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In Vivo and In Vitro Roles of IL-21 in Inflammation

Martin Pelletier, Amélie Bouchard, and Denis Girard

IL-21 is a cytokine known to mediate its biological activity via the IL-21R, composed of a specific chain, IL-21Rα, and the common γ-chain (CD132). Recent data suggest that IL-21 possesses proinflammatory properties. However, there is no clear evidence that IL-21 induces inflammation in vivo and, curiously, the interaction between IL-21 and neutrophils has never been investigated, despite the fact that these cells express CD132 and respond to other CD132-dependent cytokines involved in inflammatory disorders. Using the murine air pouch model, we found that IL-21 induced inflammation in vivo, based on recruitment of neutrophil and monocyte populations. In contrast to LPS, administration of IL-21 into the air pouch did not significantly increase the concentration of IL-6, CCL5, CCL3, and CXCL2. We demonstrated that HL-60 cells expressed IL-21Rα, which is downregulated during their differentiation toward neutrophils, and that IL-21Rα is not detected in neutrophils. Concomitant with this, IL-21 induced Erk-1/2 phosphorylation in HL-60 cells, but not in neutrophils. To eliminate the possibility that IL-21 could activate neutrophils even in the absence of IL-21Rα, we demonstrated that IL-21 did not modulate several neutrophil functions. IL-21-induced Erk-1/2 phosphorylation was not associated with proliferation or differentiation of HL-60 toward neutrophils, monocytes, or macrophages. IL-21Rα was detected in human monocytes and monocyte-derived macrophages, but IL-21 increased CXCL8 production only in monocyte-derived macrophages. We conclude that IL-21 is a proinflammatory cytokine, but not a neutrophil agonist. We propose that IL-21 attracts neutrophils indirectly in vivo via a mechanism independent of IL-6, CCL3, CCL5, and CXCL2 production. The Journal of Immunology, 2004, 173: 7521–7530.

Interleukin-21 is a recently identified cytokine known to mediate its biological activity through the IL-21R composed of a specific chain, IL-21Rα, and the common γ-chain (CD132) shared by receptors to IL-2, IL-4, IL-7, IL-9, and IL-15. IL-21Rα is a member of the type I cytokine receptor superfamily that has the highest amino acid sequence similarity to IL-2/15Rβ (CD122) and IL-4Rα (CD124). Northern blot analysis revealed IL-21Rα transcripts mainly in lymphoid tissues (1), and also in PHA-activated PBMCs (2). Western blot analysis confirmed that IL-21Rα is expressed in resting lymphoid cells, activated PBMCs, and in some cell lines of T, B, and NK origins. The γc has also been demonstrated to be an indispensable subunit of the functional IL-21R complex (3, 4).

IL-21 has close structural similarities to IL-2, IL-4, and IL-15, and is known to regulate proliferation of mature T and B cells in response to activating stimuli. IL-21 mediates NK cell expansion from bone marrow (1), and inhibits dendritic cell activation and maturation (5). It was also found to regulate Ig production from B cells (6) and to prevent Ag-induced IgE production of IL-4-stimulated B cells (7). IL-21 was also shown to be a growth and survival factor for human myeloma cells (8) and to display antitumor activity (9). Recently, Wang et al. (10) demonstrated that IL-21 possesses antitumor activity in vivo, mediated by NK cells, suggesting that this cytokine may have therapeutic potential.

As with other CD132-dependent cytokines, IL-21 was found to activate the Jak/STAT pathway. In particular, IL-21 activates Jak-1 and 3 and STAT1, STAT3, STAT5 in different human T cell and B cell lines and in YT NK cells (2–4). In addition to the Jak/STAT pathway, IL-21 was found to activate the MAPK pathway, because it induces phosphorylation of Erk1/2 (p44/42 MAPK), at least in myeloma cells (8).

An increasing number of studies have indicated that human neutrophils can be activated by several CD132-dependent cytokines and to express several, but not all, of the corresponding receptor components (see Ref. 11 for a review). Interestingly, human neutrophils were found to express γc (CD132; Ref. 12), IL-2/15Rβ (CD122; Ref. 13), IL-4Rα (CD124; Ref. 14), and IL-15Rα (15) on their surface, but not IL-2Rα (CD25; Ref. 13), IL-7Rα (CD127; Ref. 16), and IL-9Rα (15). Neutrophil expression of IL-21Rα is not yet known.

