Development of Autoimmune Diabetes in the Absence of Detectable IL-17A in a CD8-Driven Virally Induced Model

Tom L. Van Belle, Enric Esplugues, Jeanette Liao, Therese Juntti, Richard A. Flavell and Matthias G. von Herrath

*J Immunol* 2011; 187:2915-2922; Prepublished online 10 August 2011;
doi: 10.4049/jimmunol.1000180
http://www.jimmunol.org/content/187/6/2915

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2011/08/10/jimmunol.1000180.DC1

**References**
This article cites 49 articles, 24 of which you can access for free at:
http://www.jimmunol.org/content/187/6/2915.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Development of Autoimmune Diabetes in the Absence of Detectable IL-17A in a CD8-Driven Virally Induced Model

Tom L. Van Belle,*† Enric Esplugues,† Jeanette Liao,* Therese Juntti,* Richard A. Flavell,‡* and Matthias G. von Herrath*

Recent studies have shown that IL-17 can contribute beneficially to pathogen defense but also that excessive IL-17 levels are associated with chronic inflammation and autoimmune disorders. To date, the role of IL-17 in viral infections and type 1 diabetes is ambiguous. In this study, we used IL-17A enhanced green fluorescent protein bicistronic reporter mouse strains to analyze in situ production of IL-17A. Upon Klebsiella pneumoniae bacterial infection, CD4+ and γδ T cells produce IL-17A. In contrast, CD4+ or CD8+ T cells do not produce IL-17A in response to acute or protracted viral infection with lymphocytic choriomeningitis virus or during autoimmune diabetes development in the CD8-driven lymphocytic choriomeningitis virus-induced model of type 1 diabetes. We conclude that viral elimination and type 1 diabetes can occur in the absence of detectable IL-17A production, suggesting IL-17A is not essential in these settings. The Journal of Immunology, 2011, 187: 2915–2922.

Upon activation by APCs, CD4 Th cells undergo clonal expansion and differentiation into cytokine-secreting effector T cells. Besides Th1, Th2, and follicular helper T cells, a subset of polarized effector cells, Th17, is characterized by the production of IL-17 and other cytokines (1). IL-17–producing CD8 T cells (Tc17) have also been described and shown to harbor colitogenic properties (2). We have begun to appreciate the importance of Th17 cells in host defense against certain pathogens (3), especially in bacterial [e.g., Klebsiella pneumoniae (4)] and fungal [e.g., Candida albicans (5)] infections. IL-17 can be protective by initiating granulopoiesis and orchestrating neutrophil trafficking (6). In contrast, data on the role of IL-17 in antiviral immunity is rather limited. For example, after primary challenge with influenza A, Th17 and Tc17 effector cells are found in the lung, and blocking IL-17 during influenza A infection increases weight loss and reduces survival (7). Moreover, IL-10 deficiency unleashes an influenza-specific Th17 response and enhances survival against high-dose challenge (8). On the contrary, Th17 cells can also contribute to viral persistence, as is the case for Th17’s murine encephalomyelitis virus (9). For other viruses, the data at best suggest an indirect link between IL-17 and infection. As such, during the immune response to lymphocytic choriomeningitis virus (LCMV) infection, cytotoxic T cells produce IFN-γ and TNF-α, but CD4 T cell-derived IL-21 (10) is crucial to control LCMV infection (11–13). IL-21 can initiate and amplify Th17 differentiation in vitro (14, 15), but it remains to be seen whether IL-17 is also produced during LCMV infection.

