Published in final edited form as:
J Clin Immunol. 2011 October ; 31(5): 811–826. doi:10.1007/s10875-011-9549-z.

A Distinct Role of CD4+ Th17- and Th17-Stimulated CD8+ CTL in the Pathogenesis of Type 1 Diabetes and Experimental Autoimmune Encephalomyelitis

Manjunatha Ankathatti Munegowda,
Research Unit, Saskatchewan Cancer Agency, Department of Oncology, Saskatoon Cancer Center, 20 Campus Drive, Saskatoon, Saskatchewan S7N 4H4, Canada. Department of Pathology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

Yulin Deng,
Research Unit, Saskatchewan Cancer Agency, Department of Oncology, Saskatoon Cancer Center, 20 Campus Drive, Saskatoon, Saskatchewan S7N 4H4, Canada

Rajni Chibbar,
Department of Pathology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

Qingyong Xu,
Department of Pathology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

Andrew Freywald,
Department of Pathology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

Sean J. Mulligan,
Department of Physiology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

Sylvia van Drunen Littel-van den Hurk,
Vaccine and Infectious Disease Organization, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

Deming Sun,
Doheny Eye Institute, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA

Sidong Xiong, and
Institute of Medical Sciences, Soocow University, Suzhou, China

Jim Xiang
Research Unit, Saskatchewan Cancer Agency, Department of Oncology, Saskatoon Cancer Center, 20 Campus Drive, Saskatoon, Saskatchewan S7N 4H4, Canada. Department of Pathology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

Jim Xiang: jim.xiang@saskcancer.ca

Abstract

Both $CD4^+$ Th17-cells and $CD8^+$ cytotoxic T lymphocytes (CTLs) are involved in type 1 diabetes and experimental autoimmune encephalomyelitis (EAE). However, their relationship in pathogenesis of these autoimmune diseases is still elusive. We generated ovalbumin (OVA)- or myelin oligodendrocyte glycoprotein (MOG)- specific Th17 cells expressing RORγt and IL-17 by

Correspondence to: Jim Xiang, jim.xiang@saskcancer.ca.

in vitro co-culturing OVA-pulsed and MOG_{35-55} peptide-pulsed dendritic cells (DC_{OVA} and DC_{MOG}) with $CD4^+$ T cells derived from transgenic OTII and MOG-T cell receptor mice, respectively. We found that these Th17 cells when transferred into C57BL/6 mice stimulated OVA- and MOG-specific CTL responses, respectively. To assess the above question, we adoptively transferred OVA-specific Th17 cells into transgenic rat insulin promoter (RIP)-mOVA mice or RIP-mOVA mice treated with anti-CD8 antibody to deplete Th17-stimulated $CD8^+$ T cells. We demonstrated that OVA-specific Th17-stimulated CTLs, but not Th17 cells themselves, induced diabetes in RIP-mOVA. We also transferred MOG-specific Th17 cells into C57BL/6 mice and H-$2K^{b-/-}$ mice lacking of the ability to generate Th17-stimulated CTLs. We further found that MOG-specific Th17 cells, but not Th17-activated CTLs induced EAE in C57BL/6 mice. Taken together, our data indicate a distinct role of Th17 cells and Th17-stimulated CTLs in the pathogenesis of TID and EAE, which may have great impact on the overall understanding of Th17 cells in the pathogenesis of autoimmune diseases.

Keywords

Th17; pMHC I complex; $CD8^+$ CTL; type 1 diabetes; experimental autoimmune encephalitis

Introduction

$CD4^+$ Th17 lymphocytes have unique cytokine expression profile, transcriptional regulation, and biological function, and represent an independent lineage of $CD4^+$ Th cells [1, 2]. The discovery of $CD4^+$ Th17 subset not only changes the classical Th1/Th2 paradigm of Th differentiation, but also markedly facilitates our understanding of immune responses under both physiological and pathological conditions [3, 4]. The differentiation and regulation of Th17 cells have been extensively studied. Transcriptional factors RORγt and STAT3 are critical and are required for the development of Th17 cells [5, 6], and cytokine transforming growth factor (TGF)-β, IL-6, and IL-21 are critical for initiation and differentiation of Th17 cells [7–10].

Accumulated data suggest that Th17 cells play an important role in host defense against microbial infections and in the pathogenesis of autoimmune diseases such as type 1 diabetes (T1D) and experimental autoimmune encephalomyelitis (EAE) [11]. In T1D, the involvement of $CD8^+$ T cells in pathogenesis has been recognized. T1D is caused by autoimmune destruction of insulin-producing islet β cells of the pancreas [12]. Antigen-specific $CD8^+$ T cells have been found in the peripheral blood of T1D patients [13]. Studies in a nonobese diabetic (NOD) mouse model of T1D have indicated that $CD8^+$ T cells inflict damage to islet β cells both at the early stage in diabetes development and at the final effector phase of the disease [14–16]. On the contrary, there is some preliminary evidence showing that Th17 cells may be considered as a contributing factor in the pathogenic process of T1D. For example, it has been found that IL-17 is expressed in the pancreas of T1D mouse model [17], and the reduction of Th17 cells with the induction of IFN-γ inhibited IL-17 production and restored normoglycemia at the prediabetic stage [18]. However, the relative contribution of Th17 cells and CTLs in T1D has not been addressed.

