (Tagaya 1996).

Cytokine detection

DCs (1x10⁶/ ml) in 24 well plates were stimulated for 24h with LPS (10ng/ml). Culture supernatants were collected and analyzed for mouse cytokines using a Bio-PlexTM kit (Bio-Rad, Munich, Germany). 50 μ l supernatant per sample was analyzed on the Luminex 100TM according to manufacture's instruction.

Analysis of endocytosis by flow cytometry

To quantify the endocytic activity the uptake of FITC-dextran (MW: 70.000, Molecular Probes, Göttingen, Germany) was monitored by FACS analysis as described by Stumbles *et al* (Stumbles 1998). In brief, 5×10^5 DCs were re-suspended in 100 μ l RPMI/FCS containing 0.5mg/ml FITC-dextran for 30min respectively 60 or 90min at 37°C or on ice. Cells were washed twice with ice-cold PBS/BSA and fluorescence intensity was determined by FACS.

DC activation by LPS

DCs were generated by culturing BM cells for 8 days with GM-CSF as described above. These DCs were cultured for another 24h in medium or with low dose LPS (10ng/ml) to induce DC activation and maturation. To analyze the effects of IL-15 and IL-21 on this *in vitro* maturation process, DCs were incubated with a combination of LPS and IL-15 or IL-21 (100ng/ml). Cytokine and LPS concentrations were chosen out of several titration experiments. As control, the anti-inflammatory cytokine IL-10 (100ng/ml) was used, which has been shown to inhibit DC maturation (Takayama 2001). After 24h, the surface phenotype of the DCs was analyzed by FACS.

DC activation by various stimuli

DCs were generated by culturing BM cells for 8 days as described. All DC types were cultured for another 24h with 10 μ g/ml OVA protein (Sigma), with 2 μ g/ml OVA³²³⁻³³⁹ peptide or with low dose LPS (10ng/ml) to induce DC activation and maturation. In addition all DCs were labeled with FITC (12,5 μ g/ml) as described in detail below. OVA-protein, -peptide and LPS concentrations were chosen out of several titration experiments. As control only medium was supplemented to the cultures. After 24h the surface phenotype of the different DCs was analyzed by FACS.

Proliferation assays

T cells were isolated from lymph nodes (LN) of OTI and OTII TCR transgenic mice. 1×10^5 DCs (IL-15^{+/+} and IL-15^{-/-}) were aliquoted into 96-well flat-bottom culture plates (Costar Corning, Cambridge, MA) and allowed to adhere for 2h. CD4⁺ cells were added at 2×10^5 cells per well. 0.1 μ M OVA⁽³²³⁻³³⁹⁾ peptide (optimal concentration defined by titration experiments) was supplemented to a final volume of 200 μ l. The cultures were incubated for 72h and labeled for additional 18h with 0.2 μ Ci/well [³H]thymidine.

Antigen specific CD8⁺ T cell proliferation assays were performed as follows: DCs were labeled with 2µg OVA⁽²⁵⁷⁻²⁶⁴⁾ peptide for 2h at 37°C in 500µl medium, washed twice and plated-out into 96-well round-bottom culture plates (Greiner, Hamburg, Germany) at a density of 1x10⁴ cells per well along with 1x10⁵ LN cells. After 48 hours, plates were pulsed for 18h with 0.2µCi/well [³H]thymidine. [³H]thymidine uptake was analyzed by liquid scintillation counting (Wallac/ PerkinElmer, Freiburg, Germany). The experiments were repeated three times with six replicates each.

***In vitro* differentiation of CD8⁺ T cells**

To analyze the CD8⁺ T cell stimulation *in vitro*, 2x10⁵ DCs from IL-15^{+/+} and IL-15^{-/-} mice were labeled with OVA⁽²⁵⁷⁻²⁶⁴⁾ peptide as described above and plated in 24 well plates together with 2x10⁶ spleen cells from OVA CD8⁺ T cell transgenic OTI mice in 1ml RPMI for 48h. Cells were harvested, and CD8⁺ T cells were analyzed by FACS for induction of memory cells or apoptosis using Annexin V/Pi kit (BenderMed Systems, Vienna, Austria).

Confocal Microscopy

IL15^{+/+}, IL15^{-/-}, and IL-15Rα^{-/-} DCs after 8 days culture were seeded in concentration of 5x10⁴ cells/well in 4-wells cover glass system (Lab-Tek II, Nalge Nunc Int., Naperville, USA) the day before staining. Next day cells were fixed with 2% paraformaldehyde for 10min at room temperature, permeabilized by 0.25% Triton X-100 for 10min and stained with 1:100 dilutions of antibodies recognizing IL-15Rα (H-109) (Santa Cruz Biotechnology, Santa Cruz, USA) in combination with anti-IL-15 antibodies (L-20) (Santa Cruz Biotechnology) for 30min at room temperature. Alexa Fluor-488 donkey anti-goat IgG (H+L) and Alexa Fluor-546 goat anti-rabbit IgG (H+L) (Molecular probes, Leiden, Netherlands) in dilution of 1:100 were used as second antibodies. Nuclei were stained using TOTO-3 dye (Molecular probes). To stain cell membranes, fixed cells were incubated with wheat germ agglutinin (WGA, Molecular Probes) for 15min at room temperature, washed and permeabilized with 0.25% Triton X-100 followed by staining with primary and secondary antibodies. Cells were analyzed by scanning confocal microscopy (Leica, Ruel-Malmason, France).

***In vivo* DC migration**

In order to examine whether the differently generated DCs migrate with equal efficiency, local draining lymph nodes (DLN) were examined. DCs were labeled with FITC as described below and 5x10⁵ labeled DCs were injected s.c. in the hind footpad. To assess that FITC was

not taken up by other cells, in additional experiments, FITC labeled DCs were fixed with 0.1% glutaraldehyde and injected s.c in the hind footpad (Eggert 1999). After 24h local DLN were removed and prepared as described (Vremec 1997). 1×10^6 cells were stained with α CD11c-PE Ab and 1×10^5 cells were analyzed by flow cytometry.

Sensitization to picryl chloride (PCI) and elicitation of CHS

IL-15^{+/+} or IL-15^{-/-} mice were sensitized as described (Rückert 2002) on the shaved abdominal skin by a single painting with 50 μ l of 1% (w/v) picryl chloride (2,4,6-trinitrochlorobenzene; Tokyo Kasei Co., Tokyo, Japan) in acetone/ethanol (1:3) on day 0. Negative controls were painted with diluents. For the elicitation of the CHS, 1% PCI solved in olive oil (20 μ l) was painted on both sides of one earlobe on day 5 after sensitization. Ear thickness was measured with a spring-loaded micrometer (Mitutoyo, Elk Grove Village, U.S.A.) before challenge and 24, 48 and 72h after challenge(**Fig. A**). For each mouse, the ear swelling response was expressed as the mean increment in thickness above the basal control value.

Where indicated, mice were injected i.p. with 50 μ g of soluble IL-15R α (Ruchatz 1998) as IL-15 antagonist in 200 μ l PBS, 30min before sensitization and once daily until day 5, when the mice were challenged for the first time. A second challenge was performed at day 19 and ear swelling was measured again 24, 48 and 72h after the second challenge.

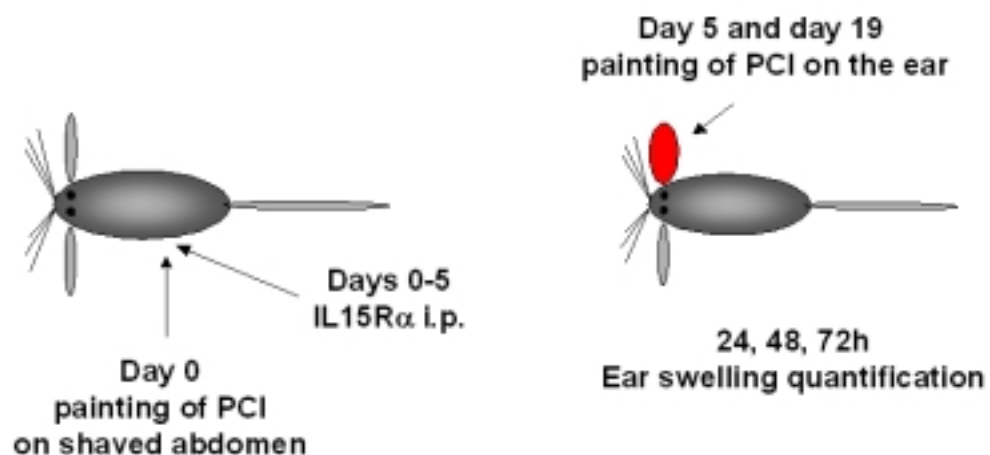


Figure A. CHS Model to picryl chloride (PCI).

Alternatively, 72h after the first challenge mice were sacrificed and cells from DLNs were isolated and stained for FACS as described above to estimate CD4⁺/ CD8⁺ ratios as well as the expression of activation and memory markers on T cells.

Contact hypersensitivity model to FITC

To estimate the capacity of DCs to induce antigen specific T cell sensitization *in vivo*, BMDCs (1×10^6 cells/ml) from IL-15^{+/+}, IL-15^{-/-}, or IL-15R α ^{-/-} mice were labeled with 12.5 μ g/ml of FITC (Sigma) or FITC-conjugated OVA (Harlow 1988) in PBS for 20min at 37°C as previously described (Macatonia 1986). Alternatively, IL15^{-/-}-DCs or IL-15R α ^{-/-}-DCs were incubated for 30 min with 20ng/ml IL-15 at 37°C after FITC labeling. Short-time effects of IL-15 and IL-21 were analyzed by incubation IL-15^{+/+}-DCs for 2h with 100ng/ml of the cytokines before FITC labeling. Cells were washed 3 times with ice-cold RPMI/FCS medium, resuspended and injected in one footpad with a Hamilton syringe (5×10^5 cells in 50 μ l PBS). After 5 days, mice were challenged by applying 50 μ l FITC (3.5mg/ml in acetone:dibutylphthalate, 1:1, Sigma) on the dorsal and ventral sides of the right ear. As control the left ear was painted with an equal amount of the diluent. In addition, unsensitized mice were painted with FITC. CHS response was determined by measuring the ear swelling at 24, 48 and 72h after challenge as described above.

Irritant dermatitis to croton oil

To analyze the capacity of IL-15^{+/+} and IL-15^{-/-} mice to mount an unspecific, antigen-independent, irritant contact dermatitis (Zhang 2000), croton oil (Sigma) at 0.8% (v/v) dissolved in acetone was painted on one ear. Painting of acetone alone served as negative control. Ear swelling was analyzed at 24, 48, and 72h.

Th1 model of DTH reaction to SRBC

As previously described (Kunzendorf 1996), mice were sensitized by i.v. injection of 4×10^5 sheep red blood cells (SRBC) in 100 μ l PBS and challenged at day 5 by injecting 2×10^8 SRBC in 50 μ l PBS into the left hind footpad. Non-immunized mice were injected with the same dose of SRBC to determine non-specific swelling. Where indicated, mice were injected i.p. with 50 μ g of sIL15R α in diluent 30min before sensitization, and once daily until day 5, i.e. when mice were challenged for the first time (**Fig. B**). 24, 48 and 72h after challenge the footpad swelling was measured as above described. A second challenge was performed at day 19. Control groups were injected with diluent.

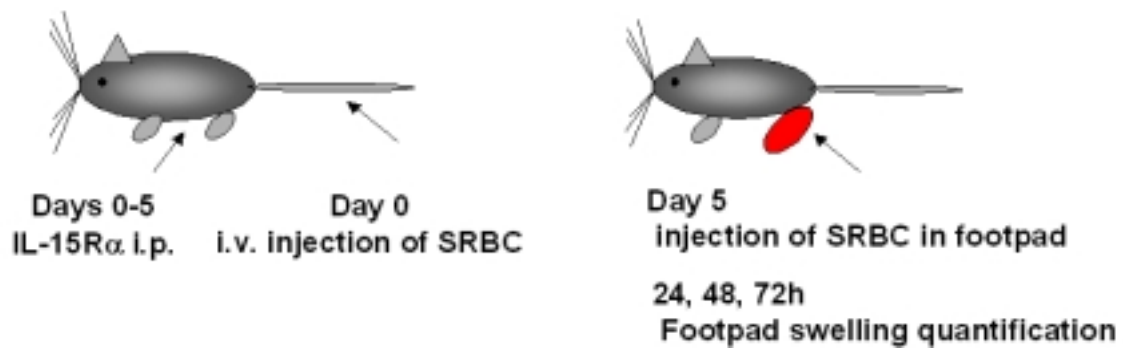


Figure B. DTH model to sheep red blood cells (SRBC).

Eight days after primary immunization, serum was collected, and hemagglutinin titers to SRBC were measured as described (Kunzendorf 1996). In brief, the serum was incubated at 56°C for 30min and diluted using NaCl 150mM with or without 0.78% mercaptoethanol in serial log 2 dilutions. 200 μ l of a 0.5% SRBC solution was added to 200 μ l of the serum dilutions, incubated at 37°C for 30min, at room temperature for 2h and at 4°C for additional 12h. The serum hemagglutinin titers are the highest log 2 dilution steps in which hemagglutination could still be determined.

Homing and activation of APC

Mice were painted on the shaved abdomen with 400 μ l of 0.35% FITC solution (Sigma) in acetone: dibutylphthalate (1:1) as previously described (Macatonia 1986). 24h later, the mice were sacrificed and their axillary, cervical, and inguinal DLN removed, pooled and 100.000 cells were analyzed by FACS for FITC uptake. Dendritic cells (DCs) were stained with APC labeled anti-CD11c and PE labeled anti-CD80 and -CD86 antibodies.

Statistical analysis

Results are presented as mean \pm SD from pooled data of 2-3 identical experiments, (using a total of 10-15 mice/group) all of which gave comparable results. FACS data from one representative experiment of at least five are shown. The Student's t-test for unpaired samples was used for the determination of statistical differences (* $p \leq 0.05$; ** $p \leq 0.01$).

Results

CD11c⁺ DCs differentiate in the presence of IL-15 or IL-21 *in vitro*

IL-21 and IL-15 are structural relatives and belong, like IL-4, to the 4 α -helix bundle cytokine family. All three cytokines share the common γ -chain in their receptor complexes but have their own, private receptor unit (IL-21R, IL-15R α , IL-4R α). Recent studies revealed that IL-15 is critical for APC activation (Mattei 2001) and essential in the development of lymphoid cell lineages (Kennedy 2000). IL-21 was found to act synergistic to IL-15 in the generation and activation of lymphoid cells (Parrish-Novak 2000). On the other hand it is well accepted that the expansion of *in vitro* generated bone marrow (BM)-derived DCs is highly promoted by IL-4, (Sallusto 1994). Taken together it is evident that all these cytokines have a specific influence on stem-cell differentiation and their further maturation.

As IL-4, IL-15 and IL-21 receptors share the common γ -chain, we analyzed whether IL-15 and IL-21, just like IL-4, may modulate the GM-CSF-induced differentiation of BM stem cells into DCs. Therefore, freshly isolated BM cells were cultured following established protocols (Inaba 1992; Lutz 1999) with GM-CSF alone (subsequently designated DCs) or GM-CSF combined with 20ng/ml IL-15 (named IL-15DCs) or 20ng/ml IL-21 (IL-21DCs). In a typical experiment, after 8 days of culture 60-80% of the cells appear as loosely adherent clumps or isolated floating cells with the typical dendritic cell morphology. DCs were transferred into microchamber slides at day 8 and allowed to adhere for 48h. After Pappenheim staining the differentially generated DCs displayed a characteristic DC phenotype such as cytoplasmic protrusions (“dendrites”) (**Fig. 1**). In FACS analysis after 8 days of culture, the IL-15DCs and IL-21DCs showed comparable size and granularity like DCs indicated by same forward-(FSC), sideward-scatter (SSC) properties (**Fig. 1**). Further, the percentage of CD11c⁺ cells, a specific marker for murine DCs, was similar in all conditions (**Fig. 1**). Moreover we assessed by FACS, that adding IL-15 or IL-21 to BM cells in combination with GM-CSF did not lead to increased differentiation of other cell types like B cells (CD45/B220⁺), NK cells (NK1.1⁺), T cells (CD3⁺), granulocytes (Gr1⁺), macrophages (F4/80⁺) or CD8 α ⁺ lymphoid DCs (not shown). Thus, IL-15 and IL-21 neither did alter the purity of the generated DCs nor the total yield of DCs compared to GM-CSF alone (mean of 7.5x10⁶ cells per petridish out of 2x10⁶ seeded for all conditions). Given IL-15 and IL-21 alone (without GM-CSF) to BM cells did not induce differentiation of DCs (not shown).

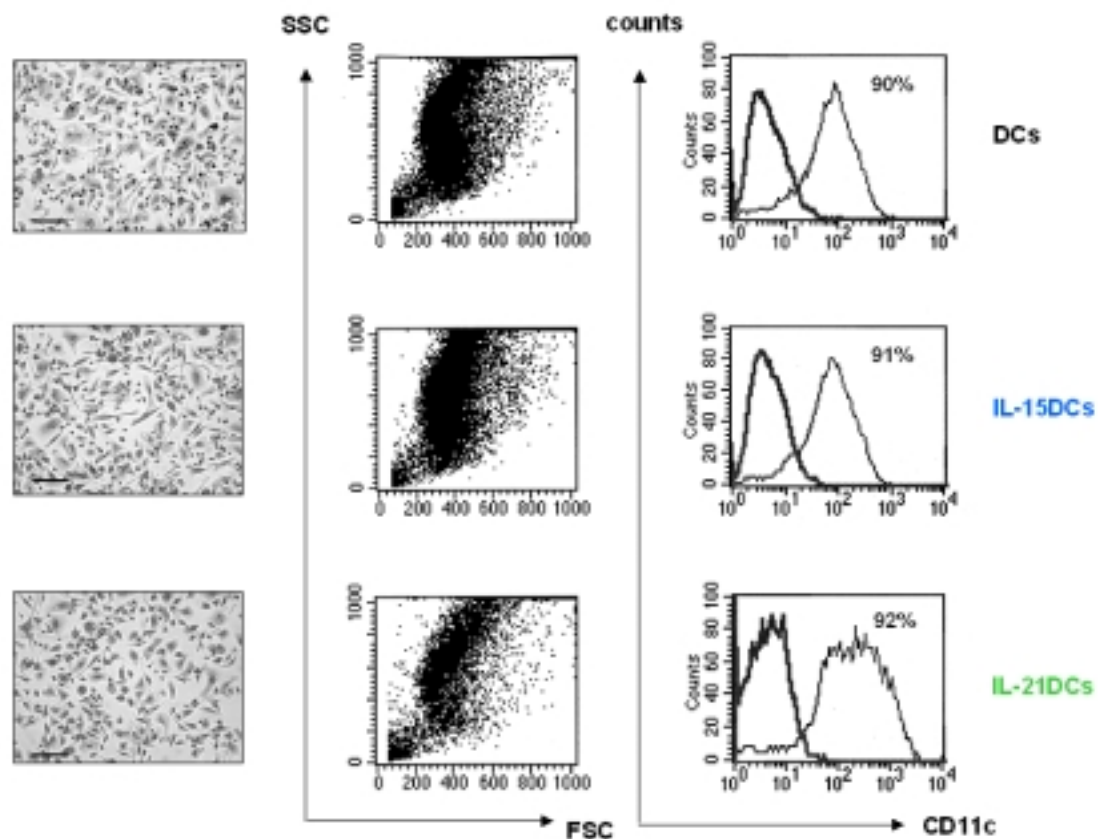


Figure 1. Generation of DCs. Murine BM cells were induced to differentiate to DCs by GMCSF (DCs), or by addition of IL-15 (IL-15DCs), or IL-21 (IL-21DCs) in combination with GMCSF during the entire culture period of 8 days. Morphology was assessed by culturing the DCs for 48h on micro chamber slides following Pappenheim staining. The scale bar is equal to 100µm. Further; cells were analyzed by FACS at day 8. All cells showed no differences in their size and granularity (2nd column, forward- and sideward-scatter, FSC, SSC). Additionally, cells were stained for CD11c expression. The 3rd column shows that about 90% of all cells expressed CD11c and therefore are considered to be DCs. Unstained cells served as negative controls (bold lines). Shown is one representative out of 10 experiments.

