Interleukin-21 Is a Critical Cytokine for the Generation of Virus-Specific Long-Lived Plasma Cells

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Long-lived plasma cells that reside in the bone marrow constitutively produce antibody in the absence of antigen and are the cellular basis of durable humoral immunity. The generation of these long-lived plasma cells depends upon a series of highly orchestrated interactions between antigen-specific CD4 T cells and B cells and the formation of germinal centers (GCs). In this study, we have examined the role of the cytokine interleukin-21 (IL-21) in regulating humoral immunity during acute viral infections. Using IL-21 receptor-deficient (IL-21R−/−) mice, we found that virus-specific CD4 T cells were generated after infection with lymphocytic choriomeningitis virus (LCMV) and that these CD4 T cells differentiated into T follicular helper (TFH)–like cells in the absence of IL-21 signaling. There was also no defect in the formation of GCs, although after day 15 these GCs disappeared faster in IL-21R−/− mice than in wild-type mice. Isotype switching and the initial LCMV-specific IgG response were normal in IL-21R−/− mice. However, these mice exhibited a profound defect in generating long-lived plasma cells and in sustaining antibody levels over time. Similar results were seen after infection of IL-21R−/− mice with vesicular stomatitis virus and influenza virus. Using chimeric mice containing wild-type or IL-21R−/− CD4 T cells and B cells, we showed that both B and CD4 T cells need IL-21 signaling for generating long-term humoral immunity. Taken together, our results highlight the importance of IL-21 in humoral immunity to viruses.
shown that CD4 TFH cells also express high levels of the IL-21 receptor (IL-21R) (17, 19). Using protein antigens, it was shown that IL-21 presumably acts as an autocrine factor for TFH cells (20, 21). However, recent studies have questioned the involvement of IL-21 in regulating CD4 TFH cells and humoral immunity after viral infection needs further study. To address these questions, we used three different viral systems, lymphocytic choriomeningitis virus (LCMV), influenza virus, and vesicular stomatitis virus (VSV), to evaluate the role of IL-21 signaling in long-term humoral immunity.

**MATERIALS AND METHODS**

**Mice and infections.** Six- to 8-week-old C57BL/6 (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). IL-21R-deficient (IL-21R−/−) mice have been described previously and were maintained and bred at the animal facility of Emory University (22). For infections, mice received 2 × 10^6 PFU of LCMV Armstrong intraperitoneally (i.p.), 2 × 10^6 PFU of the VSV Indiana serotype i.p., or 200 PFU of influenza virus PR8 intranasally (i.n.). Animals were housed at the Emory University School of Medicine animal facility, and experiments were performed according to approved IACUC protocols.

**Antibodies and flow cytometry.** The MHC class II tetramer specific for the LCMV glycoprotein epitope from residues 66 to 77 (IAβ-gp66-77) was provided by the NIH tetramer core facility at Emory University. For direct ex vivo staining, 2.0 μg/ml of tetramer was used in RPMI (10% fetal calf serum [FCS]) with 10 mM HEPES. Splenocytes (10^6) were resuspended at 300 μl/well in 96-well round-bottom plates and incubated at 37°C for 3 h. After this tetramer staining, the cells were stained with other surface antibodies. For intracellular cytokine staining, 10^6 splenocytes were incubated with a peptide of LCMV consisting of residues 66 to 81 of GP (GP66-81) in the presence of brefeldin A for 5 h at 37°C. Following staining with surface antibodies, intracellular cytokines were stained and cells were fixed in Cytofix/Cytoperm (BD Biosciences). PD-1 antibody was purchased from Biolegend. Antibodies to Bcl-6, CXCR5, CD95, ICOS, CD4, B220, CD44, gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), and IL-2 were purchased from BD Biosciences. Bcl-6 and CXCR5 staining was performed as described by Eto et al. (23). PNA was procured from Vector Laboratories. Cells were acquired on a FACSCanto II cytometer (BD Biosciences) and analyzed on FlowJo software (Tree Star, Inc.).