EAE is a rodent model that has been valuable for the characterization of the immunopathogenic processes of human multiple sclerosis (MS). Attention has originally been focused on the role of $CD4^+$ T cells in the induction of EAE because susceptibility to MS is associated with MHC class II genes [19–21] and the critical role of $CD4^+$ Th17 cells in pathogenesis of EAE has, eventually, been demonstrated [22–26]. Recently, both $CD4^+$ Th17 and $CD8^+$ T cells have been identified in active lesions in brains of MS patients [27]. It has been shown that myelin oligodendrocyte glycoprotein (MOG)-specific $CD8^+$ T cell

responses are involved prior to and after the onset of EAE [28, 29], and adoptive transfer of MOG-specific $CD8^+$ T cells can also induce EAE [30–32]. However, (a) the potential relationship between the pathogenic $CD4^+$ Th17 and $CD8^+$ T cells and (b) the extent of their relative involvement in the pathogenesis of T1D and EAE are still not very clear.

Intercellular membrane transfer through trogocytosis plays an important role in immune modulation [33]. We have recently demonstrated that ovalbumin (OVA)-specific T cell receptor (TCR) transgenic OT II mouse $CD4^+$ Th1 cells in vitro activated by OVA-specific dendritic cells (DC_{OVA}) acquired DC_{OVA} 's peptide-MHC (pMHC) I and co-stimulatory molecules and became capable of directly stimulating antigen-specific $CD8^+$ cytotoxic T lymphocyte (CTL) responses leading to antitumor immunity in wild-type C57BL/6 mice [34, 35] and diabetes in transgenic rat insulin promoter (RIP)-mOVA mice expressing islet β-cell antigen OVA [36, 37]. Based on the phenomenon of trogocytosis, we speculate that $CD4^+$ Th17 cells may similarly acquire pMHC I by APC activation and become capable of stimulating $CD8^+$ T cell responses in autoimmune diseases such as T1D and EAE.

In this study, we generated OVA-specific $CD4^+$ Th17 cells and MOG-specific $CD4^+$ MOG-TCR-Th17 cells by cultivating OVA-pulsed dendritic cells (DC_{OVA}) or MOG peptide-pulsed dendritic cells (DC_{MOG}) with $CD4^+$ T cells derived from T cell receptor transgenic OTII mice or MOG-TCR transgenic mice, respectively. We also generated MOG-specific $CD4^+$ Th17 by culturing MOG_{35-55} peptide-pulsed splenocytes with $CD4^+$ T cells purified from C57BL/6 mice with MOG_{35-55} immunization-induced EAE in presence of IL-6/IL-23/TGF-β. Through the utilization of this experimental model, we found that $CD4^+$ Th17 cells acquired pMHC I in the process of activation by dendritic cells (DCs) and became capable of stimulating OVA or MOG-specific $CD8^+$ CTL responses, when transferred into the mice. To assess the pathogenic behavior of $CD4^+$ Th17- and Th17-induced $CD8^+$ T cells in T1D and EAE, we transferred OVA- and MOG-specific $CD4^+$ Th17 cells into RIP-mOVA and C57BL/6 mice, where both $CD4^+$ Th17- and Th17-stimulated $CD8^+$ CTL populations co-existed. We also used anti-CD8 antibody treatment in RIP-mOVA mice to deplete Th17-stimulated $CD8^+$ T cells or employed H-$2K^{b-/-}$ mice lacking the endogenous $CD8^+$ population to independently assess the effect of $CD4^+$ Th17 cells or of Th17-stimulated $CD8^+$ CTLs in pathogenesis of T1D and EAE. These experiments have clearly showed a distinct role of $CD4^+$ Th17- and Th17-stimulated $CD8^+$ CTLs in pathogenesis of autoimmune diseases demonstrating that T1D is directly mediated by $CD8^+$ lymphocytes, whereas EAE appears to be induced by the $CD4^+$ Th17 cells.

Materials and Methods

Reagents, Antibodies, Cell Lines, and Animals

The biotin-labeled anti-CD4 (GK1.5), CD11c (HL3), CD25 (7D4), CD40L (TRAP1), CD69 (H1.2 F3), FasL (CD178), and major histocompatibility complex (MHC) class I (K^b) (AF6-88) and II (Ia^b) (KH74) antibodies (Abs) were purchased from BD-Biosciences (San Diego, CA). Anti-mouse IFN-γ (XMG1.2), IL-17 (TC11-18H10), and perforin (δG9) Abs were obtained from Pharmingen Inc. (Mississauga, Ontario, Canada). The PE-conjugated anti-mouse T-bet (4B10) and RORγt (RORg2) Abs were purchased from BioLegend (San Diego, CA). The FITC-conjugated avidin was obtained from Jackson Immuno Research Laboratory Inc, West Grove, PN. Chicken OVA protein was purchased from Sigma, St. Louis, MO. Various peptides including OVAI ($OVA_{257-264}$, SIINFEKL) peptide specific for H-$2K^b$, OVAII ($OVA_{323-339}$, ISOAVHAAHAAHAEINEAGR) peptide specific for Ia^b, 3LL lung carcinoma antigen Mut1 (FEQNTAQP) peptide specific for H-$2K^b$, MOG peptide (MOG_{35-55}, MEVGWYRSPFSRVVHLYRNGK) specific for both H-$2K^b$ and Ia^b, and MOGI (MOG_{41-50}, RSPFSRVVHL) peptide specific for H-$2K^b$ were synthesized by Multiple Peptide Systems (San Diego, CA). The FITC-labeled anti-CD8 Ab and PE-H-$2K^b$/