Unregulated Th17 responses or overwhelming IL-17 production from T cells and other sources is associated with chronic (autoimmune) inflammation and severe immunopathologic conditions (16) with evidence that Th17 cells are involved in rheumatoid arthritis, psoriasis, multiple sclerosis (17), and inflammatory bowel disease. Recent studies also provide evidence of increased frequencies of IL-17–secreting cells in the lymphocyte populations of long-term type 1 diabetes (T1D) patients (18) and children with T1D (19). Studies in mouse models of T1D report conflicting results. In an early study, Vukkadapu et al. (20) showed that levels of serum IL-17 are elevated in very young NOD mice but not at later ages or at diabetes onset. In contrast, recently it was shown that the levels of IL-17A/F transcripts increase as diabetes develops (21). Th17 cells are also elevated in the gut of young NOD mice, a phenomenon that can be ended by an anti-diabetogenic diet (22). Blockade of IL-17A by anti–IL-17A mAb delays diabetes in NOD mice when administered late prediabetic (10 wk) but not at a young age (23). Moreover, Jain et al. (24) reported that treatment with a fusion protein consisting of IgG and GAD peptide 206–220 confers diabetes protection to hyperglycemic NOD mice, correlating with a reduced number of IL-17–producing cells present in the spleen and induction of IFN-γ–producing cells. Although these data support a role for IL-17 in T1D cause or pathogenesis, data from transfer models shed a different light on this. Th17 cells generated from “islet-specific” BDC2.5 TCR transgenic CD4 T cells induce diabetes but convert to Th1 upon transfer to NOD/SCID (21, 25). Notably, blockade of IFN-γ but not IL-17A delayed Th17 transfer-induced diabetes (25), indicating that IL-17 is not essential for the diabetogenic properties of the transferred BDC2.5 effector cells. Recent studies even show a protective role for IL-17–producing γδ T cells in NOD mice (26). Also, transfer of bulk splenocytes isolated from CFA-treated NOD mice and activated in the presence of TGF-β and IL-6 delayed diabetes in the NOD/SCID transfer model.
LACK OF IL-17 DURING LCMV INFECTION OR AUTOIMMUNE DIABETES

(27). Similarly, IL-17–producing CD8+ T cells differentiated with TGF-β and IL-6 are not diabetogenic, whereas IL-23–treated cells potently induce diabetes (28).

In this study, we assessed the expression of IL-17A during autoimmune diabetes development in the CD8-driven rat insulin promoter–lymphocytic choriomeningitis virus (RIP-LCMV) model. We used an enhanced green fluorescent protein (eGFP) reporter system to allow eventually the real-time in vivo imaging of IL-17A–producing cells within the pancreas using two-photon imaging technology and purify IL-17A–producing cells from diabetic mice for further functional studies. For this purpose, we used transgenic models based on IRES-driven eGFP IL-17A (IL-17A eGFP) bicistronic reporter strains (29). Our results confirm that the IL-17A eGFP reporter system can be used for direct detection of IL-17A and show that IL-17A is produced by T cells during bacterial K. pneumoniae infection. However, IL-17A remained undetectable during viral LCMV infection and during the development of autoimmune diabetes in the CD8-driven RIP-LCMV model.

Materials and Methods

Mice and infections

IL-17A eGFP knock-in mice (IL-17A eGFP; allele symbol, Il17a< tm1.1Ftv>; allele accession ID, MGI:5006665) are described elsewhere (29) and were used as such or crossed to rat insulin promoter–lymphocytic choriomeningitis virus–glycoprotein (RIP-LCMV-GP) (C57BL/6) mice to generate IL-17A eGFP RIP-LCMV-GP mice. IL-17A eGFP Smartha mice were obtained by crossing IL-17A eGFP mice with Smartha/SjL (C57BL/6) mice. The LCMV gp61-80 specific CD4 TCR transgenic Smarta cells, capable of reporting in situ LCMV infection, were used for transfers to the LCMV-induced RIP-LCMV-GP (C57BL/6) model of T1D.

Plaque-purified viral isolates of the Armstrong and clone 13 strains of LCMV were propagated in NIH-21 cells. The titers of viral stocks were determined by plaque assay. To establish acute infections, B6 mice received 10^4 PFU LCMV Armstrong (low dose) or 2 × 10^5 PFU LCMV Armstrong (high dose) in a volume of 0.1 ml by i.p. or i.v. inoculation, respectively. Protracted infections were established by i.v. inoculation with 2 × 10^5 PFU LCMV clone 13 in a volume of 0.1 ml. For diabetes experiments, mice were infected i.p. with 10^5 PFU LCMV Armstrong. All animal experiments were performed with permission from the institutional animal care and use committee at the La Jolla Institute for Allergy and Immunology and Yale University.

K. pneumoniae infection

K. pneumoniae ATCC 43816 was incubated overnight with aeration in brain heart infusion media and then diluted in sterile PBS to the desired dose. IL-17A-IRES-eGFP mice were lightly anesthetized using methoxyflurane and then intranasally instilled with 1 × 10^6 CFU in a volume of 50 μl. Mice were sacrificed at the indicated time postinfection, and lungs, livers, and spleens were harvested and processed as described previously. Expression of IL-17A (eGFP) was analyzed by flow cytometry.