**ELISA.** LCMV-specific antibodies were measured by enzyme-linked immunosorbent assay (ELISA). LCMV clone 13-infected BHK-21 cell lysate was used as capture antigen. Ninety-six-well Polysoor plates (Nunc, Rochester, NY) were coated with sonicated lysate for 2 days before performing the ELISA. Threefold serial dilutions of serum samples were incubated and detected with isotype-specific hors eradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin (Southern Bio tech.). o-Phenylenediamine dihydrochloride (OPD) was used as a substrate, and the reaction was stopped with HCl and read at 490 nm. For detecting VSV-specific IgG, the same ELISA protocol was used, with VSV-infected BHK-21 cell lysate used as the capture antigen. For the influenza virus PR8-specific ELISA, PR8 virus was used as the capture antigen. To determine relative affinity, after the antibody binding, the ELISA plates were incubated with 8 M urea to detect the endpoint shift of LCMV nucleoprotein (NP)-specific antibody.

**ASC (ELISPOT) assay.** LCMV-specific antibody-secreting cells (ASCs) were quantitated by enzyme-linked immunosorbent spot (ELISPOT) assay. LCMV clone 13-infected BHK-21 cell lysate was used as the capture antigen. After coating the plates for 2 days, cells were incubated for 8 h and then detected using biotinylated goat anti-mouse IgG (Caltag Laboratories) and HRP-conjugated avidin D (Vector Laboratories Inc.). Using the same protocol, VSV-infected BHK-21 cell lysate was used for detecting VSV-specific ASCs and PR8 virus was used for detecting influenza virus-specific ASCs.

**Recombinant LCMV NP.** The entire coding sequence of the nucleoprotein gene, minus the stop codon, was cloned from LCMV Armstrong strain RNA by reverse transcription-PCR (RT-PCR). The gene was sequenced and subcloned into vector pET-41b (Novagen) using the NdeI and NotI sites. This eliminated the upstream glutathione S-transferase tag and put the nucleoprotein gene in frame with a C-terminal 8× histidine tag. The protein was expressed in Rosetta2(DE3) cells (Novagen) by overnight induction at room temperature and purified under native conditions on Ni-nitrilotriacetic acid Superflow agarose (Qiagen). The eluted protein was buffer exchanged into phosphate-buffered saline (PBS).

**HAI assay.** Serum was treated with receptor-destroying enzyme (RDE) and serially diluted (2-fold) in a 96-well V-bottom plate. Eight hemagglutination units (HAU) of PR8 was incubated for 30 min, and 0.5% turkey red blood cells (RBCs) was added to each well. The hemagglutination inhibition (HAI) endpoint was taken as the highest serum dilution in which no agglutination was observed.

**Neutralization assay.** Heat-inactivated serum was serially diluted (2-fold) and incubated with 100 PFU of VSV at 37°C for 1 h. The virus antibody mixture was then transferred onto Vero cell monolayers and incubated at 37°C for 45 min and subsequently overlaid with 1% agarose in 2× Dulbecco modified Eagle medium. The plaques were enumerated after the monolayers were fixed with 20% ethanol and stained with 1% crystal violet.

**Quantitative real-time RT-PCR.** Total RNA was isolated using an RNeasy kit (Qiagen) according to the manufacturer’s protocols. RNA was reverse transcribed using a QuantiTect reverse transcription kit from Qiagen. Real-time PCR was performed on an iCycler apparatus (Bio-Rad) using SYBR green (Qiagen). Oligonucleotides for Bcl-6 and IL-2 were also purchased from Qiagen. Results were normalized to the β-actin mRNA levels and represented using the comparative threshold cycle method.

**Statistical analysis.** Data were analyzed using Prism (version 6.0) software (GraphPad). Statistical analysis between the experimental groups was assessed using a two-tailed unpaired Student t test.