OVAI tetramer were obtained from Beckman Coulter, Mississauga, Ontario, Canada. PE-H-2D^b/MOGI pentamer was obtained from Proimmune, Oxford, UK. The recombinant granulocyte macrophage colony-stimulating factor (GM-CSF), IL-2, IL-4, IL-6, IL-23, TGF-β, and anti-IL-2 antibody were obtained from R&D Systems Inc, Minneapolis, MN. Thymoma cell lines EL4 and OVA-expressing EG7 were obtained from American type culture collection (ATCC, Rockville, MD). Wild-type C57BL/6 mice were obtained from Charles River Laboratories (St. Laurent, Quebec, Canada). The OVA-specific TCR transgenic OTII mice, and Ia$^{b-/-}$, H-2K$^{b-/-}$, and perforin$^{-/-}$ knockout (KO) mice were purchased from the Jackson Laboratory (Bar Harbor, MA). Homozygous OTII/H-2K$^{b-/-}$ mice were generated by backcrossing the designated H-2K$^{b-/-}$ KO mice onto OTII background for two generations. The homozygosity was confirmed through PCR analysis according to Jackson laboratory's protocols. Transgenic RIP-mOVA mice with C57BL/6 background were obtained from Dr. W. Heath, Walter and Eliza Hall Institute of Medical Research (Melbourne, Australia). These transgenic RIP-mOVA mice express OVA under the control of RIP. They are transgenic for truncated OVA gene that is expressed as a membrane bound molecule in pancreatic islets, kidney proximal tubules, and testis of male mice [38]. All mice were housed in the animal facility at the Saskatoon Cancer Center and treated according to Animal Care Committee guidelines of University of Saskatchewan.

Preparation of Dendritic Cell and OVA-Specific CD4$^+$ Th1 and CD8$^+$ Tc1 Cells

Bone marrow-derived DCs were generated in presence of GM-CSF (20 ng/ml) and IL-4 (20 ng/ml) as described previously [39]. DC derived from wild-type C57BL/6 mice were pulsed with OVA (0.3 mg/ml) overnight at 37°C, and termed as DC_{OVA}. DC derived from MOG-TCR transgenic mice were pulsed with MOG_{35-55} peptide (20 μg/ml) for 2 h at 37°C, and termed as DC_{MOG}. OVA-pulsed DC generated from H-2K$^{b-/-}$ was referred to as ($K^{b-/-}$)DC_{OVA}. The preparation of DC_{OVA}-activated OVA-specific OT II CD4$^+$ type 1 help T (Th1) and OT I CD8$^+$ type 1 cytotoxic T (Tc1) cells was previously described [35, 40].

OVA- and MOG-specific CD4$^+$ Th17 Cell Preparation

Spleens were removed from OT II, transgenic MOG-TCR, and EAE-induced mice, and were mechanically disrupted to obtain a single-cell suspension. The erythrocytes were lysed using 0.84% ammonium chloride. Naïve T cells were enriched by passing through nylon wool columns (C&A Scientific Inc, Mannose, VA). Naïve OVA-specific CD4$^+$ T cells were then purified by negative selection using anti-mouse CD8 (Ly2) paramagnetic beads (DYNAL Inc, Lake Success, NY). To generate OVA-specific CD4$^+$ Th17 cells, naïve CD4$^+$ T cells (2×10^5 cells/ml) from OT II mice were stimulated for 3 days with irradiated (4,000 rads) DC_{OVA} (1×10^5 cells/ml) in the presence of IL-6 (10 ng/ml), IL-23 (10 ng/ml), TGF-β (5 ng/ml), and anti-IFN-γ antibody (20 μg/ml). These in vitro-activated CD4$^+$ Th17 cells were separated by Ficoll-Paque (Sigma) density gradient centrifugation, further purified by positive selection using CD4-microbeads (Miltenyi Biotech, Auburn, CA). In vitro ($K^{b-/-}$)DC_{OVA}-activated CD4$^+$ T cells derived from OTII mice with K$^{b-/-}$ KO were termed CD4$^+$ ($K^{b-/-}$)Th17 cells. The cytokine profiles of the above ($K^{b-/-}$)Th17 were similar to CD4$^+$ Th17 cells (data not shown). MOG-specific CD4$^+$ Th17 cells were generated by incubation of irradiated (4,000 rad) wild-type C57BL6 mouse splenocytes with CD4$^+$ T cells purified from spleens of MOG_{35-55} immunization-induced EAE mice with clinical score ≥2.5 at 1:1 ratio in presence of MOG_{35-55} peptide (20 μg/ml), IL-6 (10 ng/ml), IL-23 (10 ng/ml), and TGF-β (5 ng/ml). MOG-specific CD4$^+$ MOG-TCR-Th17 cells were generated by co-stimulating naïve CD4$^+$ T cells (2×10^5 cells/ml) from MOG-TCR transgenic mice with irradiated (4,000 rads) DC_{MOG} (1×10^5 cells/ml) in the presence of IL-6 (10 ng/ml), IL-23 (10 ng/ml), TGF-β (5 ng/ml), and anti-IFN-γ antibody (20 μg/ml) for 3 days. Three days subsequent to incubation, MOG-specific CD4$^+$ Th17 and CD4$^+$ MOG-TCR-Th17 cells were purified by positive selection using CD4-microbeads.

Phenotypic Characterization of OVA- and MOG-specific CD4$^+$ Th17 Cells

The above CD4$^+$ Th17 cells were stained with a panel of Abs and analyzed for expression of various cell-surface molecules by flow cytometry. To measure intracellular expression of cytokines, CD4$^+$ Th17 cells were processed using a commercial kit (Cytofix/CytoPerm Plus with GolgiPlug; BD-Biosciences, San Diego, CA) and stained with PE-conjugated anti-IFN-γ, IL-17, perforin, T-bet, and RORγt Abs. Culture supernatants of these OVA-specific and MOG-specific CD4$^+$ Th17 cells re-stimulated with irradiated (4,000 rad) OVAII peptide-pulsed and MOG peptide-pulsed LB27 cells, respectively, were analyzed for cytokine expression using ELISA kits (Endogen, Cam-bridge, MA).

