Interleukin-21 Inhibits Dendritic Cell-Mediated T Cell Activation and Induction of Contact Hypersensitivity *In Vivo*

Katja Brandt, Silvia Bulfone-Paus, André Jenckel, Donald C. Foster,* Ralf Paus,† and René Rückert

Research Center Borstel, Department of Immunology and Cell Biology, Borstel, Germany; *Department of Cytokine Biology, ZymoGenetics, Inc., Seattle, Washington, USA; †Department of Dermatology, University-Hospital Hamburg-Eppendorf, University Hamburg, Hamburg, Germany

Interleukin (IL)-21 is a newly described cytokine that is produced by activated T cells and displays structural homology to IL-4 and IL-15. We here analyzed the role of IL-21 in dendritic cell (DC)-induced, T cell-mediated contact hypersensitivity (CHS) *in vivo* and on T cell activation and unspecific mixed lymphocyte reaction *in vitro*. By PCR, we demonstrate here constitutive expression of the specific IL-21 receptor and the common γ-chain in DC, which together are able to mediate IL-21 signaling. Short-time incubation of *in vitro* generated DC with IL-21 significantly reduced their potential to induce an antigen-specific CD8+ T cell proliferation. Interestingly, 2h incubation of these DC with IL-21 before injection completely inhibited the potential of these DC to induce a CHS reaction to the hapten fluorescein 5-isothiocyanate *in vivo*. Mice injected with IL-21-treated DC even failed to mount a CHS response after repetitive injection of non-IL-21-treated DC 2 weeks later, suggesting that an antigen-specific unresponsiveness can be induced by IL-21-treated DC. Our data demonstrate that IL-21 is a new modulator of DC–T cell interaction with the potential to induce DC-mediated antigen-specific tolerance. *Key words: dendritic cells/cytokines/inflammation/T cells. J Invest Dermatol 121: 1379–1382, 2003*

Dendritic cells (DC) are professional antigen-presenting cells that initiate T cell-mediated immune responses. The capacity of mature DC to prime naïve T cells and promote their differentiation is not only attributed to upregulation of costimulatory and adhesion molecules but also to the ability to secrete cytokines. In contrast, polarizing signals from the microenvironment or from T cells during DC–T cell interaction directly shape the outcome of an immune response during antigen presentation (Jonuleit *et al*, 2001; Guermonprez *et al*, 2002).

One new candidate to modulate DC–T cell interaction is the recently described cytokine interleukin (IL)-21, whose expression is restricted to T cells (Parrish-Novak *et al*, 2000). IL-21 is a member of the four-helix bundle family and has high structural similarities with IL-15. Like IL-15, IL-21 binds to its private high-affinity receptor chain and to the common γ-chain (Asao *et al*, 2001). This functional heterodimeric IL-21 receptor (IL-21R) has been shown to be expressed by T, B, and NK cells so far (Parrish-Novak *et al*, 2000).

These observations raise the possibility that IL-21 is involved in T cell-dependent immune responses mediated by professional antigen-presenting cells like DC. Here we have explored this hypothesis *in vitro*, using coculture of DC with antigen-specific CD8+ T cells and in vivo by active sensitization using hapten-labeled, *in vitro*-generated DC to induce a contact hypersensitivity (CHS) response in mice. Apart from being the experimental counterpart for a variety of dermatologic disorders of great clinical relevance (Grabbe and Schwarz, 1998), the study of CHS responses provides a unique approach to assess multiple functions of DC *in vivo*, where the overall T cell-mediated CHS response represents the net result of both the antigen-presenting and the T cell-activating functions of DC at multiple stages.

To study the effects of IL-21 on DC-mediated induction of T cell priming, we incubated DC with IL-21 before *in vitro*/*in vivo* use. IL-15, which is in contrast to IL-21 produced by DC itself and which mediates their activation (Mattei *et al*, 2001), was used for comparison. We show here that DC express the complete IL-21 receptor and that IL-21 has the potential to inhibit DC-induced antigen-specific, but not unspecific, T cell activation and differentiation *in vitro* and *in vivo*.