**RESULTS**

The LCMV-specific antibody response is not sustained in IL-21R−/− mice. To determine the effect of IL-21 signaling on B cell responses, we used a mouse model of acute LCMV infection. Wild-type (WT) and IL-21R−/− mice were infected with LCMV Armstrong and serially bled to measure LCMV-specific IgG in the serum using ELISA. As shown in Fig. 1A, the IgG responses to LCMV in IL-21R−/− mice were comparable to those in wild-type mice at days 8 and 15 postinfection, but there was a dramatic decline in the levels of LCMV-specific IgG after day 15 postinfection. At memory phase (days 90 and 300 postinfection), the IL-21R−/− mice had minimal to no detectable LCMV-specific IgG, whereas wild-type mice sustained the expected high titers. In wild-type mice, at day 8 after LCMV infection, the majority of the LCMV-specific IgG present was produced by extrafollicular SLPCs and was of lower affinity. As we saw no difference in LCMV antibody titers between the wild-type and IL-21R−/− mice at day 8 after LCMV infection (Fig. 1A), we wanted to ascertain if their affinities were comparable. To determine this we used conventional ELISA combined with a urea wash after the antibody had been bound to the LCMV NP antigen on the plate. Urea, being a chaotropic agent, dissociates the binding of low-affinity but not high-affinity antibody, which is reflected as a shift in the endpoint dilution. At day 8, there was a drop in the endpoint titer of serum antibody after the urea wash in both WT and IL-21R−/− mice (Fig.
Later, as the immune response progressed, WT mice generated higher-affinity antibody. However, there was no further improvement in the affinity of antibody produced in IL-21R−/−mice. Collectively, these results show that there is a defect in long-term antibody maintenance and affinity maturation in the absence of IL-21 signaling.

Defective humoral immune responses in IL-21R-deficient mice after influenza virus and VSV infections. We next examined if the defect in maintaining virus-specific antibody levels in the absence of IL-21 signaling occurred with other viral infections. To determine this, we infected IL-21R−/−mice with either influenza virus or VSV, which induce mucosal and systemic infections, respectively. Wild-type and IL-21R−/−mice were infected with influenza virus and then bled serially to determine HAI titers in serum. As shown in Fig. 2A, the wild-type mice showed robust HAI titers that were maintained at high levels. Analogous to the LCMV study (Fig. 1A), we found that the IL-21R−/−mice had HAI titers comparable to those of wild-type mice at day 8 postinfection but the HAI titer did not increase further and in fact slightly declined. Similar results were obtained when we measured the PR8-specific IgG level in serum of wild-type and IL-21R−/−mice (Fig. 2B). To further extend these observations, we then used a third viral system infecting IL-21R−/−mice with VSV. As shown in Fig. 2C, in wild-type mice, the VSV-specific IgG level increased from day 0 to day 24 postinfection, at which point the level plateaued and was then sustained for the time frame of the experiment (75 days following infection). In contrast, the IL-21R−/−mice had significantly lower levels of VSV-specific IgG at all the time points tested. Anti-VSV neutralizing antibody levels were significantly lower in IL-21R−/−mice at memory phase (Fig. 2D).

Antibody levels at early time points were variable, depending on the type of infection; LCMV and influenza virus infection induced comparable levels of antibody in both WT and IL-21R−/−mice, whereas after VSV infection, the defect was much more pronounced in IL-21R−/−mice. In summary, the kinetics of the antiviral IgG responses for all three viral infections showed that the lack of IL-21 signaling profoundly affected the induction of long-lived antibody responses.
Analysis of LCMV-specific CD4 T cell response in IL-21R−/− mice. To examine the role of IL-21 signaling during the generation and maintenance of CD4 T cell responses, we first analyzed antigen-specific CD4 T cells using MHC class II tetramer specific for the LCMV glycoprotein epitope from residues 66 to 77 (IAb-gp66-77). As shown in Fig. 3A, there was no defect in generating LCMV-specific CD4 T cells in IL-21R−/− mice. In fact, if anything, the CD4 T cell response appeared to be slightly higher in the IL-21R−/− mice than the WT mice. Next, we asked if CD4 T cells in the IL-21R−/− environment produced Th1 cytokines. For this, we performed intracellular cytokine staining to measure IFN-γ, TNF-α, and IL-2 after in vitro stimulation of splenocytes with the GP61-80 peptide at various time points after infection. The frequency of IFN-γ-positive (IFN-γ+), TNF-α-positive (TNF-α+) and IFN-γ+ IL-2-positive (IL-2+) CD4 T cells was slightly higher at day 8 and day 15 in IL-21R−/− mice than the wild-type mice (Fig. 3B and C). At later time points, the frequency of IFN-γ+ TNF-α+ and IFN-γ+ IL-2+ CD4 T cells was comparable between the IL-21R−/− mice and wild-type mice. These data clearly demonstrate that Th1 responses are intact in the absence of IL-21 signaling.