Some data suggest that IL-21 is a proinflammatory cytokine. Mice overexpressing IL-21 have shown inflammatory infiltrates in different tissues consisting of macrophages and neutrophils (17). Furthermore, administration of IL-21 plasmid DNA into mice producing high levels of circulating IL-21 in vivo resulted in altered populations of immune cells in the spleen and in peripheral blood (10). Interestingly, the percentages of CD11b and Gr-1 stained cells were increased in IL-21-expressing mice (10). TS/A murine mammary adenocarcinoma cells genetically modified to secrete IL-21 (TS/A-IL-21) and develop small tumors were rejected when injected in syngeneic mice (18). Interestingly, 5 days after injection, TS/A-IL-21 tumors showed infiltrating cells, including granulocytes and macrophages.

The above observations prompted us to study potential proinflammatory effects of IL-21 in vivo as well as to investigate the interaction of IL-21 with human neutrophils and PBMC. Using the murine air pouch model, we found that IL-21 induced an accumulation of neutrophils and monocytes.
neutrophil and mononuclear cells. In vitro studies indicated that IL-21 had no direct effect on several neutrophil functions, but activated immature promyelocytic HL-60 cells based on phosphorylation of Erk-1/2. Interestingly, we found that neutrophils do not express IL-21Ra, whereas this component receptor was expressed in HL-60 cells but was down-regulated following maturation toward a neutrophil-like phenotype, as induced by DMSO. IL-21 did not promote cell differentiation of HL-60 cells toward neutrophils, monocytes, or macrophages. However, we found that IL-21 induced monocyte-derived macrophages to secrete CXCL8, a potent neutrophil chemotactant, suggesting that macrophages may play a role in the recruitment of neutrophils in vivo.

Materials and Methods

Chemicals, agonists, and Abs

Recombinant human (rh) IL-21 was kindly provided by D. C. Foster from ZymoGenetics (Seattle, WA). Recombinant murine IL-21 was purchased from R&D Systems (Minneapolis, MN), rhGM-CSF and rHL-15 were purchased from PeproTech (Rocky Hill, NJ). The monoclonal anti-actin clone AC-40 from mouse ascites fluid (mouse IgG2a), the monoclonal anti-Bcl-2 clone Bcl-2-100 from mouse ascites fluid (mouse IgG1), the monoclonal anti-CD14 clone UCHM-1 FITC conjugate (mouse IgG2a), and the monoclonal anti-human CD11b clone 44 (mouse IgG1) were purchased from Sigma-Aldrich (St. Louis, MO). The monoclonal anti-human IL-21R clone 152504 (mouse IgG1) was from R&D Systems. The FITC-rat anti-mouse F4/80 Ag (rat IgG2b), the FITC-rat anti-mouse neutrophil clone 7/4 (rat IgG2a), and the FITC-rat IgG2a negative control were from Serotec (Raleigh, NC). The FITC-conjugated goat anti-mouse IgG and HRF-conjugated goat anti-mouse IgG were from Jackson ImmunoResearch Laboratories (Mississauga, Ontario, Canada). FITC-Annexin V was purchased from BioSource International (Camarillo, CA) and FITC-mouse anti-human CD16 mAb was purchased from BD Pharmingen (Mississauga, Ontario, Canada). LPS, propidium iodide (PI), nitro blue tetrazolium (NBT), PHA-P, purified human IgG, and 1α,25-dihydroxyvitamin D3 (Vit D3), FITC, and PI were purchased from Sigma-Aldrich.

Air pouch experiments

CD1 mice (6- to 8-wk- old) were obtained from Charles River Laboratories (St. Constant, Canada). The mice (≥5/group) were anesthetized with isoflurane and sterilized air (3 ml filtered through a 0.2-μm Millipore filter; Billerica, MA) was injected s.c. in the back using a 26-gauge needle to make an air pouch on days 0 and 3. On day 6, 1 ml of IL-21, LPS (positive control), or the diluent (PBS) was injected into the air pouches of mice 3, 6, or 9, or 24 h before the mice were killed by CO2 asphyxiation (19). The air pouches were washed once with 1 ml and then twice with 2 ml of HBSS containing 10 mM EDTA, and the exudates were centrifuged at 100 × g for 10 min at room temperature. Supernatants were collected and stored at −20°C for further analysis. The cells were resuspended in 1 ml of HBSS-EDTA and counted. The cells (2 × 100 6) were centrifuged, spread on microscope slides, and stained with Hema-Stain to allow quantification of monocyte/macrophages. Human neutrophils and monocytes were identified by morphology and by positive anti-CD16, human CD16 mAb for 30 min at 4°C (light protected) directed against monocyte/macrophages. Analysis was performed using a FACScan (BD Biosciences, San Jose, CA).

Detection of murine IL-6, CCL3, CCL5, and CXCL2

Fluids were harvested from air pouches after 3 and 6 h of treatment with buffer, LPS (1 μg/ml), or IL-21 (100 ng/ml). IL-6, CCL3, CCL5, and CXCL2 were quantified using the following commercially available ELISA kits according to the manufacturer’s recommendations: murine IL-6 (sensitivity of <3 pg/ml; BioSource International), murine CCL3, CCL5, and CXCL2 (sensitivity of <2, <1.5, and <1.5 pg/ml, respectively; R&D Systems). All samples were tested at least in duplicate.