MATERIALS AND METHODS

Mice C57BL/6 mice were purchased from Charles River. OTI^{tg} mice, transgenic for a CD8+ restricted TCR-recognizing OVA$^{(257–264)}$ peptide were provided by A. von Bonin (BNI, Hamburg, Germany). All mice were used at 8 to 10 wk and were maintained in pathogen-free conditions. *In vivo* experiments were performed in compliance with national and institutional guidelines.

DC preparation Bone marrow-derived DC were generated as previously described (Lutz *et al*, 1999). On day 8, cells were harvested and analyzed further. Purity was routinely >95% CD11c+ DC as determined by FACS analysis.

RT-PCR Total RNA was extracted from 5×10^6 cells after 8 d of culture using RNAzol (Life Technologies, Gaithersburg, MD) following the manufacturer's instructions. cDNA was synthesized using random hexanucleotide primers and the Superscript preamplification system II (Life Technologies).

Primer sequences Primer sequence were as follows: IL-21R sense 5′-CTCAGCCAGGCACTTCATTCAGG-3′ and IL-21R antisense 5′-

Manuscript received January 16, 2003; revised June 2, 2003; accepted for publication July 6, 2003

Reprint requests to: René Rückert, MD, Research Center Borstel, Department of Immunology and Cell Biology, Parkallee 22, 23845 Borstel, Germany. Email: rrueckert@fz-borstel.de

Abbreviations: CHS, contact hypersensitivity; DC, dendritic cell; FITC, fluorescein 5-isothiocyanate; IL, interleukin; IL-21R, IL-21 receptor.

ATCACAGGAAGGGCATTTAGC-3′; IL-15Rα sense 5′-AACATCCACC CTGATTGAGTGT-3′ and IL-15Rα antisense 5′-GTTTCCATGGTTTCC ACCTCAA-3′; γ_c sense 5′-GTCGACAGAGCAAGCACCATGTTGAAA CTA-3′ and γ_c antisense 5′-GGATCCTGGGATCACAAGATTCTGTAG GTT-3′; and β-actin sense 5′-GTGGGGCGCCCCAGGCACCA-3′ and β-actin antisense 5′-CTCCTTAATGTCACGCACGATTTC-3′ (all from Metabion, Lexington, KY). To exclude contaminations, all experiments were run with a mock PCR. β-Actin message was used to normalize cDNA amount.

Proliferation assays T cells were isolated from lymph nodes (LN) of OTI TCR-transgenic mice. DC were labeled with 2 μg $OVA^{(257–264)}$ peptide and IL-15 or IL-21 (100 ng/mL, Tebu-Bio, Offenbach, Germany; and Zymogenetics, Seattle, WA, respectively) for 2 h at 37°C in 500 μL of RPMI, washed, and plated with six replicates into 96-well plates (Greiner, Longwood, FL) at 1×10^4 cells per well along with increasing amounts of lymph node cells (6.25×10^4–1×10^5). In an additional setup, an anti-IL-21 antibody (5 μg/mL, R&D Systems, Minneapolis, MN) was added to the culture. After 48 h, cells were pulsed for 18 h with 0.2 μCi per well [^{3}H]thymidine, and proliferation was analyzed by liquid scintillation counting (Wallac/Perkin-Elmer, Boston, MA). A mixed leukocyte reaction was set up by incubating the DC from C57BL/6 mice and lymph node cells from BALB/c mice (1×10^4–1×10^6 cells) for 5 d, and proliferation was assessed as above.

