The induction of antibody production by IL-6 is indirectly mediated by IL-21 produced by CD4+ T cells

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Interleukin (IL) 6 is a proinflammatory cytokine produced by antigen-presenting cells and nonhematopoietic cells in response to external stimuli. It was initially identified as a B cell growth factor and inducer of plasma cell differentiation in vitro and plays an important role in antibody production and class switching in vivo. However, it is not clear whether IL-6 directly affects B cells or acts through other mechanisms. We show that IL-6 is sufficient and necessary to induce IL-21 production by naive and memory CD4+ T cells upon T cell receptor stimulation. IL-21 production by CD4+ T cells is required for IL-6 to promote B cell antibody production in vitro. Moreover, administration of IL-6 with inactive influenza virus enhances virus-specific antibody production, and importantly, this effect is dependent on IL-21. Thus, IL-6 promotes antibody production by promoting the B cell helper capabilities of CD4+ T cells through increased IL-21 production. IL-6 could therefore be a potential coadjuvant to enhance humoral immunity.

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plasmocytosis, suggesting that IL-6 can promote the differentiation of B cells into plasma cells (12). Likewise, IL-6-deficient mice show reduced antigen-specific IgG1, IgG2a, and IgG3 levels upon immunization with a T cell-dependent antigen, although IgM levels were not affected (13). Further evidence for a role of IL-6 in IgG production has come from experiments using a transgenic mouse expressing a truncated form of gp130. These mice are unable to activate STAT3 upon IL-6 exposure and show reduced levels of most antibody isotypes after immunization with a T-dependent antigen (14). However, expression of the transgene is not restricted to B cells, leaving open the possibility that other cells may require a functional gp130 receptor. Likewise, B cell-specific deletion of STAT3 results in impaired plasma cell differentiation and diminished antibody responses (15), but this transcription factor is also activated by other cytokines.

The cytokine IL-21 has been shown to play a major role in antibody production by promoting the differentiation of B cells into plasma cells both in mice and humans (16, 17). Accordingly, IL-21 promotes the production of IgG1, IgG2a, and IgG3, but it has an inhibitory effect on IgE production (18). The inhibition of IgE is mediated by IL-21-induced up-regulation of Id2 that negatively regulates class switching to IgE (19). Similar to IL-6, IL-21 activates predominantly STAT3, but through binding to its specific receptor and the common ν chain signal transducing unit that it shares with other members of the IL-2 family of cytokines (20). In B cells, IL-21-induced STAT3 activation down-regulates B cell lymphoma 6 and up-regulates expression of B lymphocyte-induced maturation protein 1, thereby promoting plasma cell differentiation (21).

In this report, we identify IL-21 as the only cytokine that is specifically induced in CD4+ T cells by IL-6 early upon antigen stimulation. IL-6 is necessary to mediate IL-21 production in naive CD4+ T cells during antigen stimulation. Furthermore, we show that IL-6 promotes antibody production in B cells indirectly by up-regulation of IL-21 expression in CD4+ T cells and that this IL-21 then acts on B cells.

RESULTS AND DISCUSSION
Regulation of early gene expression by IL-6 during the activation of CD4+ T cells
IL-6 has been associated with different aspects of T cell activation, differentiation, and survival (2). However, little is known about the changes in gene expression induced by IL-6 stimulation early during T cell activation. To further characterize early effects of IL-6 on CD4+ T cells, we performed microarray analysis. Sorted CD4+ NK1.1+ T cells were activated with anti-CD3 and anti-CD28 mAbs in the absence or presence of IL-6 for 16 h. For each condition, three independent preparations of RNA were used for hybridization of gene arrays. For analysis of microarray data, we explored the use of the 1% contour on the joint distribution of fold change and p-value derived from sample label permutations (Fig. 1 A), which resulted in significance at P = 0.1, as well as identification of 343 genes, with a false discovery rate of 13%. 132 differentially regulated genes (190 probe sets) were found when using a twofold threshold on the probe set intensity ratio, IL-6 treated to untreated (Fig. 1 B; and Fig. S1 and Table S1, available at http://www.jem.org/cgi/content/full/jem .20081571/DC1). The microarray results were validated by real-time RT-PCR of several genes positively or negatively affected by the IL-6 treatment (Fig. S2).