Human neutrophil and PBMC isolation, monocytes, and monocyte-derived macrophages

Neutrophils were isolated from venous blood of healthy volunteers by centrifugation over Ficoll-Paque (Amersham Pharmacia Biotech, Baie d’Urfe, Quebec, Canada) as previously described (19, 20). Blood donations were obtained from informed and consenting individuals according to institutionally approved procedures. Cell viability was monitored by trypan blue exclusion, and the purity (>98%) was verified by cytology from cytocentrifuged preparations colored by the Hema-Stain staining kit. Human PBMC were harvested during neutrophil isolation. PBMCs were also incubated in RPMI 1640–10% autologous serum for 2 h at 37°C in plastic dishes and adherent cells were identified as monocytes, based on positive α-naphyl acetate esterase tests (Sigma Diagnostics, St. Louis, MO). Monocytes were further incubated for 7 days in RPMI 1640–10% FCS supplemented with 2 ng/ml GM-CSF to obtain monocyte-derived macrophages (21).

HL-60 cell differentiation

HL-60 cells (American Type Culture Collection, Manassas, VA) were treated with 1.25% (v/v) DMSO or with 100 nM 1,25-(OH)2 vitamin D3 for 6 days for differentiation toward neutrophil-like or monocyte-like cells, respectively, or with 10 nM PMA for 3 days for differentiation toward macrophage-like cells. Differentiation was evaluated by morphology, by cell cycle analysis using PI and by the NBT assay (22). Cell surface expression of CD11b (2 μl/tube) and CD14 (2 μl/tube) were performed by flow cytometric analysis (10,000 events) using a FACScan (BD Biosciences).

Cell cycle analysis using PI

Cells were harvested and washed twice with PBS. Cells were then fixed in cold 70% ethanol for at least 30 min at 4°C. Cells were washed twice with PBS and treated with 100 μg/ml RNase. PI (50 μg/ml) was added and cells were analyzed by flow cytometry.

Immunoblotting

Cells were lysed in 2× Laemmli’s sample buffer and aliquots corresponding to 0.5 × 106 cells (from 0.5 to 2 × 106 cells for neutrophils) were loaded onto 10% SDS-PAGE and transferred to gel polyvinylidene difluoride membranes. Nonspecific sites were blocked with 5% nonfat dry milk (Carnation, Don Mills, Ontario, Canada) in TBS-Tween (25 mM Tris-HCl, pH 7.8, 190 mM NaCl, 0.15% Tween 20) for 1 h at room temperature. Membranes were then washed with TBS-Tween and incubated overnight at 4°C with mouse anti-IL-21R at 1 μg/ml in TBS-Tween. Membranes were then washed with TBS-Tween and incubated for 1 h at room temperature with a goat anti-mouse HRP secondary Ab (Jackson ImmunoResearch Laboratories) at 1/20,000 in TBS-Tween + 5% nonfat dry milk, followed by washes. Membranes were revealed with ECL. Membranes were stripped and re-probed with a mouse anti-IL-21R Ab (Jackson ImmunoResearch Laboratories) at 1/20,000 in TBS-Tween + 5% nonfat dry milk, followed by washes. Membranes were revealed with ECL. In other experiments, immunoblotting was performed with the same Abs. Actin and/or Bl-2 expression was monitored in parallel using specific Abs.

Assessment of neutrophil apoptosis by cytology, CD16 expression, and annexin V

Freshly isolated human neutrophils (100 μl of a 10 × 106 cells/ml suspension in RPMI 1640 supplemented with 10% autologous serum) were incubated for 24 h in the presence or absence of increasing concentrations of IL-21, and apoptosis was evaluated by cytology as previously described (19). The results were expressed as the percentage of neutrophils in apoptosis.

CD16 expression and annexin V were used to confirm the inability of IL-21 to modulate the neutrophil apoptotic rate. After 24 h of incubation in the presence or absence of IL-21, the cells were suspended at concentrations of 1.5 × 105/ml, washed, and preincubated for 30 min at 4°C (light protected) with 2 μl of FITC-Annexin V for 15 min at room temperature (light protected) before FACS analysis. Flow cytometric analysis (10,000 events) was performed using a FACScan (BD Biosciences).
O$_2^-$ production

O$_2^-$ production was performed by colorimetric assay (reduction of cytochrome c), as previously published (20). For the priming experiments, cells were incubated for 30 min with IL-21 or 65 ng/ml GM-CSF (positive control) as above, followed by a 5-min incubation with 10$^{-7}$ M fMLP. The absorbance of cytochrome c was monitored at 550 nm and the number of O$_2^-$ anions produced was calculated by the difference between corresponding wells with or without O$_2^-$ dismutase using an extinction coefficient of 21.1.