CHS to fluorescein 5-isothiocyanate (FITC) To estimate the capacity of DC to induce antigen-specific T cell sensitization *in vivo*, bone marrow-derived DC (1×10^6 cells/mL) from C57BL/6 mice were incubated for 2 h with 20 ng per mL IL-21 (ZymoGenetics) or IL-15 (Tebu-Bio) at 37°C and were labeled during the last 30 min with FITC (Sigma Chemical Co., St. Louis, MO) as described (Macatonia *et al*, 1986). DC were washed and injected in one footpad with a Hamilton syringe (5×10^5 cells in 50 μL phosphate-buffered saline). After 5 d, mice were challenged using 50 μL of FITC in acetone:dibutylphthalate (1:1, Sigma Chemical Co.) (Macatonia *et al*, 1986) on the right ear. The left ear was painted with the diluent as control. In addition, unsensitized mice were painted with FITC. The CHS response was determined by measuring ear swelling at 24 h after challenge using a micrometer (Mitutoyo, Aurora, IL). A second injection of FITC-labeled DC (without any cytokine incubation) was performed 14 d after first injection. Mice were challenged on the left ear 5 d after the second injection (day 19) and swelling was measured 24 h later.

Statistical analysis Results are presented as means ± SD from data of two to three experiments (using a total of 8–12 mice/group). PCR data from one representative experiment are shown. A Student's *t* test for unpaired samples was used for the determination of statistical differences ($^*p \leq 0.05$; $^{**}p \leq 0.01$).

RESULTS AND DISCUSSION

The principal method to generate DC *in vitro* was adapted in our laboratory from a previous publication (Lutz *et al*, 1999) to yield in high amounts of pure, CD11c+ DC. Expression of the IL-21R and the high-affinity IL-15Rα was analyzed by PCR. DC express the complete IL-21R message, which consists of the common γ-chain and a private IL-21R under unstimulated conditions (**Fig 1, *first column***). These products are not upregulated by the strong DC stimulus lipopolysaccharide (**Fig 1, *second column***). As previously reported (Mattei *et al*, 2001), the high-affinity IL-15Rα is expressed weakly in unstimulated DC but is upregulated upon lipopolysaccharide stimulation (**Fig 1**). To validate the biologic function of the IL-21R, we verified the uptake of IL-21 into DC by confocal microscopy after 30 min of incubation with IL-21 (not shown). These data revealed that murine DC express a functional receptor for the newly described cytokine IL-21.

To estimate the function of IL-21 during DC–T cell interaction we first studied its effects on T-cell activation mediated by DC *in vitro*. To this purpose we used OVA peptide-specific, transgenic CD8+ T cells. The effects of IL-21 were compared to the structural homolog IL-15. First, DC were pulsed for 2 h with OVA peptide alone, or additionally IL-15 or IL-21 were added in parallel to OVA peptide. After being washed, T cells from lymph nodes of OTI mice, expressing the CD8+ T cell-restricted, transgenic T cell receptor, which specifically recognizes OVA peptide, were given to the peptide/cytokine-labeled DC.

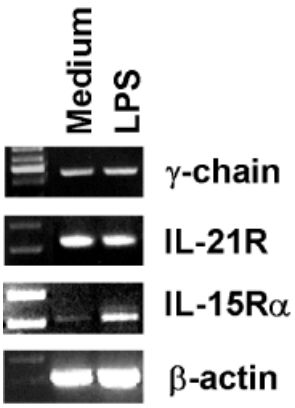

Figure 1. DC express the common γ-chain and high-affinity IL-21/-15 receptor subunits. DC were generated for 8 d and stimulated with 10 ng per mL lipopolysaccharide for 24 h or left unstimulated (medium).