CD8$^+$ T Cell Proliferation Assays

To assess the functional effect of OVA-specific CD4$^+$ Th17, we performed in vitro CD8$^+$ T cell proliferation assay. Irradiated (4,000 rad) DC_{OVA}, CD4$^+$ Th17, CD4$^+$ Th17 with anti-Il-2 antibody, and ($K^{b-/-}$)Th17 cells (0.4×10^5 cells/well) were co-cultured with a constant number of naive OT I CD8$^+$ T cells (1×10^5 cells/well). After culturing for 48 h, an overnight thymidine incorporation was determined by liquid scintillation counting. In in vivo proliferation assay, irradiated (400 rad) RIP-mOVA or perforin$^{-/-}$ mice (six mice per group) were i.v. adoptively transferred with DC_{OVA} (1×10^6 cells), CD4$^+$ Th17 cells (3×10^6 cells), or CD4$^+$ ($K^{b-/-}$)Th17 cells. Six days subsequent to adoptive transfer, mouse tail blood samples and pancreatic lymph node cell suspensions were stained with FITC-anti-CD8 Ab and PE-H-2K^b/OVAI tetramer, and analyzed by flow cytometry. In another set of experiments, tail blood samples of mice immunized with MOG_{35-55} peptide or injected with MOG-specific CD4$^+$ Th17 cells (3×10^6 cells) and CD4$^+$ MOG-TCR-Th17 cells (3×10^6 cells) were stained using FITC-anti-CD8 Ab and PE-H2-D^b/RSPFSRVVHL pentamer (Proimmune, Oxford, UK) and analyzed by flow cytometry 6 days after MOG_{35-55} peptide immunization or Th17 cell injection.

Cytotoxicity Assays

In in vitro cytotoxicity assay, Th17-activated OTI CD8$^+$ Tc1 cells were used as effector (E) cells, while ^{51}Cr-labeled EG7 and EL4 cells were used as target (T) cells in a chromium release assay. For testing the killing mechanisms, the effector cells were preincubated with CMA (1 μM) and emetin (5 μM) for 2 h before incubation with the target cells to prevent perforin and Fas/FasL interaction-mediated cytotoxicity. Specific killing was calculated as: 100× [(experimental cpm–spontaneous cpm)/(maximal cpm–spontaneous cpm) as previously described [34]. In in vivo cytotoxicity assay, C57BL/6 mouse splenocytes were incubated with either high (3.0 μM, $CFSE^{high}$) and low (0.6 μM, $CFSE^{low}$) concentrations of CFSE, and pulsed with OVAI or MOGI peptide and Mut1 peptide, respectively, and i.v. injected at 1:1 ratio into the OVA-specific or MOG-specific CD4$^+$ Th17 cell-transferred and MOG peptide-immunized mice. Sixteen hours after target cell delivery, the spleens were removed, and residual $CFSE^{high}$ and $CFSE^{low}$ target cells remaining in recipient spleens were sorted and analyzed by flow cytometry.

RT-PCR

Total RNA was extracted with Qiagen RNeasy purification kit (Qiagen, Mississauga, Ontario, Canada) as per manufacturer's protocol. Qiagen quantitative reverse transcription kit (Qiagen) was used to synthesize cDNA. Later, RORγt and GAPDH were analyzed by conventional PCR analysis. Following primer pairs were used for RT-PCR; RORγt: 5′ GCGGAGCAGACACACTTACA 3′, 5′ TTGGCAAACTC CACCACATA 3′ and GAPDH: 5′ CAGGTTGTCTCCTGC GACTT 3′, 5′ CTTGCTCAGTGTCCTTGCTG.3′. The protocol employed for amplification of mRNA comprised: 1 cycle of 94°C (5 min) and 25 cycles of

94°C (1 min), 52°C (1 min), and 72°C (1 min). All PCR reaction products were resolved using ethidium bromide stained 1% agarose gels.

EAE Induction

EAE was induced in wild-type C57BL/6 mice (15 mice/group) by s.c. injection over four sites in the flank with MOG_{35-55} peptide (200 μg/mouse) emulsified in CFA containing 0.6 mg mycobacterium tuberculosis (BD-Biosciences, San Diego, CA). To assess whether $CD4^+$ or $CD8^+$ T cells are the major pathogenic effector T cells in EAE, $Ia^{b-/-}$, and H-$2K^{b-/-}$ gene KO mice (ten mice/group) lacking $CD4^+$ and $CD8^+$ T cells were similarly s.c. immunized with MOG_{35-55} peptide. Two days after immunization, these MOG-sensitized mice were i.p. injected with 400 ng pertussis toxin (PT; Sigma) [31]. EAE was also induced by i.v. adoptive transfer of MOG-specific $CD4^+$ Th17 and $CD4^+$ MOG-TCR-Th17 cells (5×10^6 cells) into C57BL/6 or H-$2K^{b-/-}$ mice. Mice were examined daily for clinical signs. Mice were scored on scale of 0 to 5: 0, no clinical sign; 0.5, partially limp tail; 1, limp/flaccid tail; 2, moderate hind limb weakness; 2.5, one hind limb paralyzed; 3, both hind limbs paralyzed; 3.5, hind limbs paralyzed and weakness in forelimbs; 4, forelimbs paralyzed; and 5, moribund/death [31]. The analysis was performed on the raw data that included all clinical scores for each mouse at each time point in each group.

Histopathology

Pancreas was collected in 10% formalin from RIP-mOVA mice injected with $CD4^+$ Th17 and PBS. Pancreatic tissue sections were stained with hematoxylin and eosin (H&E) and slides were assessed for inflammatory cell infiltration and tissue destruction in a blind fashion. EAE mice were extensively perfused with ice-cold PBS with 2 U/ml heparin (Sigma), and spinal cords were harvested and fixed in formalin. Sections (6 mm) were stained with Luxol fast blue (myelin stain) along with H&E counterstaining. Slides were assessed in a blind fashion for inflammation: 0, none; 1, a few inflammatory cells; 2, organization of perivascular infiltrates; and 3, increasing severity of perivascular cuffing with extension into adjacent tissue. For demyelination: 0, none; 1, rare foci; 2, a few areas of demyelination; and 3, large (confluent) areas of demyelination.