Diabetes induction and blood glucose values

For diabetes induction, RIP-LCMV-GP or derived strains were infected with low-dose (10^4 PFU) LCMV Armstrong 53b by i.p. inoculation. Blood glucose was monitored with OneTouch Ultra at 3- to 5-d intervals. Diabetes onset was dated on the first of two consecutive readings >300 mg/dl.

In vitro Th17/Tc17 culture conditions

Spleens were removed from 8-wk-old mice, and whole splenocytes were cultured at 1 × 10^6 cells/ml after RBC removal in RPMI 1640 medium in the presence of anti-CD3 (1 μg/ml plate-bound, 145-2C11; BD) and anti-CD28 (10 μg/ml plate-bound, 37.51; BD) as well as IL-6 (20 ng/ml; PeproTech), IL-23 (20 ng/ml; PeproTech), TGF-β (1 ng/ml; R&D), anti–IFN-γ (5 μg/ml; BD), anti–IL-4 (5 μg/ml; BD), and anti–IL-2 (5 μg/ml, JS66-1A12; BD). Cells were harvested at day 7, stained for CD4 or CD8, and samples were acquired on an LSRII flow cytometer.

Cell preparations

Spleen, pancreas, and pancreas–draining lymph node (LN) cells were isolated from (recipient) mice at indicated times. Spleen and pancreatic LN cells were mechanically disrupted and washed twice. Cells from the pancreas were prepared as follows: after mincing and incubating in 1 mg/ml Collagenase P (Roche) plus 1 μg/ml DNase (Sigma) for 20 min at 37°C, the mixture was passed through a 350-μm screen and washed twice with HBSS. Density centrifugation for 15 min at 800 × g using Histopaque 1077 (Sigma) was followed by two washes with HBSS. Single-cell suspensions were obtained by incubation in nonenzymatic cell dissociation buffer (Invitrogen) for 6 min at 37°C. Cells were washed twice before staining or restimulation assays.

Flow cytometry staining and fixation

Abs used for flow cytometry were from eBioscience: CD25 (PC61), CD44 (J37), CD62L (MEL-14), CD4 (RM-4.5), and CD8 (53-6.7). Neoautoantigen–specific CD8 T cells were detected by incubating for 1 h at room temperature with Pro5 MHC Pentamer (Proimmune, Oxford, U.K.) LCMV/gp33-41/H-2d or LCMV/hnp396-404/H-2Dd. For intracellular staining of cytokines using mAbs, in vitro restimulation was necessary (data not shown) and done by PMA and ionomycin for 3 h, gp33-41 peptide for 4 h, or gp61-80 peptide for 7 h. For PMA/ionomycin and gp33-41 stimulation, brefeldin A was added immediately, and for gp61-80 after 2 h of culture. For intracellular cytokine staining (ICS) or overnight (16-h) storage, fixation tests showed that resuspending and incubating in BD Cytofix for 15 min at room temperature is superior to 1% paraformaldehyde for 15 min at 6°C (see Supplemental Fig. 1). After two washes, cells were stored until acquisition or incubated with anti–IL-17A, anti–TNF-α, or anti–IFN-γ mAbs in BD Cytoperm buffer for 45 min at 6°C, washed twice, and immediately acquired on an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software.

Results

In vitro validation of IL-17A eGFP reporter mice: Th17/Tc17 cultures

Recently, novel reporter strains of mice have become available to track in vivo expression of IL-17 isoforms. For example, the CrelokP–based strain IL-17F-CreEYFP (30) or IRES-driven red fluorescent protein IL-17F reporter mice (31) accurately and sensitively report IL-17F. In this study, we track in situ production of IL-17A using recently generated IL-17A eGFP mice (29).