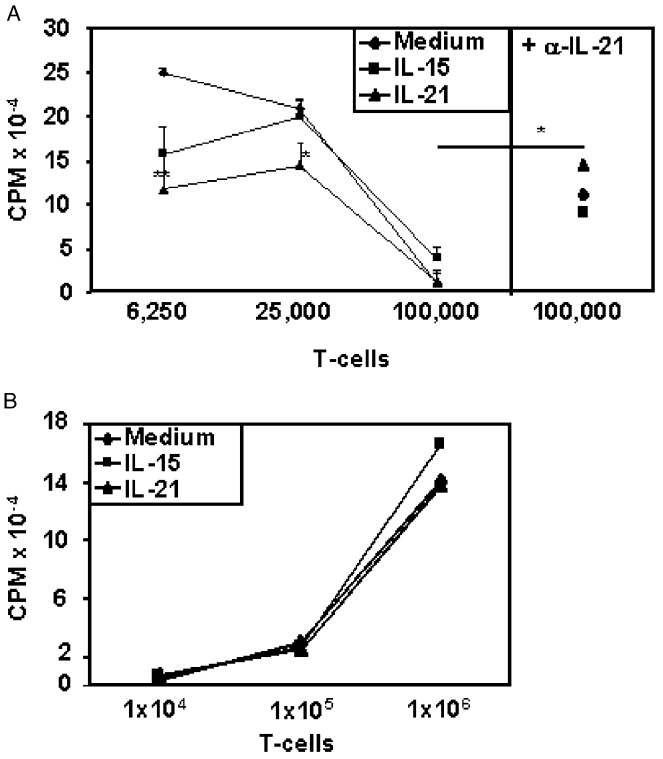

Figure 2. IL-21 reduces antigen-specific CD8+ T cell proliferation but not mixed leukocyte reaction *in vitro*. (*A*) DC were incubated in parallel to $OVA^{(257–264)}$ peptide with IL-15 or IL-21 for 2 h. To neutralize the effects of T cell-secreted IL-21, anti-IL-21 antibodies were added to the culture. Peptide-specific, transgenic T cells were added for an additional 72 h. T cell proliferation was estimated by [^{3}H]thymidine uptake. The experiment was repeated twice with six replicates for each experiment; shown is the median ± SD. Significance was calculated using Student's *t* test ($^{**}p \leq 0.01$; $^*p \leq 0.05$). *CPM*, counts per minute. (*B*) DC generated from C57BL/6 mice were incubated with allogenic T cells from BALB/c mice: DC were labeled with cytokines as described above. After 72 h, proliferation was assessed through [^{3}H]thymidine incorporation.

As depicted in **Fig 2*A***, OVA peptide-labeled DC induced a strong, antigen-specific T cell proliferation. Strikingly only 2 h of DC stimulation with IL-21 high significantly suppressed the DC-mediated CD8+ T cell proliferation (**Fig 2*A***, IL-21). Addition of IL-10, which has recently been identified as an important

inhibitory cytokine for DC activation (Müller *et al*, 2002), could not further suppress T-cell proliferation compared to IL-21 in this assay (not shown). Interestingly, using a high number of T cells (100,000) strongly inhibited the proliferation of T cells under all conditions, probably due to a T cell-secreted factor (**Fig 2*A***). Because T cell-derived IL-21 obviously inhibited the antigen-specific DC-induced T cell proliferation, a blocking anti-IL-21 antibody was added to the culture and could significantly enhance the T cell proliferation in this assay (**Fig 2*A, right panel,* + α *-IL-21***).

This adds new aspects to the role of IL-21. In the human system, it has been shown that IL-21 can enhance proliferation of α-CD3 stimulated T cells and IL-15-stimulated NK cells *in vitro*; nevertheless, it rather inhibits expansion of murine NK cells, suggesting species-specific differences of IL-21 action. Nevertheless, during the complex process of T cell activation by murine DC IL-21 obviously inhibits T cell priming and DC maturation, which could also be observed using human DC (T. Musso and S. Bulfone-Paus, unpublished observations).

In contrast to the antigen-specific reaction, IL-21-preincubated DC did not reduce T cell proliferation after unspecific allorecognition in a mixed leukocyte reaction (**Fig 2*B***). These data support the fact that IL-21 is likely an important regulator of acquired immune responses rather than it could influence innate immune reactions. This notion is also promoted by our findings in which IL-21 also inhibits functions related to antigen presentation in macrophages (K. Brandt and R. Rückert, unpublished data).

These results prompted us to test whether blocking of DC-mediated, specific T cell reactions by short-time addition of IL-21 is also relevant during CHS *in vivo*, using a well-established model of FITC-induced murine CHS response. DC were incubated with IL-15 and IL-21 for 2 h and in parallel labeled with the hapten FITC and injected in the hind footpad of syngenic mice as previously described (Macatonia *et al*, 1986). After 5 d, mice were challenged by applying 50 μL of FITC on the dorsal and ventral sides of the right ear, and ear swelling was measured at indicated time points.