Phagocytosis of sheep erythrocytes

Sheep RBC (SRBC) were opsonized with a final 1:200 dilution of rabbit IgG anti-SRBCs Ab (Sigma-Aldrich) followed by incubation for 45 min at 37°C as previously described (19). Neutrophils (10$^6$ cells/ml in RPMI 1640) pretreated 30 min with buffer or IL-21, were incubated with 50 $\times$ 10$^6$ opsonized SRBCs for 45 min as above. The pollutant toxaphene was used a positive control in this assay (23). The samples were centrifuged at 200 $\times$ g at 4°C for 10 min. Supernatants were discarded and, to eliminate noningested SRBCs, osmotic shock was performed on the pellets by treating them with 300 $\mu$l of H$_2$O for 20 s followed immediately by the addition of 4.5 ml of ice-cold PBS (1×). The samples were washed twice with ice-cold PBS and the final pellets were suspended to a final concentration of 10$^6$ cells/ml. Duplicate cytocrifuged preparations were prepared in aliquots of .200 $\mu$l and processed essentially as previously documented (19, 21). Phagocytosis was measured as percentage of neutrophils ingesting at least one opsonized SRBC.

Chemotaxis

In vitro chemotaxis was performed in a 48-well Microchemotaxis chamber (NeuroProbe, Gaithersburg, MD) using a 3-$\mu$m polycarbonate membrane filter as previously described (24, 25). CXCL8 was used as a positive control (26).

Production of human CXCL8 and IL-1$\beta$

Freshly isolated human neutrophils (10 $\times$ 10$^6$/ml in RPMI 1640 supplemented with 5% FCS) were incubated with buffer, GM-CSF (65 ng/ml), or increasing concentrations of IL-21 (100, 250, or 500 ng/ml) for 24 h. The concentration of CXCL8 and IL-1$\beta$ released into the external milieu were quantified by commercial ELISA kits (BioSource International; sensitivity of .5 and <1 pg/ml, respectively) according to the manufacturer’s instructions. All samples were tested at least in duplicate.

Results

IL-21 induces an inflammatory response in vivo

We decided to use the murine air pouch model to evaluate the potential proinflammatory effect of IL-21 in vivo. Increasing concentrations of murine IL-21 were injected into the air pouch and the number of infiltrated cells was quantified after 6 h, an optimal condition in this model (27). As a positive control, we used LPS-induced pouches, a condition known to attract several millions of cells into the air pouch. The maximum number of cells attracted by IL-21 (2.4 ± 0.5 $\times$ 10$^6$ cells/pouch) was observed at a concentration of 100 ng/ml IL-21. As expected, LPS caused a significant accumulation of cells with an average number of 6.6 ± 0.9 $\times$ 10$^6$ cells/pouch. Cytocentrifuged preparations were performed to characterize the leukocyte subpopulations migrating into the air pouch (Fig. 1B). When compared with LPS, IL-21 attracted a higher proportion of mononuclear cells in addition to neutrophils. We identified ~12% of mononuclear cells in response to LPS, whereas this number increases to ~35% in response to IL-21. The mononuclear cells observed by cytology were mainly of the monocytic lineage, as confirmed by the α-naphthyl acetate esterase enzymatic test (Fig. 1B) and by the binding of the F4/80 Ab by flow cytometry (Fig. 1C).

Knowing that IL-21 attracted a significant number of cells other than neutrophils, we performed kinetic experiments to answer whether or not these cells arrive before or after neutrophils. As illustrated in Fig. 2A, the injection of buffer, LPS, or IL-21 into the air pouches did not increase significantly the total number of cells collected after 3 h. Moreover, the cell types found in the collected fluids did not significantly differ between the three groups, although granulocytes already formed the majority of inflammatory
cells identified (Fig. 2, B and C). Again, the number of cells was significantly increased after 6 h of treatment with 100 ng/ml IL-21. The response started to decline after 9 h, whether cells were attracted by IL-21 or LPS. More importantly, the inflammatory response induced by IL-21 or by LPS was transient, and was completely resolved after 24 h, because neither the neutrophils nor the mononuclear cells persist following the treatment.

