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Autocrine Regulation of IL-21 Production in Human T Lymphocytes[1]

Flavio Caprioli,* Massimiliano Sarra,* Roberta Caruso,* Carmine Stolfi,* Daniele Fina,* Giuseppe Sica,† Thomas T. MacDonald,‡ Francesco Pallone,* and Giovanni Monteleone[2]*

IL-21 has pathologic function in immune-inflammatory diseases. IL-21 mediates its functions through a heterodimeric receptor, composed of a specific subunit, termed IL-21R, and the common γ-chain. IL-21 is mostly produced by $CD4^+$ T cells, but molecular mechanisms that regulate IL-21 synthesis are not fully understood. The fact that $CD4^+$ T cells express high levels of IL-21R and are capable of functionally responding to IL-21 raises the possibility that IL-21 may regulate its own production. We here show that IL-21 enhances IL-21 RNA and protein expression in human peripheral blood $CD3^+$ T cells in a dose- and time-dependent fashion. Additionally, both IL-7 and IL-15, but not IL-4, induce IL-21, thus suggesting that common γ-chain signals are not sufficient to promote IL-21 synthesis. Analysis of molecular mechanisms underlying IL-21 induction reveals that IL-21 activates Stat3 and enhances its recruitment to IL-21 gene promoter. Pharmacologic inhibition and knockdown of Stat3 by small interference RNA largely prevent IL-21 induction in IL-21-treated cells. Consistently, IL-21 is inducible in T cells by IL-6, another cytokine that activates Stat3. Finally, we show that IL-21 positively regulates its own expression in human intestinal $CD3^+$ lamina propria lymphocytes, and blockade of endogenous IL-21 in cultures of $CD3^+$ lamina propria lymphocytes isolated from patients with Crohn's disease, a chronic inflammatory bowel disease characterized by high IL-21, down-regulates Stat3 activation and IL-21 expression. These data suggest the existence of a positive autocrine loop that could help to amplify and stabilize IL-21-driven, T cell-mediated responses. ***The Journal of Immunology,*** **2008, 180: 1800–1807.**

The $CD4^+$ T cells play a decisive role in the pathogenesis of various immune inflammatory and allergic diseases. This is strongly supported by the demonstration that $CD4^+$ T cells accumulate into the affected tissues and synthesize high levels of inflammatory cytokines (1–3) and that therapies targeting $CD4^+$ T cell-derived cytokines can be used with clinical success in patients with such diseases (4–7). Moreover studies in animal models of T cell-dependent inflammation have shown that either deletion or over-expression of specific $CD4^+$ T cell gene products can profoundly alter the course of immune- and allergen-mediated diseases (8–10).

IL-21 is a newly discovered cytokine that is most homologous to IL-15 and also has significant homology to IL-2 and IL-4. IL-21 is able to exert a variety of regulatory effects on both lymphoid and nonlymphoid cells, which are dependent not only on cell type but also on the differentiation or activation status of each cell type (11, 12). In particular, IL-21 augments the proliferation of $CD4^+$ and $CD8^+$ T lymphocytes (13–15), regulates the survival (13) and the profile of cytokines secreted by these cells (16, 17), drives the differentiation of B cells into memory cells and terminally differentiated plasma cells (18), and enhances the activity of NK cells (19). High production of IL-21 has been described in various immune-mediated diseases, including inflammatory bowel diseases (20), celiac disease (21), and *Helicobacter pylori*-related gastritis (22). Moreover, both in vitro and in vivo studies have shown that IL-21 is able to trigger and/or amplify several inflammatory pathways that sustain pathologic responses (20, 23–26).

IL-21 biological functions are mediated by a heterodimeric receptor, formed by the common γ-chain subunit, that is shared with IL-2, IL-4, IL-7, IL-9, IL-13, and IL-15 receptors as well as its own unique receptor (designated IL-21R), a member of the class I cytokine receptor family (12, 27, 28). Binding of IL-21 to its receptor leads to the activation of the JAK family protein tyrosine kinases JAK1 and JAK3 (27, 29) and, subsequently, to the activation of Stat1, Stat3, and to a lesser degree Stat4, Stat5, and Stat6 (16, 27).

Although several studies have shown that IL-21 is preferentially made by $CD4^+$ T cells, but not $CD8^+$ T cells (19, 28), little is known about the molecular mechanisms that regulate IL-21 production in these cell types. The fact that IL-21R is highly expressed by $CD4^+$ T cells (19, 28, 30) and that these cells functionally respond to IL-21 prompted us to explore the possibility that IL-21 may regulate its own production. We here show that IL-21 positively regulates its own expression in human peripheral blood and intestinal $CD3^+$ T cells through a mechanism that involves Stat3 activation.

Materials and Methods

Peripheral blood $CD3^+$ T cell isolation and culture

Leukocyte-rich buffy coats were obtained from healthy blood donors (University Tor Vergata Hospital, Blood Transfusion Service, Rome,

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Italy). PBMC were isolated by density gradient centrifugation using Ficoll-Paque (Amersham Biosciences). Monocytes were removed by adherence, and T cells were further purified by positive MACS separation using $CD3^+$ cell isolation kits (Miltenyi Biotec). The resulting cell preparations contained >95% $CD3^+$ T cells as assessed by flow cytometry. Additionally, T cells were purified by negative selection using CD14, CD20, CD138, and CD56 microbeads (Miltenyi Biotec). $CD4^+$ T cells were also isolated from peripheral blood by using a $CD4^+$ multisort kit and then used to purify $CD45RO^+$ cells by $CD45RO^+$ microbeads (Miltenyi Biotec).

Peripheral blood $CD3^+$ T lymphocytes were resuspended in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) (Invitrogen Life Technologies). Cells were cultured in the presence or absence of graded doses of rh-IL-21 (Biosource Europe) with or without the initial addition of IL-4 (25 ng/ml), IL-6 (25 ng/ml), IL-7 (25 ng/ml), IL-15 (25 ng/ml) (all from PeproTech), and IL-23 (25 ng/ml) (R&D Systems) for the indicated time points.