As expected (Macatonia *et al*, 1986), injection of FITC-labeled DC resulted "after FITC-challenge" in a profound ear swelling. Incubation with IL-15 for 2 h failed to further elevate the induction of this T cell-mediated CHS response compared to DC (**Fig 3*A***).

In contrast, IL-21 incubation of the DC for only 2 h before injection completely abrogated the capacity of these DC to induce an antigen-specific type IV immune response *in vivo*, and the FITC-induced ear swelling did not even exceed that of the non-immunized controls (**Fig 3*A***). To confirm the fact that the sensitization was based on active migration of the injected, viable DC from the footpad to the draining lymph nodes, control mice were injected with FITC-labeled glutaraldehyde fixed (i.e., dead) DC (Eggert *et al*, 1999), which failed to induce any CHS response (not shown). Therefore, the absent T cell stimulatory capacity of IL-21-treated DC is most likely attributed to their low immunogenic phenotype and not due to a homing defect (i.e., migration to the draining lymph nodes). To exclude the fact that the DC simply undergo cell death after IL-21 stimulation, we incubated them with IL-21 from 1 to 200 ng per mL and analyzed apoptosis by annexin V/propidium iodide staining using FACS. Nevertheless, neither IL-15 nor IL-21 induced apoptosis above the levels of DC cultured in medium (data not shown). Cells were also evaluated by morphologic criteria using microscopy and FACS, revealing no differences after short-time incubation with IL-21. These effects and the intact migration of DC to dendritic lymph node cells suggest that IL-21 mediates a specific, suppressive effect on DC-induced antigen-specific immune reactions rather than leading to a "null event."

To further analyze whether IL-21-treated DC induce a state of antigen-specific unresponsiveness, mice that had been injected with the cytokine-primed (DC + IL-15; DC + IL-21), FITC-labeled DC were injected 14 d later again with FITC-labeled, but not cytokine-incubated, DC. Interestingly, injecting DC in previous DC + IL-21-sensitized mice failed to induce an ear swelling above control levels (**Fig 3*B***). In contrast, mice that had been treated with DC + IL-15 and 14 d later with DC, mounted a significantly enhanced ear swelling compared to mice sensitized twice with DC.

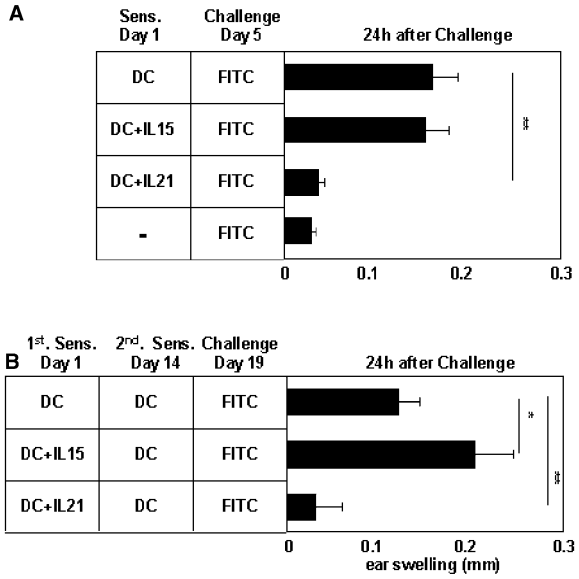

Figure 3. Short-time IL-21 labeling blocks CHS induction by DC. (*A*) DC were incubated with IL-15 and IL-21 for 2 h and in parallel labeled with the hapten FITC. A total of 5×10^5 DC were injected in the hind footpad of C57BL/6 mice (day 1). Five days later, mice were challenged on the right ear, and swelling was measured after 24 h. As control, one ear was painted with diluent and unsensitized mice were challenged with FITC. Significance was calculated between DC and DC + IL-21 using Student's *t* test ($^{**}p \leq 0.01$). (*B*) A second injection of FITC-labeled DC was performed at day 14 after first injection. Mice were challenged on the left ear 5 d after the second injection (day 19) and swelling was measured 24 h later.