Statistical Analysis

Mouse survival and clinical score were analyzed using Log rank and Mann–Whitney *U* test [41, 42], respectively, and all other experiments were tested for statistical differences using unpaired, two tailed, Student's *t* test. Differences were considered significant if $p<0.05$.

Results

$CD4^+$ Th17 Cells Acquire pMHC I Complexes from DC_{OVA} in the Course of Activation

To activate naïve OT II $CD4^+$ T cells, we co-incubated them with irradiated DC_{OVA} in the presence of the IL-23/IL-6/TGF-β/anti-IFN-γ antibody cocktail. While naïve OT II $CD4^+$ T cells did not express CD25, CD40L, CD69 and Ia^b, the co-incubated $CD4^+$ lymphocytes acquired the above molecules (Fig. 1a), which clearly confirmed their activation status. The activated $CD4^+$ also expressed the cell-surface FasL, intranuclear RORγt [43], and intracellular perforin, IL-17 (Fig. 1a, b), but not IL-4, indicating that they represented the $CD4^+$ Th17 cells. To further confirm this, we performed RT-PCR analysis to show that these cells express transcription factor RORγt (Fig. 1c), but not T-bet (data not shown). ELISA assays also revealed the $CD4^+$ Th17 nature of the activated cell, since they proved to secrete the IL-2 (2.8 ng/ml), IL-6 (4.5 ng/ml), IL-17 (1.8 ng/ml), and TGF-βγ(0.2 ng/ml) cytokines. No $CD11c^+$ DC_{OVA} contamination could be observed in these $CD4^+$ Th17 cell populations (Fig. 1d). We previously showed that $CD4^+$ Th1 cells acquired DC's pMHC

complexes in the course of DC activation [35]. In this study, we also showed that $CD4^+$ Th17 cells resulting from DC_{OVA} activation did display some DC's molecules such as pMHC I complexes (Fig. 1a), whereas $CD4^+$ ($K^{b-/-}$) Th17 cells obtained by co-incubation with pMHC I-deficient ($K^{b-/-}$)DC_{OVA} did not (Fig. 1e) but were activated similar to $CD4^+$ Th17 cells (data not shown), indicating that $CD4^+$ T cells acquire pMHC I complexes from DC_{OVA} upon co-culturing.

$CD4^+$ Th17 Cells Stimulate Effector $CD8^+$ CTL Responses In Vitro

Our further work showed that DC_{OVA}-activated $CD4^+$ Th17 cells with acquired pMHC I also stimulated in vitro OT I $CD8^+$ Tcell proliferation in a dose-dependent fashion (Fig. 2a). Interestingly, $CD4^+$ ($K^{b-/-}$)Th17 cells without acquired pMHC I failed in stimulation of $CD8^+$ T cell proliferation. To assess whether $CD4^+$ Th17-activated $CD8^+$ T cells have any functional effect, we performed a chromium release assay, in which $CD4^+$ Th17-activated $CD8^+$ T cells and OVA-expressing EG7 tumor cells were used as effector and target cells, respectively. We found that $CD4^+$ Th17-activated $CD8^+$ T cells showed killing activity to OVA-expressing EG7 tumor cells, but not to the control EL4 tumor cells without OVA expression (Fig. 2b), indicating that their killing activities are specific for OVA. To assess the pathway responsible for the killing activity of $CD8^+$ T cells, we preincubated effector $CD8^+$ T cells with CMA or emetin to prevent perforin- and Fas/FasL interaction-mediated cytotoxicity. We found that CMA but not emetin treatment significantly abolished $CD8^+$ T cells' killing activity ($p<0.05$), indicating that the killing activity of $CD4^+$ Th17-stimulated CTLs was mediated by the perforin pathway.

$CD4^+$ Th17 Cells Stimulate Effector $CD8^+$ CTL Responses In Vivo in RIP-mOVA Mice

To assess the ability of $CD4^+$ Th17 cells to induce in vivo $CD8^+$ T cell proliferation, we performed an OVA-specific tetramer staining assay in transgenic RIP-mOVA mice adoptively transferred with $CD4^+$ Th17 cells [35]. As shown in Fig. 2c, $CD4^+$ Th17 cells stimulated in vivo proliferation of OVA-specific $CD8^+$ T cells accounting for 0.68% and 1.18% of total $CD8^+$ T cell population in peripheral blood and pancreatic lymph nodes, respectively. To investigate the role of acquired pMHC I, we repeated the above assay using ($K^{b-/-}$)DC_{OVA}-activated $CD4^+$ ($K^{b-/-}$)Th17 cells, lacking acquired pMHC I. We found that $CD4^+$ ($K^{b-/-}$)Th17 cells completely lost their in vivo stimulatory effect, indicating that the acquired pMHC I complexes play an important role in targeting $CD4^+$ Th17's stimulatory effect onto $CD8^+$ T cells. To assess the influence of $CD4^+$ Th17 cell-induced $CD8^+$ T cell differentiation into CTLs, we performed the in vivo cytotoxicity assay. This assay monitored eradication of an adoptively transferred target population of splenocytes in RIP-mOVA mice adoptively transferred with $CD4^+$ Th17 cells. Six days following the adaptive transfer of $CD4^+$ Th17, these mice were infused with syngeneic splenocytes pulsed with OVA I peptide and labeled with a high concentration of CFSE ($CFSE^{high}$) or pulsed with an irrelevant Mut1 peptide and labeled with low concentration of CFSE ($CFSE^{low}$) as OVA-specific or control target cells at a 1:1 ratio [35]. Sixteen hours later, the remaining CFSE-labeled target cells were enumerated and their numbers compared with the reference population. We found that there was substantial loss of the OVA-specific and $CFSE^{high}$-labeled target cells in Th17 cell-immunized (43.9%) mice (Fig. 2c), indicating that $CD4^+$ Th17 cells can stimulate $CD8^+$ T cell differentiation into effector CTLs with killing activity for OVA I-pulsed target cells. In addition, the $CD4^+$ ($K^{b-/-}$)Th17 cell-vaccinated mice did not display any killing activity for the OVA-specific and $CFSE^{high}$-labeled target cells in cytotoxicity assay. To assess the pathway responsible for the killing activity of $CD4^+$ Th17-stimulated $CD8^+$ T cells in vivo, we repeated the above experiments using perforin$^{-/-}$ mice in tetramer staining and in vivo cytotoxicity assays. We found that OVA-specific $CD8^+$ T cell responses in C57BL/6 and perforin$^{-/-}$ mice with transfer of $CD4^+$ Th17 cells were similar (Fig. 2d). However, $CD8^+$ T cell-induced killing activity to OVA-specific $CFSE^{high}$ target cells was lost in perforin$^{-/-}$