**IL-21-induced cell attraction in vivo is not linked to CCL3, CCL5, CXCL2, or IL-6 production**

The production of chemokines by neutrophils or by other cells, including those already present at the injection site at the time of stimulation, could explain the recruitment of neutrophils and mononuclear cells by IL-21 in vivo. We verified the presence of increased protein expression of three important chemotactic factors, namely CCL3, CCL5, and CXCL2 and the cytokine IL-6 by ELISA. We selected this latter cytokine because it was found to be increased by LPS in some similar circumstance (30, 31). As illustrated in Fig. 3, IL-21 treatment did not increase the production of the three chemokines and IL-6 but, as expected, LPS increased significantly the production of CCL5, CCL3, CXCL2, and IL-6. These results indicate that the presence of neutrophils and mononuclear cells in IL-21-stimulated mice is not due to local production of CCL3, CCL5, CXCL2, or IL-6. In two separate experiments, we have verified the ability of the different collected air pouch fluids (n ≥ 3 animals/group) to induce cell attraction in vivo and we found that none of the collected fluids attracted cells in vivo (data not shown).

**Human PBMC express IL-21Rα but not mature neutrophils**

Because we found that IL-21 induced neutrophil and monocyte-macrophage cell attraction in vivo, we reasoned that these cells could be important targets of this cytokine. IL-21 is known to act via its receptor, composed of the specific chain IL-21Rα and γc. Although γc is known to be expressed in these cells, the presence of IL-21Rα in neutrophils, unlike PHA-P-induced PBMC (2), is unknown. As illustrated in Fig. 4A, IL-21Rα was detected by immunoblotting in PHA-P-induced PBMC after 24–72 h, but not in naive PBMC cells. IL-21Rα was not detected in freshly isolated human neutrophils, even if we increased cell loading up to 2 × 10^6 cells/lane (note the corresponding increased quantity of actin). We next try to detect IL-21Rα in 24 h-aged neutrophils that were incubated or not with known neutrophil agonists such as fMLP, LPS, IL-15, PMA, or with IL-21. As illustrated in Fig. 4B, IL-21Rα was not detected under these conditions, but as expected, IL-21Rα was expressed in PHA-P-induced PBMC.
**FIGURE 4.** Human neutrophils do not express the IL-21Rα. Freshly isolated neutrophils or PHA-P-stimulated PBMC (A) or neutrophils stimulated for 24 h with the indicated agonists (B), were lysed, subjected to SDS-PAGE, and immunoblotted with the anti-human IL-21Rα or anti-human actin as described in Materials and Methods. Results are representative of three different experiments. MW, m.w. standards. PHA-P, FMLP, LPS, IL-15, IL-21, and PMA were used at 2 ng/ml, 250 ng/ml, 100 ng/ml, 250 ng/ml, and 10^{-7} M, respectively.

**FIGURE 5.** IL-21 activates Erk-1/2 in promyelocytic HL-60 cells, but not neutrophils: expression of IL-21Rα is down-regulated during differentiation of HL-60 cells toward neutrophils. Unreated or HL-60 treated with 1.25% DMSO (v/v) for the indicated times, and untreated neutrophils (A). GMCSF- or IL-21-induced HL-60 and neutrophil cells (B) were lysed, subjected to SDS-PAGE, and immunoblotted with the respective Abs as described in Materials and Methods. Data shown are representative of at least three different experiments.

**Figure 6.** IL-21 is not a maturation agent for HL-60 cells}

Activation of Erk-1/2 has been implicated in several cellular processes, including cell proliferation and differentiation (37). Because phosphorylation of Erk-1/2 occurs in HL-60 cells following stimulation with IL-21, we verified whether IL-21 could induce the proliferation or differentiation of these cells toward neutrophils, monocytes, or macrophages. HL-60 cells were treated for several days with either 10, 100, or 1000 ng/ml IL-21 or DMSO (neutrophil differentiation), Vit D3 (monocyte differentiation), or PMA (macrophage differentiation), and characteristic markers of proliferation and differentiation were analyzed. As illustrated in Fig. 8A, the cell cycle was not altered with IL-21, as compared with DMSO, Vit D3, or PMA treatment, where the majority of the cells were in the G_{1}/G_{0}, phase and fewer cells were in the S and G_{2}/M phases. Microscopic observations (Fig. 8A, panels 1–5) illustrated HL-60 cells, but its expression decreased over time during differentiation toward neutrophils. The expression of IL-21Rα is not completely abolished after 6 days of differentiation; this is explained by the fact that ~75% of cells had differentiated, according to the NBT assay test (data not shown). The different bands detected by the anti-IL-21Rα Ab can be explained by the five N-linked glycosylation sites present in IL-21Rα, resulting in a number of potential O-linked glycosylation sites leading to appearance of different polypeptides ranging from 60 to 100 kDa (2). In contrast to actin, expression of the anti-apoptotic Bcl-2 protein decreased over time, indicating efficiency of the differentiation process toward neutrophils (32–34). As expected, actin, but not IL-21Rα and Bcl-2, was detected in mature human neutrophils. As with other χ_{c} (CD132)-dependent cytokines, IL-21 has been found to activate the Jak-STAT pathway (2–4). Recently, the MAPK family members Erk 1/2 were also found to be activated by IL-21 in the IL-6-dependent myeloma cell lines (8). We investigated potential activation of Erk-1/2 by IL-21 in HL-60 cells, knowing that these cells express both χ_{c} (35) and IL-21Rα. As illustrated in Fig. 5B, IL-21 induced phosphorylation of Erk-1/2 in HL-60 cells, but not in mature neutrophils. Neutrophils were, however, responsive, because GM-CSF induced phosphorylation of Erk-1/2 (20, 36).