IL-21 and IL-15 receptors are expressed in BM derived DCs

The IL-15 receptor complex, which is composed of a private α -subunit, the IL-2/IL-15R β - and the common γ -chain, is expressed on DCs (Mattei 2001, Fukao 2000). Using northern blot analysis, expression of the private IL-21R subunit was previously found to be most

abundant in lymphoid tissues (Parrish-Novak 2000). In addition, both private receptor chains for IL-15 and IL-21 are expressed in BM stromal cells.

In order to elucidate whether myeloid DCs express the private receptor subunits and the common γ -chain at mRNA level and are therefore susceptible for this cytokines, we performed RT-PCR analysis. We found both private receptor chains, IL-15R α and IL-21R, expressed after 8 days of culture in BM derived myeloid DCs (**Fig. 2a,b lane 2**). Adding IL-15 or IL-21 to the culture did not change the expression levels of the IL-21R subunit (**Fig. 2a lane 3,4**). Analyzing the expression of IL-15R α mRNA in the differently generated DC types revealed that all conditions showed IL-15R α transcripts (**Fig. 2b lane 2-4**). Freshly isolated BM cells *ex vivo* expressed low levels of IL-15R α (**Fig. 2b lane 1**).

We next determined whether the endogenous expression of IL-15 and IL-21 was modulated by the different culture conditions since IL-15 mRNA up-regulation was identified during an increase of DC activation (Mattei 2001). Transcripts for IL-15 were expressed in all three differentiated DC types at comparable levels, however IL-21 expression was only very weak (**Fig. 2c,d lane 2-4**). In freshly isolated BM cells neither IL-15 nor IL-21 expression was detectable (**Fig. 2c,d lane 1**).

Besides its high affinity receptor, IL-15R α , IL-15 is also able to signal through the low affinity IL-2R β -chain (Grabstein 1994). For both cytokines, IL-15 and IL-21, the common γ -chain is an indispensable subunit (Giri 1994, Asao 2001). To see whether IL-15 or IL-21 have modulatory capacity on the expression of other subunits of the receptor complexes we also examined the expression of the β - and γ -chains and in addition the IL-2R α -chain (CD25). IL-2R α mRNA was only low expressed in IL-21DCs compared to DCs or IL-15DCs (**Fig. 2e lane 2-4**). Interestingly, the mRNA expression of the β -subunit was restricted to IL-15DCs (**Fig. 2f lane 3**) whereas in DCs or IL-21DCs no expression was evident. All DC types had comparable mRNA levels for the common γ -chain (**Fig. 2g**). Shown are representative data out of 5 experiments. Positive controls exhibit the expected results.

Taken together, BM derived myeloid DCs expressed the private receptor chains IL-15R α and IL-21R plus the common γ -chain and therefore may respond to IL-21 and IL-15, where the latter was expressed by DCs itself.

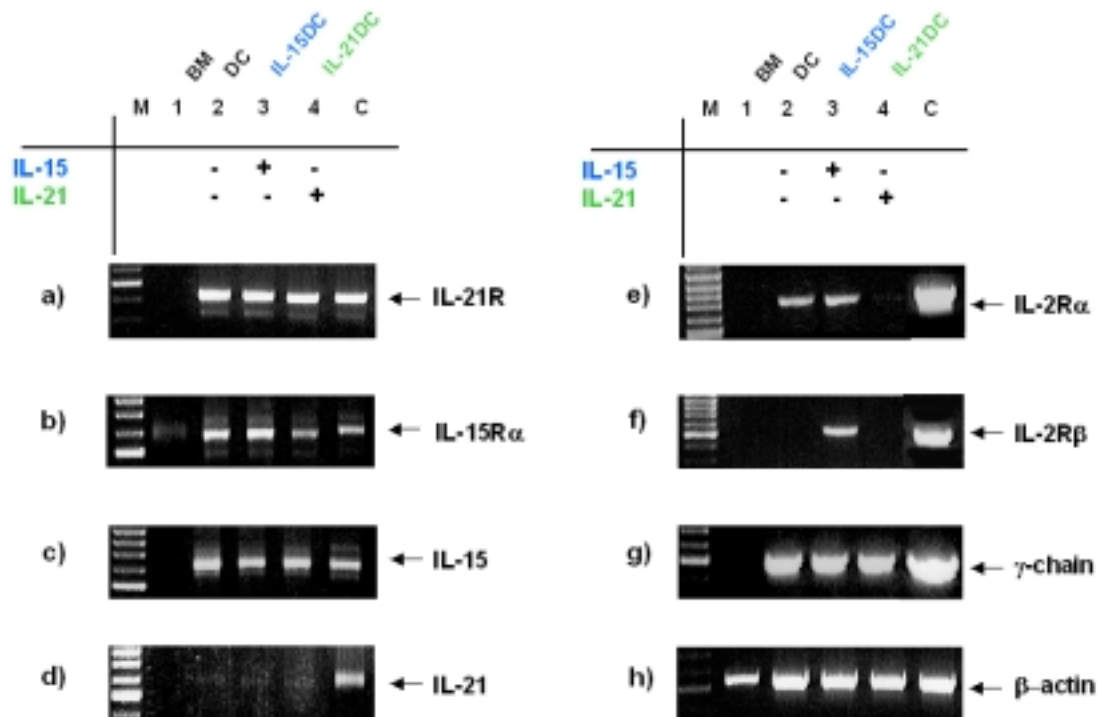


Figure 2. mRNA expression of IL-15 and IL-21 and their receptors. Freshly isolated bone marrow (BM, lane 1) as well as DCs (lane 2), IL-15DCs (lane 3) and IL-21DCs (lane 4) were analyzed by RT-PCR at day 8 for mRNA expression of IL-21R, IL-15R α , IL-15, IL-21, and IL-2R α - and β -chain as well as for the common γ -chain. β -actin message expression was used to normalize the cDNA amount. To exclude contaminations all experiments were run with a mock PCR and found negative (not shown). The correct size of PCR product was assessed by a marker (M). As positive controls (C) for IL-21 and its receptor cDNA from activated CD4⁺ T cells from C57BL/6 mice was isolated. For IL-15 and its receptor we used cDNA from L929 fibroblasts and CTLL-2 cells were the positive reference for all IL-2R chains. Shown is one representative experiment out of 5.

IL-21 reduced MHC class II expression

Antigen presentation to and co-stimulation of T cells by DCs in innate and acquired immunity are mediated by MHC- and a variety of co-stimulatory molecules on the surface of DCs. To assess the expression-repertoire of surface molecules we did surface phenotyping by FACS analysis after 8 days of culture. All three differently generated DCs showed comparable high

levels of CD80 whereas CD86 expression was reduced in IL-15DCs as well as in IL-21DCs (**Fig. 3a**). Moreover, generation in the presence of IL-21 resulted in a significantly decreased expression of MHC class II molecules (**Fig. 3a**) when compared with DCs or IL-15DCs.

a

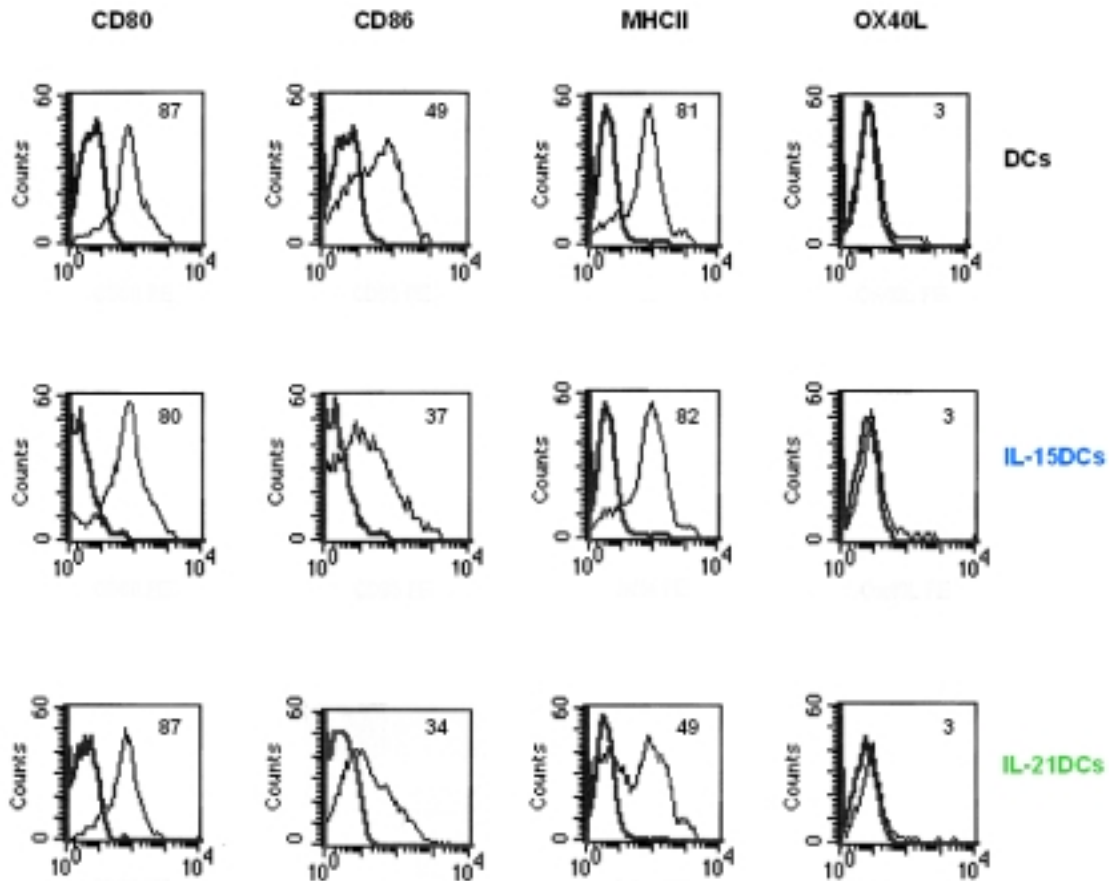
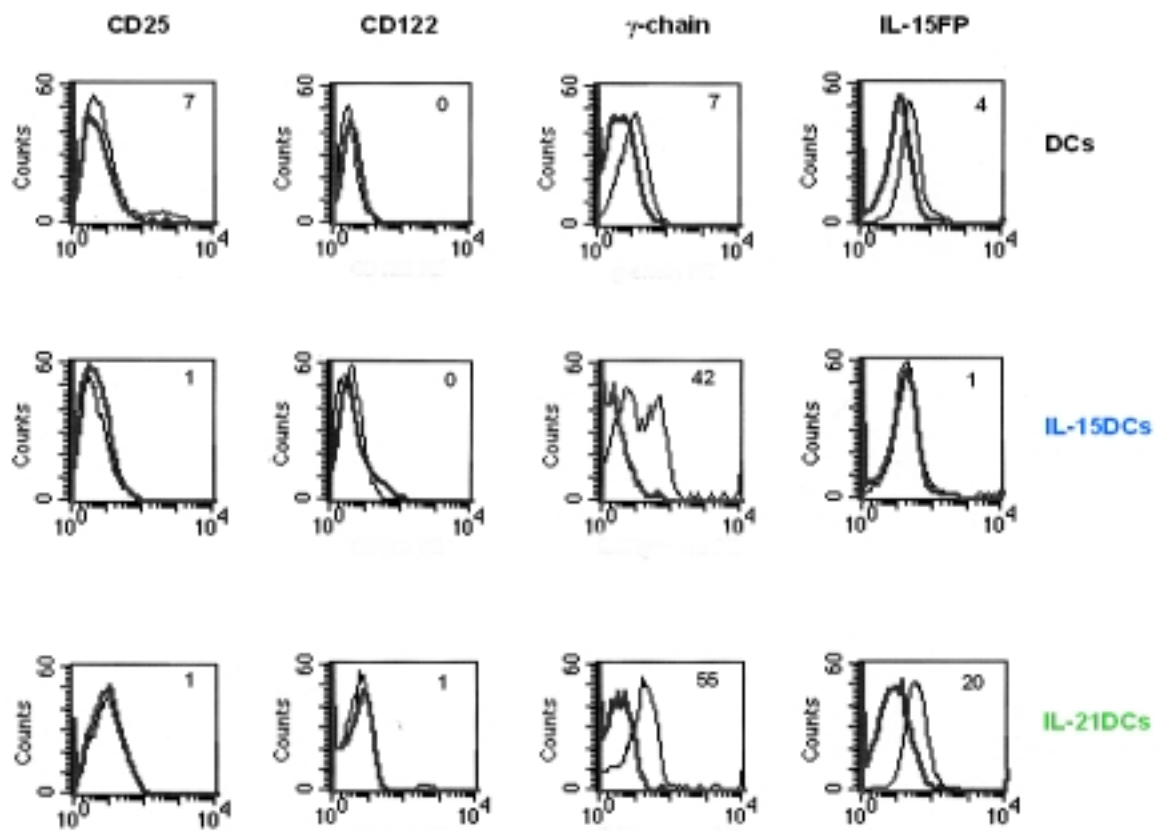


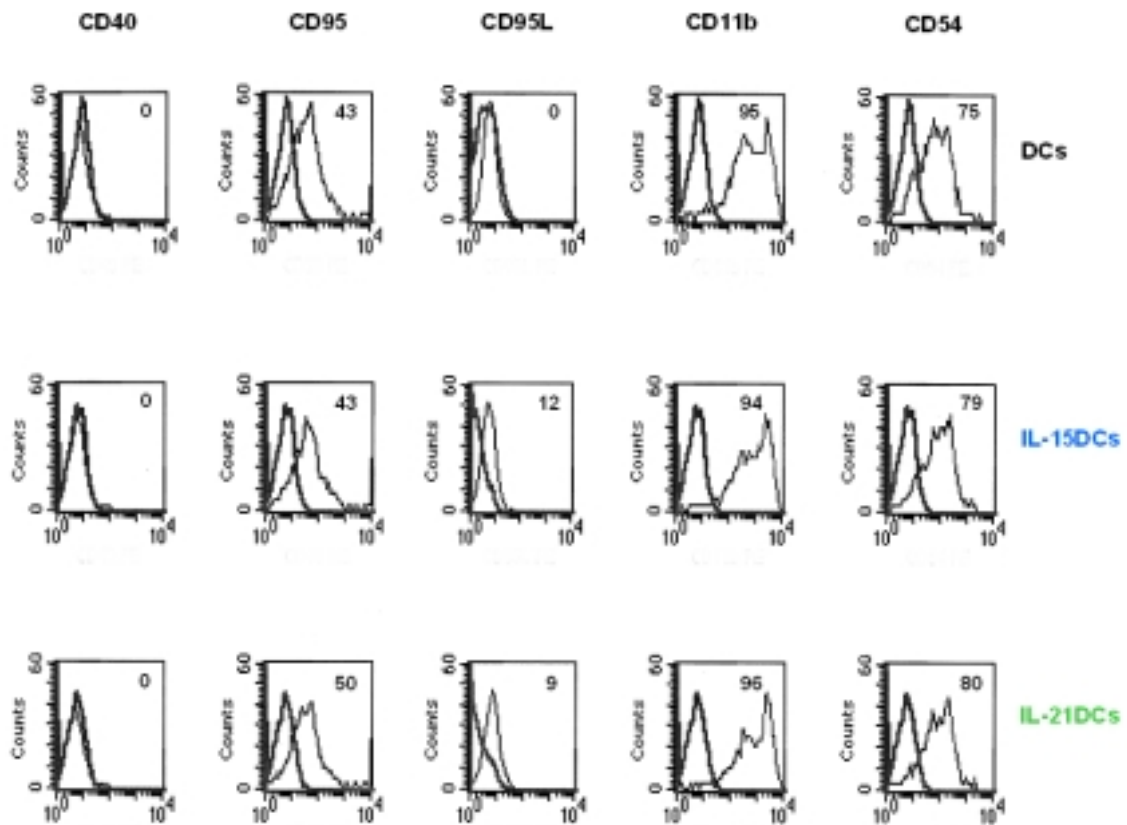
Figure 3. Surface phenotyping by FACS. DCs, IL-15DCs, and IL-21DCs were analyzed by multicolor flow cytometry (gated on CD11c positive cells). Shown are representative data sets out of 10 experiments for **a**) co-stimulatory molecule), **b**) IL-2/IL-15 receptor chains and **c**) activation/adhesion molecules. Given in each histogram is the percentage of positive cells. Unstained cells were used as negative controls (shown in bold lines).

Both, IL-15DCs and IL-21DCs, did not differ in the weak expression of the co-stimulatory molecule OX40L in comparison to DCs. IL-15DCs, respectively IL-21DCs, displayed no CD25 (IL-2R α) and CD122 (IL-2R β) on the cell surface (**Fig. 3b**). In contrast, the expression of the common γ -chain in comparison to DCs was increased in IL-15DCs and in IL-21DCs. In addition, the expression of IL-15 binding sites (as analyzed with an IL-15 fusion protein, Bulfone-Paus 1999) was down modulated in IL-15DCs whereas in IL-21DCs a strong increase was evident.

b



c



All DC types showed similar high expression of the adhesion molecules CD54 and CD11b and of CD95 (Fas) and had no CD40 on their surface (**Fig. 3c**). CD95L (FasL) was only expressed moderately in IL-15DCs and IL-21DCs (**Fig. 3c**).

Although the quantity of DCs given in cell number and morphology was not altered by adding IL-21 or IL-15 during generation, the phenotype of the DCs shown by expression of functional relevant antigen-presenting and co-stimulatory molecules was modulated.

Increased antigen uptake by IL-21DCs contrasted by low uptake by IL-15DCs

The phenotypic changes of DCs generated in the presence of IL-21 described above, particularly the significantly reduced expression of the MHCII molecule, point to different maturational stages. Immature DCs are characterized by low expression of accessory molecules especially MHCII and their increased antigen-capture activity.

To assess whether low expression of MHCII on IL-21DCs also refers to altered Ag-uptake we studied the endocytic activity using a fluorescent model-antigen. FITC dextran uptake was monitored after 8 days of DC culture. After 30min of incubation IL-21DCs showed a significantly increased FITC dextran uptake at 37°C (mean fluorescence: 495) (**Fig. 4**).

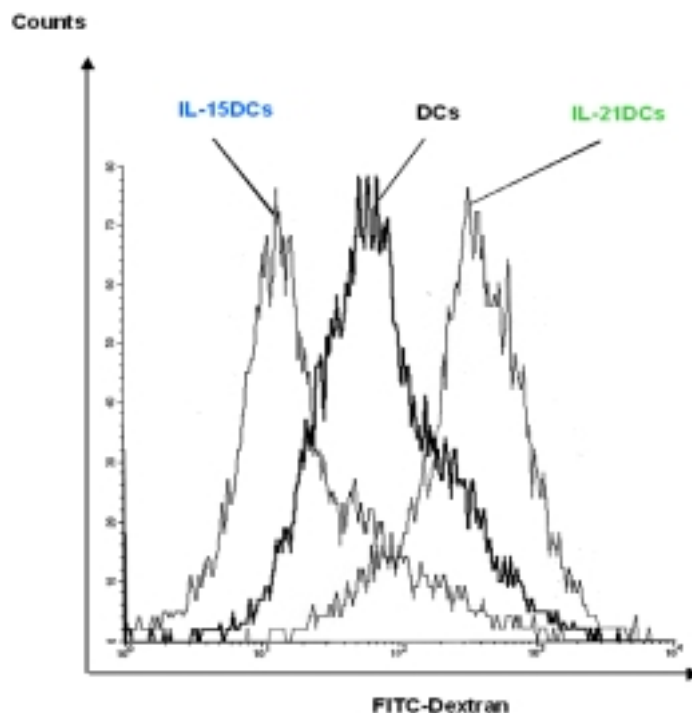


Figure 4. Antigen uptake (endocytosis) of FITC dextran at 37°C.