mice (Fig. 2d), indicating that the in vivo $CD4^+$ Th17-stimulated $CD8^+$ T cell-induced killing activity to OVA-specific target cells is also via perforin-dependent pathway.

$CD4^+$ Th17 Cell-Induced Diabetes in Transgenic RIP-mOVA Mice is Mediated by Th17-Stimulated $CD8^+$ CTLs

Interestingly, all (6/6) RIP-mOVA mice adoptively transferred with $CD4^+$ Th17 cells carrying acquired pMHC I developed diabetes, while none (0/6) of the mice adoptively transferred with $CD4^+$ ($K^{b-/-}$)Th17 cells without acquired pMHC I complexes developed diabetes (Fig. 2e). As expected, pancreatic islet tissues were destroyed and infiltrated with numerous lymphocytes in the diabetic mice (Fig. 2f). To assess the importance of $CD8^+$ T cells in pathogenesis of diabetes, we treated adoptively transferred mice with anti-CD8 Ab to deplete $CD4^+$ Th17-stimulated OVA-specific $CD8^+$ T cells. We found that this treatment completely prevented diabetes development in the experimental animals (0/6). The effect proved to be very specific, since similar treatment with control irrelevant antibody has not suppressed disease onset (Fig. 2e), indicating that $CD4^+$ Th17 cell-induced diabetes in transgenic RIP-mOVA mice is mainly mediated by Th17-stimulated $CD8^+$ CTLs.

EAE Induction by MOG_{35-55} Peptide Immunization is Mainly by $CD4^+$ T Cells

To induce EAE, we s.c. administered MOG_{35-55} peptide to C57BL/6 mice, following previously described procedures [30]. All treated animals were found to develop chronic-progressive EAE with apparent clinical scores subsequent to MOG_{35-55} immunization (Fig. 3a). To assess the MOG-specific $CD8^+$ T cell responses, we performed MOG-specific PE-pentamer staining using tail blood from mice 2 weeks subsequent to the immunization with MOG_{35-55} peptide. We found that MOG_{35-55} peptide immunization stimulated in vivo proliferation of MOG-specific $CD8^+$ T cells accounting for 0.56% of total $CD8^+$ T cell population (Fig. 3b), and these MOG-specific $CD8^+$ T cells are cytotoxic effector cells since there was a substantial loss of the MOG-specific and $CFSE^{high}$-labeled target cells (38%) in MOG_{35-55}-immunized mice (Fig. 3c). Consistent with the above clinical finding, the histological examination of the spinal column revealed dramatic pathological changes in the immunized mice, with multiple inflammatory foci and extensive demyelination in the white matter of the spinal cord (Fig. 3d). Mean inflammation and demyelination scores were 2.6 and 1.5, respectively (Fig. 3e), which were significantly higher than in control animals ($p<0.01$). To assess whether MOG-specific $CD4^+$ or $CD8^+$ T cells play a major role in EAE pathogenesis, we immunized H-$2K^{b-/-}$ and $Ia^{b-/-}$ KO mice with MOG peptide for EAE induction. As shown in Fig. 3a, $Ia^{b-/-}$ and H-$2K^{b-/-}$ mice, lacking $CD4^+$ and $CD8^+$ T cells, revealed practically no clinical score ($p<0.01$) and slightly lower clinical scores ($p<0.05$), respectively, compared to the control MOG_{35-55}-immunized C57BL/6 mice, indicating that $CD4^+$ T cells, but not $CD8^+$ T cells, are predominantly involved in EAE induction.