**IL-21 is not a human neutrophil agonist in vitro**

The presence of neutrophils and mononuclear cells in the IL-21-induced air pouch suggested that IL-21 may activate neutrophils and/or mononuclear cells. Although we found that neutrophils do not express IL-21Rα and that IL-21 does not activate Erk-1/2, we decided to investigate potential effects of IL-21 on different neutrophil functions, because we do not know whether neutrophils express any unidentified IL-21-binding component. As illustrated in Fig. 6, IL-21 does not modulate human neutrophil apoptosis as assessed by the FITC-Annexin V binding assay, cell surface expression of CD16, and cytokology. Because this response is measured after 24 h, we decided to study the potential effects of IL-21 on neutrophil responses occurring more rapidly. IL-21 did not induce neutrophil O_{2}^{-} production after 5 (Fig. 7A) or 30 min (Fig. 7B) of stimulation. We verified also whether IL-21 could have a priming effect by increasing the fMLP response. In these experiments, cells were pretreated with IL-21 and then activated with fMLP. As illustrated in Fig. 7C, IL-21, unlike GM-CSF, did not increase the fMLP response. In addition, we demonstrated that IL-21 did not modulate phagocytosis (Fig. 7D), chemotaxis (Fig. 7E), production of CXCL8 (IL-8) (Fig. 7F), and IL-1β (Fig. 7G). In addition, we also verified whether IL-21 modulated RNA synthesis as assessed by [3H]uridine uptake (19). We found that IL-21 did not modulate this response (Fig. 7H). Collectively, these data demonstrated clearly that IL-21 is not a neutrophil agonist in vitro.

**IL-21 is not a maturation agent for HL-60 cells**

Activation of Erk-1/2 has been implicated in several cellular processes, including cell proliferation and differentiation (37). Because phosphorylation of Erk-1/2 occurs in HL-60 cells following stimulation with IL-21, we verified whether IL-21 could induce the proliferation or differentiation of these cells toward neutrophils, monocytes, or macrophages. HL-60 cells were treated for several days with either 10, 100, or 1000 ng/ml IL-21 or DMSO (neutrophil differentiation), Vit D3 (monocyte differentiation), or PMA (macrophage differentiation), and characteristic markers of proliferation and differentiation were analyzed. As illustrated in Fig. 8A, the cell cycle was not altered with IL-21, as compared with DMSO, Vit D3, or PMA treatment, where the majority of the cells were in the G_{1}/G_{0}, phase and fewer cells were in the S and G_{2}/M phases. Microscopic observations (Fig. 8A, panels 1–5) illustrated...
that IL-21, in contrast to DMSO, PMA, and Vit D3, had no effect on HL-60 cells, because cell morphology was identical to that of control cells. For simplicity, only the results obtained with 100 ng/ml IL-21 and after 6 days of treatment are illustrated, because the results were similar with the other conditions.

Changes in cell surface marker expression have been reported during differentiation of HL-60 cells treated with DMSO, Vit D3, and PMA (38). To further demonstrate that IL-21 had no effect on HL-60 cell differentiation, cell surface expression of CD11b and CD14 was analyzed after each treatment. As indicated in Fig. 8B, IL-21 did not alter CD11b and CD14 expression, whereas, as expected, CD11b was up-regulated with DMSO, PMA, and Vit D3 treatment (38). CD14 was up-regulated only with Vit D3 treatment (38). These data demonstrate that IL-21 is not a maturation factor for HL-60 cells.

Expression of IL-21Rα by monocytes and monocyte-derived macrophages

Because we demonstrated that human neutrophils do not express IL-21Rα and that IL-21 had no effect on human neutrophil functions, we next verified whether IL-21Rα is expressed in monocytes and macrophages, because these cells were the other major cell types attracted by IL-21 in the murine air pouch. In parallel, we studied the expression of IL-21Rα in HL-60 cells differentiated toward monocytes or macrophages after treatment with Vit D3 or PMA, respectively. As illustrated in Fig. 9A, human monocytes (Fig. 9A) and monocyte-derived macrophages (Fig. 9B) express the IL-21Rα. The expression of IL-21Rα was maintained during differentiation of HL-60 cells toward monocytes or macrophages. Therefore, both monocytes and macrophages may be direct targets of IL-21.