Among the 132 genes whose expression was affected by IL-6, the cytokine IL-21 was especially remarkable, with a >200-fold induction in CD4+ T cells activated in the presence of IL-6. The induction of IL-21 by IL-6 was confirmed by real-time RT-PCR using RNA from CD4+ T cells activated for 16 h (Fig. 1 C). Similar results were obtained by ELISA, where IL-21 production could only be detected in the presence of IL-6 (Fig. 1 D). Although IL-6 has been shown to induce the expression of IL-4 and IL-17 (7, 8, 10, 22), their expression was not increased by IL-6 stimulation early during activation. These results also confirm a recent study showing that induction of IL-21 by IL-6 is independent of TGF-β stimulation and Th17 cell differentiation (23). Moreover, among known cytokines IL-21 is the most potently induced one by IL-6 early during the activation of CD4+ T cells, as indicated by its high up-regulation, especially when compared with other soluble factors (Table S1).

IL-6 is required for IL-21 expression in CD4 T cells upon TCR stimulation
Given the high induction of IL-21 by IL-6, we examined whether other cytokines have a similar effect. CD4+ T cells were activated in the presence of IL-4, IL-12, or IFN-γ, but none of those cytokines were able to promote IL-21 production (Fig. 2 A). Neither growth and survival factors such as IL-2 and IL-7 (Fig. 2 A and not depicted) nor other proinflammatory cytokines such as TNF-α and IL-1 were able to up-regulate IL-21 production (Fig. 2 A and not depicted). IL-11, another cytokine that signals through gp130 (1), also failed to promote IL-21 production (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20081571/DC1). Moreover, IL-6 is a very efficient inducer of IL-21 expression, as a dose–response analysis revealed that IL-6 levels as low as 0.3–0.8 ng/ml were able to readily induce measurable IL-21 production (Fig. 2 B).

To determine whether IL-6 was required for IL-21 production by CD4+ T cells, spleen cells from wild-type or IL-6−/− mice were treated with LPS, and the conditioned media were added to CD4+ T cells activated with anti-CD3 and anti-CD28 mAbs. Only CD4+ T cells activated in the presence of the LPS-conditioned media from wild-type splenocytes expressed IL-21, whereas this expression was abrogated in the presence of a neutralizing anti-IL-6 mAb (Fig. 2 C). In addition, conditioned media from IL-6−/− splenocytes stimulated with LPS did not promote IL-21 expression (Fig. 2 C). As a control, LPS alone added to activated CD4+ T cells did not induce IL-21 expression (Fig. 2 C). Thus, APC-derived IL-6 is required for IL-21 production by CD4+ T cells upon activation in vitro. To show that IL-6 is required for IL-21 expression in vivo, IL-6−/− and wild-type mice were immunized with OVA, and CD4+ T cells were analyzed for IL-21 production by ELISPOT analysis.
The number of IL-21–producing CD4+ T cells was severely impaired in IL-6−/− compared with wild-type mice (Fig. 2 D). Collectively, these results demonstrate that IL-6 is required for the production of IL-21 by CD4+ T cells upon antigen stimulation in vitro and in vivo. Although a recent paper reported that IL-21 can induce its own expression through STAT3 (24), our results indicate that the initial IL-21 expression in CD4+ T cells is mediated by IL-6.