Taken together, our data imply that mice sensitized with IL-21-labeled DC are unable to establish antigen-specific responses. Whether this is due to the induction of regulatory T cells or to the inability of IL-21-treated DC to instruct memory T cells has not been clear until now. In this regard, previous reports pointed out that IL-21 inhibits the IL-15-mediated proliferation of murine CD8+ memory T cells and the subsequent upregulation of cytokine receptors for IL-2, IL-15, and IFN-γ (Parrish-Novak *et al*, 2002), which could also be an explanation for the suppressive effects of IL-21 in our experiments. It is also reasonable to speculate that from T cells released IL-21 (during or after contact with antigen-presenting DC) may inhibit activation and expression of costimulatory signals, thereby providing a negative feedback signal keeping DC in an immature, low immunogenic state. Subsequently antigen presentation by these immature DC might induce differentiation of naïve T cells toward suppressor/regulatory phenotype.

Other cytokines in the extracellular environment, notably from T cell-released IL-10, have been implicated in impeding DC maturation, resulting in the retention of an "immature" phenotype, which was recently demonstrated also for DC-mediated *in vivo* reactions (Müller *et al*, 2002). Thus, we show here for the first time that the newly described cytokine IL-21 has comparable inhibitory effects on DC-induced T-cell activation like IL-10. This makes IL-21R-mediated signaling an intriguing novel target for the pharmacologic manipulation of clinically relevant CHS responses.

Taken together we showed for the first time that short-time incubation of DC with IL-21 was able to abrogate antigen-specific T cell responses *in vitro* and *in vivo*.

REFERENCES

Asao H, Okuyama C, Kumaki S, Ishii N, Tsuchiya S, Foster D, Sugamura K: Cutting edge: The common gamma-chain is an indispensable subunit of the IL-21 receptor complex. *J Immunol* 167:1–5, 2001

Eggert AA, Schreurs MW, Boerman OC, *et al*: Biodistribution and vaccine efficiency of murine dendritic cells are dependent on the route of administration. *Cancer Res* 59:3340–3345, 1999

Grabbe S, Schwarz T: Immunoregulatory mechanisms involved in elicitation of allergic contact hypersensitivity. *Immunol Today* 19:37–44, 1998

Guermonprez P, Valladeau J, Zitvogel L, Thery C, Amigorena S: Antigen presentation and T cell stimulation by dendritic cells. *Annu Rev Immunol* 20:621–667, 2002

Jonuleit H, Schmitt E, Steinbrink K, Enk AH: Dendritic cells as a tool to induce anergic and regulatory T cells. *Trends Immunol* 22:394–400, 2001

Lutz MB, Kukutsch N, Ogilvie AL, Rossner S, Koch F, Romani N, Schuler G: An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods* 223:77–92, 1999

Macatonia SE, Edwards AJ, Knight SC: Dendritic cells and the initiation of contact sensitivity to fluorescein isothiocyanate. *Immunology* 59:509–514, 1986

Mattei F, Schiavoni G, Belardelli F, Tough DF: IL-15 is expressed by dendritic cells in response to type I IFN, double-stranded RNA, or lipopolysaccharide and promotes dendritic cell activation. *J Immunol* 167:1179–1187, 2001

Müller G, Müller A, Tüting T, *et al*: Interleukin-10-treated dendritic cells modulate immune responses of naive and sensitized T cells *in vivo*. 119:836–841, 2002

Parrish-Novak J, Dillon SR, Nelson A, *et al*: Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. *Nature* 408:57–63, 2000

Parrish-Novak J, Foster D, Holly RD, Clegg C: IL-21 and the IL-21 receptor: Novel effects of NK and T cell responses. *J Leukoc Biol* 72:856–863, 2002