Cells were incubated 30min with FITC labeled dextran, washed and analyzed for FITC dextran incorporation by FACS. Shown is one representative out of 3 experiments.

Prolonged incubation for additional 30 and 60min did not lead to increased uptake of FITC dextran (not shown). In contrast to IL-21DCs, FITC dextran uptake was significantly reduced in IL-15DCs (mean fluorescence: 59) compared to normal DCs (mean fluorescence: 156) (**Fig. 4**). To estimate passive FITC diffusion, all experiments were done on ice, showing very low uptake (not shown). To exclude surface binding, internalization was further confirmed by examination of the FITC dextran uptake by fluorescence microscopy (not shown). Thus, the presence of IL-21 *in vitro* leads to the differentiation of less mature DCs, which are characterized by high unspecific antigen uptake, whereas IL-15 induces DCs with a more mature phenotype, accompanied by lower unspecific antigen uptake.

IL-21DCs keep their immature phenotype after antigen uptake and LPS stimulation

The high FITC dextran uptake, which we have shown above, indicates that IL-21DCs have besides an immature phenotype also functional alterations. To study this more in detail we investigated the activation of all generated DCs by different stimuli. It is well known that antigen uptake (They 2001) as well as bacterial products like LPS (Resigno 1999) are strong stimuli for APCs *in vivo* and *in vitro*: They induce DC activation and maturation, accompanied by up-regulation of MHC and co-stimulatory molecules.

To examine whether IL-21DCs are blocked in activation by keeping their immature phenotype even after strong stimulation by LPS or after antigen uptake/ processing, we incubated all DC types with the small model antigen (hapten) FITC (**Fig. 5a**), complete OVA protein (**Fig. 5b**), OVA peptide (**Fig. 5c**) and with low dose LPS (**Fig. 5d**) for 24h. FACS analysis of costimulatory surface markers like CD80 and MHCII revealed that IL-21DCs showed a significantly reduced expression of these representative molecules in all conditions (**Fig. 5a-d**). Given are the percent of DCs with high expression (fluorescence channel $>10^2$) of the indicated molecules, correlating with their surface density /number. This is different from our previous (**Fig. 3**) settings where we included also low and intermediate expression. In contrast, DCs and IL-15DCs clearly showed an up-regulation of all these molecules indicating that IL-21DCs were blocked in their expression and hence less mature.

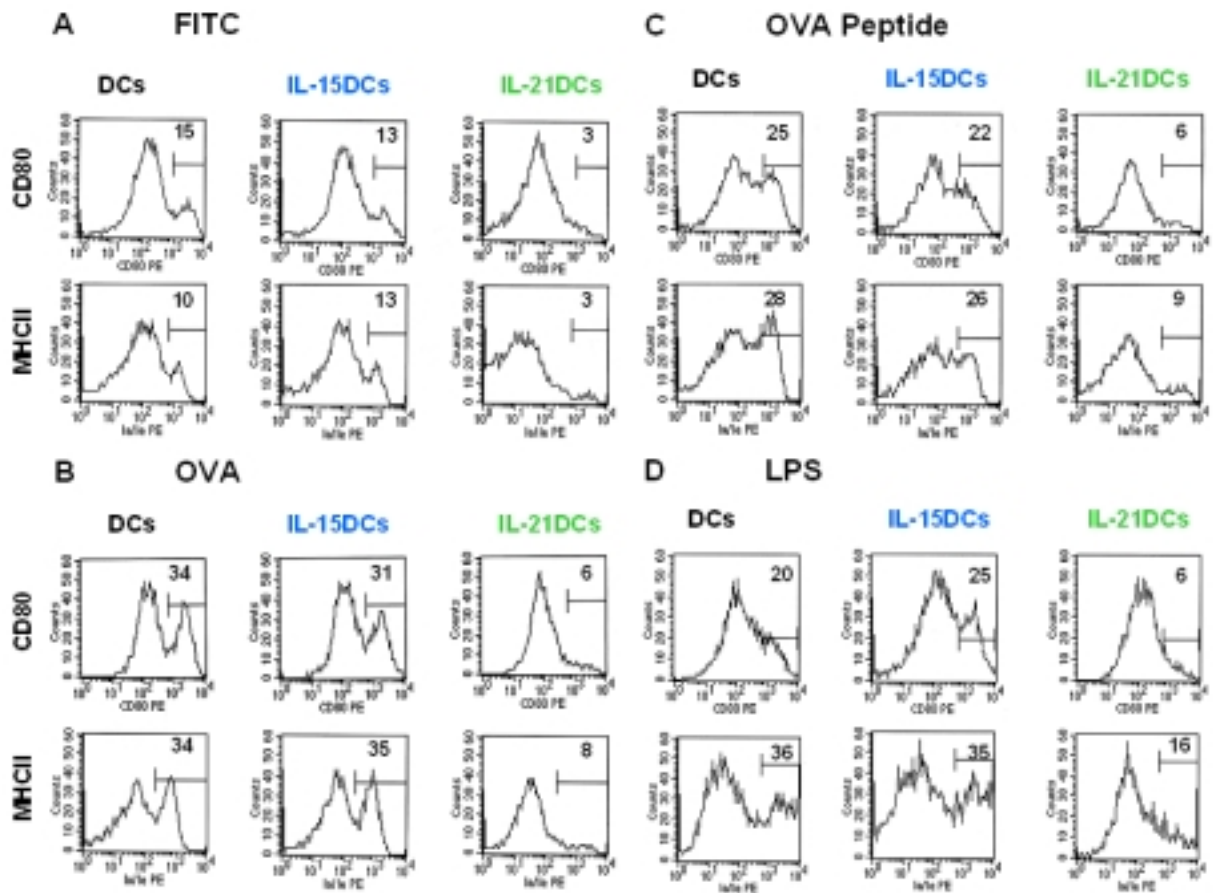


Figure 5. Blocked maturation by various stimuli in IL-21DCs. All three generated DC types were stimulated with a) FITC, b) OVA protein, c) OVA peptide and d) LPS for additional 24h after 8 days of culture. Surface expression of co-stimulatory molecules CD80 and MHCII (gated on CD11c⁺ cells) were analyzed by FACS. Given is the percentage of high positive cells, defined by the marker. Shown is one representative out of two experiments.

IL-21DCs induce low antigen specific CD4⁺ T cell proliferation

The capacity to induce specific T cell activation and proliferation is a functional *in vitro* (and *in vivo*) hallmark of mature DCs whereas immature DCs fail to prime T cell responses. To further characterize the immature stage of IL-21DCs and to assess the functional consequences out of their impaired MHCII expression we set up an antigen specific T cell proliferation assay.

Therefore, the different DCs were pulsed after 8 days of culture with OVA⁽³²³⁻³³⁹⁾ peptide and co-cultured for 72h with freshly isolated T cells from lymph nodes of syngenic OTII^{tg} mice, which have a transgenic T cell receptor for OVA⁽³²³⁻³³⁹⁾ peptide. We compared the T cell stimulatory capacity of DCs that had been cultured with GMCSF alone or in combination with IL-15 or IL-21. As shown in **Fig. 6** IL-15DCs showed a high significantly increased ability to prime T cell proliferation (blue bars), compared to DCs (white bars), with a maximum at OVA⁽³²³⁻³³⁹⁾ peptide concentration of 0.3μM. In contrast IL-21DCs (green bars) induced significantly lower T cell proliferation rates.

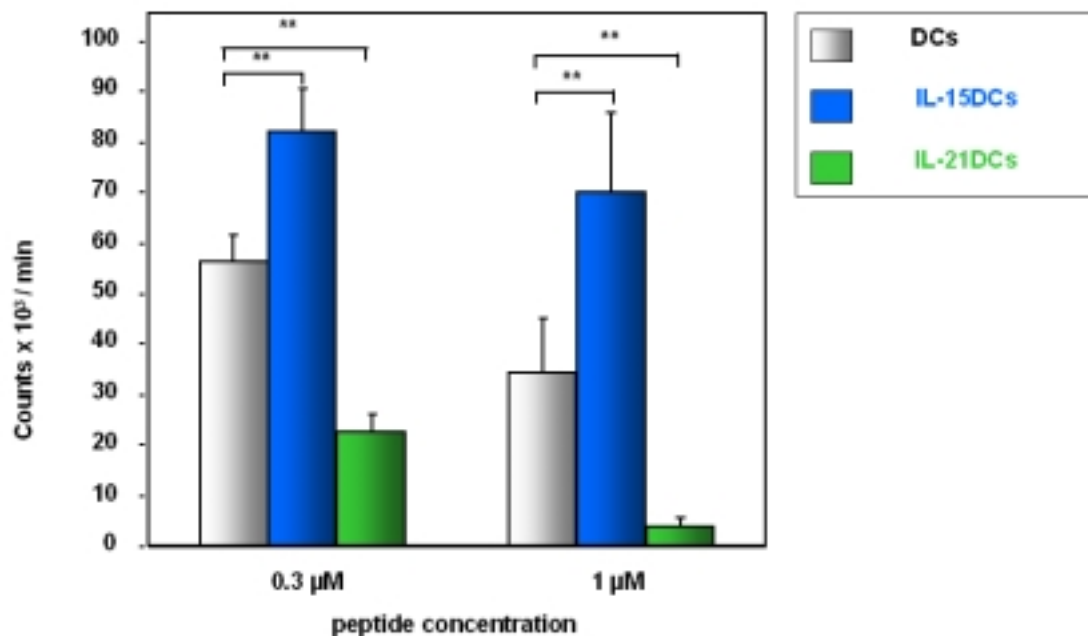


Figure 6. Co-stimulation by the different DCs. 100.000 DCs, IL-15DCs and IL-21DCs were labeled with different OVA⁽³²³⁻³³⁹⁾ peptide concentrations, washed, and incubated with 200.000 lymph-node cells from OT II mice with a transgenic CD4⁺ restricted T cell receptor, which recognizes the OVA⁽³²³⁻³³⁹⁾ peptide presented by MHCII. After 72h incubation cells were labeled with 0.2μCi [³H]thymidine and analyzed 12h later. Shown is one representative experiment out of 3. Significance compared to DCs was calculated using student's t-test (** p≤0.01).

This reduced ability of IL-21DCs to prime specific T cells could refer to the above described reduced MHCII expression which could not be raised (even not after incubation with strong

stimuli like LPS) supporting the deduction that DCs generated in the presence of IL-21 are less mature and - most important – do not acquire a mature phenotype after Ag uptake.

IL-21DCs are unable to prime *in vivo* contact hypersensitivity reaction to FITC

Small molecules, which act as antigens after protein binding are designated as haptens, and induce a contact hypersensitivity (CHS) in the skin. Following primary application to skin, epidermal DCs take up hapten-protein complex, process it and migrate towards the regional lymph nodes, to prime an antigen-specific T cell response there. During this process, DCs convert from an immature resting into an activated functional (mature) state (reviewed by Grabbe 1998).

In comparison to our *in vitro* data, we wanted to examine whether the three types of DCs had modulated capabilities to prime T cells *in vivo* and especially whether IL-21DCs were again prevented from undergoing maturation and therefore less immunogenic. For this purpose we labeled the different DCs *in vitro* with the fluorescent hapten FITC and injected them subcutaneous in the footpad of syngenic C57BL/6 wildtype mice (sensitization phase of CHS). After 5 days mice were challenged (elicitation phase of CHS) at the dorsal and ventral site of one ear with FITC (the other ear with diluent only) and the ability to initiate an antigen-specific, T cell mediated immune response was examined by measuring the FITC-specific ear swelling 24, 48 and 72h after challenge. Mice, which had not been sensitized, but challenged on the ear, served as negative controls for unspecific ear swelling. Mice that had been actively sensitized with FITC labeled IL-15DCs (blue) showed a high significant increase in the CHS ear swelling response 24h after challenge compared to mice, which had been injected with DCs (**Fig. 7a**). In contrast injection of IL-21DCs (green) resulted in a high significantly reduced CHS response at all three time points, i.e. no swelling above unsensitized controls was observed (**Fig. 7a**).

To confirm that the sensitization was based on active migration of the injected, viable DCs from the footpad to the DLN, additional control mice were injected with FITC labeled, glutaraldehyde fixed (dead) DCs (Eggert 1999). These fixed cells failed to induce any CHS response (not shown).

Two weeks after the first challenge, a second challenge was performed by painting FITC on the other ear and analyzing ear swelling as before (**Fig. 7b**). The mice, which had been sensitized by DCs and IL-15DCs showed in repetition a strong ear swelling, even more pronounced than after the first challenge. However, mice sensitized with IL-21DCs again did not mount a significant ear swelling compared to negative controls.

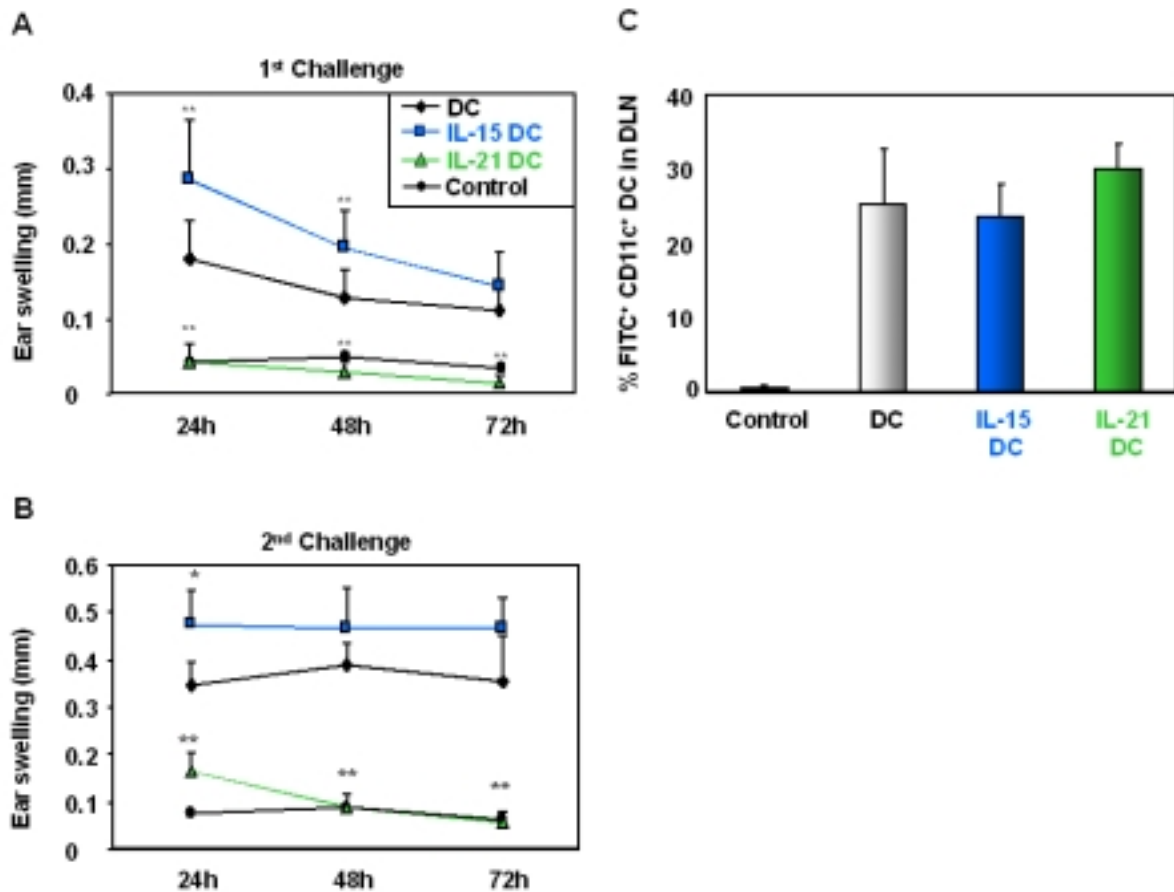


Figure 7. In vivo priming of T cell mediated immune reaction. DCs, IL-15DCs and IL-21DCs were labeled in vitro with FITC, washed and injected into the hind footpad of syngenic C57BL/6 mice (5×10^5 cells/mouse). **a:** 1st challenge: 5 days later, mice were challenged with FITC painting on the earlobe and after 24, 48, and 72h the ear swelling reaction was analyzed. Painting of un-sensitized mice served as control. **b:** 2nd challenge: 14 days after the first challenge, the mice were painted again on the other ear, and swelling was analyzed as before. **c:** Migration of DCs was analyzed by FACS of the draining lymph nodes, 24h after injection of 5×10^5 FITC labeled DCs. Cells were double stained for CD11c. Shown are the CD11c/FITC⁺ DCs as percent of total CD11c⁺ cells in the DLN. Controls were injected with unlabelled DCs. The experiments were repeated at least twice, shown is the median \pm SD of

12 mice. Significant differences between IL-15DCs and IL-21DCs compared to DCs were calculated using the student's t-test (* $p \leq 0.05$; ** $p \leq 0.01$).

This clarifies that the mice sensitized with IL-21DCs were not able to establish antigen-specific or memory T cells after the first FITC challenge in contrast to the other two groups, which even showed a stronger recall induced inflammation with higher ear swelling after the second challenge.

These findings are completely in line with our previous *in vitro* data stressing the fact that combining GM-CSF and IL-15 generates mature, "high immunogenic" DCs while IL-21 induces "functional immature" DCs, which are unable to mature to fully effective APCs after antigen uptake neither *in vitro* nor *in vivo*.

Comparable migration of all DC types to DLN

To rule out that the differences in CHS responses are due to altered migratory capacities of the DCs, we investigated their abilities to enter draining lymph nodes (DLN) following transfer *in vivo* (Lappin 1999).

For this purpose we labeled 0.5×10^6 DCs with the fluorescent cell tracker FITC in PBS for 20min at 37°C and after washing subsequently injected the cells in the footpad. After 24h cell suspensions from the inguinal DLNs were prepared as described (Vremec 1997), stained with α CD11c antibody and analyzed by flow cytometry. Double positive cells (FITC⁺/CD11c⁺), which migrated into the DLN, are given in percent of total CD11c⁺ DCs (**Fig. 7c**). As control, unlabeled DCs were injected. The differently generated DCs showed similar migratory capacities; 25-30% of the total lymph node CD11c⁺ DCs migrated within 24h from the periphery into DLNs. This exhibits that the reduction of CHS responses by IL-21DCs *in vivo* is not attributed to limited migration. In contrast, footpad-injected glutaraldehyde-fixed (dead) FITC labeled DCs did not reach the DLN and no FITC uptake by other DCs of recipient mice was observed, indicating that the observed migration into DLN is an active and specific process (not shown).

IL-21 inhibits LPS induced activation of normal DCs

Now that we found, that IL-21, when present during DC differentiation, prevent their maturation, we subsequently wanted elucidate whether IL-21 could act also in short time course on DC activation.