**Figure 1. Effects of IL-6 on the gene expression profile of CD4+ T cells early during activation.** (A) FACS-sorted CD4+ T cells were activated with anti-CD3 and anti-CD28 in the absence or presence of IL-6 for 16 h. The gene expression profile from total RNA was examined by Affymetrix GeneChip analysis. In the volcano plot, open circles represent probe sets for which expression is changed more than twofold up or down after IL-6 treatment, whereas dots represent all other probe sets. The number of genes exceeding the threshold was the largest among the 10 datasets obtained by permutation of sample labels, yielding statistical significance of differential expression at P = 0.1 (the minimum value) and a false discovery rate < 19% (the minimum being 10%). The blue line encloses 99% of the probe sets under the null hypothesis, obtained by permutation of sample labels. The IL-21 gene is marked with an arrow. (B) Heat maps representing the relative expression of genes among IL-6–treated and control samples. Genes were sorted within each group based on their fold change (top, highest fold induction; bottom, highest fold down-regulation). Expression statistics for each gene were centered, scaled, and mapped to a color scale. Red represents relatively low expression, whereas green represents relatively high expression. (C) Cells were purified and activated as in A, and IL-21 expression was measured by quantitative real-time RT-PCR. The means ± SEM of three experiments are shown. (D) CD4+ T cells were activated with anti-CD3 and anti-CD28 mAbs in the absence or presence of IL-6 for the indicated periods of time. Cell-culture supernatants were analyzed for IL-21 production by ELISA. The means ± SEM of four experiments are shown.
Figure 2. IL-6 is sufficient and necessary to induce IL-21 expression during the activation of naive and memory CD4+ T cells. (A and B) FACS-sorted CD4+ T cells were activated with anti-CD3 and anti-CD28 mAbs in the absence or presence of the indicated cytokines (A) or the indicated IL-6 concentrations (B). After 3 d, cell-culture supernatants were analyzed for IL-21 production by ELISA. The means ± SEM of three (A) or four (B) experiments are shown. (C) FACS-purified CD4+ T cells were activated with anti-CD3 and anti-CD28 mAbs in the presence of medium alone, LPS, conditioned media (CM) from LPS-stimulated mouse splenocytes, CM plus 10 μg/ml of a neutralizing anti–IL-6 (αIL-6) mAb, or CM from LPS-stimulated IL-6/fl−/− splenocytes. IL-21 expression was measured by quantitative real-time RT-PCR. One representative experiment out of two is shown (error bars represent SD). (D) C57BL/6 and IL-6/fl−/− mice were immunized s.c. with 200 μg OVA in CFA. CD4+ T cells prepared from spleens and lymph nodes were analyzed 6 d later for the frequency of IL-21–producing cells by ELISPOT. The means ± SD of three immunizations are shown (P < 0.05). (E) FACS-purified CD4+ CD25− NK1.1− CD44low naive cells were activated with anti-CD3 and anti-CD28 mAbs in the absence or presence of IL-6 for the indicated periods of time. IL-21 expression was measured by quantitative real-time RT-PCR. One representative experiment out of two is shown (error bars represent SD). (F) CD4+ T cells were isolated from AND TCR transgenic mice, and activated with 5 μM cyt c peptide and APCs in the absence or presence of IL-6 for the indicated periods of time. IL-21 production was examined by ELISA. One representative experiment out of two is shown (error bars represent SD of a triplicate determination). (G) FACS-purified CD4+ CD25− NK1.1− CD44high memory cells were activated with anti-CD3 and anti-CD28 mAbs in the absence or presence of IL-6 for 24 h. Cytokine expression was measured by quantitative real-time RT-PCR. Values are presented as fold induction over naive CD4+ T cells activated for 24 h. One representative experiment out of two is shown.
IL-21 has been shown to be produced by differentiated Th2 and Th17 effector CD4+ T cells upon restimulation as well as by NKT cells (25–29). To further demonstrate the direct effect of IL-6 on IL-21 production by naive CD4+ T cells, we activated sorted CD4+ CD44low NK1.1− T cells in the presence or absence of IL-6. IL-21 expression was essentially undetectable in purified naive CD4+ T cells activated in the absence of IL-6 even after 3 d of stimulation (Fig. 2 E). In contrast, high IL-21 expression could be detected in activated naive CD4+ T cells treated with IL-6 as soon as 24 h after initial stimulation (Fig. 2 E). Similarly, naive CD4+ T cells from AND TCR transgenic mice (recognizing pigeon cytochrome c [cyt c]) produce IL-21 only in the presence of IL-6 upon stimulation with cyt c peptide and APCs (Fig. 2 F).