To examine the role of Stat3 in the control of IL-21 production, $CD3^+$ PBL were preincubated with AG490, a JAK2/Stat inhibitor (200 μM; Inalco), or vehicle (DMSO; Sigma-Aldrich) for 1 h and then were stimulated with IL-21 50 ng/ml for 20 min to 24 h. Additionally, cells were cultured with or without human Stat3 or control small interfering RNA (siRNA)[3] according to the manufacturer's instructions (Santa Cruz Biotechnology). After 48 h, an aliquot of cells was used for analyzing total Stat3 by Western blotting, while the remaining were washed and cultured with or without IL-21, IL-7, and IL-15 for 3 h further.

Intestinal $CD3^+$ lamina propria lymphocytes (LPL) isolation and culture

Intestinal mucosal samples were taken from eight patients with moderate to severe Crohn's disease undergoing colonic resection for a chronically active disease poorly responsive to medical treatment. Moreover, colonic specimens were taken from macroscopically and microscopically unaffected areas of six patients undergoing colectomy for colorectal cancer. The study received approval from the local ethical committee.

Lamina propria mononuclear cells were prepared as described previously (31), and used to purify $CD3^+$ T-LPL by using a $CD3^+$ cell isolation kit (Miltenyi Biotec). The resulting cell preparations contained >93% $CD3^+$ T-LPL as assessed by flow cytometry.

Normal $CD3^+$ LPL were cultured in the presence or absence of IL-21 (50 ng/ml) with or without the addition of AG490 or DMSO. AG490 and DMSO were preincubated for 1 h before adding IL-21. At the indicated time points, cells were harvested and used for RNA or protein extraction. Additionally, normal and Crohn's disease $CD3^+$ T-LPL were stimulated with anti-CD3 and anti-CD28 Ab-coated beads (Miltenyi Biotec) for 48 h, and cell supernatants were then analyzed for IL-21 content by ELISA using a commercially available kit (eBioscience). To evaluate the role of endogenous IL-21 in the control of IL-21 and phosphorylated (p)-Stat3 expression in Crohn's disease, $CD3^+$ T-LPL isolated from Crohn's disease patients were cultured in the presence or absence of a blocking anti-IL-21 or control IgG (23). After 12 and 24 h, cells were harvested and used for RNA and protein extraction, as well as to assess the rate of apoptosis.

Protein extraction and Western blot analysis

Proteins were extracted using a buffer containing 10 mM HEPES (pH 7.9), 1 mM EDTA (pH 8.0), 60 mM KCl, Nonidet P40 0.2%, supplemented with 1 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM Na_3VO_4, and 1 mM NaF (Sigma-Aldrich). Lysates were clarified by centrifugation at 4°C for 30 min at 12,000 × *g*. Equal amounts of total proteins (60 μg/sample) were separated on a 10% SDS-PAGE.

IL-21 expression was analyzed in total extracts of T cells as previously indicated (20). After detection of IL-21, blots were stripped and incubated with a mouse anti-human β-actin Ab (Sigma-Aldrich; final dilution 1/5,000) followed by a goat anti-mouse Ab conjugated to HRP (DakoCytomation; final dilution 1/20,000). p-Stat1, p-Stat3, and p-Stat5 were evaluated using mouse anti-human mAbs (p-Stat1 and p-Stat3 Abs were from Santa Cruz Biotechnology; p-Stat5 Ab was from Upstate Biotechnology), followed by a goat anti-mouse Ab conjugated to HRP (DakoCytomation; final dilution 1/20,000), and the reactions were detected with a chemiluminescence kit (Pierce). At the end, blots were stripped and incubated with a mouse anti-human Stat1, Stat3, or Stat5 Ab (Santa Cruz Biotechnology), followed by a goat anti-mouse Ab conjugated to HRP (DakoCytomation; final dilution 1/20,000). Computer-assisted scanning densitometry (TotalLab; AB.EL Science-Ware Srl) was used to analyze the intensity of the immunoreactive bands.

[3] Abbreviations used in this paper: siRNA, small interfering RNA; LPL, lamina propria lymphocyte; GAS, IFN-γ activated site; AV, annexin V; PI, propidium iodide.