We first determined the sensitivity of the IL-17A eGFP reporter strain by stimulating splenocytes from homozygous (IL-17KI) or heterozygous (IL-17Het) IL-17A eGFP or wild-type (WT) littermates under Th17 conditions for 3 d. We found clear and strong IL-17A/eGFP expression by both CD4 T cells (±63% of CD4+) and CD8 T cells (±72% of CD8) in the cultures of IL-17KI and IL-17Het splenocytes but not WT splenocytes (Supplemental Fig. 1A). We also found that fixation using 1–2% paraformaldehyde is not ideal for eGFP reporter signal detection. However, formaldehyde-based fixation (e.g., BD Cytofix) maintains the signal intensity of eGFP and allows detection of intracellular molecules on permeabilized cells as well as overnight (16-h) storage (Supplemental Fig. 1B, 1C). Also, whereas detection of the eGFP signal can be done right after culture without the need for an in vitro restimulation, restimulation is needed for intracellular cytokine detection (i.e., ICS) by mAb (data not shown and Supplemental Fig. 1C). This is because visualization of IL-17A expression via detection of the eGFP signal is not only more sensitive than the Ab-based detection approach but also does not require an accumulation of cytokines because eGFP produced in parallel with the cytokine is not immediately secreted during the effector T cell response.

From this we concluded that the IL-17A eGFP reporter is capable of strongly reporting IL-17 expression and that the eGFP signal can be used with caution in combination with formaldehyde fixation for the duration of standard intracellular staining protocols used in some of the experiments described later.

Direct ex vivo validation of IL-17A eGFP reporter mice: K. pneumoniae infection

It has been previously established that IL-17A production is induced in response to infection with K. pneumoniae (32, 33). This in turn leads to the production of chemokines in infected tissues followed by the recruitment of neutrophils. To validate the IL-17A
cells in vitro overestimates the frequency of IL-17A + cells directly ex vivo, we infected IL-17A eGFP mice with *K. pneumoniae* and examined the expression of eGFP in lung lymphocytes as *K. pneumoniae* infection initiated in the lung (Fig. 1). Kinetics of eGFP expression in the infected mice revealed a peak of IL-17A expression in both CD4+αβ and γδ T cells at 24 h after *K. pneumoniae* infection. However, a direct comparison between CD4+αβ and γδ T cells indicated an increased frequency of eGFP+ cells in the γδ T cell (35%) over the CD4+αβ T cell (8%) compartment (Fig. 1). Notably, a gradual reduction of eGFP-expressing cells in both CD4+αβ and γδ T cell compartments was observed at later time points.

eGFP signal corresponds with detection of IL-17A by ICS

Having shown that eGFP can be detected directly ex vivo, we validated that this eGFP signal reports IL-17A similar to standard ICS detection methods. The IL-17A eGFP signal corresponds with IL-17A detection by the conventional intracellular staining for cytokines, at least from in vitro cultures (Supplemental Fig. 1A). We next determined whether this also holds true for the direct ex vivo measurement. We infected IL-17A eGFP or nonreporter littermate mice with 10^4 PFU LCMV and harvested splenocytes after 1 d. In vitro restimulation by PMA/ionomycin in the presence of eGFP + cells in the lung peaks 24 h postinfection with *K. pneumoniae*. IL-17A knock-in mice infected intranasally with 10^4 CFU *K. pneumoniae*. At the indicated time points, animals were sacrificed, and lungs were harvested and analyzed for the presence of eGFP+ cells. The isolated cells were gated on CD4+ T cells (A) and γδ T cells (B). A representative FACS plot for each analyzed time point is shown.

Lack of T cell-derived IL-17A during acute and protracted LCMV infection

CD4 T cells can augment the functional activity of pathogen-specific CD8 T cells (34, 35). The main inflammatory cytokines produced by CD8 T cells are IFN-γ and TNF-α, but IL-21 is crucial to control LCMV infection (10–13) and can initiate and amplify Th17 differentiation (14, 15). Thus, to determine whether Th17 or Tc17 cells are present at any stage during an anti-LCMV T cell response, we infected IL-17A eGFP C57BL/6 mice and assessed the in situ production of IL-17A at various time points postinfection. Prior to infection, splenocytes from IL-17A eGFP reporter mice produce negligible fractions of IL-17A directly ex vivo (<0.05%; Fig. 3A) or upon in vitro restimulation (<0.2%; Supplemental Fig. 2, day 0). In response to an acute infection with a low dose of LCMV Armstrong, CD4 T cells become activated, as determined by the increase in CD44 (data not shown) and the loss of CD62L expression, but fail to produce IL-17A, as measured directly ex vivo (Fig. 3A, top row). To show that this is not an issue with the IL-17A eGFP reporter system, standard ICS with mAbs and in vitro restimulation with LCMV-gp61-80 peptide or PMA/ionomycin showed that CD4 T cells increased the production of IFN-γ, but not of IL-17A, after LCMV infection (Supplemental Fig. 2).