Memory CD4+ T cells are known for their ability to produce high amounts of effector cytokines such as IFN-γ and IL-4 rapidly after antigen stimulation (30). To test whether IL-21 expression was similar to other effector cytokines up-regulated early in memory CD4+ T cells, sorted CD4+ CD44high T cells were stimulated with anti-CD3 and anti-CD28 mAbs in the absence or presence of IL-6. As expected, memory CD4+ T cells expressed relatively high levels of IFN-γ and IL-4 regardless of the presence of IL-6 (Fig. 2 G). However, similar to naive CD4+ T cells, memory cells did not produce IL-21 in the absence of IL-6 but did have high levels of expression when IL-6 was present (Fig. 2 G). Thus, production of IL-21 by naive and memory CD4+ T cells upon antigen stimulation requires the presence IL-6.

Induction of antibody production by IL-6 requires up-regulation of IL-21 expression in CD4+ T cells

Both IL-6 and IL-21 have been shown to promote antibody production by B cells. Several studies demonstrated a direct effect of IL-21 on plasma cell differentiation through up-regulation of

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**Figure 3.** IL-6 requires IL-21 expression in CD4+ T cells to induce IgG1 production. (A) Total splenocytes from wild-type and IL-6−/− mice were stimulated with anti-CD3 mAb for 7 d. IgG1 levels in the cell supernatant were measured by ELISA. Results are representative of three experiments performed in triplicate. (B) B220+CD19+ B cells and CD4+ T cells from the spleens of C57Bl/6 mice were analyzed for IL-6Rα and IL-21R expression (bold-line histograms) by flow cytometry. Shaded histograms are control-stained cells. Representative profiles of one mouse out of five analyzed are shown. (C) Total splenocytes of IL-6−/− mice were activated with anti-CD3 mAb and 100 ng/ml IL-6 or 100 ng/ml IL-21 where indicated. After 7 d, IgG1 levels in the supernatant were determined by ELISA. (D) CD4+ T cells from IL-21−/− mice or wild-type littermates were activated with anti-CD3 mAb in the presence of B cells from IL-6−/− mice and the indicated cytokines. After 7 d, IgG1 levels in the supernatant were determined by ELISA. (E) CD4+ T cells from wild-type mice were activated with anti-CD3 mAb in the presence of B cells from IL-21R−/− mice or wild-type littermates and the indicated cytokines. After 7 d, IgG1 levels in the supernatant were determined by ELISA. The means ± SEM of a triplicate determination from one representative experiment out of three performed are shown in A and C–E.
the transcription factor B lymphocyte–induced maturation protein 1 (16, 17, 21). Although in vitro experiments using T–B cell co-culture and in vivo studies indicate that IL-6 is required for antibody production (13), the molecular mechanism remains unknown, and it is unclear whether this is a direct effect on B cells. Because IL-6 promotes IL-21 production by CD4+ T cells, it was possible that these two pathways are closely linked to provide T cell help to B cells. The analysis of antibody production in vitro with wild-type or IL-6−/− total spleen cells activated with anti-CD3 mAb confirmed the contribution of IL-6 to IgG1 production (Fig. 3 A). However, analysis of IL-6Ra cell-surface expression showed that resting splenic B cells had almost undetectable levels, whereas it was present on a substantial fraction of CD4+ T cells (Fig. 3 B). In contrast, IL-21R expression could be detected on B cells as well as CD4+ T cells, in accordance with a previous paper (20). In addition, IL-21 promoted IgG1 production in total splenocytes from IL-6−/− mice activated with anti-CD3 mAb, similar to IL-6 (Fig. 3 C). Collectively, these results suggest that IL-6 mediates antibody production by inducing IL-21 expression in CD4+ T cells. To test for this possibility, we established a co-culture of CD4+ T cells activated with anti-CD3 mAb and B cells in the presence of exogenous IL-6. CD4+ T cells were isolated from wild-type and IL-21−/− mice, whereas B cells were obtained from IL-6−/− mice, because IL-6 is known to be also produced by B cells. In the absence of IL-6, IgG1 levels were practically undetectable (Fig. 3 D). IL-6 strongly increased IgG1 production when B cells were cultured together with CD4+ T cells from wild-type mice but failed to induce IgG1 in the presence of IL-21−/− CD4+ T cells (Fig. 3 D). Thus, the effect of IL-6 on antibody production appears to be dependent on IL-21 produced by CD4+ T cells. Addition of exogenous IL-21 could compensate for IL-21 deficiency, indicating that IL-21−/− CD4+ T cells are functional despite their lack of IL-21 production (Fig. 3 D).