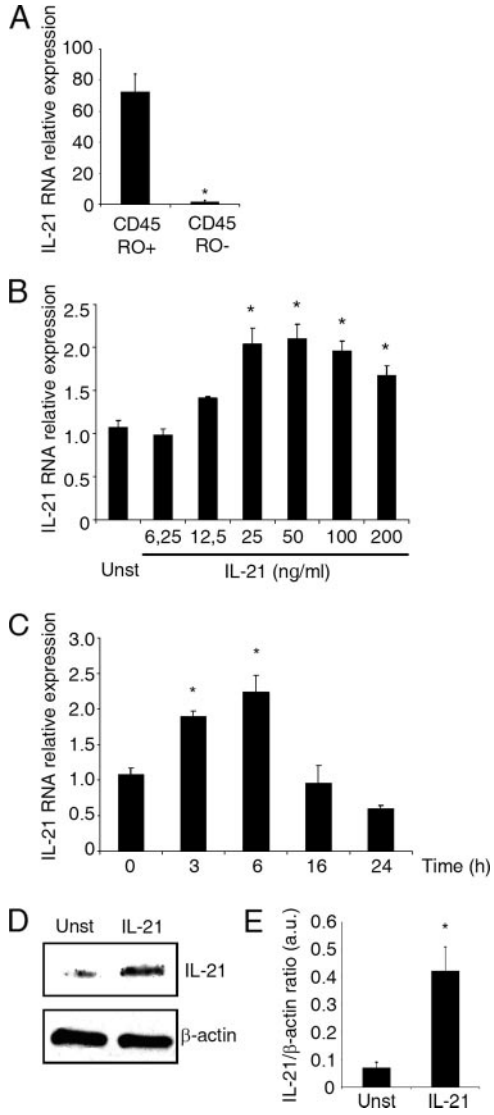

FIGURE 1. *A*, IL-21 RNA is expressed in human blood $CD45RO^+$ T cells. $CD45RO^+$ and $CD45RO^-$ T cells were isolated from blood samples of normal subjects and analyzed for the content of IL-21 RNA by real-time PCR. Data indicate mean ± SD of four separate experiments. *, $p < 0.001$. *B–E*, IL-21 induces IL-21 in $CD3^+$ PBL in a dose- and time-dependent manner. *B* and *C*, $CD3^+$ PBL were either left unstimulated (Unst) or stimulated with the indicated doses of rhIL-21 for 3 h (*B*) or with rhIL-21 (50 ng/ml) for the indicated time points (*C*). IL-21 RNA was then analyzed by real-time PCR. Data indicate mean ± SD of four separate experiments. *, $p < 0.05$, IL-21 vs unstimulated. *D*, Representative Western blots showing IL-21 and β-actin in $CD3^+$ PBL were either left unstimulated or stimulated with rhIL-21 (50 ng/ml) for 24 h. One of four representative Western blots is shown. *E*, Quantitative analysis of IL-21/β-actin protein ratio, as measured by densitometry scanning of Western blots. Values are expressed in arbitrary units (a.u.) and are the mean ± SD of four experiments. *, $p < 0.02$, IL-21 vs unstimulated.

Oligonucleotide DNA precipitation

PBL were either left unstimulated or stimulated with IL-21 (50 ng/ml) for 1, 2, 4, or 6 h. Cell lysates were then prepared, as indicated above, and incubated with streptavidin-agarose beads coupled to 5′-biotinylated

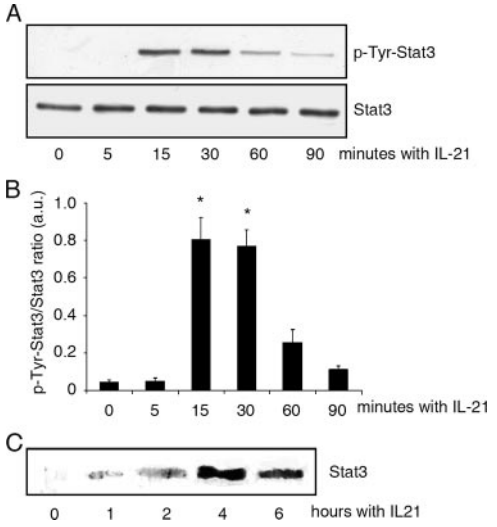

FIGURE 2. IL-21 induces activation of Stat3 in $CD3^+$ PBL. *A*, Representative Western blots showing p-Tyr-Stat3 and total Stat3 in $CD3^+$ PBL either left unstimulated or stimulated with rhIL-21 (50 ng/ml) for the indicated time points. *B*, Quantitative analysis of p-Tyr-Stat3/Stat3 protein ratio, as measured by densitometry scanning of Western blots. Values are expressed in arbitrary units (a.u.) and are the mean ± SD of four experiments. *, $p < 0.01$, IL-21 vs unstimulated. *C*, $CD3^+$ PBL were either left unstimulated or stimulated with rhIL-21 (50 ng/ml) for the indicated time points; after which, total protein extracts were precipitated with IL-21 promoter GAS oligonucleotides and analyzed by Western blotting with anti-Stat3 Ab. The results are representative of three separate experiments.

oligonucleotide, corresponding to the human IL-21 promoter IFN-γ activated site (GAS), located in the promoter region from −504 to −468 before the starting codon (5′-GGATCCGACATAGTTATTACCATA AGAAAAAGTCCT-3′) (Sigma-Aldrich). As a control, proteins were immunoprecipitated using a scrambled 5′-biotinylated oligonucleotide. The binding reactions were performed for 2 h at 4°C in binding buffer containing 10 mM HEPES, 133 mM KCl, 10% glycerol, 2 mM EDTA, 1 mM EGTA, 0.01% Triton X-100, 0.5 mM DTT, 1 mM $NaVO_4$, and protease inhibitors. After washing, the oligonucleotide-bound proteins were released in SDS sample buffer and separated on 10% SDS-PAGE. Blots were then incubated with a mouse anti-human Stat3 or Stat5 Ab (1/500; Santa Cruz Biotechnology), followed by a goat anti-mouse Ab conjugated to HRP (DakoCytomation; final dilution 1/20,000), and the reaction was detected with a chemiluminescence kit (Pierce).

RNA extraction, cDNA preparation, and real-time PCR

RNA was extracted by TRIzol according to the manufacturer's instructions (Invitrogen Life Technologies). A constant amount of RNA (500 ng/sample) was retro-transcribed into cDNA. One μl of cDNA/sample was then amplified using the following conditions: denaturation 1 min at 95°C, annealing 30 s at 58°C for 18S, followed by 30 s of extension at 72°C. Primers sequence was as follows: 18S (forward: 5′-CGT CTG CCC TAT CAA CTT TCG-3′ and reverse: 5′-GAG AAA CGG CTA CCA CAT CCA-3′). IL-21 was evaluated using a commercially available TaqMan probe (Applied Biosystems). Real-time PCR was performed using the IQ SYBR Green Supermix (Bio-Rad).

Analysis and quantification of cell death

To score cell death, Crohn's disease $CD3^+$ LPL were either left untreated or treated with anti-IL-21 or control Ab (25 μg/ml) for 12–24 h. Cells were then collected, washed twice in 1× PBS, stained with FITC-annexin V (AV; 1/100 final dilution) according to the manufacturer's instructions (BD Biosciences), incubated with 5 μg/ml propidium iodide (PI) for 30 min at 4°C, and their fluorescence was measured using FL-1 and FL-2 channels of FACSCalibur using Cell Quest Pro software.

Statistical analysis

Values are expressed as mean ± SD, and differences were calculated using the Student *t* test.