Similarly, acute infection with high-dose LCMV Armstrong or a protracted infection with clone 13 also did not reveal IL-17A/eGFP signal directly ex vivo in CD4 T cells during the expansion phase (day 5), peak (day 9), or after contraction (day 33) of the response (Fig. 3B, top row). Moreover, both bulk and LCMV-specific CD8 T cells failed to produce IL-17A during the expansion, peak, or contraction phases of the anti-LCMV response,

**FIGURE 1.** IL-17A expression in CD4 and γδ T cells in the lung peaks 24 h postinfection with *K. pneumoniae*. IL-17A knock-in mice were infected intranasally with 10^4 CFU *K. pneumoniae*. At the indicated time points, animals were sacrificed, and lungs were harvested and analyzed for the presence of eGFP+ cells. The isolated cells were gated on CD4+ T cells (A) and γδ T cells (B). A representative FACS plot for each analyzed time point is shown.

**FIGURE 2.** IL-17A-IRES-eGFP mice accurately report IL-17 production capacity of CD4 T cells. A, Comparison of IL-17A detection by eGFP signal or by ICS for IL-17A using mAb. Splenocytes from day 1 LCMV-infected mice were restimulated in vitro for 3 h with PMA/ionomycin plus brefeldin A. Cells were fixed with BDFix, then stained for intracellular IL-17A and IFN-γ by mAb in BDPerm buffer. B, In vitro restimulation by PMA/ionomycin overestimates the in vivo production of IL-17A cytokine by CD4 T cells. eGFP levels were measured by flow cytometry after in vitro restimulation with PMA/ionomycin plus brefeldin A or without restimulation.
IL-17A is not produced by T cells during an anti-LCMV response. A and B, The in situ production of IL-17A by splenic T cells was determined at various time points after acute low-dose LCMV infection (10^3 PFU LCMV Armstrong; A), acute high-dose LCMV infection (2 × 10^6 PFU LCMV Armstrong; B, left side), or protracted infection (2 × 10^6 PFU LCMV clone 13; B, right side) of IL-17A eGFP C57BL/6 mice. Cells were freshly stained for the indicated surface markers and freshly acquired without prior in vitro restimulation, inhibition of protein secretion, cell fixation or permeabilization. Graphs shown are gated on CD4 T cells (top rows) or CD8 T cells (lower three rows). n = 4 per time point per infection condition. gp33-41/Db and np396-404/Db are pentamers to detect gp33-41-specific and np396-404-specific CD8 T cells, respectively. Numbers in graphs denote the percentages of cells in the respective gates.

**FIGURE 3.** IL-17A is not produced by T cells during an anti-LCMV response. NOD mice develop diabetes (21). Serum levels of IL-17 protein, however, are elevated in very young NOD mice and decline with age (20). We were interested in studying the role of IL-17A production during the diabetic effector phase in the pancreas for real-time imaging studies, but also to find out if IL-17A–producing cells were pathogenic and how their diabetogenic potential could be curbed. Despite the lack of IL-17A production directly ex vivo during the systemic response to LCMV infection, we anticipated, based on the data in NOD mice and experimental autoimmune encephalitis (3), that organ-specific autoimmunity would create more favorable conditions for IL-17A production. Indeed, the activation of T cells in the pancreatic LN and the specific microenvironment in the pancreas both can alter the T cell response during diabetogenesis. In the RIP-LCMV mouse model for virally induced T1D (36), mice express viral Ags from LCMV under the control of the RIP selectively on pancreatic β-cells and, in some lines, in the thymus (37). In C57BL/6 RIP-LCMV-GP mice, used in the current study, the viral Ag is not expressed in the thymus, and infection with LCMV induces rapid CD8 T cell–dependent diabetes. We crossed the RIP-LCMV-GP strain to IL-17A eGFP reporter mice to track the in situ production by conventional CD4 T cells, regulatory T cells, as well as autoantigen–specific CD8 T cells during virally induced T1D.