CD4 T<sub>FH</sub> cells are generated in the absence of IL-21 signaling after infection with LCMV. We next investigated if the failure to induce long-lived antibody responses in the absence of IL-21 signaling was due to a defect in the generation of virus-specific CD4 T<sub>FH</sub> cells. As shown in Fig. 4A, LCMV-specific CD4 T cells from spleen were stained with class II tetramer (IAb-gp66-77) at day 12 after infection. Coexpression of CXCR5 and ICOS on LCMV-specific CD4 T cells was comparable between wild-type and IL-21R−/− mice. In addition, PD-1 expression was also similar on LCMV-specific CD4 T cells from both the groups. Naive cells from the same mouse were gated and used as a negative control for all the three TFH cell markers. Bcl-6 has been shown to be an important transcriptional regulator of T<sub>FH</sub> cell differentiation (24). Interestingly, there was also no defect in the expression of Bcl-6 at the protein and transcript levels in CD4 T cells in the absence of IL-21 signaling (Fig. 4B). We next measured the level of the IL-21 cytokine transcript in sorted CD4 T<sub>FH</sub> cells using RT-PCR. As shown in Fig. 4C, CD4 T<sub>FH</sub> cells from IL-21R−/− mice had abundant levels of IL-21 mRNA. In summary, these data clearly show that a lack of IL-21 signaling does not affect CD4 T<sub>FH</sub> cell differentiation in response to LCMV infection.

GC B cells are generated in IL-21R-deficient mice but are not sustained. Germinal centers are essential for the generation of memory B cells and LLPCs that reside in the bone marrow. We have shown that CD4 T<sub>FH</sub> cells, a critical cellular component of the GC reaction, are generated in the absence of IL-21 signaling (Fig. 4). We next addressed if IL-21 signaling is essential for the generation of GC B cells, the other critical cellular component of the GC reaction. Surprisingly, at day 8 after LCMV infection, there was an approximately 3-fold increase in the frequency of GC B cells.
IL-21 and Long-Term Humoral Immunity

Lack of IL-21 signaling does not affect short-lived plasma cell generation but results in a profound defect in generation of long-lived plasma cells in the bone marrow. We have shown that IL-21 signaling is required for the maintenance of long-term antibody after LCMV, influenza virus, and VSV infection (Fig. 1 and 2). We next determined if this defect was due to a defect in the generation of LLPCs, the producers of long-term antibody. To do this we used an ELISPOT assay to determine the number of LCMV-specific ASCs generated in spleen and bone marrow after LCMV infection. At day 8 following infection, a robust plasma-
blast response was observed in the spleens of IL-21R−/− mice, although the response was about 2-fold lower than that in wild-type mice (Fig. 6, top). LCMV-specific ASCs were still detectable in the spleens of the IL-21R−/− at days 15 and 22 postinfection, albeit they were 11- and 16-fold lower, respectively, than those in the spleens of wild-type mice. Strikingly, by day 38, no LCMV-specific ASCs were detected in the bone marrow of the IL-21R−/− mice. We next analyzed the bone marrow, where LLPCs reside. As expected, at day 8 after infection there were no ASCs in the bone marrow in wild-type or IL-21R−/− mice (Fig. 6, bottom). ASCs were also detectable in the IL-21R−/− mice at days 15 and 22 postinfection but were significantly reduced in numbers (~13-fold lower) (Fig. 6, bottom). By day 38, there were no detectable LCMV-specific ASCs present in the bone marrow of the IL-21R−/− mice. This long-term ASC defect was not specific to LCMV infection. As shown in Fig. 7, similar results were obtained after influenza virus PR8 and VSV infection. At day 143 after influenza virus or VSV infection, the numbers of influenza virus-
specific (Fig. 7A) or VSV-specific (Fig. 7B) ASCs in bone marrow were significantly reduced. Together, these data demonstrate that IL-21 signaling is dispensable for the differentiation of extrafollicular SLPCs but critical for the generation and maintenance of LLPCs in bone marrow after viral infection.