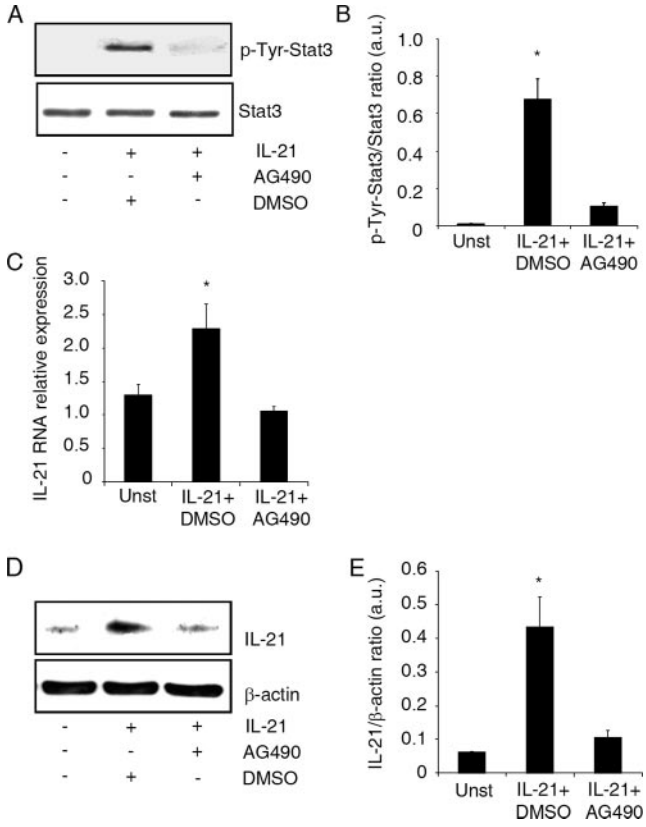

FIGURE 3. Treatment of IL-21-stimulated $CD3^+$ PBL with AG490, a JAK2/Stat inhibitor, prevents IL-21 induction. *A*, Representative Western blots showing p-Tyr-Stat3 and total Stat3 in $CD3^+$ PBL cultured in the presence or absence of AG490 (100 μM) for 1 h, followed by stimulation with IL-21 (25 ng/ml) for 20 min further. One of three representative Western blots is shown. *B*, Quantitative analysis of p-Tyr-Stat3/Stat3 protein ratio, as measured by densitometry scanning of Western blots. Values are expressed in arbitrary units (a.u.) and are the mean ± SD of three experiments. *, $p < 0.01$, IL-21 plus DMSO vs unstimulated or IL-21 plus AG490. *C*, $CD3^+$ PBL were cultured in the presence or absence of AG490 (100 μM) for 1 h, followed by stimulation with IL-21 (25 ng/ml) for 3 h further. IL-21 RNA was then analyzed by real-time PCR. Data indicate mean ± SD of four separate experiments. *, $p < 0.05$, IL-21 plus DMSO vs unstimulated or IL-21 plus AG490. *D*, Representative Western blots showing IL-21 and β-actin in $CD3^+$ PBL cultured in the presence or absence of AG490 (100 μM) for 1 h, followed by stimulation with IL-21 (25 ng/ml) for 24 h further. One of four representative Western blots is shown. *E*, Quantitative analysis of IL-21/β-actin protein ratio, as measured by densitometry scanning of Western blots. Values are expressed in arbitrary units (a.u.) and are the mean ± SD of four experiments. *, $p < 0.01$, IL-21 plus DMSO vs unstimulated or IL-21 plus AG490.

Results

IL-21 positively regulates its own production in human $CD3^+$ PBL

IL-21 RNA transcripts were barely detectable in T-PBL purified by negative selection, although they were present in positively selected resting $CD3^+$ PBL. It is, thus, possible that CD3 beads used for the positive selection stimulated T cells to express IL-21. IL-21 RNA was detectable in freshly isolated $CD4^+CD45RO^+$ but not $CD45RO^-$ T-PBL (Fig. 1*A*).

To study the effect of IL-21 on IL-21 gene expression, $CD3^+$ PBL were stimulated with graded doses of exogenous IL-21 for 3 h and then analyzed for IL-21 RNA transcripts by real-time PCR. IL-21 dose-dependently enhanced its own RNA expression, with

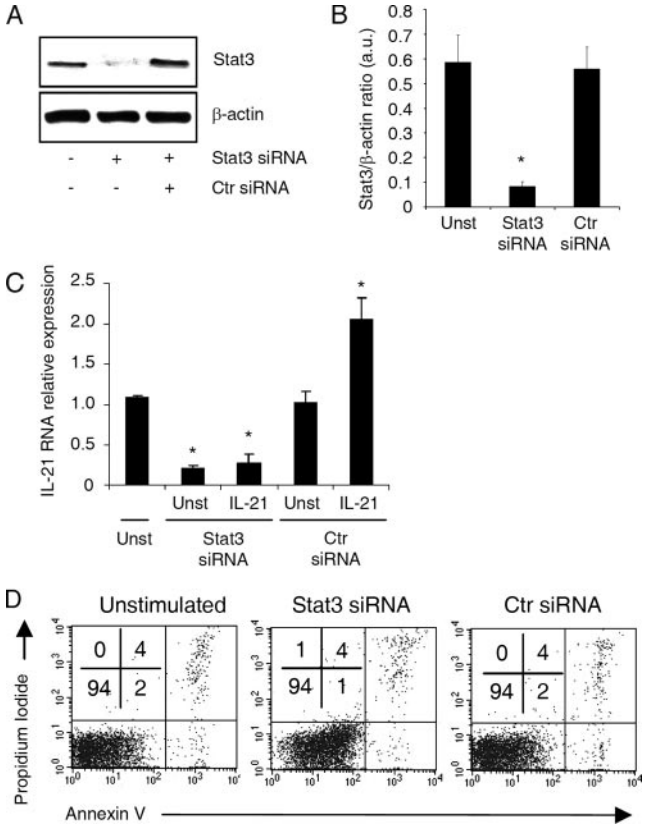

FIGURE 4. IL-21-mediated induction of IL-21 requires Stat3. *A*, Representative Western blots showing total Stat3 and β-actin in $CD3^+$ PBL cultured in the presence or absence of a specific human Stat3 or control (Ctr) siRNA for 2 days. One of three representative Western blots is shown. *B*, Quantitative analysis of Stat3/β-actin protein ratio, as measured by densitometry scanning of Western blots. Values are expressed in arbitrary units (a.u.) and are the mean ± SD of three experiments. *, $p < 0.01$, Stat3 siRNA-treated cells vs untreated or control siRNA-treated cells. *C*, $CD3^+$ PBL were cultured as indicated in *A*, then extensively washed and cultured with or without IL-21 for an additional 3 h. IL-21 RNA was then analyzed by real-time PCR. Data indicate mean ± SD of three separate experiments. *, $p < 0.05$, IL-21 in Stat3 siRNA-treated cells vs unstimulated; *, $p < 0.01$, IL-21 in Stat3 siRNA-treated cells vs IL-21 in control siRNA-treated cells. *D*, Representative flow cytometry dot plots showing AV- and/or PI-positive cells after culture in the presence or absence of a specific human Stat3 or control siRNA for 2 days. Numbers indicate the percentage of cells in the designated gates. One of four representative experiments is shown.