Therefore we again activated DCs, which differentiated for 8 days with GMCSF only, for additional 24h with low dose LPS (10ng/ml) alone or in combination with 100ng/ml of IL-15 or IL-21. As a control we used the anti-inflammatory cytokine IL-10 that is known to inhibit DC activation (Takayama 2001) by inhibiting up-regulation of co-stimulatory molecules and blocking IL-12 expression (Corinti 2001). It was evident that the addition of IL-21 showed similar properties like IL-10 in blocking the LPS induced up-regulation of CD80, CD86 and I_A/I_E (MHCII) (**Fig. 8a**). This observation strongly supports the evidence that IL-21 prevents also *in vitro* induced activation by LPS. Supplement of IL-15 to LPS did not further enhance the effects on co-stimulatory molecules mediated by LPS alone. CD40 expression was induced by LPS and not altered by the influence of any cytokine.

a

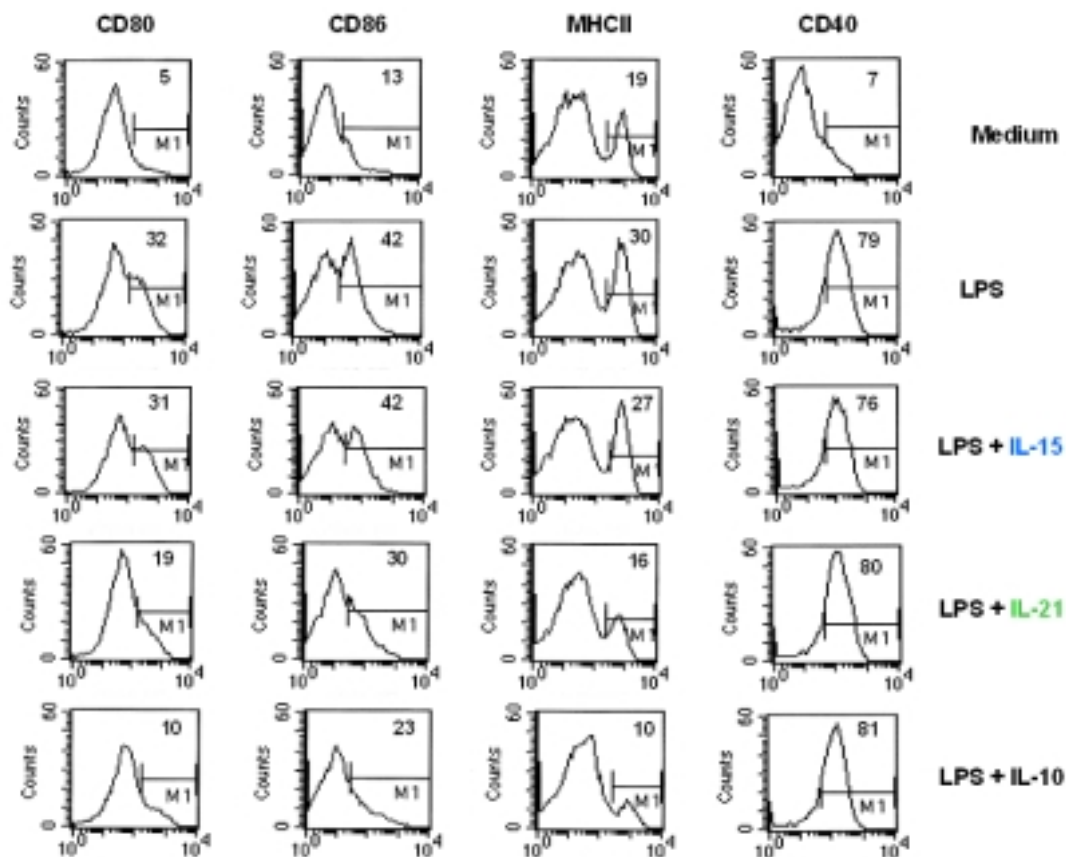


Figure 8a. Inhibition of LPS-induced DC activation.

Immature DCs were activated with low-dose LPS (10ng/ml) or with LPS plus IL-15, IL-21 or IL-10 for 24h. Surface expression of co-stimulatory molecules (Fig.8a) or IL-2 / IL-15 receptors (Fig. 8b) were analyzed by FACS. Given is the percentage of high positive cells, defined by the marker M1. Shown is one representative out of four experiments.

We further analyzed the modulation of the IL-2R subunits and the IL-15R α by LPS (**Fig. 8b**). To provide an activation marker for internal comparison, CD25 expression was studied. LPS induced the expression of CD25 nearly twofold. Addition of IL-15 to LPS led to no further induction of CD25. Supplemented IL-21 or IL-10 to LPS reduced CD25 expression to unstimulated medium levels. The IL-2R β -chain remained unaffected in all conditions, whereas the γ -chain exhibited an increased expression especially in the presence of IL-21 and IL-10. As expected (Kumaki 1996) IL-15R binding capacity was down-regulated with IL-15 below un-stimulated medium values but surprisingly up-regulated on the cell surface when IL-21 or IL-10 was added to LPS.

b

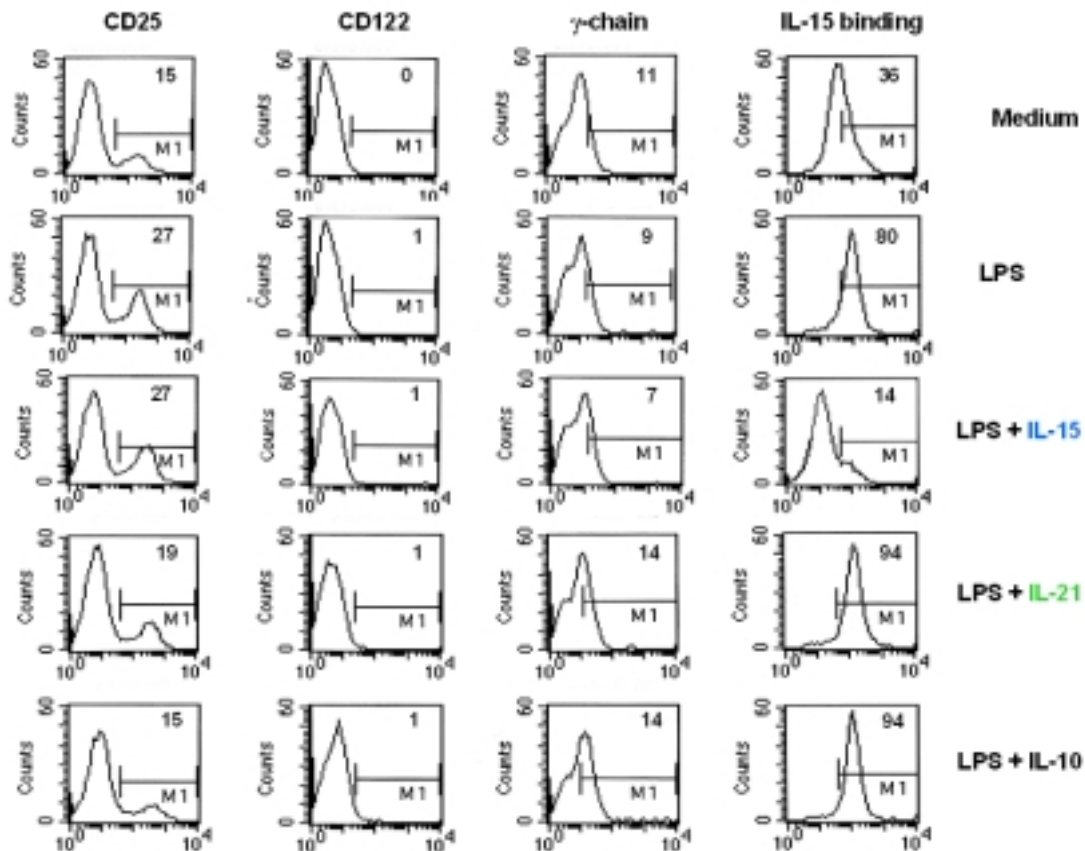


Figure 8b.

As a control we also checked the effects of the cytokines alone. For this purpose we stimulated DCs with the cytokines IL-15, IL-21 and IL-10 (100ng/ml) for 24h and subsequently did a FACS analysis. We found no pronounced up-regulation of the indicated markers due to the stimulation with solely cytokines (not shown).

In conclusion, IL-21 not only inhibits DC maturation when present during the generation but also the activation of DCs in the presence of high potent stimuli such as LPS.

Short time incubation with IL-21 inhibits DC-mediated CD8⁺ T cell response *in vitro* and *in vivo*

The first evidences we gained from our *in vitro* stimulation studies, revealing that IL-21 blocks also DC activation when present in parallel to LPS for a short time only, invited us to examine the modulatory capacities of short time added IL-15 and IL-21 on DC mediated T cell reactions *in vitro* and *in vivo*.

First we pulsed DCs for 2h with the CD8⁺ restricted TCR specific OVA⁽²⁵⁷⁻²⁶⁴⁾ peptide and added IL-15 and IL-21 in parallel to peptide pulse. Subsequently, T cells from OTI mice, which specifically recognize the pulsed OVA peptide, were given to the DCs and co-cultured for 72h. Proliferation was assessed by [³H]thymidine incorporation. As depicted in **Fig. 9a**, DCs (white bar) induced a high Ag-specific T cell proliferation, which was not further elevated through the short time labeling with IL-15 (blue bar). Strikingly only 2h labeling with IL-21 (green bar) high significantly suppressed the DC-mediated CD8⁺ T cell proliferation.

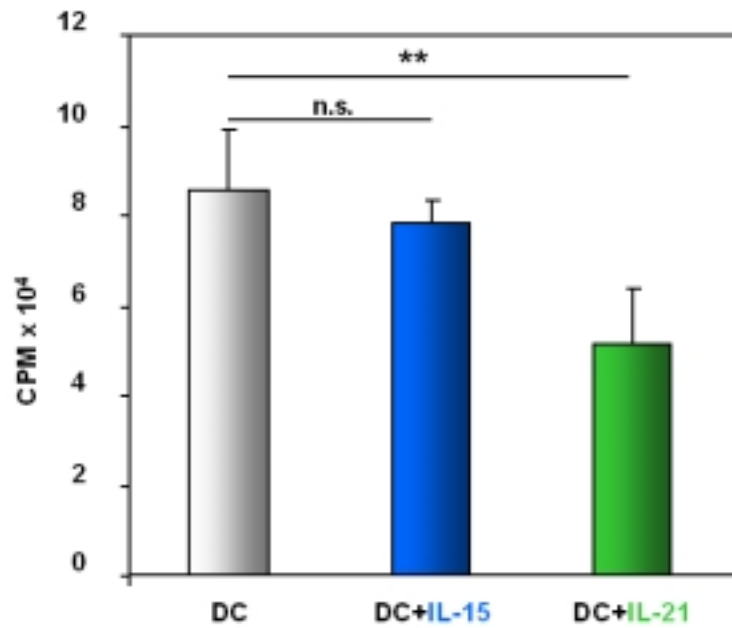


Figure 9a. Short time IL-21 stimulation blocks CD8⁺ T cell proliferation in vitro. DCs were incubated in parallel to peptide with IL-15 or IL-21 for 2h. Peptide specific T cells were added and co-cultured for another 72h. T cell proliferation was estimated by [³H]thymidine uptake. Significance was calculated using student's t-test (** $p \leq 0.01$; ns: not significant).

This findings prompted us to test whether blocking of DC mediated, specific T cell reactions by short time addition of IL-21 is also relevant in an *in vivo* situation like in CHS. For this purpose, we incubated DCs with IL-15 (blue) and IL-21 (green) for 2h and in parallel labeled them with FITC prior injection into the footpad. After 5 days mice were challenged with FITC on the ear and swelling were measured at indicated time points. As shown in **Fig. 9b**, IL-15 incubation for 2h could not further elevate the induction of the T cell mediated CHS response compared to DCs. In contrast, IL-21 incubation for only 2h prior injection completely abrogated the capacity of these DCs to induce an antigen-specific immune response. Thus, the resulting ear swelling did not exceed the non-immunized controls.

Taken together we clearly could show that IL-21 not only influences DC differentiation by establishing low immunogenic DCs when added during generation phase but also is able to abrogate DC activation hence suppresses DC mediated T cell response when present only for a short time.

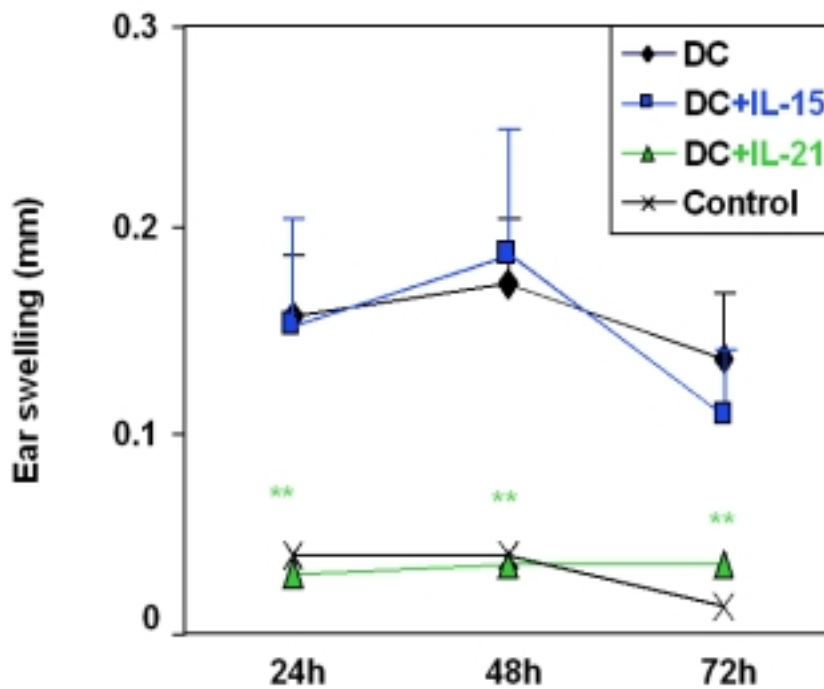


Figure 9b. Short-time IL-21 stimulation blocks CHS induction by DCs. DCs were incubated with IL-15 or IL-21 for 2h, in parallel labeled in vitro with FITC, washed and injected into the hind footpad of syngenic C57BL/6 mice (5×10^5 cells/mouse). 5 days later, mice were challenged with FITC painting on the earlobe and after 24h, 48h, and 72h the ear swelling reaction was analyzed. Painting of un-sensitized mice served as control. All experiments were repeated at least 2 times, * $p \leq 0.05$.

IL-15 is essential for a hapten-specific CHS response, but dispensable for irritant contact dermatitis

With our previous data we outlined that besides the inhibitory effects of IL-21 on DC activation and maturation, IL-15 was able to increase immunogenic features of DCs when present during generation. Our *in vitro* generated IL-15DCs could prime highest T cell responses *in vitro* (**Fig. 6**) and *in vivo* (**Fig. 7a**). Based on these findings and with the knowledge from literature that IL-15 is produced by DCs (Mattei 2001) we further asked which role DC-derived IL-15 could play in the context of antigen specific T cell reactions *in vivo* and *in vitro*. To clarify this we blocked the function of IL-15 *in vivo* by a soluble IL-15R α . To distinguish between effects of IL-15 and of the interplay with its specific high affinity IL-15R α , we extended these studies by the availability of IL-15^{-/-} and IL-15R α ^{-/-} knockout mice.

To verify whether IL-15 indeed has promoting effects on DC-mediated T cell responses, we analyzed in our first experiments, how the CHS response is established in IL-15 deficient mice. To this end we sensitized IL-15^{+/+} (Wt) and IL-15^{-/-} mice to the low molecular weight hapten picrylchloride (PCl) by painting it on the shaved abdomen and challenged 5 days later by painting PCl on the left ear. In addition, one group of IL-15^{+/+} mice was treated during the sensitization phase with a potent and selective IL-15 antagonist, the soluble IL-15R α (Ruchatz 1998). Application of hapten induced contact hypersensitivity, which was mainly CD8⁺ T cell mediated (Grabbe 1998). After PCl challenge resulting ear swelling response was analyzed at 24, 48, and 72h and showed that both IL-15^{-/-} (yellow) and sIL-15R α ^{-/-} (dark green) treated IL-15^{+/+} mice failed to mount an inflammatory reaction above control levels (**Fig. 10a**), giving a first hint that IL-15 is essential for the development of this type of DTH immune response.

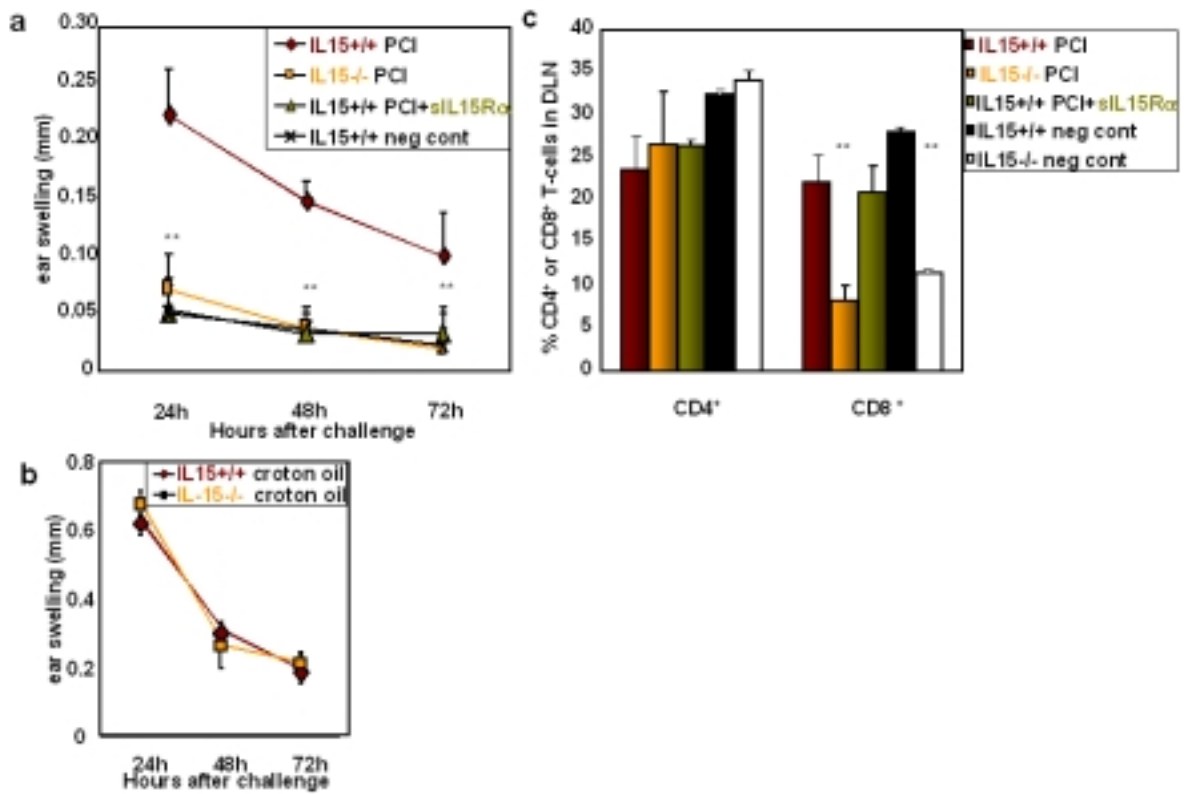


Figure 10. IL-15 is essential for contact hypersensitivity immune response. Mice were sensitized by painting the hapten picrylchloride (PCI) on the shaved abdomen at day 1 and CHS was induced by challenging the mice after 5 days by painting PCI on the left ear. **a:** Ear swelling was analyzed at 24, 48, and 72h after challenge in IL-15^{+/+}, IL-15^{-/-} mice and IL-15^{+/+} mice, which had been treated by i.p. injection with sIL-15R α (50 μ g/day) 2h before sensitization until hapten-challenge on day 5. Negative controls are un-sensitized mice. Given is the ear swelling as increment in thickness above the right ear, which had been painted with diluent only. **b:** Irritant contact dermatitis after croton oil application in IL-15^{+/+} and IL-15^{-/-} mice. Mice were painted on one ear with the irritant contact sensitizer croton oil and ear swelling was analyzed as above described. **c:** 72h after the challenge DLN of PCI sensitized mice were analyzed by FACS for CD4⁺ and CD8⁺ T cells. Given is the percentage of cells in the DLN. All experiments were repeated at least 3 times, ** $p \leq 0.01$.