In addition to its effects on B cells, IL-21 has been shown to promote Th2 cell differentiation by inhibiting Th1 cell differentiation (25). It can also induce Th17 cell differentiation when TGF-β is present (27–29). To exclude that IL-6–induced IL-21 promoted antibody production through its effect on the differentiation of CD4+ T cells rather than acting directly on B cells, we activated wild-type CD4+ T cells with anti-CD3 mAb and co-cultured them in the presence of B cells from wild-type and IL-21R−/− mice. IL-6 augmented IgG1 production in wild-type B cells compared with the medium control (Fig. 3 E). In contrast, B cells that were deficient for IL-21R did not produce IgG1 when stimulated with IL-6 despite co-cultured CD4+ T cells that were capable of receiving IL-21 signals (Fig. 3 E). Accordingly, IL-21 promoted...
IgG1 production only when IL-21R was present on B cells but not in IL-21R−/− B cells, suggesting that IL-21 induced by IL-6 acted directly on B cells to increase IgG1 levels (Fig. 3 E). These results show that IL-6 can promote antibody production by inducing IL-21 expression in CD4+ T cells, and IL-21 subsequently acts on B cells.

Administration of IL-6 during influenza immunization promotes specific IgG production through IL-21

The indirect effect of IL-6 on antibody production through IL-21 generated by CD4+ T cells suggested that IL-6 could be a potent enhancer of the humoral immune response, because small amounts of IL-6 could result in an increased and prolonged production of IL-21, which would increase antibody production by B cells. We therefore investigated whether administration of IL-6 during viral immunization could boost the antiviral specific antibody response. We immunized wild-type mice with inactive PR8 influenza virus (in the absence of adjuvant) in combination with IL-6 or PBS, and analyzed virus-specific antibody production. Influenza-specific IgM serum levels were not affected by the administration of IL-6 (Fig. 4 A), indicating that IL-6 did not provide overall survival signals to B cells. In contrast, the presence of IL-6 during immunization substantially enhanced the levels of influenza-specific IgG1 (Fig. 4 A). Thus, IL-6 enhances the in vivo antibody response.

To determine whether the effect of IL-6 on in vivo antiviral antibody response is also dependent on IL-21, we immunized wild-type and IL-21−/− mice with inactive influenza virus in the presence or absence of IL-6. Influenza-specific IgM levels were comparable in IL-21−/− and wild-type mice (Fig. 4 B), in agreement with previous studies showing independence of IgM from IL-21 (18). In contrast, IL-6 promoted the production of IgG1 in wild-type mice, but it had no effect in IL-21−/− mice (Fig. 4 B). Collectively, these results show that administration of IL-6 enhances antibody response, but this effect is dependent on endogenous IL-21 production.