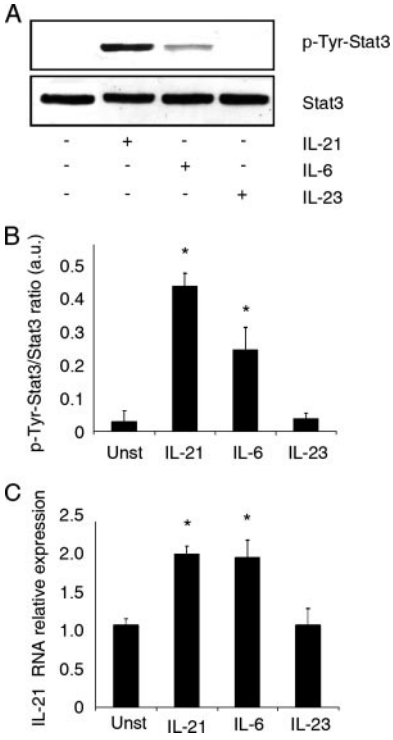

FIGURE 5. IL-6, but not IL-23, induces IL-21 in human $CD3^+$ PBL. *A*, Representative Western blots showing p-Tyr-Stat3 and total Stat3 in $CD3^+$ PBL cultured in the presence or absence of IL-21 (25 ng/ml), rhIL-6 (25 ng/ml), or IL-23 (25 ng/ml) for 20 min. One of three representative Western blots is shown. *B*, Quantitative analysis of p-Tyr-Stat3/Stat3 protein ratio, as measured by densitometry scanning of Western blots. Values are expressed in arbitrary units (a.u.) and are the mean ± SD of three experiments. *, $p < 0.02$, IL-21 or IL-6 vs unstimulated. *C*, $CD3^+$ PBL were either left unstimulated (Unst) or stimulated with the indicated cytokines for 3 h. IL-21 RNA was then analyzed by real-time PCR. Data indicate mean ± SD of three separate experiments. *, $p < 0.05$, IL-21 or IL-6 vs unstimulated.

significant induction seen at 25 ng/ml or higher doses (Fig. 1*B*). Time course studies also revealed that induction of IL-21 RNA occurred as early as 3 h after cell stimulation, peaked at 6 h, and then declined at later time points (Fig. 1*C*).

The induction of IL-21 RNA in IL-21-treated cells led us to investigate whether IL-21 protein was also being induced. To this end, $CD3^+$ PBL were either left unstimulated or stimulated with IL-21 for 24 h. We cannot use a standard ELISA approach to analyze IL-21 protein because of the impossibility to differentiate the exogenous IL-21 used for stimulating cells and the endogenous IL-21 released in the culture supernatants. Therefore, IL-21 protein expression was assessed by Western blotting. Immunoreactive bands for IL-21 were seen in total extracts of both unstimulated and IL-21-stimulated cells, but their intensity was more pronounced in IL-21-treated cells (Fig. 1*D*). IL-21 expression quantitated by densitometry and normalized by β-actin content was significantly increased in IL-21-stimulated cells as compared with untreated cells (Fig. 1*E*). Taken together, these data indicate that IL-21 positively regulates its own expression in human $CD3^+$ PBL.

IL-21-mediated induction of IL-21 requires Stat3

Previous studies have identified putative GAS elements in the human IL-21 promoter (32). Because GAS elements are known to bind Stat transcription factors and IL-21 is known to activate Stat3 in T cells (27), we examined whether Stat3 is involved in the induction of IL-21 by exogenous IL-21. To this end, we first performed a time-course analysis of Stat3 tyrosine phosphorylation in IL-21-treated $CD3^+$ PBL. As expected, p-Tyr-Stat3 was clearly induced by IL-21, and this was evident as early as 15 min after IL-21 stimulation, persisted at 30 min, and then declined (Fig. 2, *A* and *B*). In contrast, no induction of p-Tyr-Stat1 or p-Tyr-Stat5 was seen in the same cell culture after IL-21 stimulation (not shown). Second, we performed oligonucleotide precipitation experiments to examine whether Stat3 was recruited to IL-21 promoter GAS upon IL-21 stimulation. Resting $CD3^+$ PBL were either left unstimulated or stimulated with IL-21 for 1–6 h, after which, cell lysates were prepared. Specific proteins were precipitated with oligonucleotides containing IL-21 promoter GAS sequence and analyzed by Western blotting with anti-Stat3 Ab. IL-21 induced binding of Stat3 to GAS oligonucleotides in resting PBL (Fig. 2*C*). No immunoreactive band for Stat3 was evident when proteins extracted