IL-21 induces human monocyte-derived macrophages to produce CXCL8, but not monocytes

Knowing that human neutrophils do not express IL-21Rα and that IL-21 did not induce CXCL8 production in these cells, we questioned whether IL-21 could induce CXCL8 production by human monocytes and/or monocyte-derived macrophages. As illustrated in Fig. 10, macrophages, but not monocytes, increased CXCL8 production in response to IL-21. As expected, LPS induced CXCL8 production in both cell types (data not shown). These results clearly indicate that although monocytes were not activated by IL-21, they were responsive and produced CXCL8.

Discussion

The CD132-dependent cytokines IL-2, IL-4, and IL-15 are proinflammatory cytokines known to play important roles in inflammatory disorders, including rheumatoid arthritis (39). The major aim of the present study was to elucidate the in vivo and in vitro proinflammatory properties of the recently identified CD132-dependent
cytokine, IL-21. In particular, knowing that IL-2, IL-4, and IL-15 activate human neutrophils, and that these cells express CD132 (11), which is an IL-21R subunit, we were interested in knowing whether these cells are important targets of IL-21.

Using the murine air pouch model we have demonstrated that IL-21 induces a neutrophilic and mononuclear inflammation response in vivo, suggesting that these cells are targets to IL-21. However, we found that human neutrophils do not express the IL-21Rα component. This could explain why IL-21 does not modulate O$_2^-$ production, phagocytosis, CXCL8 and IL-1β production, chemotaxis, and RNA synthesis. Because IL-21 was not a chemoattractant for neutrophils in vitro, but attracted them in vivo, we hypothesized that recruitment of neutrophils into air pouches occurred via the production of chemokines by other cell types, including the monocytes and macrophages, which are also attracted in vivo. Although it was beyond the scope of this study to determine which chemokines are involved in IL-21-induced cell attraction, we nevertheless investigated the role of CCL3, CCL5, and

**FIGURE 7.** IL-21 does not modulate human neutrophil responses. Freshly isolated neutrophils ($1 \times 10^6$ cells/ml) were treated for 5 min (A) or 30 min (B) with buffer (Ctrl), PMA $10^{-7}$ M (positive control), or increasing concentrations of IL-21 (100, 250, or 500 ng/ml), and superoxide production was measured by the reduction of cytochrome $c$ as described under Materials and Methods. Results are means ± SEM ($n \geq 4$). C. Cells were treated for 30 min with buffer (Ctrl), GM-CSF 65 ng/ml, or IL-21, and then incubated for 5 min with fMLP ($10^{-7}$ M) before measuring superoxide production to verify potential priming effects. D. Phagocytosis was evaluated by determining ingestion of opsonized SRBCs by neutrophils pretreated for 30 min with buffer (Ctrl), 10 μg/ml toxaphene (Tox), or IL-21. Results are expressed as the percentage of phagocytosis (cells ingesting at least one opsonized SRBC/number of cells counted $\times 100$). E. Neutrophils were stimulated with CXCL8 (IL-8) (200 ng/ml) or increasing concentrations of IL-21 and in vitro chemotaxis was performed in a 48-well microchemotaxis chamber, as described in Materials and Methods. F and G. Production of CXCL8 and IL-1β were measured by ELISA and RNA synthesis (H) was performed as previously described (14). E and H, results are expressed as a stimulation index (number of cells from tested group/number of cells from control). * $p < 0.05$ vs control (Ctrl) by ANOVA.
CXCL2, three excellent potential candidates. These chemokines are known to attract neutrophils (40, 41), monocytes, and macrophages (42, 43). However, in contrast to LPS, we did not observe increased expression of these chemokines in the exudates following IL-21 treatment. IL-6 was also another interesting potential candidate, because this cytokine is known to be involved in pneumolysin-induced lung inflammation in mice characterized by a dose-dependent influx of neutrophils in bronchoalveolar lavage fluids (30). In addition, IL-6 was recently found to be highly expressed in the exudates of hemozoin-induced murine air pouches, characterized by an influx of neutrophil and monocyte populations (31). Again unlike LPS, IL-6 expression was not increased in response to IL-21. These results strongly suggest that IL-21 attracts cells in a manner different from LPS, and by a mechanism that is independent of CCL3, CCL5, CXCL2, and IL-6. This discrepancy is of interest, because IL-21 attracts an important proportion of mononuclear cells into the air pouch as compared with LPS induction. Although the collected fluids from IL-21-induced animals were devoid of chemotactic activity, we do not rule out the possibility that, at a particular time point, some chemotactic factors would be expressed at higher concentrations. However, it is clear

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**FIGURE 8.** IL-21 is not a maturation agent for HL-60 cells. HL-60 cells were treated with 100 ng/ml IL-21, or with 1.25% DMSO (v/v) for neutrophil differentiation; 100 nM Vit D3 for monocyte differentiation; or 10 nM PMA for macrophage differentiation. Cells were harvested after 6 days for DMSO or Vit D3 treatment and after 3 days for PMA treatment. A, Cell cycle analysis was evaluated using PI as described in Materials and Methods. Right panels illustrate corresponding morphological observations monitored by optical microscopy (magnification, ×200). B, HL-60 cell surface expression of CD11b and CD14 (filled curve) was monitored by flow cytometry as described in the Materials and Methods. Results are representative of three different experiments.