Role of IL-21 and IL-6 in antibody response to influenza infection

Despite the established role of IL-21 in generation of plasma cells and antibody production, it remains unknown whether IL-21 plays a role in antibody response during influenza infection or any other viral infection. We therefore examined...
the contribution of endogenous IL-21 to the production of virus-specific antibodies in response to a sublethal dose (0.1 LD_{50}) of the live PR8 influenza virus using wild-type and IL-21-deficient mice. 14 d after infection, influenza-specific antibody titers in serum were determined. Influenza-specific IgG1 and IgG2c levels were severely reduced in infected IL-21^{−/−} mice compared with wild-type mice (Fig. 5A). Thus, IL-21 plays a role in antibody response to influenza. We also examined the contribution of endogenous IL-6 to the antibody production against infection with PR8 infection using IL-6^{−/−} mice. Interestingly, we found in repeated experiments that IL-6-deficient mice were highly susceptible to influenza virus infection, because a sublethal dose of live PR8 was sufficient to cause a relatively high rate of mortality in these mice but not in wild-type mice (Fig. 5B), indicating that IL-6 is essential for protection against influenza virus. Unlike infected wild-type mice, infected IL-6^{−/−} mice could not recover the loss cost caused by the acute viral infection (Fig. 5C), suggesting that these mice could not clear or control the virus. Analysis of PR8-specific antibody production in the surviving IL-6^{−/−} mice showed a reduction in virus antibody titers (Fig. 5D). IL-6 is therefore a key cytokine for resolution of influenza virus infection.

In summary, we show that IL-6 is a very specific and effective inducer of IL-21 production by CD4^{+} T cells, and that it is sufficient and required for IL-21 production by naive (and probably memory) CD4^{+} T cells upon antigen stimulation in vitro and in vivo. Although it was proposed that IL-21 is preferentially expressed by effector Th17 cells (24, 27–29), a recently published study (23), as well as our results in this report, show that IL-6, in the absence of TGF-β, can induce the production of IL-21 in CD4^{+} T cells. IL-21 is also produced by the T follicular helper (Tfh) subset of CD4^{+} T cells that localizes in B cell follicles and provides help to B cells (31). Because follicular dendritic cells produce significant amounts of IL-6 (13), it is possible that the presence of IL-6 in follicles can contribute to the production of IL-21 by these Tfh cells. A study published at the time of submission of this manuscript supports the need of IL-6 for the generation of Tfh cells (32).

Although IL-6 has been long considered as a B cell growth factor and mediator of plasma cell differentiation, our data in this report show the requirement of IL-21 for IL-6 to induce antibody production in vitro and in vivo. We propose that IL-6 enhances antibody production indirectly by acting on naive (and likely memory) antigen-specific CD4^{+} T cells to promote their differentiation into Tfh cells that produce high levels of IL-21. Accordingly, we also show that both IL-6 and IL-21 play a role in the immune response during influenza infection, and administration of IL-6 during viral immunization substantially increased virus-specific antibody response without the need of additional coadjuvants, but in an IL-21-dependent manner. The use of IL-6 as coadjuvant during immunization may therefore be a promising tool to achieve successful vaccination against specific pathogens whose clearance is highly dependent on humoral immunity.
TaqMan system with Assay-on-Demand sets (Applied Biosystems) and normalized to the levels of B2-microglobulin present in the same sample.

Microarray. Fragmented biotinylated antisense cRNA was prepared according to the Affymetrix standard labeling protocol and hybridized to Mouse 430a 2.0 GeneChips (Affymetrix) at the Microarray Core Facility of the University of Vermont. Microarray data have been deposited in the Gene Expression Omnibus under accession no. GSE7459.

Data analysis. The signal intensity for each probe on each chip was calculated from scanned images using GeneChip Operating Software (Affymetrix). Signal intensities were analyzed using R (38) and BioConductor (39). Probe intensities were normalized using the Qspline method (40), a cubic spline normalization using quantiles. An expression statistic was calculated for each probe set on each chip using the robust multichip average method (41, 42). The difference of population means, IL-6 treated minus control, was considered significant if the mean signal ratio was at least twofold change in gene expression level after IL-6 treatment and the microarray analysis. Fig. S3 shows the lack of IL-21 production by CD4+ T cells after IL-11 treatment. Table S1 lists all genes with an at least twofold change in gene expression level after IL-6 treatment during the activation of CD4+ T cells. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20081571/DC1.

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