We found that both male and female IL-17A eGFP × RIP-LCMV-GP mice develop hyperglycemic upon LCMV infection and develop diabetes with comparable kinetics of WT RIP-LCMV-GP C57BL/6 mice (Fig. 4A and data not shown). Next, we harvested spleens, pancreatic LNs, and pancreata from diabetic IL-17A eGFP RIP-LCMV-GP mice to determine in situ IL-17A expression by CD4 (Th17) or CD8 (Tc17) T cells. We could not detect in situ IL-17A expression by conventional CD4 T cells (CD4^+CD25^−) or regulatory T cells (CD4^+CD25^+) from spleen, pancreatic LN, or pancreas, regardless of the expression of CD62L (Fig. 4B) or CD44 (data not shown). Moreover, autoantigen (glycoprotein)-specific CD8 T cells, detected by GP33-41/Db pentamer, did not produce IL-17A/eGFP, despite being activated (CD62L^low^) (Fig. 4B). Similarly, LCMV-specific CD8 T cells, detected by NP396-404/Db pentamer, lacked IL-17A/eGFP signal. These data indicate that IL-17A is not present in situ and is unlikely to be required for diabetes development in the RIP-LCMV model.

Direct ex vivo measurement of cells isolated from diabetic RIP-LCMV-GP mice did not reveal IL-17A/eGFP signal. It can be argued that in vitro restimulation can increase the IL-17A/eGFP signal. Therefore, we restimulated cells from spleen and pancreatic LN pharmacologically using PMA/ionomycin or autoantigen–specifically using MHC class I–restricted gp33-41 or MHC class II–restricted gp61-80 peptides. Next, we determined whether IL-17A was produced via eGFP signal detection, in combination with TNF-α and IFN-γ detection via standard intracellular staining. As expected, CD4 T cells produced IFN-γ in response to PMA/ionomycin and, to a lesser extent, to gp61-80 peptide (Fig. 4C). However, CD4 T cells did not show any measurable IL-17A/eGFP signal (Fig. 4C). Moreover, we found that both polyclonal and Ag-specific restimulation elicited TNF-α production by CD8 T cells from spleen and pancreatic LN (Fig. 4D). However, as in CD4 T cells, we could not detect IL-17A/eGFP in CD8 T cells (Fig. 4D). Because the formaldehyde fixation preserves the eGFP signal during an additional ICS (see Supplemental Fig. 1), we conclude that T cells from diabetic RIP-LCMV-GP mice do not produce IL-17A, even after restimulation in vitro.

**T cells from prediabetic RIP-LCMV-GP mice do not produce IL-17A.**

On the transcript level, IL-17A and especially IL-17F increase as diabetes develops (24). To address whether IL-17A is produced earlier in the diabetes development, we induced diabetes in IL-17A eGFP RIP-LCMV-GP mice and analyzed IL-17A/eGFP expression at day 7, that is, when autoantigen–specific T cells can be detected in the pancreas and pancreatic LN (data not shown). Detection of IL-17A/eGFP was performed directly ex vivo as well as upon in vitro restimulation with PMA/ionomycin or with autoantigen–specific peptides.

We found that CD4 T cells did not express any IL-17A/eGFP when freshly measured after isolation from day 7 prediabetic mice (Fig. 5A, “No Stim”). Moreover, autoantigen–specific restimulation of CD4 T cells in vitro by MHC class II–restricted gp61-80 peptide did not elicit IL-17A/eGFP signal in spleen or in pancreatic LN (Fig. 5A). Even strong stimulation with PMA/
ionomycin did not produce strong IL-17A/eGFP signals in CD4 from spleen, pancreatic LN, or pancreas, regardless of whether they were of naive (CD44low) or effector/memory (CD44 high) phenotype (Fig. 5A). Similarly, CD8 T cells from prediabetic animals did not produce IL-17A in situ or upon stimulation with MHC class I-restricted gp33-41 peptide or PMA/ionomycin (Fig. 5B). This indicates that during the effector response leading up to diabetes in the RIP-LCMV model, IL-17A is unlikely to play a driving role in the autoimmune process.