To examine whether the pro-inflammatory cytokine IL-15 is also involved in a non-specific inflammation, croton oil was applied to one ear, which induces an antigen-independent, irritant contact dermatitis, mainly mediated by neutrophil granulocytes (Zhang 2000). Both,

IL-15^{+/+} (dark red) and IL-15^{-/-} (yellow) mice showed a profound ear swelling, which demonstrates that irritant contact dermatitis develops independently of IL-15 (**Fig. 10b**).

As already mentioned CD8⁺ T cells are known to be the main mediators of CHS (Watanabe 2002), we next analyzed by FACS the distribution and number of T cells in the local draining lymph nodes (DLN). We found that the percentage as well as the total numbers of CD4⁺ T cells within DLN was not significantly different between the studied groups (**Fig. 10c**). However, as expected (Kennedy 2000), IL-15^{-/-} mice (yellow bar) showed a high significantly reduced number of CD8⁺ T cells. Interestingly, treatment with the sIL-15R α (dark green bar) did not affect the number of CD8⁺ T cells compared to IL-15^{+/+} control mice (**Fig. 10c, dark red bar**), although this antagonist treatment completely blocked the CHS response.

This suggests that the role of IL-15 in CHS is not primarily related to its effect on CD8⁺ T cell *numbers* but rather on T cell priming/activation. Therefore, besides total T cell numbers, activation and induction of regulatory or memory T cells in DLN were assessed. No significant induction of CD4⁺/CD25⁺, i.e. so called “regulatory T cells” (Cavani 2001) was observed in all tested groups. However, in IL-15^{-/-} and sIL-15R α treated mice, CD8⁺ T cells were significantly less activated (as assessed by their CD69 expression); in addition, CD8⁺ memory T cells (as recognized by their high expression level of CD44) were reduced in both groups compared to untreated IL-15^{+/+} mice (**Table 1**).

	IL15 ^{+/+} PCI	IL15 ^{-/-} PCI	IL15 ^{+/+} PCI + sIL15R α	IL15 ^{+/+} neg cont	IL15 ^{-/-} neg cont
CD4 ⁺ /CD25 ⁺	6.8±1.0	6.6±0.8	7.2±0.4	5.8±0.9	5.6±1.0
CD8 ⁺ /CD69 ⁺	2.6±0.8	1.0±0.3**	1.7±0.2*	1.9±0.4	0.9±0.5
CD8 ⁺ / CD44 ^{hi}	22±2.4	9±2.3**	17±4.0*	7.8±4.4	4.3±1.1

Table 1. Regulatory T cells and activated or memory CD8⁺ T cells in DLN. FACS analysis of T cell activation and induction of memory T cells in the local ear-draining lymph nodes 72h after PCI challenge. Given is the percentage in relation to all CD4⁺ or CD8⁺ T cells (set to 100%). Shown is the mean \pm SD. Significance was calculated using Student’s t-test (* $p \leq 0.05$; ** $p \leq 0.01$).

In conclusion, IL-15 is essential for a hapten-specific *in vivo* CD8⁺ T cell mediated CHS reaction, including the activation as well as differentiation of memory T cells but is dispensable for an non-specific, irritant contact dermatitis. This was further stressed by treatment with the IL-15 antagonists, sIL-15R α , which efficiently inhibited contact hypersensitivity *in vivo*.

DTH footpad swelling, but not antibody production, is suppressed by IL-15 knockout or IL-15 antagonist

In order to evaluate the role of IL-15 in both CD4⁺ and CD8⁺ T cell as well as B cell mediated Th1 immune response, a murine DTH model of footpad swelling reaction to sheep red blood cells (SRBC; Kunzendorf 1996) was employed. In comparison to CHS, which is mainly mediated by CD8⁺ T cells, DTH to SRBC represents a both T- and B cell-dependent immune response, which has often been used as an instructive research model for identifying immune-regulatory factors (Kunzendorf 1996; Kalish 1999). Sensitization to SRBC induces activation of T cells in the peripheral lymphoid tissues and subsequently specific antibody production by B cells. After local antigen challenge in the footpad, activated T cells migrate to the site of challenge and induce a footpad-swelling reaction, which correlates with the extent of the DTH reaction (Kalish 1999).

Footpad swelling was analyzed 24, 48 and 72h after SRBC challenge in IL-15^{+/+} (dark red) and IL-15^{-/-} mice (yellow), or IL-15^{+/+} mice that had been treated with an IL-15 antagonist (sIL-15R α , dark green). Non-immunized mice served as negative- (open circles), IL-15^{+/+} mice as positive controls. As shown in **Fig. 11**, blocking the function of IL-15 by systemic sIL-15R α treatment high significantly suppressed the footpad swelling compared to the positive control at all three time points. In comparison IL-15^{-/-} mice also showed a significantly reduced footpad swelling (**Fig. 11**). Negative controls, instead, only had a slight, non-specific increase in footpad thickness, and no differences between IL-15^{+/+} (open circles) and IL-15^{-/-} (crosses) negative control mice were detected.

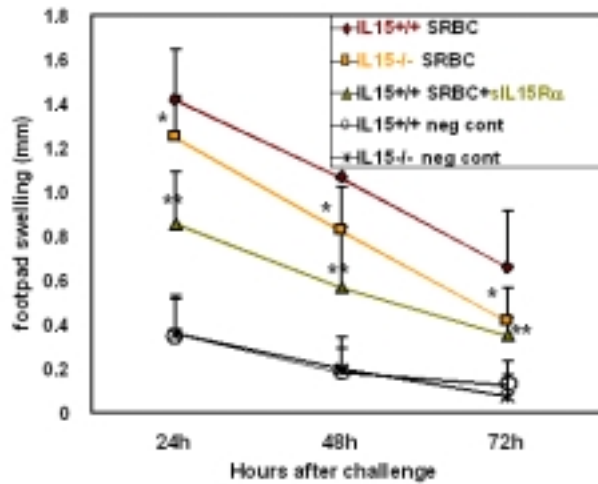


Figure 11. IL-15 mediates Th1 DTH footpad swelling reaction to sheep red blood cells. IL-15^{+/+} and IL-15^{-/-} mice were left un-sensitized or were immunized i.v. with 4×10^5 SRBC at day 0. IL-15^{+/+} mice were left untreated as positive control or treated with sIL-15R α (50 μ g/day) i.p. every 24h from day 0 until challenge on day 5. Challenge was performed by injection of SRBC in the left hind footpad, the right footpad was injected with diluent to calculate the specific swelling reaction: swelling was determined 24, 48 and 72h after challenge with a micrometer. Experiments were repeated 3 times. * $p \leq 0.05$; ** $p \leq 0.01$.

In contrast to the reduced footpad swelling after SRBC challenge, SRBC-specific hemagglutinating antibodies (7S and 7S+19S) were detectable in the absence of functional IL-15 in both IL-15^{-/-} mice and in sIL15R α -treated IL-15^{+/+} mice, at levels comparable to IL-15^{+/+} mice (positive control) (**Table 2**).

	IL15 ^{+/+} SRBC	IL15 ^{-/-} SRBC	IL15 ^{+/+} SRBC + sIL15R α	IL15 ^{+/+} neg cont	IL15 ^{-/-} neg cont
19S + 7S	10.3 \pm 1.3	10.2 \pm 1.0	9.3 \pm 0.4	5.2 \pm 1.4	6.8 \pm 0.9
7S	9.0 \pm 1.0	9.7 \pm 0.8	9.6 \pm 0.8	3.6 \pm 0.5	5.2 \pm 0.4

Table 2. Antibody titers after SRBC injection. Serum titers of hemagglutinating total (7S+19S) and IgG (7S) antibodies against SRBC were determined at day 8, 72h after SRBC challenge in the footpad. Non-immunized mice were challenged without prior immunization.

A second SRBC challenge on the other foot 14 days after the first SRBC injection again revealed a significantly suppressed footpad swelling in sIL-15R α -treated and IL-15 $^{-/-}$ mice, but comparable antibody titers (not shown).

These data suggest that IL-15 is essential for T cell mediated effects also in this type of DTH model. Notably, just blocking endogenous IL-15 activity with a suitable antagonist during the sensitization phase suffices to elicit a long-lasting DTH suppression. In contrast, our results show that IL-15 is dispensable for B cell activation and antibody production in this example of an *in vivo*-Th1 immune response.

***In vivo* antigen-uptake and migration/ activation of DCs are IL-15-independent**

In the following, we focused on the CHS model of DTH, since it facilitates dissection of the underlying, T cell specific mechanisms of CHS inhibition in the absence of IL-15. For this purpose, we analyzed the antigen-uptake by epidermal DCs in the skin and their migration to the local DLNs as well as the up-regulation of CD80 and CD86 co-stimulatory molecules on DCs, which are critically involved in CHS reactions (Xu 1997).

In order to address which of these steps may be influenced by IL-15, we used again FITC as model antigen, since it can easily be visualized by FACS (Macatonia 1986). As shown in **Table 3**, 24h after abdominal FITC painting, the percentage of FITC $^{+}$ /CD11c $^{+}$ -DCs (i.e. cells, which migrated in this time from the skin, where they took up Ag) in DLN was comparable in all FITC-painted groups (the total cell number in each lymph node was also comparable in all groups; not shown). Negative controls (without FITC painting) showed a lower amount of DCs in the DLN (around 2.2%, compared to over 5% CD11c $^{+}$ after FITC painting). Finally, we analyzed the activation of DCs by quantifying the expression of the co-stimulatory molecules CD80 and CD86, which play an important role in CHS responses (Katayama 1997). No significant differences in the expression of both molecules on antigen-bearing cells that were FITC positive could be detected between all FITC painted groups. DCs from untreated IL-15 $^{+/+}$ or IL-15 $^{-/-}$ mice expressed low levels of CD80 and CD86.

	IL15+/+ FITC	IL15-/- FITC	IL15+/+ + sIL15Rα FITC	IL15+/+ Control	IL15-/- Control
CD11c⁺	5.5	5.3	5.3	2.2	2.1
CD11c⁺/CD80⁺	41.7	40.3	43.0	11.2	14.0
CD11c⁺/CD86⁺	60.9	59.5	56.5	18.8	27.1

Table 3. IL-15 is not mandatory for cutaneous antigen uptake by and activation of DCs. *IL-15+/+ or IL-15-/- mice were painted on the abdomen with FITC or were injected 30min before painting with sIL-15R α (50 μ g) i.p. Negative controls and left untreated. 24h later, the DLN were analyzed by FACS (100.000 cells) for total number of CD11c positive DC (1st row, given is the total percentage of DC in lymph nodes). Furthermore, activation of the FITC⁺ DCs after migration from the skin was analyzed by FACS. Expression of the co-stimulatory molecules CD80 (2nd row) and CD86 (3rd row) was estimated using PE labeled antibodies (given is the percentage of high (fluorescence channel > 10²) CD80 and CD86 positive cells on CD11c⁺ FITC⁺ DCs in the DLN). Negative controls (without FITC painting) were analyzed for total CD11c⁺ DCs in DLN.*

Bone marrow derived DCs (BMDCs) from IL-15+/+ and IL-15-/- mice (see below) also displayed no significant differences in endocytic antigen-uptake *in vitro* as analyzed by the model-antigen FITC-dextran (not shown).

Taken together, DCs from IL-15+/+ and IL-15-/- mice take up antigen (*in vivo* and *in vitro*) and migrate to local DLN with comparable efficiency, indicating that IL-15 is not essential in modulating these important steps of CHS response.

Bone marrow derived DCs from IL-15+/+ and IL-15-/- mice differ in their T cell stimulatory capacity

In order to unravel the above described specific defects of IL-15-/- mice in T cell immunity and memory, and to elucidate whether DC-derived IL-15 is the key in the induction of Th1 immune responses like CHS, bone marrow derived DCs (BMDCs) from IL-15+/+ or IL-15-/- mice were employed, which had been generated 8 days *in vitro* by culturing with GM-CSF. Surface phenotyping after 8 days revealed similar purity (>95%) of CD11c⁺ DCs. Also, the

co-stimulatory molecules CD80, CD86, MHC II, and CD25 were expressed at comparable levels (**Fig. 12a**).

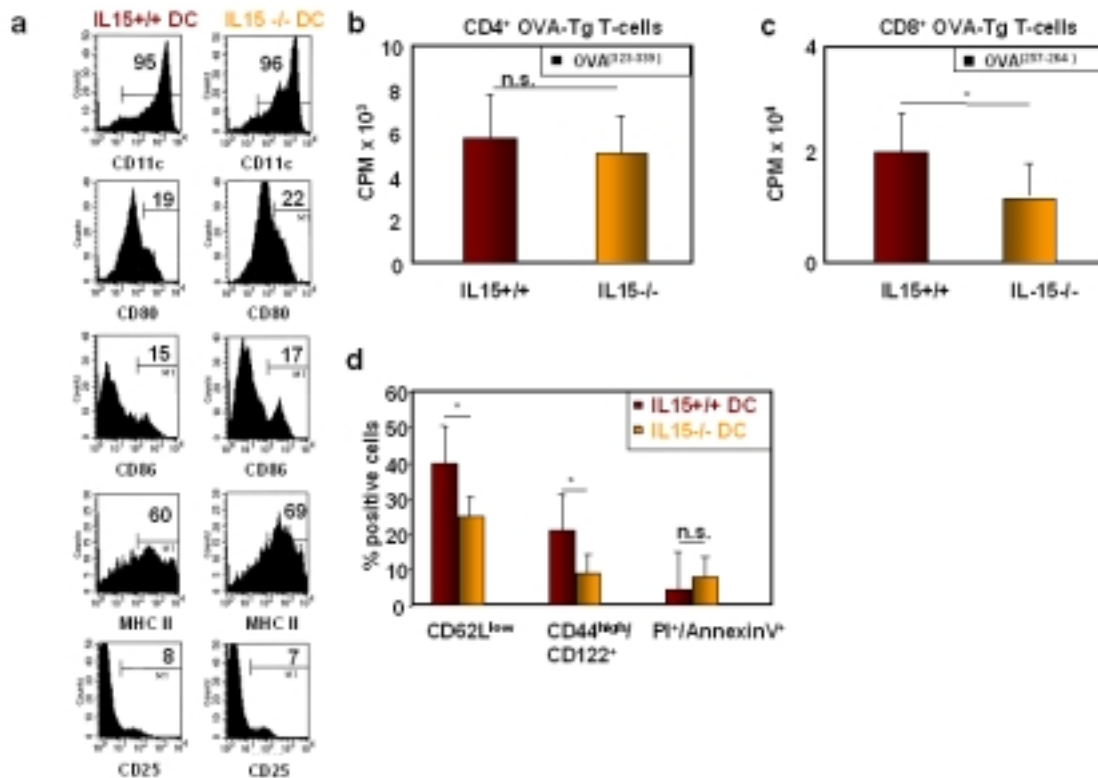


Figure 12. DC derived IL-15 mediates CD8⁺ T cell proliferation and memory cell differentiation in vitro. **a:** Bone marrow derived DCs from IL-15^{+/+} and IL-15^{-/-} mice were analyzed for different co-stimulatory molecules on day 8 by FACS. Shown is one representative out of 3 experiments. Given is the percentage of high positive (fluorescence channel >10²) cells except for CD11c, where all positive cells after 8 days culture are given. **b:** BMDCs from IL-15^{+/+} and IL-15^{-/-} mice were co-cultured with CD4⁺ T cells from OT II mice with an CD4-restricted OVA transgenic T cell receptor and with OVA⁽³²³⁻³³⁹⁾ peptide. Proliferation was analyzed by ³[H]thymidine incorporation (CPM: counts per minute; n.s.: not significant). **c:** Co-culture as in 12b, but with CD8⁺ T cells from OTI mice with a CD8-restricted OVA transgenic T cell receptor, with OVA⁽²⁵⁷⁻²⁶⁴⁾ peptide (*p≤0.05). **d:** Induction of CD8⁺ memory cells and apoptosis was analyzed in the co-culture experiments with IL-15^{+/+} and IL-15^{-/-} BMDCs and CD8⁺ OVA-TCR transgenic cells after 48h. Given are the percent CD62L^{low} and CD44^{high}/CD122⁺ (memory) cells as well as dead cells (positive for PI/Annexin V). Significance was analyzed using Student's t-test (n.s.: not significant; *p≤0.05).

This suggests that IL-15 is not essential for the *in vitro* generation of BMDCs, which is supported by the fact that no known abnormalities in DC subtypes and number have been noted in IL-15^{-/-} or IL-15R α ^{-/-} mice (Kennedy 2000; Lodolce 1998; Ohteki 2001).

DCs are specialized for antigen capture, migration and antigen-presentation to CD4⁺ and CD8⁺ T cells (Cella 1997; Lambrecht 2001). To analyze the effect of IL-15 on DC/T cell interactions, and in order to specifically dissect the immuno-stimulatory capacity of DCs influenced by IL-15, BMDCs from IL-15^{+/+} or IL-15^{-/-} mice were studied in a co-culture assay with syngenic T cells. The DCs were labeled with peptide fragments from ovalbumine and were cultured with T cells *in vitro*. These T cells expressed a CD4⁺ T cell restricted, transgenic (tg) T cell receptor (from OTII mice), which recognize the OVA⁽³²³⁻³³⁹⁾ peptide (Robertson 2000), and CD8⁺ transgenic T cells (from OTI mice), which recognizes the OVA⁽²⁵⁷⁻²⁶⁴⁾ peptide (Rotzschke 1991). As shown in **Fig. 12b**, peptide-labeled DCs from IL-15^{+/+} (dark red bars) or IL-15^{-/-} mice (yellow bars) induced comparable proliferation of CD4⁺ tg T cells, suggesting that this T cell subpopulation proliferates independently from DC-derived IL-15. These *in vitro* data correspond to the CD4⁺ T cell mediated production of hemagglutinating antibodies *in vivo*, which were equal in IL-15^{+/+} and IL-15^{-/-} mice, as shown above (**Table 2**).

In view of previous reports, indicating that IL-15 acts mainly on CD8⁺ T cells (Schluns 2002; Fehniger 2001), we also studied activation of CD8⁺ tg T cells. Indeed, IL-15^{-/-} BMDCs induced significantly lower antigen-specific CD8⁺ T cell proliferation compared to IL-15^{+/+} BMDCs (**Fig. 12c**): CD8⁺ T cell proliferation was diminished when specific Ag was presented by IL-15 deficient DCs, even though the level of MHC class I expression was comparable on IL-15^{+/+} and IL-15^{-/-} DCs (not shown). Reduced levels of CD8⁺ specific T cell proliferation induced by IL-15^{-/-} DCs could be restored by exogenous given IL-15 (not shown).

These findings confirm that IL-15 produced by DCs is a key-player in DC mediated CD8⁺ T cell priming.

DC-derived IL-15 mediates differentiation of memory T cells *in vitro*

Following recognition of Ag presented by DCs, naive T cells become activated and are polarized to differentiate into effector and subsequently memory T cells (Tuma 2002; Weninger 2002). Studies in IL-15^{-/-} mice revealed that IL-15 plays a role in establishing CD8⁺ memory (Kennedy 2000; Lodolce 1998). However, it is not known at which stage memory cell induction is disrupted. We next analyzed the development of these cells *in vitro*, focusing on the influence of DC-derived IL-15.