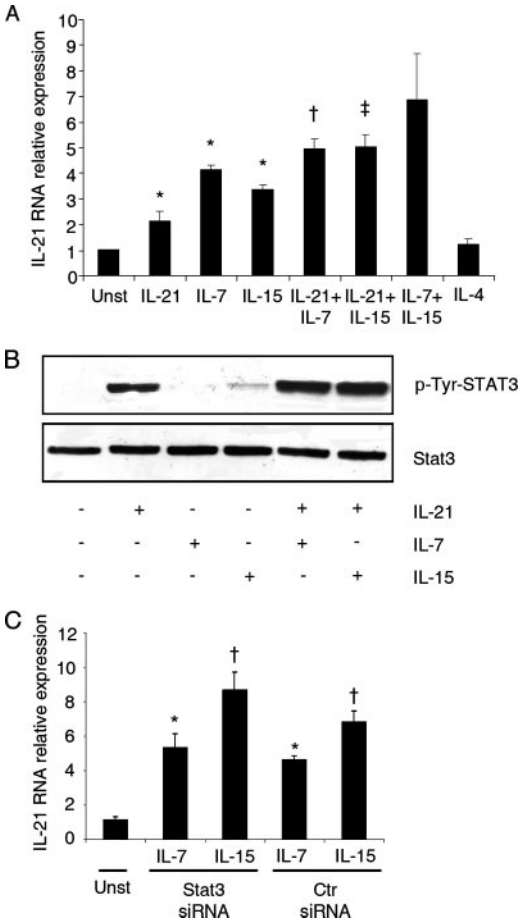

FIGURE 6. IL-21 expression is not induced by all common γ-chain-dependent cytokines. *A*, $CD3^+$ PBL were either left unstimulated (Unst) or stimulated with the indicated cytokines (25 ng/ml) for 3 h. IL-21 RNA was then analyzed by real-time PCR. Data indicate mean ± SD of three separate experiments. ∗, $p < 0.05$ unstimulated vs IL-21, IL-7, or IL-15; †, $p < 0.03$, IL-21 plus IL-7 vs IL-7; ‡, $p < 0.01$, IL-21 plus IL-15 vs IL-15. *B*, Representative Western blots showing p-Tyr-Stat3 and total Stat3 in $CD3^+$ PBL either left unstimulated or stimulated with the indicated cytokines (25 ng/ml) for 20 min. *C*, $CD3^+$ PBL were cultured in the presence or absence of a specific human Stat3 or control (Ctr) siRNA for 2 days then extensively washed and cultured with or without the selected cytokines (25 ng/ml) for an additional 3 h. IL-21 RNA was then analyzed by real-time PCR. Data indicate mean ± SD of three separate experiments. ∗, $p < 0.02$ vs Unst; †, $p < 0.01$ vs Unst.

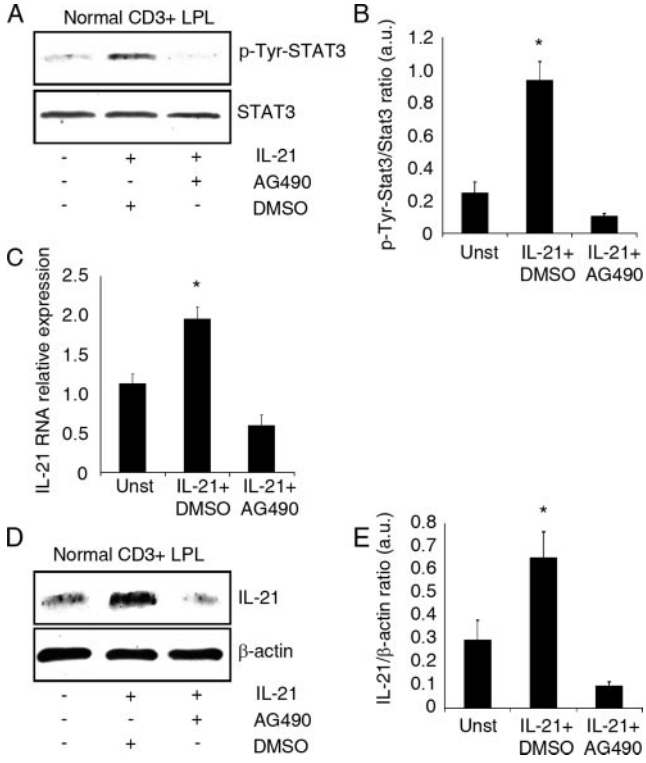

FIGURE 7. IL-21 induces IL-21 in normal intestinal $CD3^+$ LPL. *A*, Representative Western blots showing p-Tyr-Stat3 and total Stat3 in $CD3^+$ LPL cultured in the presence or absence of AG490 (100 μM) for 1 h, followed by stimulation with IL-21 (25 ng/ml) for 20 min further. One of three representative Western blots is shown. *B*, Quantitative analysis of p-Tyr-Stat3/Stat3 protein ratio, as measured by densitometry scanning of Western blots. Values are expressed in arbitrary units (a.u.) and are the mean ± SD of three experiments. ∗, $p < 0.01$, IL-21 plus DMSO vs unstimulated or IL-21 plus AG490. *C*, LPL were cultured in the presence or absence of AG490 (100 μM) for 1 h, followed by stimulation with IL-21 (25 ng/ml) for 3 h further. IL-21 RNA was then analyzed by real-time PCR. Data indicate mean ± SD of four separate experiments. ∗, $p < 0.05$, IL-21 plus DMSO vs unstimulated or IL-21 plus AG490. *D*, Representative Western blots showing IL-21 and β-actin in normal $CD3^+$ LPL cultured in the presence or absence of AG490 (100 μM) for 1 h, followed by stimulation with IL-21 (25 ng/ml) for 24 h further. One of four representative Western blots is shown. *E*, Quantitative analysis of IL-21/β-actin protein ratio, as measured by densitometry scanning of Western blots. Values are expressed in arbitrary units (a.u.) and are the mean ± SD of four experiments. ∗, $p < 0.01$, IL-21 plus DMSO vs unstimulated or IL-21 plus AG490.

from IL-21-treated cells were precipitated with a scrambled GAS oligonucleotide (not shown). Similarly, no band was seen in proteins immunoprecipitated with oligonucleotides containing IL-21 promoter GAS sequence and probed with anti-Stat1 and anti-Stat5 (not shown).