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**FIGURE 9.** IL-21Rα is expressed by human monocytes and monocyte-derived macrophages. Monocytes and 7-day monocyte-derived macrophages were isolated as described in Materials and Methods. HL-60 cells were treated with 100 nM 1,25-dihydroxyvitamin D3 (Vit D3) for monocyte differentiation or 10 nM PMA for macrophage differentiation for the indicated periods of time, were lysed, subjected to SDS-PAGE, and immunoblotted with the Ab. Results are from one representative experiment of three.
that the chemokine profiles induced in response to IL-21 and LPS are distinct and this needs to be addressed.

In vitro, stimulation of human monocyte-derived macrophages by IL-21 resulted in a concentration-dependent production of CXCL8. Interestingly, we did not observe increased CXCL8 expression in IL-21-induced human monocytes. CXCL8 is a potent chemoattractant of human neutrophils and our results suggest that macrophages may be partly responsible for the recruitment of neutrophils in vivo. However, because CXCL8 is not found in mice, additional experiments are needed to identify the human CXCL8-like chemokine(s) involved in neutrophil and mononuclear cell recruitment. The fact that IL-21 does not directly activate neutrophils does not indicate an absence of response by these cells to IL-21. Indirectly, neutrophils could respond to IL-21 via cytokines or chemokines produced by macrophages or other cell types present at the inflammatory site.

The exact role of IL-21/IL-21R interaction in immature HL-60 cells is currently unclear. However, it is clear that binding of IL-21 to its receptor induces phosphorylation of Erk-1/2. Activation of Erk-1/2 is frequently associated with the first phases of proliferation and differentiation of these cells (44–47). However, this does not appear to be the case in IL-21-induced HL-60 cells. More surprising, IL-21 did not induce HL-60 cell differentiation/matura-

![FIGURE 10. IL-21 increases CXCL8 production by monocyte-derived macrophages but not by monocytes. Monocytes (A) and 7-day monocyte-derived macrophages (B) (both at 1 x 10^6 cells/ml) were incubated for 24 h with buffer (Ctrl) or increasing concentrations of IL-21 (10–500 ng/ml). Supernatants were harvested and CXCL8 (IL-8) concentration was measured using a commercial ELISA kit, as described in Materials and Methods. Results are expressed as stimulation indices (CXCL8 produced by stimulated cells/CXCL8 produced by unstimulated cells x 100) (n = 4). * p < 0.05 by ANOVA. Results varied from experiment to experiment ranging broadly from 25 to 304 pg/ml in untreated vs 54 to 827 in IL-21 (500 ng/ml)-induced macrophages and 14 to 353 pg/ml in untreated vs 13 to 290 in IL-21 (500 ng/ml)-induced monocytes.](http://www.jimmunol.org/content/181/9/7529/F10)

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The presence of neutrophils at tumor sites and their role in tumor rejection is a relatively new concept in immunobiology. Although we have demonstrated that IL-21 is not a neutrophil agonist, we do not rule out the possibility that IL-21 could activate these cells indirectly. The antitumoral role of IL-21 is now well established and there is evidence that neutrophils might be involved in the rejection process induced by IL-21 (18). Di Carlo et al. (18) have shown that IL-21-induced tumor rejection required the presence of CD8^+ T lymphocytes and of granulocytes. Recruitment of neutrophils and macrophages at tumor sites by IL-21 strongly suggests an active role of these cells in the rejection process. Interestingly, absence of granulocytes was found to correlate with a delay in the rejection process (18).

The results of the present study support a potential indirect role of IL-21 on neutrophils via the stimulation of mononuclear cells. We believe that IL-21 plays important roles in inflammation and during activation and/or development of nonlymphoid cells, including promyelocytes, neutrophils, and mononuclear cells. The importance of IL-21R expression in IL-21-mediated biological action has been previously demonstrated (50). Herein, our results obtained with neutrophils clearly demonstrate that the sole expression of CD132 is insufficient to induce cell signaling in response to IL-21. We conclude that IL-21 is not a direct human neutrophil agonist, but we propose to include IL-21 into the growing list of CD132-dependent cytokines having proinflammatory properties in vivo and in vitro.

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