In Vitro-Generated $CD4^+$ MOG-TCR-Th17 Cells Play a Major Role in Pathogenesis of EAE

To assess whether $CD4^+$ Th17 cells have a pathogenic function in EAE, we generated MOG-specific $CD4^+$ MOG-TCR-Th17 cells in vitro by co-incubating DC_{MOG} with $CD4^+$ T cells derived from MOG-TCR transgenic mice in presence of the IL-23, IL-6, TGF-β cytokines, and anti-IFN-γ antibody. The resulting activated $CD4^+$ T cells were subjected to flow cytometry, ELISA, and RT-PCR analysis. These $CD4^+$ T cells proved to be, indeed, efficiently activated as they strongly expressed CD25 and CD69 on their membranes (Fig. 4a). They also produced intracellular IL-17, but not IL-4, and expressed intranuclear RORγt, but not T-bet, indicating that they represent the $CD4^+$ Th17 subset (Fig. 4a). The RT-PCR analysis also revealed RORγt expression, thus further confirming the $CD4^+$ Th17 nature of the obtained population (Fig. 4b). As expected, they secreted the IL-2 (1.8 ng/ml), IL-6 (3.3 ng/ml), IL-17 (1.5 ng/ml), and TGF-βγ(0.1 ng/ml), confirming that they belong to $CD4^+$ Th17 cells. To assess their potential stimulatory effect, we i.v. injected them into C57BL/6

mice and performed MOG-specific PE-pentamer staining assay. As shown in Fig. 4c, MOG-specific $CD8^+$ T cells were detected in $CD4^+$ MOG-TCR-Th17-immunized mice and accounted for 0.36% of the total $CD8^+$ T cell population, indicating that MOG-specific $CD4^+$ MOG-TCR-Th17 cells are capable of stimulating MOG-specific $CD8^+$ T cell responses. To assess MOG-specific $CD8^+$ T cell killing activity, we performed the in vivo cytotoxicity assay. A moderate (20%) loss of the MOG-specific, $CFSE^{high}$-labeled target cells was observed in $CD4^+$ MOG-TCR-Th17 cell-transferred mice (Fig. 4d), indicating that $CD4^+$ MOG-TCR-Th17 immunization was likely to stimulate $CD8^+$ T cell differentiation into effector CTLs with cytotoxic activity specific for MOG peptide-pulsed target cells. To examine their ability to induce EAE, we injected C57BL/6 mice with the in vitro-amplified $CD4^+$ MOG-TCR-Th17 cells. All mice injected with MOG-specific $CD4^+$ Th17 cells, but not with OVA-specific Th17 control, developed chronic-progressive EAE, indicating that $CD4^+$ Th17-induced EAE was MOG specific (Fig. 4e). In addition, no significant difference in EAE initiation between C57BL/6 mice and H-$2K^{b-/-}$ mice lacking $CD8^+$ T cells could be observed (Fig. 4e), suggesting that $CD4^+$ Th17 cells, but not Th17-stimulated $CD8^+$ T cells, played a major role in EAE pathogenesis. Consistent with this clinical finding, histological examination of CNS tissues revealed pathological changes in C57BL/6 mice adoptively transferred with MOG-specific $CD4^+$ MOG-TCR-Th17 cells (Fig. 4f). In these adoptively transferred mice, multiple inflammatory foci and demyelination were observed in the white matter of the spinal cord, and mean inflammation and demyelination scores were 1.6 and 0.6, respectively, which was significantly higher than the control mice ($p<0.01$; Fig. 4g).

$CD4^+$ Th17 Cells Derived from EAE Mice also Play a Major Role in Pathogenesis of EAE

It has been demonstrated that in vivo-generated $CD8^+$ T cells derived from MOG_{35-55} peptide-immunized mice can induce EAE after they are amplified in vitro by MOG_{35-55} peptide stimulation and then i.v. transferred into C57BL/6 mice [31]. To assess whether the in vivo-generated $CD4^+$ Th17 cells derived from MOG_{35-55} peptide-immunized mice could also induce EAE, we amplified the MOG-specific $CD4^+$ Th17 cells by culturing $CD4^+$ T cells obtained from MOG_{35-55}-immunized mice with MOG_{35-55} peptide-pulsed splenocytes in the presence of IL-6, IL-23, and TGF-β. The amplified $CD4^+$ T cells were purified using CD4-microbeads and phenotypically analyzed by flow cytometry, ELISA, and RT-PCR approaches. The purified $CD4^+$ T cells proved to express CD25 and CD69 and produced IL-17 and RORγt, but not IL-4 or T-bet, confirming that they are active $CD4^+$ Th17 cells (Fig. 5a, b). In agreement, they also secreted IL-2 (1.2 ng/ml), IL-6 (3.0 ng/ml), IL-17 (1.3 ng/ml), and TGF-β (0.1 ng/ml). To assess their potential stimulatory effect, we injected MOG-specific $CD4^+$ Th17 cells into C57BL/6 mice and followed it by the MOG-specific PE-pentamer staining assay. As shown in Fig. 5c, MOG-specific $CD8^+$ T cells accounting for 0.46% of the total $CD8^+$ T cell population were detected in the injected mice, indicating that in vivo-generated MOG-specific $CD4^+$ Th17 cells are also capable of stimulating MOG-specific $CD8^+$ T cell responses. To assess MOG-specific $CD8^+$ T cell killing activity, we performed the in vivo cytotoxicity assay. We observed a 22% reduction within the MOG-specific, $CFSE^{high}$-labeled target cells in the MOG-$CD4^+$ Th17 transferred mice (Fig. 5d), showing again that MOG-$CD4^+$ Th17 cells could stimulate $CD8^+$ T cell differentiation into effector CTLs specifically targeting MOG peptide-pulsed target cells. To determine whether they are capable of inducing EAE, we injected C57BL/6 mice with the in vitro-amplified MOG-$CD4^+$ Th17 cells originally obtained from MOG_{35-55}-immunized mice. As shown in Fig. 5e, all C57BL/6 mice developed chronic-progressive EAE with apparent clinical scores occurring subsequent to adoptive transfer of MOG-specific $CD4^+$ Th17 cells, but not OVA-specific Th17 control. In addition, there is no significant difference in EAE between C57BL/6 mice and $CD8^+$ T cell-deficient H-$2K^{b-/-}$ mice, indicating that $CD4^+$ Th17 cells, rather than Th17-stimulated $CD8^+$ T cells, play a central role in EAE pathogenesis. Consistent with this clinical finding, histological examination of CNS tissues

revealed pathological changes in C57BL/6 mice immunized with MOG-specific $CD4^+$ Th17 cells (Fig. 5f). In particular, multiple inflammatory foci and demyelination were observed in the white matter of the spinal cord of the immunized animals, with mean inflammation and demyelination scores of 1.1 and 0.6, respectively, which was significantly higher than the scores in control mice ($p<0.01$; Fig. 5g).