The requirement of IL-21 signaling for sustaining humoral immune responses is both B and T cell autonomous. As shown in Fig. 4, a lack of IL-21 signaling did not affect the generation of CD4 Tfh cells, as assessed by the conventional Tfh markers CXCR5, ICOS, and PD-1. Even Bcl-6 and IL-21 were expressed at comparable levels in IL-21R−/− CD4 Tfh cells and wild-type cells. Hence, we sought to investigate if the failure to induce LLPCs was intrinsic to CD4 T cells or B cells using splenic chimeras. As shown in Fig. 8A, B cells and T cells were purified from the spleens of wild-type and IL-21R−/− mice and mixed in equal proportions (25 x 10^6 cells each) and transferred into irradiated naive recipients, resulting in 4 different chimeras: wild-type T cells and wild-type B cells (TWT BWT), wild-type T cells and IL-21R−/− B cells (TWT BKO, where KO indicates knockout), IL-21R−/− T cells and wild-type B cells (TKO BWT), and lastly, wild-type T cells and IL-21R−/− B cells (TWT BKO). These chimeric mice were then infected with LCMV and serum samples were collected at the indicated time points. Figure 8B shows the ELISA profiles of LCMV-specific IgG in serum from two independent experiments. In experiment I, as expected, on both day 15 and day 30 postinfection, a significant amount of LCMV-specific IgG was seen in the TWT BWT chimeras. In contrast, a greatly reduced or undetectable LCMV-specific IgG response was seen in the TKO BKO, TKO BWT, and TWT BKO mice. In a second independent experiment (experiment II), there was a complete abrogation of LCMV-specific IgG on day 22 and day 35 postinfection in all the chimeras except TWT BWT (Fig. 8B). These results clearly indicate that IL-21 signaling is required for both CD4 T and B cells to induce long-term humoral immune responses.

**DISCUSSION**

In this study, we evaluated the role of IL-21 signaling in regulating humoral immune responses after acute viral infections using IL-21R−/− mice. We found that SLPCs, GC B cells, and antigenspecific Tfh cells are generated in IL-21R−/− mice. However, the GC B cells are not sustained in the absence of IL-21 signaling. The early collapse of the GCs may contribute to the profound defect in the generation of LLPCs and the subsequent maintenance of long-term antibody. These findings were observed in both systemic
LCMV and VSV) and mucosal (influenza virus) viral infections. Using chimeric mice, we were able to demonstrate that the requirement for IL-21 signaling to generate effective long-term humoral immunity is intrinsic to both CD4 TFH and B cells.