These studies showed that, indeed, co-culturing of CD8⁺ tg T cells with BMDC from IL-15^{+/+} mice induced CD8⁺ T cells with a memory phenotype, as evidenced by their characteristic surface marker pattern, i.e. low expression of CD62L and CD44^{high}/CD122⁺ (**Fig. 12d, dark red bar**). Instead, co-culture with IL-15^{-/-} BMDCs resulted in significantly reduced induction of the memory T cell phenotype (**Fig. 12d yellow bar**). By PI/Annexin staining, only few T cells were demonstrated to have undergone apoptosis, and no significant differences between IL-15^{+/+} and IL-15^{-/-} BMDCs in this respect were detected (**Fig. 12d, right columns**). This makes it very unlikely that the lack of IL-15 (a potent anti-apoptotic agent; Bulfone-Paus 1997) only resulted in premature apoptosis of activated CD8⁺ cells, and can thus not be invoked as a convincing explanation for the defect in memory T cell induction in the absence of IL-15-secreting DCs.

In conclusion, our *in vitro* assays showed that the absence of DC-derived IL-15 leaves the expression of co-stimulatory molecules on DCs unaffected, but significantly reduces the ability of DCs to induce CD8⁺ T cell proliferation, and differentiation towards a memory phenotype.

BMDC migration *in vivo* is IL-15-independent

To elucidate whether IL-15 might affect DC functions *in vivo*, we applied a model of active sensitization with FITC labeled DCs. First, we examined whether the complex migration process of DCs *in vivo* depends on endogenous, DC-derived IL-15 or on exogenous IL-15 from the surrounding tissue. This was done by comparing the ability of BMDCs from IL-15^{+/+} or IL-15^{-/-} mice to enter DLN in IL-15^{+/+} or IL-15^{-/-} mice following BMDC injection *in vivo* (Lappin 1999). IL-15^{+/+} or IL-15^{-/-} BMDCs were labeled with the fluorescent cell tracer FITC and subsequently injected into the footpad of IL-15^{+/+} or IL-15^{-/-} mice. After

24h, cell suspensions were prepared from the DLNs (Vremec 1997), stained with anti-CD11c antibody and analyzed by FACS. Double positive cells, which had migrated in the DLN (FITC⁺/CD11c⁺), were expressed in percent of total CD11c⁺ DCs. As control, unlabeled DCs were injected.

As shown in **Fig. 13a**, the IL-15^{+/+} BMDCs had similar migratory capacities in both IL-15^{+/+} (dark red bar) and IL-15^{-/-} mice (red/yellow bar). IL-15^{-/-} BMDC had no altered pattern of migration to the DLNs (yellow bar). Within 24h, 16-18% of the total lymph node CD11c⁺ DCs migrated from the periphery into DLNs.

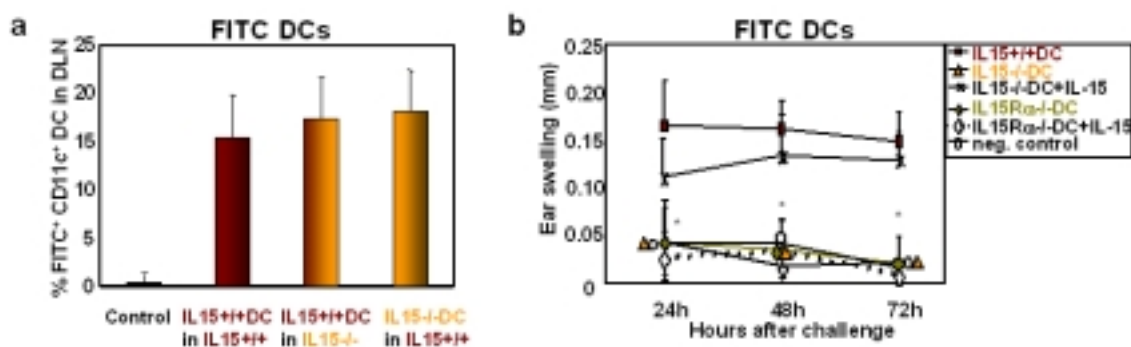


Figure 13. Comparable migration but different induction of T cell immunity by IL-15^{+/+} and IL-15^{-/-} BMDCs. *a*: Migration of DCs was analyzed by FACS of the draining lymph nodes, 24h after injection of 5×10^5 FITC labeled DCs. *In vitro* generated DCs from IL-15^{+/+} and IL-15^{-/-} were labeled with FITC, washed and injected into the hind footpad (5×10^5 cells/mouse) in IL-15^{+/+} and IL-15^{-/-} mice and after 24h the DLNs were analyzed for FITC⁺ cells. Given are percent of double stained CD11c⁺/FITC⁺ cells out of all CD11c⁺ DCs. Controls were injected with unlabeled DCs. *b*: In addition, mice injected with the FITC labeled DCs were challenged by painting FITC on the left earlobe at day 5 for induction of CHS and ear swelling was analyzed 24, 48, and 72h later. Painting of un-sensitized mice served as control.

In contrast, glutaraldehyde-fixed, FITC labeled DCs (i.e. dead cells) did not reach the DLNs after footpad injection, and no FITC uptake by other DCs of recipient mice was detected (not shown), confirming that the observed migration into DLNs is an active and specific process (Eggert 1999). These results strongly support our *in vivo* data presented above (**Table 3**), which had suggested that the migration/homing of DCs from peripheral tissue sites depends

neither on the autocrine production of IL-15 by DCs itself, nor on exogenous IL-15 secreted by the DC microenvironment or delivered via the serum.

DC-derived IL-15 and the IL-15R α on DCs are essential for inducing a T cell mediated CHS response to FITC

In order to determine directly to which extent the capacity of DCs for T cell priming and sensitization *in vivo* depends on endogenous, DC-derived IL-15, IL-15^{+/+}, IL-15^{-/-} and IL-15R α ^{-/-} DCs were therefore labeled *in vitro* with FITC, and injected subcutaneously in the footpad of IL-15^{+/+} mice. Five days after this active sensitization, mice were challenged at the dorsal and ventral site of one ear with FITC (the other ear with diluent only) and the ability to initiate an antigen-specific, T cell mediated CHS response was examined by measuring the FITC-induced ear swelling 24, 48 and 72h after challenge.

Mice, which had not been sensitized, but were challenged with FITC on the ear, served as negative controls for unspecific ear swelling (open quadrangles). Un-sensitized mice responded with small unspecific ear swelling that was equal in IL-15^{+/+} and IL-15^{-/-} (**Fig. 13b, opened circles**). IL-15^{+/+} mice that had been actively sensitized with IL-15^{+/+} DCs served as positive controls (**Fig. 13b, dark red quadrangles**). To confirm that the sensitization is based on active migration of the injected, viable DCs from the footpad to the DLN, control mice were injected with FITC labeled, glutaraldehyde fixed DCs (Eggert 1999). As expected, these fixed cells, incapable of migration failed to induce any CHS response (not shown).

Injection of IL-15^{-/-} DCs resulted in a highly significantly reduced CHS response that was indistinguishable from un-sensitized controls all three time points (**Fig. 13b, yellow triangles**). However, when the lack of endogenous IL-15 was substituted by incubating IL-15^{-/-} DCs prior to injection for only 30min with recombinant IL-15, their ability to induce T cell sensitization and subsequent induction of a CHS response was almost restored to the level of IL-15^{+/+} DCs (**Fig. 13b, crosses**).

Using BMDCs from IL-15R α ^{-/-} mice, which lack the high affinity IL-15R α chain but produce IL-15, could not induce a CHS response above negative control values, either (**Fig. 13b, dark green diamonds**). As expected, loading these IL-15R α ^{-/-} DCs prior to injection

with IL-15 (**Fig. 13b, opened diamonds with dotted line**), did not restore the capacity to prime a T cell response.

These data clarify that endogenous IL-15, produced by DCs as well as IL-15R α expression by DCs, are required for the initiation of an efficient DTH response *in vivo*.

DC-derived IL-15 is dispensable for B cell priming to OVA/FITC

To support the findings from the DTH to SRBC experiments shown above, namely that IL-15 mediates T cell activation but is not mandatory for B cell function, we used a FITC-labeled, complete protein-antigen (Harlow 1988) to actively sensitize the mice by injecting OVA/FITC DCs. This antigen induces CD4⁺ and CD8⁺ T as well as B cell activation.

As shown in **Fig. 14a**, a significant ear swelling to FITC was induced when labeled IL-15^{+/+} DCs were injected into IL-15^{+/+} mice after challenge with FITC on day 5 (as described above). As expected, OVA/FITC labeled IL-15^{-/-} DCs failed to induce T cell priming and therefore CHS swelling reaction in IL-15^{+/+} mice (**Fig. 14a, dark red diamonds**). To analyze the B cell function in these mice, the animals were bled 7 days after sensitization by OVA/FITC labeled DCs, and anti-OVA-IgM titers were determined by ELISA. As shown in **Fig. 14b**, both IL-15^{-/-} (yellow quadrangles) and IL-15^{+/+} DCs were capable of inducing anti-OVA antibody production at comparable levels.

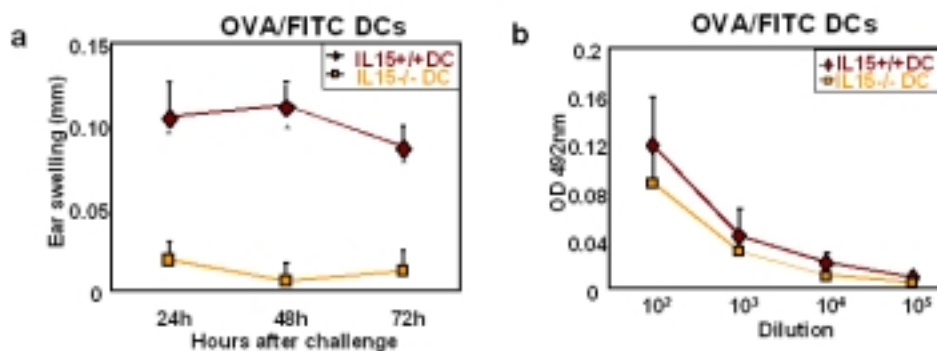


Figure 14. DC-derived IL-15 is dispensable for B cell priming. *a*: DCs from IL-15^{+/+} and IL-15^{-/-} mice were labeled with OVA/FITC and injected in IL-15^{+/+} mice, which in addition to T cells also activates B cells. Ear swelling was induced and analyzed as in Fig. 13b. *b*: B

cell activation was analyzed by detecting serum anti-OVA-IgM antibodies at day 8 using ELISA. Given is the optical density (OD at 492 nm) of serial serum dilutions.

These findings are fully in line with the *in vivo* data presented above and underscore the concept that DC-derived IL-15 is essential for the induction of CD8⁺ T cell activation and differentiation after antigen-contact, but is dispensable for B cell priming. In addition, the presence of the high affinity IL-15R α chain on DC was found to be crucial in inducing CD8⁺ T cell response.

Compared to IL-15^{+/+} DCs, IL-15^{-/-} DCs produce higher levels of anti-inflammatory and lower levels of pro-inflammatory cytokines

It remained to be determined, how IL-15/IL-15R α mediated signaling affects the T cell priming by DCs. It is appreciated, that cytokine production by DCs critically modulates the outcome of immune responses during antigen-presentation to T cells (Liu 2001a) and that this cytokine secretion pattern of DCs is modulated by many different stimuli as antigen-processing and -presentation, DC-T cell interactions (Guermónprez 2002; Lanzavecchia 2001) and pathogens like microbial cell-wall components (Schuurhuis 2000). In response to these signals, DCs are either activated to enter maturation, which transforms them into specialized cytokine-secreting cells, or are kept in an immature state by anti-inflammatory cytokines like IL-10 (Corinti 2001). Since IL-15 can alter the secretion of first-line defense cytokines like type I interferons from DCs (Mattei 2001), it was of interest to compare our DCs generated *in vitro* from IL-15^{+/+} and IL-15^{-/-} mice with respect to their production of pro-inflammatory- (IL-1 β , TNF- α , IL-12) and anti-inflammatory- (IL-10) cytokines.

First, we analyzed cytokines from co-cultures of DCs and CD8⁺ T cells (as described above). Pro-inflammatory, T cell derived cytokines like IL-2 and IFN- γ were found to be reduced when Ag was presented by IL-15^{-/-}-DCs (not shown), which suggests an important role of DC-derived IL-15 on the secretion of T cell cytokines during antigen presentation by DCs.

To distinguish between DC-derived and T cell derived cytokines we, next, also stimulated DCs solely by low-dose LPS (Reis e Sousa 1999) and measured cytokine production. As shown in **Fig. 15**, IL-15^{+/+} DCs (red bars) produced significantly higher amounts of IL-1 β and TNF- α than IL-15^{-/-} DCs (yellow bars), whereas the latter produced significantly more anti-inflammatory IL-10 *in vitro* (**Fig. 15**). The failure of IL-15^{-/-}-DCs to induce a Th1 CHS

response was not mediated due to reduced IL-12 production, since the levels of the Th1-inducing cytokine IL-12 (Liu 2001a), secreted by IL-15^{+/+} and IL-15^{-/-} DCs were comparable (not shown), suggesting that the reduced CD8⁺ T cell activation is not indirectly mediated by reduced IL-12 release.

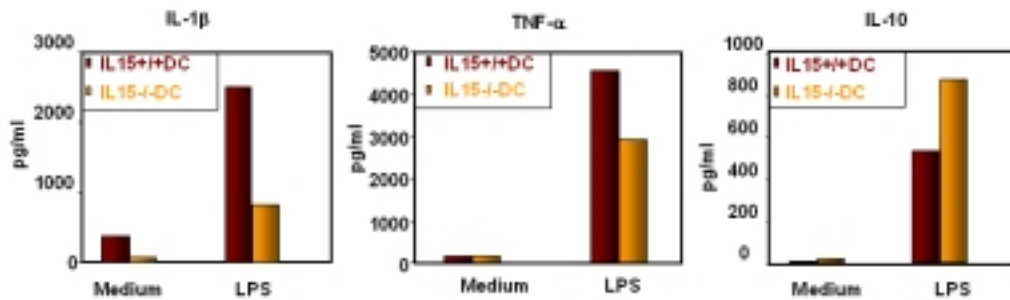


Figure 15. BMDCs from IL-15^{+/+} mice produce more pro-inflammatory cytokines after LPS activation. DCs from IL-15^{+/+} and IL-15^{-/-} mice were either activated *in vitro* for 24h with low dose LPS (10ng/ml) or were incubated with medium alone. Concentrations of pro- and anti-inflammatory cytokines (as indicated) were detected in cell-free supernatants.

These data suggests, that DC-derived IL-15 could modulate the T cell-priming functions of DCs in an autocrine fashion by boosting the secretion of pro-inflammatory TNF- α and IL-1 β , and by down-regulating the anti-inflammatory IL-10 release by DCs. This provides further evidence that IL-15 is directly and critically involved in fine-tuning the DC-cytokine network towards the mounting of an efficient, CD8⁺-mediated Th1 immune responses.

IL-15 is internalized via IL-15R α and co-localizes with IL-15R α in DCs

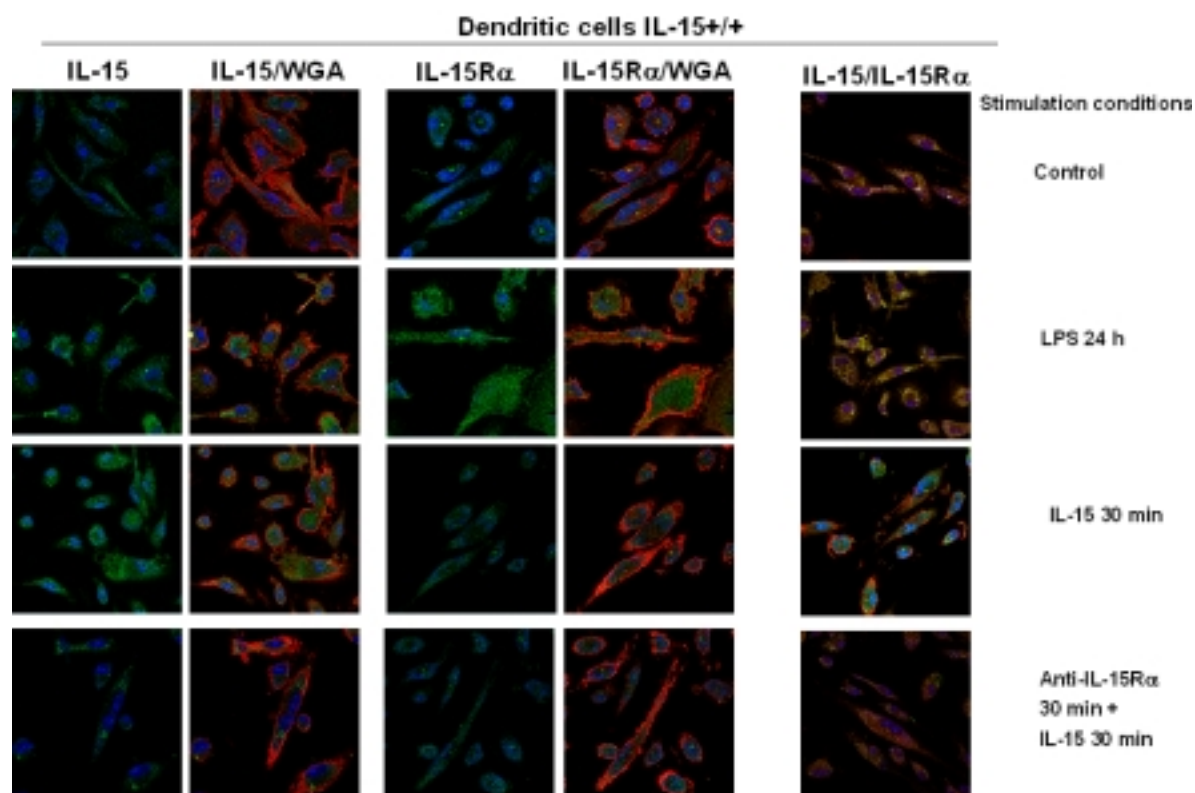
Finally, we addressed whether membrane or intracellular IL-15-IL-15R α interactions are critical in this respect. As a first step towards clarifying this, IL-15/IL-15R α (co-) localization was analyzed by confocal microscopy. IL-15^{+/+}, IL-15^{-/-} and IL-15R α ^{-/-} DCs were cultured for 24h on micro chamber slides in medium or activated with low-dose LPS. Alternatively, they were incubated for 30min with IL-15 or with IL-15 in the presence of a receptor-blocking anti-IL-15R α antibody to study which role the high-affinity receptor plays in the uptake of IL-15.