We then examined whether inhibiting Stat3 activation prevented the IL-21-mediated induction of IL-21. $CD3^+$ PBL were cultured with or without AG490, a JAK2/Stat inhibitor, for 60 min and then treated with IL-21 for an additional 20 min to 24 h. As expected, AG490 markedly inhibited the IL-21-induced p-Stat3 expression (Fig. 3, *A* and *B*), and this effect was paralleled by a significant down-regulation in IL-21 RNA (Fig. 3*C*) and protein expression (Fig. 3, *D* and *E*) in IL-21-treated cells, as compared with cells treated with vehicle (DMSO). To further confirm the role of Stat3 in the regulation of IL-21 expression, $CD3^+$ PBL were transfected with a specific human Stat3 or control siRNA for 2 days, and then the content of Stat3 was monitored by Western blotting analysis of total cell extracts. As illustrated in Fig. 4, *A* and *B*, treatment of PBL with Stat3 siRNA significantly down-regulated the constitutive level of Stat3. Notably, a significant reduction of IL-21 mRNA was induced by Stat3 siRNA in both unstimulated and IL-21-stimulated cells (Fig. 4*C*). Flow cytometry analysis of AV- and PI-positive cells revealed that both Stat3 and control siRNA did not affect the rate of cell death (Fig. 4*D*). Overall these results indicate that IL-21 induces IL-21 in $CD3^+$ PBL by activating the transcription factor Stat3.

Because Stat3 can be activated by other cytokines that could act in concert with IL-21 in controlling immune responses, such as IL-6 and IL-23, we next examined whether these two cytokines regulated IL-21 expression. IL-6, but not IL-23, enhanced p-Tyr-Stat3 (Fig. 5, *A* and *B*) and IL-21 RNA expression (Fig. 5*C*).

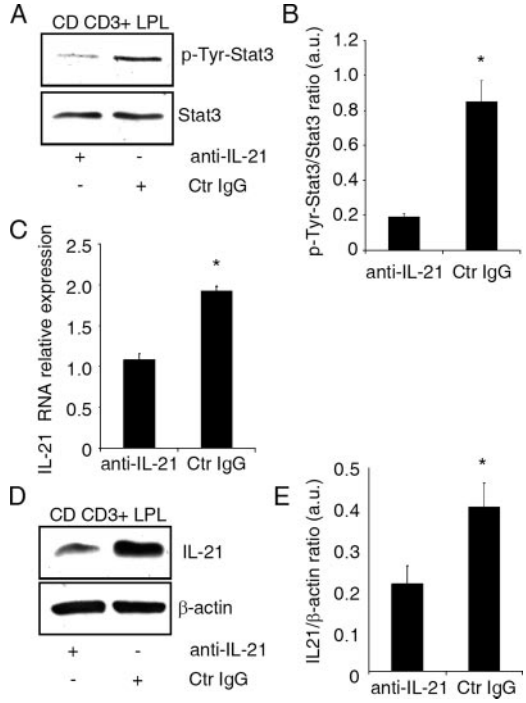

FIGURE 8. Neutralization of endogenous IL-21 reduces Stat3 and IL-21 expression in Crohn's disease intestinal $CD3^+$ LPL. Representative Western blots showing p-Tyr-Stat3 and total Stat3 in Crohn's disease $CD3^+$ LPL cultured with a blocking monoclonal anti-IL-21 Ab or control IgG for 12 h. One of four representative Western blots is shown. *B*, Quantitative analysis of pStat3/Stat3 protein ratio, as measured by densitometry scanning of Western blots. Values are expressed in arbitrary units (a.u.) and are the mean ± SD of four experiments. *, $p < 0.01$, anti-IL-21-treated cells vs control IgG-treated cells. *C*, Cells were cultured as indicated in *A*, and IL-21 RNA was analyzed by real-time PCR. Data indicate mean ± SD of four separate experiments. *, $p < 0.05$, anti-IL-21-treated cells vs control IgG-treated cells. *D*, Representative Western blots showing IL-21 and β-actin in Crohn's disease $CD3^+$ LPL cultured with a blocking monoclonal anti-IL-21 Ab or control IgG for 24 h. One of four representative Western blots is shown. *E*, Quantitative analysis of IL-21/β-actin protein ratio, as measured by densitometry scanning of Western blots. Values are expressed in arbitrary units (a.u.) and are the mean ± SD of four experiments. *, $p < 0.05$, anti-IL-21-treated cells vs control IgG-treated cells.

IL-21 expression is not induced by all common γ-chain-dependent cytokines

In subsequent experiments, we evaluated whether IL-21 expression is also regulated by other common γ-chain-dependent cytokines, which control $CD4^+$ T cell functions. To this end, $CD3^+$ PBL were cultured in the presence or absence of IL-21, IL-4, IL-7, and/or IL-15 for 3 h. At the end, cells were used for extracting RNA, and IL-21 transcripts were assessed by real-time PCR. The addition of IL-7 or IL-15 to PBL cultures resulted in a significant increase in IL-21 RNA expression (Fig. 6*A*). Of note, both these cytokines were more effective than IL-21 in enhancing IL-21 RNA transcripts. Moreover, the combined use of IL-21 and IL-7 or IL-15 caused a further increase in the expression of IL-21 RNA in comparison to that seen in cells stimulated with the single cytokines (Fig. 6*A*). In contrast, IL-4 was not able to enhance IL-21 RNA expression. IL-4 induced, however, Stat6 activation in the same cells, thus indicating that it was active in our system (not shown). Notably, the effect of IL-7 and IL-15 on the induction of IL-21 mRNA was not mediated by Stat3 activation. In fact, IL-15, but not IL-7, induced a slight increase in p-Tyr-Stat3 levels (Fig. 6*B*). Moreover, inhibition of Stat3 by siRNA did not affect the ability of IL-7 and IL-15 to enhance IL-21 RNA expression (Fig. 6*C*).

Autocrine regulation of IL-21 production occurs in intestinal lamina propria $CD3^+$ lymphocytes

IL-21 is constitutively produced in the human gut by $CD3^+$ T-LPL (20). Therefore, we next evaluated whether IL-21 production is subjected to an autocrine regulation also in human gut. Treatment of normal $CD3^+$ LPL with IL-21 enhanced p-Stat3 expression, and suppression of Stat3 activation by AG490 (Fig. 7, *A* and *B*) completely prevented the induction of IL-21 RNA and protein by exogenous IL-21 (Fig. 7, *C–E*).