It is interesting to compare our findings with those of earlier studies, as there appears to be a differential requirement of IL-21 signaling for generating TFH and GC B cells, depending on the kind of immunization system used in the study (11, 13, 20, 21, 25). Using NP-keyhole limpet hemocyanin (KLH), Zotos et al. (13) reported that IL-21 signaling is dispensable for TFH cell generation; however, in another immunization model employing sheep red blood cells (SRBCs), Vogelzang et al. (21) concluded that the absence of IL-21 signaling results in impaired TFH cell generation. Yet another study using the same SRBC model found that TFH cells do form but decline faster in the absence of IL-21 signaling (11). In light of these reported differences with various immunization strategies, it was of interest to define the role(s) of IL-21 signaling during acute viral infection. In this study, using LCMV infection, Tfh cells were detected at a comparable frequency in wild-type and IL-21R−/− mice, as measured by use of the canonical Tfh markers CXCR5, PD-1, and ICOS. Even the Tfh transcriptional regulator Bcl-6 was similarly expressed in both groups. Similar observations were made in another study using influenza virus (26). This suggests that Tfh cells are generated after a viral infection. However, we show that these Tfh-like cells with expression of known surrogate markers were functionally impaired when tested in mixed splenic chimeras. Our data indicate that, despite being phenotypically Tfh-like, these cells are functionally devoid of B helper activity and that IL-21 signaling in CD4 T cells is intrinsically required to generate fully functional Tfh cells. Currently, we do not understand the underlying defect in the Tfh cells that is generated in the absence of IL-21 signals but hypothesize that a key cognate interaction between Tfh and B cells might be missing. Comparison of the gene expression profiles of antigen-specific Tfh cells from wild-type and IL-21R−/− mice may provide insight into other key interactions that are missing in the absence of IL-21 signaling and are critical for B cell help.

As stated before, using model antigens in mice, a number of studies have addressed the role of IL-21 signaling in modulating B cell fates (11, 13, 20, 21). Apart from the type of antigen and the context of presentation, each of the studies also differed in using either IL-21−/− and/or IL-21R−/− mice. Immunization of IL-21−/− mice with KLH or SRBCs showed impaired GC formation (11, 20, 21). Likewise, using IL-21R−/− mice, Bessa et al. showed that IL-21 signaling is required for GC formation after immunization with virus-like particles (25). In contrast, and consistent with another recent report by Karnowski et al. (using influenza virus and IL-21−/− mice) (26), our results indicate that initial GC formation was unaffected in the absence of IL-21 signaling. Previously, we have shown that SAP-deficient mice harbor a severe defect in CD4 T cells that results in the absence of GCs (27). In contrast, GCs are formed in IL-21R−/− mice but are not well sustained.

In humans, in vitro studies have shown that a number of cyto-
kines, including IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, and IL-15, can induce Ig secretion by B cells in the presence of an appropriate stimulus, such as CD40L (28–38). IL-2 and IL-10 have an important role in lowering the threshold for IL-21 responsiveness of activated human B cells, suggesting an essential interplay among these three cytokines (39, 40). Mice deficient in either IL-2 or IL-4 have been shown to have normal immunoglobulin levels; however, double-knockout animals harbor defects in mounting a humoral immune response to LCMV (41). IL-6 was recently shown to be an important cytokine for IL-21 expression by CD4 T cells (42). Although these numerous cytokines have potential effects on B cell immunoglobulin secretion, our study, along with previous reports, has shown that IL-21 is the most dominant cytokine for the generation of LLPCs (11, 13, 22).

In addition to its role in long-term humoral immunity, IL-21 is also an important cytokine for CD8 T cell help during chronic infections. Using LCMV clone 13, which establishes a persistent infection in mice, it has been shown that IL-21 signaling is critical to sustain CD8 T cell responses during chronic viral infection (43–45). Since CD4 T cells are the major producers of IL-21, it will be of considerable interest to define the kind of CD4 cells that help B cells versus the kind that provide help for CD8 T cell responses.

In conclusion, we have used three different models of virus infections to demonstrate the indispensable role of IL-21 in generating long-term antiviral humoral immune responses in mice. Taken together, our study shows that the lack of IL-21–IL-21R interactions does not prevent SLPC generation, GC formation, or the generation of antigen-specific CD4 Tfh cells but results in a marked defect in the ability to generate virus-specific LLPCs. These findings extend our understanding of the important role of IL-21 signaling during antiviral humoral immune responses.

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W.J.L. and R.S. are inventors on patents and patent applications related to IL-21.

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