As revealed by double staining with wheat germ agglutinin (WGA), IL-15 is expressed in DCs mainly intracellular, but not in membrane-bound form (**Fig. 16a**). This expression

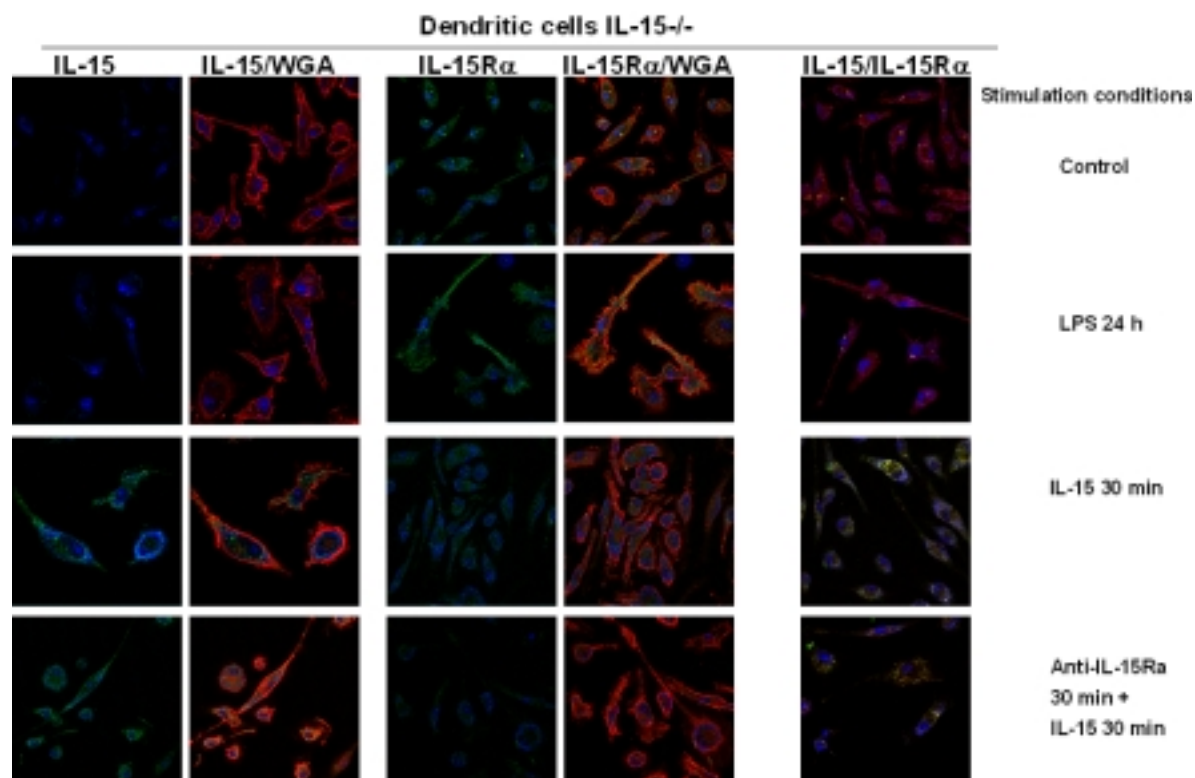
pattern differs from the one we had previously shown for monocytes (Musso 1999) and keratinocytes (Rückert 2000). Intracellular IL-15 staining was strongly up regulated in IL-15^{+/+} DCs when stimulated by LPS (**Fig. 16a**, *green fluorescence*). Exogenous IL-15 was taken up predominantly via the IL-15R α , (within 30min) since a blocking antibody against IL-15R α strongly reduced IL-15 uptake (**Fig. 16a**). Like IL-15, the IL-15R α immunoreactivity was localized more intracellular than on the membrane and its expression there could be up regulated upon stimulation with LPS (**Fig. 16**). Since the IL-15R α immunoreactivity associated with the membrane in IL-15^{+/+} mice was faint, we cannot exclude that the detection of IL-15R α -IL-15 complexes on the membrane was below detection-limit of our assay.

Furthermore, these studies showed that IL-15 was intracytoplasmatically co-localized with IL-15R α (**Fig. 16a**, IL-15/IL-15R α , *yellow fluorescence*). This intracellular complex was also found, when IL-15 was given exogenously to IL-15^{-/-} DCs (**Fig. 16b**). This underscores the tendency of IL-15 to form ligand/receptor complexes in DCs, and shows that ligand uptake by the IL-15R α is unaffected in IL-15 knockout mice. This finding also provides a convincing explanation for the restored T cell-priming ability of IL-15^{-/-} DCs after pre-incubation with IL-15 in CHS (see above, **Fig. 13b**). Although DCs from IL-15R α ^{-/-} mice produced and expressed intracellular IL-15 which was up-regulated by LPS (**Fig. 16c**), the lack of the high-affinity α -chain and therefore the missing interaction with its ligand, IL-15, led to the functional deficiency in inducing T cell mediated CHS response, which could not be restored by incubation with an excess of IL-15 (see above, **Fig. 13b**).

a



b



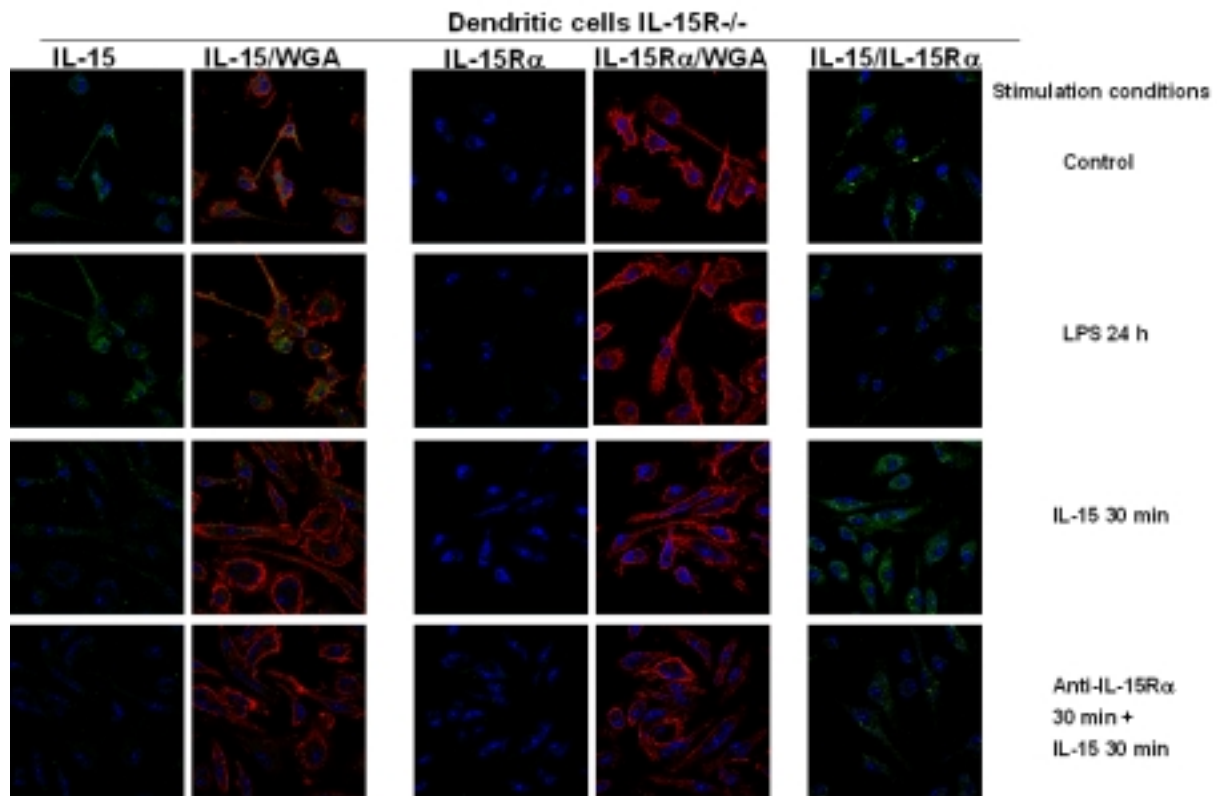
c

Figure 16. IL-15R α and IL-15 are intracellular co-localized in DCs. *a:* DCs from IL-15^{+/+} *b:* IL-15^{-/-} or *c:* IL-15R α ^{-/-} mice were stimulated for 24h with LPS or for 30min with 100ng/ml of IL-15 or treated for 30min with anti-IL-15R α antibodies prior to IL-15 stimulation for the next 30min. Un-stimulated cells were used as a control. Cells were fixed with 2% paraformaldehyde and incubated with WGA (labeled with rhodamine, **red fluorescence**) for 20min, washed, permeabilized and stained with anti-IL-15R α or with anti-IL-15 antibodies (ALEXA-488, **green fluorescence**). For co-localization of IL-15R α with IL-15, cells were fixed with 2% paraformaldehyde, permeabilized and labeled with anti-IL-15R α antibodies (ALEXA-564, **red**) in combination with anti-IL-15 antibodies (ALEXA-488, **green**) and TOTO-3 for nuclei (**blue**). **Yellow** color indicates co-localization of IL-15R α and IL-15.

Taken together our data demonstrate for the first time, that the induction of T cell immunity and CD8⁺ memory depends on IL-15-IL-15R α interaction in DCs. Hence, based on the essential role of IL-15-IL-15R α interactions in DCs, we suggest that the specific modulation of this signalling loop offers a promising, novel therapeutic target for the treatment of Th1-type autoimmune diseases, transplant rejection, and other CD8⁺-T cell dependent diseases and for DC-dependent vaccination therapy.

Discussion

Effects of IL-15 and IL-21 on generation of DCs *in vitro*

In this study we have shown that IL-15 and its structural relative, the newly described cytokine IL-21 (Parrish-Novak 2000) influence DC differentiation, maturation and function *in vitro* and *in vivo*. Given IL-15 to BM stem cells in addition to GM-CSF provided phenotypic and functional mature DCs with profound MHCII expression, decreased endocytic activity and highly increased T cell stimulatory ability *in vitro* and *in vivo*. In contrast, IL-21 mediated completely opposite effects while inhibiting maturation of DCs and down modulated DC induced Ag specific T cell response. With our first data, that IL-21 in combination to GM-CSF is able to support differentiation of myeloid BMDCs, we could not only corroborate results from literature demonstrating that IL-21 influences also lymphoid cell development (Parrish-Novak 2000), but directly report for the first time a regulatory impact of this cytokine on APC function.

Immature DCs are characterized by constitutively sampling of their surrounding fluid by endocytosis but rapidly lose their antigen uptake capacity upon maturation (Sallusto 1995). Here, IL-21DCs showed highest internalization of the model antigen FITC dextran, contrasted by very low uptake by IL-15DCs. The observed differences between IL-21DCs and IL-15DCs point to altered maturation states, supporting the deduction that IL-21 could block DC maturation. We extended this with data revealing that co-stimulatory molecules on IL-21DCs remained significantly reduced even after antigen-uptake and –processing in comparison to IL-15DCs.

Thus, IL-15 strongly promoted the differentiation of BM progenitor cells into mature DCs. The supportive effects of IL-15 in differentiation towards mature APCs are in line with previously published reports (Saikh 2001, Mohamadzadeh 2001). The divergent effects of both cytokines are likely to be mediated by different signaling pathways. Even since both use the common- γ chain, the high-affinity receptors may differ in their signal transduction after ligand binding.

The IL-21R contains a long intracellular domain with strongly conserved motifs, including classical Box1 and Box2 elements (Murakami 1991), which are important for signal

transduction, suggesting that this receptor is a signaling subunit (Parrish-Novak 2000). Even though the private IL-21R chain is not able to transduce ligand binding signals alone or as a homodimer (Parrish-Novak 2002) it is reasonable to speculate that it modulates γ -chain effects likewise. It has been reported that in cooperation with the common γ -chain, the heterodimeric IL-21R complex activates STAT (Asao 2001) and Jak (Habib 2002) pathways. The observed inhibitory effects of IL-21 on DC generation, however, remain to be elucidated by further analysis of signaling pathways in comparison to IL-15.

Despite the very short intracellular domain of IL-15R α our group already demonstrated, that TRAF2 (Bulfone-Paus 1999) as well as Syk are recruited to the intracellular receptor part (Bulanova 2001) which strongly suggests that IL-15R α is able to transduce a signal even in the absence of the γ -chain. Nevertheless, IL-15 signals also via the IL-2/15R β chain (Grabstein 1994), which is another important difference to IL-21 signaling. This could explain our observation that RNA message of IL-2/15R β chain is predominantly expressed in the IL-15DCs. In this regard it is worth noting that there are compelling evidences, which demonstrated that the IL-2/15R β -chain co-localizes with the GM-CSF receptor (Giron-Michel, personal communication). This reinforces our results that the enhanced maturational stage of IL-15DCs might be gained from synergistic effects of GM-CSF and IL-15.

It is widely accepted that DC maturation is directly linked to T cell stimulatory capacities (Lanzavecchia 2001). Indeed our experiments revealed that antigen-specific T cell response *in vitro* was significantly reduced with IL-21DCs. IL-15DCs mediated high significantly increased T cell proliferation rates. This is most probably due to altered antigen presenting capacities modulated directly by IL-15 and IL-21 during generation. However, it remained to be clarified at which stage of generation the cytokines influence stem cell differentiation in context to GM-CSF and whether this effect is reversible (“plasticity”).

That our *in vitro* generated DCs were susceptible for signals from IL-15 and IL-21 was proven by message expression of the relevant receptors on mRNA level. To confirm that IL-15 and IL-21 were taken up by DCs, we did confocal microscopy and found both cytokines internalized.

In additional experiments, I generated BM derived murine macrophages (MF), which revealed under the influence of IL-21 a similar, immature phenotype, since “IL-21MF” were also not

able to induce T cell proliferation. Therefore the here described effects of IL-21 seem to represent a general feature of this cytokine on APC differentiation.

Effects of IL-15 and IL-21 on LPS induced DC activation *in vitro*

Dendritic cells are able to respond directly to pathogens like microbial cell-wall components and indirectly sensing infection through inflammatory cytokines (Rescigno 1999). In response to these “danger” signals (Gallucci 2001), DCs are activated to enter maturation, which transforms them into highly efficient APCs.

Our data imply that IL-15 did not further increase DC activation induced by LPS, whereas in quite contrast IL-21 displayed inhibitory properties. Thus we showed that LPS induced up-regulation of functional relevant molecules like MHCII, CD80, CD86 was significantly reduced when IL-21 was given in parallel to LPS. It is known, that other cytokines, notably from T cell released IL-10, have been implicated in impeding DC maturation, which was associated with the retention of an “immature” phenotype (Morel 1997). Concomitant incubation of DCs with LPS and IL-10 also blocked, like IL-21, expression of co-stimulatory molecules, suggesting that IL-21 has comparable inhibitory effects on LPS induced DC activation. Nevertheless, main effect of IL-10 is the suppression of IL-12 production (Corinti 2001) by DCs, which we found in ELISA not to be impaired by IL-21, suggesting that other mechanisms are involved. Very interesting to that point was a hint that similar observations had been found for B cells, where IL-21 prevented LPS-induced cell proliferation. However, also in this report, the underlying mechanisms remained to be clarified (Parrish-Novak 2002). Our findings, that IL-21 inhibits DC activation, are further supported by observations in the human system. There, DCs were also abrogated in their maturation upon LPS activation when IL-21 was present (Musso, manuscript submitted).

In summary, our data provide first evidences that IL-21 is an important negative regulator of DC activation in response to LPS. In contrast IL-15 did not further enhance activation, which was most probably due to the fact that LPS already induces a maximal response in DCs.

Effects of IL-15 and IL-21 on activation of DCs *in vivo*

The CHS model provides a unique approach to assess DC induced T cell priming *in vivo* (Grabbe 1998). The outcome of the T cell mediated CHS response directly reflects the activation status of the DCs.

In accordance to our *in vitro* assays, we found that IL-15DCs induced a significantly increased CHS response. The same number of IL-21DCs failed to induce any CHS above unsensitized control mice, in line with our *in vitro* results. To exclude that the inability of IL-21DCs to induce a CHS response was due to reduced migration capacity we examined the local draining lymph nodes and found IL-21DCs homing to same extends compared to DCs and IL-15DCs. Therefore the lack of T cell stimulatory capacity by IL-21DCs is most likely attributed to their immature status with reduced MHCII expression and not due to a defect in homing to DLN. Interestingly even after a second challenge two weeks later, IL-21DCs injected mice were not able to mount a CHS response. This result argues that, IL-21DCs not only failed to undergo maturation after antigen uptake and migration to DLN but also were unable to establish a memory T cell response *in vivo*.

On the other side, IL-15 was reported to induce DC activation after short time contact (Ohteki 1999; Mattei 2001). We here present evidences that during generation of DCs, permanently given IL-15 induced high potent APCs with regard to T cell stimulatory capacities. Since IL-15 is produced *in vivo* by BM and lymph node stromal cells (Grabstein 1994), the presence of this cytokine in the extracellular matrix may shape DC phenotype during hematopoiesis and DC function during a variety of immune responses, which we discuss in detail below.

However, contrasting effects of IL-21 versus IL-15 on DCs are probably due to their unique private receptor α -chains that complete the IL-15R $\alpha\beta\gamma$ and IL-21R complexes and thereby allow differential responsiveness depending on the ligand and high affinity receptor expressed as already mentioned. Blocking of IL-21 and its private receptor and the use of IL-21/IL-21R deficient mice could give more insight into the mechanisms, which keep DCs controlled in an immature state. Moreover it is reasonable to speculate that T cells (either of naïve or memory phenotype) release IL-21 during or after contact with DCs, thereby providing a negative feedback signal keeping DCs in an immature state. Subsequently antigen presentation by

these immature DCs might induce differentiation of naïve T cells toward suppressor/regulatory phenotype (Roncarolo 2001; Jonuleit 2001).

Preliminary *in vivo* experiments revealed, that injection of IL-21 cytokine in normal mice could significantly down-regulate the expression of co-stimulatory molecules like CD80, CD86 and MHCII up to 50% in splenic DCs (not shown). The tolerogenicity of immature (IL-21)DCs could lead to therapeutical applications for instance in prolonging allograft survival (Fu 1996). Vice versa IL-15DCs might serve as potent immune stimulating DCs and possibly provide new or expanded properties for immunotherapy based on the injection of antigen-pulsed DCs (Celluzzi 1996; Porgador 1996).

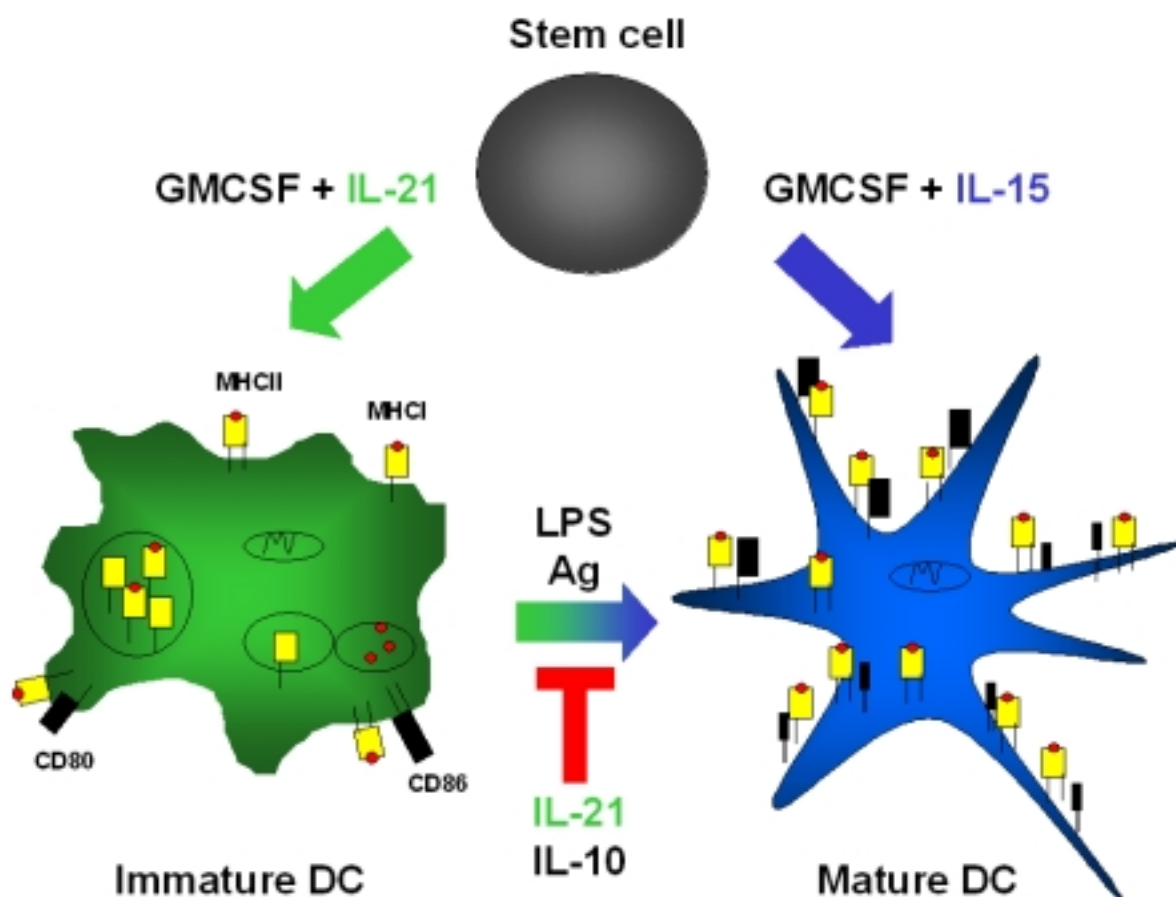


Figure A. Schematic model of IL-15 and IL-21 effects on DC generation and maturation.