We have previously shown that IL-21 is produced in excess in the inflamed gut of patients with Crohn's disease (20). To confirm further these data, we showed that $CD3^+$ LPL isolated from Crohn's disease patients produced significantly higher levels of IL-21 (3129 ± 212 pg/ml) than those made by control $CD3^+$ LPL (1739 ± 307 pg/ml; $p < 0.05$). In subsequent experiments, we assessed whether blockade of endogenous IL-21 by a neutralizing anti-IL-21 Ab in cultures of Crohn's disease $CD3^+$ LPL reduced the production of IL-21. Notably, the anti-IL-21 Ab reduced p-Stat3 (Fig. 8, *A* and *B*) and caused a marked decrease in IL-21 RNA and protein expression (Fig. 8, *C–E*). This effect was not mediated by any change in $CD3^+$ LPL survival because the anti-IL-21 did not modify the fraction of AV/PI-positive cells within the timeframe of IL-21 analysis (not shown).

Discussion

In this study, we show that IL-21 induces IL-21 in human $CD3^+$ T lymphocytes, and this effect is seen in both PBL and LPL. Induction of IL-21 is dose- and time-dependent and occurs at both the RNA and protein levels. Our data also indicate that induction of IL-21 in resting $CD3^+$ PBL is positively regulated by IL-7 and IL-15, but not IL-4, clearly indicating that signals mediated by the common γ-chain are not sufficient to enhance IL-21 gene transcription. In line with previous studies showing that IL-21 acts in concert with other common γ-chain-dependent cytokines to modulate T cell functions (17, 33), we also show that IL-21 cooperates with both IL-7 and IL-15 in enhancing the expression of IL-21 RNA in resting PBL.

In accordance with previous studies showing that Stat3 is one of the major signaling pathways initiated after binding of IL-21 to its receptors in T cells (27, 34, 35), we here show that induction of IL-21 in human $CD3^+$ T lymphocytes by IL-21 is strictly dependent on the activation of Stat3. Indeed, treatment of highly purified $CD3^+$ PBL with exogenous IL-21 enhances p-Stat3 and promotes the recruitment of Stat3 to the IL-21 gene promoter. Moreover, blockade of Stat3 activation by either a nonspecific pharmacologic compound (i.e., AG490) or selective knockdown of Stat3 by silencing completely abrogates the IL-21-induced IL-21 expression. In the same cell cultures, IL-21 expression is also inducible by IL-6, another cytokine that activates Stat3. A comparative analysis of the effect of IL-21-inducing cytokines on Stat3 shows that IL-21 is more effective than IL-6, IL-7, and IL-15 in enhancing p-Stat3 in resting PBL. By contrast, both IL-7 and IL-15 are more effective than IL-21 and IL-6 in enhancing IL-21 RNA expression, thus suggesting that signaling pathways other than Stat3 can control IL-21 expression in resting $CD3^+$ PBL following stimulation with IL-7 and IL-15.

By using intestinal $CD3^+$ LPL taken from patients with Crohn's disease, an inflammatory bowel disease characterized by high mucosal production of IL-21 (20), we show that neutralization of endogenous IL-21 reduces both p-Stat3 and IL-21 synthesis. In Crohn's disease, the mucosa is massively infiltrated with activated

$CD3^+$ LPL (36). This phenomenon is supposed to be due, at least in part, to the resistance of these cells against apoptotic stimuli (37, 38). The exact molecular mechanism underlying such a defect remains to be ascertained, but there is evidence that the sustained activation of Stat3 may play a decisive role in prolonging Crohn's disease LPL survival (39). The down-regulation of p-Stat3 seen in Crohn's disease $CD3^+$ LPL treated with the anti-IL-21 was associated with no significant change in the rate of apoptosis. Because the fraction of apoptotic cells was evaluated within the timeframe of IL-21 analysis, we can thus conclude that the down-regulation of IL-21 expression caused by anti-IL-21 is not secondary to the induction of cell death. The fact that analysis of apoptosis was limited to cells treated with the anti-IL-21 for a short time (i.e., 12 and 24 h) does not exclude, however, the possibility that in long-term Crohn's disease $CD3^+$ LPL cultures, neutralization of IL-21 may trigger apoptotic programs. Indeed, it has been recently shown that IL-21 is a survival factor for resting and activated T cells, and that IL-21 positively regulates the induction of the anti-apoptotic molecule, Bcl-2 (13). Studies are now in progress to address this issue.

Compelling evidence suggests that IL-21 plays an important role in the pathogenesis of various chronic inflammatory diseases (21, 25, 26, 40). The results presented herein suggest the existence of a positive autocrine loop that could help amplify and stabilize IL-21-driven, T cell-mediated responses. In this context, it is noteworthy that IL-21 is known to influence the differentiation/expansion of distinct lineages of inflammatory T cells. Indeed, recent studies have shown that IL-21 favors the differentiation of Th-17 cells that are implicated in the pathogenesis of immune-mediated diseases (34, 41–43). IL-21 is also able to differently regulate the production of cytokines by Th1 and Th2 cell subsets, mostly depending on the cell context analyzed. In particular, studies in murine systems have shown that an exposure of naive T cells to IL-21 results in a decrease in the production of IFN-γ, but not other Th1 cell cytokines, upon secondary stimulation (44). The inhibitory effect of IL-21 on IFN-γ production seems to rely on the ability of IL-21 to repress the expression of Eomesodermin, a T-box transcription factor. Indeed, ectopic expression of Eomesodermin protects developing Th1 cells from the inhibition of IFN-γ (45). By contrast, studies with human cells have shown that IL-21 can enhance the expression of Th1-associated transcription factors, such as Stat4 and T-bet, thereby increasing IFN-γ production (16, 17, 20). IL-21 could also make T cells resistant to the T regulatory cell-mediated suppression, further enhancing T cell activation (14). Thus, targeting IL-21 in T cell-mediated inflammatory diseases could help stop the autocrine induction of IL-21, thereby attenuating $CD4^+$ T cell activation and facilitating the resolution of tissue-damaging immune responses.

Disclosures

The authors have no financial conflict of interest.

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