Transferred autoantigen-specific CD4 T cells do not become Th17 during virally induced diabetes

We further substantiated that autoantigen-specific CD4 T cells do not produce IL-17A during diabetes development by transferring autoantigen-specific IL-17A eGFP reporter Smarta CD4 T cells into RIP-LCMV mice. Smarta T cells are CD4 TCR transgenic T cells derived from a transgenic mouse expressing an MHC class II-restricted TCR with specificity for an LCMV glycoprotein-derived Th cell epitope (gp61-80/I-A^b specific) (38). In this experiment, we crossed B6.SjL × Smarta, containing Ly5.1 allelically marked Smarta T cells, to the IL-17A eGFP reporter mouse strain. Next, transfer of purified Ly5.1+ IL-17A eGFP Smarta CD4 T cells allowed us to track CD4 T cells with a specificity for β-cell autoantigen and assess in vivo IL-17A production accurately and without manipulations or artifacts.

On days 4, 7, and 11 after diabetes induction, we harvested spleen, pancreatic LNs, and pancreas from recipient RIP-LCMV-GP mice. As expected, the Ly5.1+ Smarta CD4 population was readily

FIGURE 5. T cells do not produce IL-17A in diabetic RIP-LCMV-GP mice. IL-17A eGFP reporter RIP-LCMV-GP mice were infected with 10^5 PFU LCMV Armstrong (n = 6). A and B, At day 7 postinfection (before diabetes onset), IL-17A/eGFP production by (A) CD4 T cells and (B) CD8 T cells from spleen (top row), pancreatic LN (middle row), and pancreas (bottom row) was determined after in vitro restimulation in the presence of brefeldin A with mock, PMA/ionomycin, gp33-41 peptide, or gp61-80 peptide as indicated. Next, cells were acquired fresh without cell fixation or permeabilization. Representative graphs shown are gated on CD4 T cells or CD8 T cells in A and B, respectively.
detectable at day 4, peaked at day 7, and contracted by day 11 (Fig. 6A). However, we could not detect any IL-17A/eGFP in host CD4 T cells (Ly5.1−) or transferred Smarta (Ly5.1+) cells recovered from spleen, pancreatic LN, or pancreas (Fig. 6A). In vitro restimulation showed that recovered Smarta CD4 T cells produced IFN-γ, but not IL-17A/eGFP, in response to their cognate Ag gp61-80 at both days 7 and 11 (Fig. 6B). Similarly upon PMA/ionomycin restimulation, Smarta T cells recovered from spleen, pancreatic LN, or pancreas produced mainly IFN-γ, but not IL-17A/eGFP (Fig. 6C). Taken together, we conclude that autoantigen-specific CD4 T cells do not differentiate into Th17 during activation and clonal expansion in the type 1 diabetogenic process.

Discussion

Scientists now value the role of IL-17 in the clearance of pathogens, especially in bacterial or fungal infections (3). In this study, we first validated the use of an IL-17A eGFP bicistronic reporter mouse strain (29) for IL-17A detection directly ex vivo upon bacterial infection with K. pneumoniae. We next assessed the in situ production of IL-17A during infection with LCMV, an RNA arenavirus. The CD8 immune response to LCMV is characterized by an initial primary expansion of LCMV-specific, IFN-γ+ T cells, peaking at 7–9 d after inoculation. This is followed by a contraction phase leaving a resting pool of LCMV memory CTLs that are maintained at a similar frequency for a long period of time (39). The main inflammatory cytokines secreted by cytotoxic T cells are IFN-γ and TNF-α. CD4, in contrast, produces IL-21 during LCMV infection, which was recently shown to be crucial to control LCMV infection (10–13), and can initiate and amplify Th17 differentiation (14, 15). Therefore, it was reasonable to speculate that Th17 cells may have a role in immunity to LCMV. We addressed this by using a cytokine reporter system for IL-17A in favor of IL-17F, because IL-17F is expressed by only a subpopulation of IL-17A–expressing Th17 cells. However, in contrast to our anticipation and the data acquired in a bacterial infection setting, we could not detect T cell-derived IL-17A at any stage of the immune response to acute low- or high-dose LCMV Armstrong infection or protracted infection by LCMV clone 13. Our data on LCMV infection resemble the data on neurotropic coronavirus infection in that IL-17 levels did not exceed background (40). However, data on other viral infections show that Th17 can affect viral clearance as well as immunopathology, depending on the virus. An example of IL-17 contributing to viral clearance is the action of Tc17 and Th17 cells in response to influenza A virus (7, 8). In contrast, Th17 cells promote viral persistence after Theiler’s murine encephalomyelitis virus infection (9). IL-17 can also contribute to immunopathology. As such, elevated levels of IL-17 have been found in the corneas of mice infected with HSV-1, a DNA virus (41), but the increased immunopathological lesions upon HSV-1 infection of p19−/− mice, which lack IL-23 and thus Th17 responses, indicate that IL-17 might serve by dampening inflammatory immune responses (42). In other viral infections, IL-17 or Th17 have been detected, but their role remains to be determined. For example, hepatitis C virus infection of individuals induces Ag-specific Th17, probably to combat viral infection (43). However, hepatitis C virus nonstructural protein-4–induced TGF-β and IL-10