In conclusion, we found, that despite structural similarities, IL-15 and IL-21 have completely opposite functional consequences for DC biology including maturation and antigen presentation *in vitro* and *in vivo*. Our results indicate that IL-21 release may lead to inhibition of activation and co-stimulatory signals for DCs therefore inducing immature, low immunogenic DCs whereas IL-15 induces DCs with high immunogenic functions (**Fig. A**).

DC-derived IL-15 is essential for the induction of Th1 immune responses

From the above described data and in line with recent publications it became evident that IL-15 is a critical player in the activation of DCs. Therefore the role of IL-15 in DC-mediated T cell responses had to be focused more closely. This was done here with the use of IL-15- and IL-15R α - deficient mice and in parallel by blocking the function of IL-15 in wildtype mice with an antagonistic soluble IL-15R α (sIL-15R α , Ruchatz 1998).

Our studies elucidate that IL-15 was absolutely required for the induction of a specific CD8⁺ T cell mediated CHS reaction in mice. Analyzing the DLN revealed, that the elicitation of an inflammatory CHS response is not correlated to the absolute number of CD8⁺ T cells present in the DLN, but clearly depends on their activation status. This deduction was supported by blocking the function of IL-15 with the sIL-15R α in wildtype mice where CHS was abrogated despite normal CD8⁺ T cell numbers. Our results are consistent with the concept that one key function of IL-15 during the initiation of a CHS response is to activate CD8⁺ T cells and to establish CD8⁺/CD44^{high} T memory cells (Kennedy 2000; Lodolce 1998). Vice versa, systemic IL-15 administration, in addition to stimulating a CD8⁺ memory response, also augments the primary CD8⁺ T cell response *in vivo* and drives a Th1-biased phenotype (Rubinstein 2002). In contrast, IL-15 is not necessary for a non-specific, irritant contact dermatitis, mediated mainly by neutrophil granulocytes (Zhang 2000). Despite the fact, that IL-15 stimulates phagocytosis by these cells, our data indicate that tissue infiltration and – inflammation seem to be independent of IL-15.

Nevertheless, it was not excluded that also another mechanism of the adaptive immune response, namely the antibody production by B cells, might be affected by IL-15. To dissect between T and B cell mediated effects of IL-15; a second DTH model to SRBC revealed that IL-15 is essential only for T cell mediated inflammation. This is in line with findings, where IL-15 receptor antagonists attenuated the BSA specific DTH response in mice, and decreased T cell infiltration to the inflammation site (Kim 1998). Additionally, it is interesting to discuss my results in the context of phenotypic properties of IL-15 deficient mice. It is known that the lack of IL-15 does not affect the differentiation of CD4⁺ T cells (Kennedy 2000). This reinforces the notion that IL-15 might be dispensable for B cell activation triggered by CD4⁺ T cells and antibody production in this example of DTH response.

In order to unravel the specific defects of IL-15^{-/-} mice concerning T cell immunity and memory, and to elucidate whether DC-derived IL-15 is the key in the induction of Th1 immune responses like CHS, BMDCs from IL-15^{+/+} or IL-15^{-/-} mice were employed. The failure of IL-15^{-/-} mice to mount CHS or DTH response could be due to diminished Ag uptake and homing of DCs *in vivo*. However, DCs from IL-15^{+/+} and IL-15^{-/-} mice took up antigen in equal manner (*in vivo* and *in vitro*) and migrate to local DLN with comparable efficiency, indicating that IL-15 is not essential in modulating these important steps in mounting a CHS response. We previously published supporting findings, describing that injection of IL-15 in mice also had no effect on the migratory capacity of DCs (Rückert 2002). Also, previous works did not indicate a defect of DCs in Ag uptake and migration in the absence of IL-15 or its high-affinity receptor (Kennedy 2000; Lodolce 1998).

After Ag uptake, DCs reach the DLN, present the processed Ag to naïve T cells, which then become activated and are induced, to differentiate into memory T cells. We tested the ability of wildtype and IL-15^{-/-} DCs to stimulate proliferation and induce memory cell differentiation of CD8⁺ T cells *in vitro* and found that IL-15 produced by DCs is absolutely required for both processes. As expected, proliferation of CD4⁺ T cells was not altered in the absence of IL-15.

These findings confirm that IL-15 produced by DCs is the key-player in DC mediated CD8⁺ T cell priming. The underlying cause for the distinct responses of memory CD8⁺ (IL-15 dependent) and CD4⁺ (IL-15 independent) T cells on IL-15 for priming and survival are still unknown. Since both express the IL-15R chains (Chae 1996), it is conceivable that differences in the downstream signaling mediate these effects. It is possible however, that responding T cells were shaped by important regulatory autocrine or paracrine acting cytokines. To assess this directly, we analyzed cytokines from co-cultures of DCs and CD8⁺ T cells. Pro-inflammatory, T cell derived cytokines like IL-2 and IFN- γ were found to be reduced when Ag was presented by IL-15^{-/-} DCs. Several mechanisms may contribute to this: It is likely that DC-derived IL-15 could function in a paracrine manner either released by- or bound to the surface of DCs and therefore directly activates T cells, similar to the paracrine signaling properties of IL-15, which had previously been identified for monocytes (Musso 1999) and keratinocytes (Rückert 2000). If that was the case, T cells from DLN had to be clearly more activated (assessed by surface markers) when Ag was presented by IL-15^{+/+}DCs than by IL-15^{-/-}DCs which we could confirm. Alternatively, DC-derived IL-15 may act in an

autocrine fashion, enhancing their antigen presentation, activation and the expression of co-stimulatory signals for T cells. This is stressed by the fact that IL-15 and its high affinity chain are internalized, co-localized and even can enter the nucleus (Pereno 2000), where NF κ B, a central mediator for inducing cytokine encoding genes, is activated. Resulting autocrine stimulation and cytokine production under the influence of IL-15 has already been noted (Otheki 2001).

Taken together our data provide further evidences that IL-15 is directly and critically involved in fine tuning the DC-cytokine network towards mounting of an efficient, CD8⁺-mediated Th1 immune response.

In order to directly determine to which extent the capacity of DCs for T cell priming and sensitization *in vivo* depends on endogenous, DC-derived IL-15; IL-15^{+/+} and IL-15^{-/-} DCs were labeled *in vitro* with the fluorescent hapten FITC, and injected subcutaneously in the footpad of IL-15^{+/+} mice. Injection of IL-15^{-/-} DCs in wildtype mice completely failed to induce a CHS response comparable to unsensitized controls. Strikingly, when the lack of IL-15 was substituted by incubating IL-15^{-/-} DCs prior to injection with recombinant IL-15, their ability to induce T cell sensitization and subsequent induction of a CHS response was almost restored to the level of IL-15^{+/+} DCs. Using DCs from IL-15R α ^{-/-} mice, which lack the high affinity IL-15R α chain but produce IL-15, could not induce a CHS response above negative control values, either. As expected, loading these IL-15R α ^{-/-} DCs prior to injection with IL-15, did not restore the capacity to prime a T cell response. These data clarify that endogenous IL-15, produced by DCs as well as IL-15R α expression by DCs, are both required for the initiation of an efficient DTH response *in vivo*.

We speculated that IL-15 could be functional through autocrine and/or paracrine loops but also through intracrine loops, as has previously been reported for IL-1, IL-5 and IFN- γ (Dubois 1999). We could confirm by confocal microscopy that IL-15 indeed is internalized in DCs by binding to its high affinity IL-15R α chain and intracellular co-localized, therefore might act most likely through an intracrine loop. Indeed IL-15 and its receptor α -chain were already shown to be co-localized intracellular (Pereno 2000). Here we demonstrate for the first time the functional consequence of this co-localization for induction of T cell differentiation (**Fig. B**). Future studies will have to address, to which extent IL-15- (Meazza 1996; Onu 1997) and IL-15R α -isoforms (Dubois 1999) show differences in ligand/receptor

complexing in DCs, and which of these isoforms are differentially secreted/expressed by DCs. However, it is already evident that not only the secretion of IL-15 or membrane-bound IL-15 signaling (Musso 1999) may induce paracrine IL-15R α signaling, but also that IL-15 acts on the same cell in an autocrine fashion. Hence, it is likely, that the observed lack of CD8⁺ memory T cells in IL-15^{-/-} and IL-15R α ^{-/-} mice (Kennedy 2000; Lodolce 1998) is a consequence of missing, DC-induced differentiation of naive T cells due to the lack of these IL-15/IL-15R α interactions.

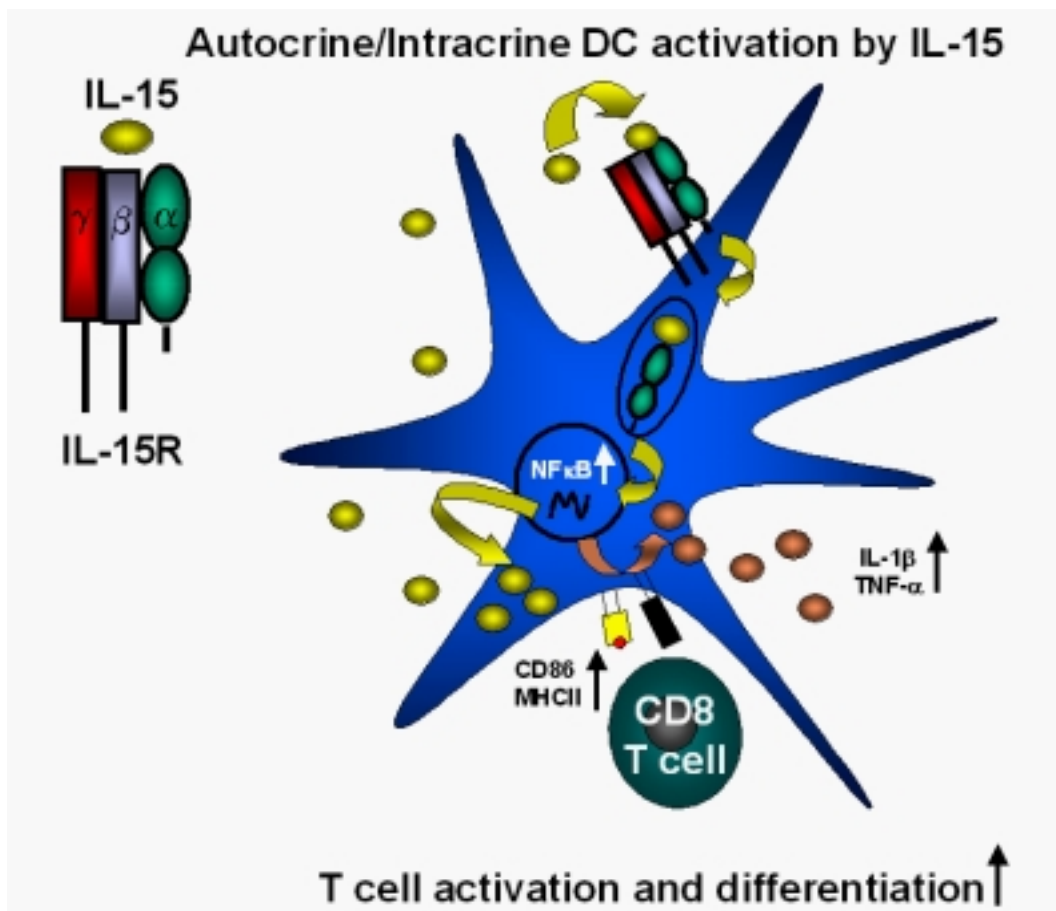


Figure B. A proposed model for autocrine/ intracrine activation of DCs by IL-15.

These data clarify that endogenous IL-15, produced by DCs as well as IL-15R α expression by DCs, are absolutely required for the initiation of an efficient, T cell mediated DTH response *in vivo*. The here presented data give considerable new insights into the mechanisms by which DCs can induce Th1 immune reactions, and allows to manipulate DC-driven T cell responses much more effectively in the future, by selectively targeting the IL-15/IL-15R α signaling.

Taken together, a complex and comprehensive analysis of the two structural related cytokines IL-15 and IL-21 revealed very interesting, although dichotomous effects on generation, maturation and function of dendritic cells. This series of studies offers novel, biological and clinical important perspectives. Based on my data, we got a deeper insight in how adaptive immune responses are shaped by the action of soluble factors on DCs, giving the opportunity to inhibit/support DC function in health and disease. This encourage one to examine, whether and how antagonists as well as agonists of IL-15 and IL-21 can be exploited to control overwhelming immune reactions for therapeutical demands.

Abbreviations

Ag:	antigen
APCs:	antigen-presenting cells
BM:	bone marrow
BMDC:	bone marrow-derived dendritic cells
CHS:	contact hypersensitivity reaction
DCs:	dendritic cells (either <i>in vivo</i> or generated <i>in vitro</i> with GMCSF from BM)
DLN:	draining lymph nodes
DTH:	delayed type hypersensitivity
FITC:	fluorescein-5-isothiocyanate
GMCSF:	granulocytes-monocyte cell stimulating factor
IL:	Interleukin
IL-15 ^{+/+} :	C57BL/6 mice
IL-15 ^{+/+} DC:	dendritic cells generated with GMCSF from C57BL/6 mice
IL-15 ^{-/-} :	IL-15 knockout mice on C57BL/6 background
IL-15 ^{-/-} DC:	dendritic cells generated with GMCSF from IL-15 knock-out mice
IL-15R α ^{-/-} :	IL-15 receptor alpha knockout mice on C57BL/6 background
IL-15R α ^{-/-} DC:	dendritic cells generated with GMCSF from IL-15R α knockout mice
IL-15DCs:	dendritic cells generated with GMCSF + IL-15
IL-21DCs:	dendritic cells generated with GMCSF + IL-21
MF:	macrophages
PCI:	picrylchloride
R:	receptor
SRBC:	sheep red blood cells
LPS:	lipopolysaccharide

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Publications

Paper

- 1.) IL-2-IgG2b fusion protein suppresses murine contact hypersensitivity *in vivo*.
R. Rückert, **K. Brandt**, U. Hofmann, S. Bulfone-Paus, R. Paus. 2002 *J Invest Dermatol* 119:370
- 2.) TSST-1 induces Th1 or Th2 differentiation in naïve CD4⁺ T-cells in a dose and APC dependent manner.
K. Brandt, Jürgen van der Bosch, Ralf Fliegert and Svante Gehring. 2002 *Scand J Immunol*, 56:572
- 3.) Interleukin-21 inhibits dendritic cell activation and maturation.
K. Brandt, S. Bulfone-Paus, R. Rückert, *Submitted for publication*
- 4.) Dendritic cell-derived IL-15 is essential for the induction of Th1 immune responses
K. Brandt*, R. Rückert*, E. Bulanova, F. Mirghomizadeh, R. Paus and S. Bulfone-Paus, (Both Authors contributed equally to this work), *Submitted for publication*
- 5.) Blocking Interleukin-15 inhibits Th2-cell differentiation, antibody production and allergic inflammation in mice.
R. Rückert, U. Herz, T. Pohl, **K. Brandt**, V. Budagian, E. Bulanova, H. Dürkop, H. Renz and S. Bulfone-Paus, *Submitted for publication*
- 6.) Interleukin-15 promotes the survival of naïve- and memory- phenotype CD8⁺ T cells
M. Berard, **K. Brandt**, S. Bulfone-Paus, and D. Tough, *Submitted for publication*
- 7.) Interleukin-21 inhibits CD8⁺ T cell activation and induction of contact hypersensitivity reaction *in vivo*.
K. Brandt, S. Bulfone-Paus, D.C. Foster, R. Paus, R. Rückert, *The Journal of Investigative Dermatology*, *Submitted for publication*

8.) IL-15 mediates activation but not generation of murine macrophages.

K. Brandt, S. Bulfone-Paus, R. Rückert, *In preparation*

Abstracts

1.) An *in vitro* model to study the influence of antigen-presenting cells on human T cell differentiation following superantigen activation.

S. Gehring, **K. Brandt**, and J. van der Bosch

2. Teupitzer Colloquium. Macrophages in Inflammation. Sept. 2.-4. 1999, Teupitz, Berlin. Germany

2.) TSST-1 induces Th1 or Th2 differentiation in naïve CD4⁺ T cells in a dose- and APC-dependent manner.

K. Brandt, J. van der Bosch, and S. Gehring

32. Annual Meeting of the German Society of Immunology, Sept. 26.-29. 2001, Dresden. Germany

Immunobiology 204: 1-2, 98, 2001

3.) Interleukin-21 inhibits dendritic cell activation and maturation.

K. Brandt, S. Bulfone-Paus, and R. Rückert

33. Annual Meeting of the German Society of Immunology. Sept. 25.-28. 2002, Marburg. Germany.

Immunobiology 206: 1-3, 20, 2002

4.) Dendritic cell-derived interleukin-15 is essential for the induction of Th1 immune reactions.

R. Rückert, **K. Brandt**, and S. Bulfone-Paus,

33. Annual Meeting of the German Society of Immunology. Sept. 25.-28. 2002, Marburg. Germany.

Immunobiology 206: 1-3, 28, 2002

5.) Interleukin-21 inhibits dendritic cell activation and maturation.

K. Brandt, S. Bulfone-Paus, and R. Rückert

25. Arbeitstagung der Norddeutschen Immunologen. Nov. 15. 2002, Borstel. Germany.

Talks

1.) Dendritic cell-derived interleukin-15 is essential for the induction of Th1 immune reactions.

K. Brandt, R. Rückert, and S. Bulfone-Paus,

33. Annual Meeting of the German Society of Immunology. Sept. 25.-28. 2002, Marburg. Germany.

Immunobiology 206: 1-3, 28, 2002

2.) Interleukin-21 inhibits dendritic cell activation and maturation.

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25. Arbeitstagung der Norddeutschen Immunologen. Nov. 15. 2002, Borstel. Germany.

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11/1998 – 05/1999

Europäische Projektstudie: „Selection and affinity maturation of recombinant phage antibodies to tumor-associated HLA-antigen/peptide complexes“ im Institut für Pharmakologie unter Betreuung von Professor Jan Engberg an der Pharmazeutischen Hochschule zu Kopenhagen, Dänemark

Promotionsarbeit

07/1999 – 02/2001

Arbeit zum Thema „Die Steuerung der T-Zelldifferenzierung durch Antigen-präsentierende Zellen *in vitro*“ unter der Leitung von Dr. van der Bosch in der Abteilung Experimentelle Immunpharmakologie des Forschungszentrums Borstel

03/2001 – 03/2003

Promotionsarbeit zum Thema „Dendritic Cell mediated immune responses are shaped by IL-15 and IL-21“ unter der Leitung von Prof. Dr. Dr. Bulfone-Paus in der Abteilung Immunbiologie des Forschungszentrums Borstel.

Preis

09/2002

Posterpreis 33rd. Annual Meeting of the German Society of Immunology
Dendritic cell-derived interleukin-15 is essential for the induction of Th1 immune reactions.
K. Brandt, R. Rückert, and S. Bulfone-Paus

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