![FIGURE 6. Transferred Smarta cells do not produce IL-17A during diabetogenesis. Ly5.1+-allelically marked IL-17A eGFP reporter Smarta CD4 T cells were transferred on day −1 to LCMV-infected RIP-LCMV-GP mice (n = 4 per time point). A, In situ IL-17A production was assessed at indicated time points in spleen, pancreatic LN, and pancreas by flow cytometry. Cells were stained fresh for surface markers and acquired fresh without prior in vitro restimulation, inhibition of protein secretion, cell fixation or permeabilization. Representative graphs are shown and displayed as IL-17A/eGFP versus Ly5.1 of the live CD4+CD8− lymphocyte gate. B and C, Transferred Smarta cells do not produce IL-17 upon in vitro restimulation. Cells recovered as in A were restimulated in vitro in the presence of brefeldin A with gp61-80 peptide (B) or PMA and ionomycin (C) before fixation, permeabilization, and intracellular staining for IFN-γ. Representative graphs (IFN-γ versus IL-17A/eGFP) shown are gated on live Ly5.1+ (B6.SjL Smarta) CD4+CD8− lymphocytes. Frequencies <0.1% are rounded to zero.](http://www.jimmunol.org/)

Downloaded from http://www.jimmunol.org/ by guest on April 20, 2017
inhibit these responses, which may represent another example of an evasion strategy of viruses to subvert protective immune responses (43). Moreover, both CD4+ and CD4+ T cells from HIV-infected individuals can produce IL-17 after in vitro stimulation with PMA and ionomycin, but the specificity of these cells is unknown (44). Notably, acute pathogenic lentiviral SIV infection of pigtailed macaques, in contrast to nonpathogenic infection of African green monkeys, results in selective loss of Th17 cells, which is an independent predictor of increased systemic immune activation (45). Importantly, this difference was explained by bacterial translocation and compromised colonic mucosal integrity (46). This implies that effects of viral infections on IL-17 responses are not necessarily direct host-to-pathogen reactions but can be a result of indirect effects involving nonviral microbial stimuli. With regard to LCMV infection, a recent report showed that both eomesodermin and T-bet block differentiation of CD8 T cells into IL-17–producing cells (47). Consequently, our data imply that the differentiation pathways governed by these transcription factors are not simultaneously impaired at any stage during acute or protracted infection. Taken together, we conclude that it is very unlikely that T cell–derived IL-17A plays a role in acute or protracted LCMV infection.

Besides a role in host defense, there is evidence that Th17 cells (or at least unregulated IL-17 production) are involved in rheumatoid arthritis, psoriasis, multiple sclerosis, and inflammatory bowel disease (16). However, the role of IL-17 in autoimmune diabetes is less clear. Recently, it was shown that treatment with anti–IL-17A mAb prevents diabetes in NOD mice when administered at late pre-diabetic stage (10 wk) but not at early stages (23). In contrast, transferred BDC2.5 Th17 cells are diabeticogenic in NOD and NOD/SCID mice but convert to IFN-γ transgenic mice for IFN-γ induced by adjuvant-free antigen restores normoglycemia in NOD mice (21, 25). These results are not inhibited by Treg cells and confirm that IL-17A is not an essential molecule in T1D. This statement is further supported by data from the laboratories of Cooke (25) and Vukวดапу (26) or Th17 (27) gd T cells is augmented in multiple sclerosis.

We are grateful to Malina McClure and the staff at La Jolla Institute for Allergy and Immunology Animal Facility for assistance with animal care and to Priscilla Colby for administrative assistance.

Disclosures
The authors have no financial conflicts of interest.

References