Discussion

T1D is an organ-specific autoimmune disease characterized by predominantly T cell-mediated destruction of insulin-producing β-cells of the islets of Langerhans, culminating in the lifelong insulin dependence [44]. Before 1990, vast range of evidence favored a sole role of $CD4^+$ T cells in T1D: (a) $CD4^+$ T cells could be detected in abundance in islet cell infiltrates, (b) transfer of $CD4^+$ T cells from NOD mice caused diabetes in disease free young mice [45], and (c) the genetic region to which the defective genes mapped was the MHC II that interacts specifically with $CD4^+$ T cells [46, 47]. The development of T1D has usually been ascribed to a $CD4^+$ Th1 response with disease transfer in animal models being mediated by Th1 clones and lines [48, 49]. In addition, a potential involvement of Th17 cells in the course of T1D has recently been demonstrated in the mouse model [17]. However, over the time, new evidence has mounted implicating $CD8^+$ T cells in T1D initiation and progression. The primacy of $CD8^+$ T cells in autoimmunity, including diabetes, came into focus with a study of human monozygotic twins and NOD mice [50, 51], that expressed low density of certain types of class I protein on the surface of APCs. Remarkably, similar studies in NOD mice also confirmed that the APCs of this spontaneously autoimmune animal model also had defects in MHC class I presentation [52]. It has been demonstrated that defects in loading of self antigens into class I polypeptides are associated with T1D pathogenesis [53, 54], indicating that defects in class I assembly and loading could lead to T1D, as a result of a negative selection defect. It has also been shown that $CD8^+$ T cells killed beta-cells expressing self-peptides in class I groove in murine models [55, 56], suggesting that $CD8^+$ T cells exert a strong role in the etiology of T1D.

To assess (a) the potential relationship between the pathogenic $CD4^+$ Th17 and $CD8^+$ T cells in T1D and (b) to determine the extent of their effect on pathogenesis of T1D, we generated RORγt- and IL-17-expressing OVA-specific $CD4^+$ Th17 cells by co-culturing OVA-specific TCR transgenic OTII $CD4^+$ T cells with OVA-pulsed DC_{OVA} in presence of IL-6, IL-23, TGF-β, and anti-IFN-γ antibody. We found that (a) OVA-specific Th17 cells stimulated OVA-specific CTL responses via IL-2 and acquired pMHC I signaling when transferred into RIP-mOVA mice, and (b) Th17-stimulated $CD8^+$ T cells were capable of killing OVA-expressing target cells via perforin pathway. To assess the pathogenesis of OVA-specific $CD4^+$ Th17 cells or $CD8^+$ CTLs in T1D, we transferred these Th17 cells into RIP-mOVA mice or RIP-mOVA mice with anti-CD8 Ab treatment to deplete Th17-stimulated $CD8^+$ T cells. We showed that Th17-stimulated $CD8^+$ CTLs, but not Th17 cells themselves were required for T1D induction in RIP-mOVA mice (Fig. 6). Our findings are consistent with some previous reports showing that (a) transfer of islet-specific Th17 cells failed in diabetes induction, though it caused an extensive insulitis [57], and (b) treatment with neutralizing IL-17-specific Abs did not prevent T1D in NOD/SCID mice, which were derived from transfer of highly purified Th17 cells from BDC2.5 transgenic mice [49]. Furthermore, autoreactive $CD8^+$ T cells have been shown to play an important role in the pathogenesis of T1D [47, 58]. $CD8^+$ CTLs kill target cells through two distinct cytolytic pathways, the perforin-dependent granule exocytosis and the Fas/FasL interaction pathways [59]. The perforin in the presence of calcium has the ability to insert into lipid bilayer membrane, polymerize, and form structural and functional pores that can lead to cell lysis, whereas the binding of FasL on CTLs to Fas initiates the death pathway of apoptosis in the Fas-bearing target cells. In this study, we demonstrated that $CD4^+$ Th17-stimulated $CD8^+$ T

cells were able to kill OVA-expressing target cells both in vitro and in vivo via perforin-dependent pathway [60], indicating that $CD4^+$ Th17 induces diabetes in RIP-mOVA mice, may be through OVA-expressing pancreatic beta-cells killing by $CD4^+$ Th17-stimulated $CD8^+$ CTLs via perforin-dependent pathway.

In addition to T1D, we used EAE in our work as a model of human multiple sclerosis induced by autoreactive $CD4^+$ Th cells that mediate tissue inflammation and demyelination in the central nervous system. EAE can be induced through adjuvant and pertussis toxin-based immunization of C57BL/6 mice with a peptide, representing a fragment of an external myelin component, the encephalitogenic MOG peptide. Following the immunization, myelin sheaths of oligodendrocytes are attacked [61]. Although the predominant evidence has shown the critical role of $CD4^+$ Th17 cells in EAE pathogenesis [22–26], a potential involvement of $CD8^+$ T cells in EAE has also been recognized [28]. Whereas, the work of Abdul-Majid et al. has previously demonstrated that both $CD4^+$ and $CD8^+$ T cells were involved in EAE pathogenesis in MOG-immunized DBA/1 mice [62].

To assess whether $CD4^+$ or $CD8^+$ T cells were involved in pathogenesis of EAE, we immunized wild-type C57BL/6 mice or H-$2K^{b-/-}$ and $Ia^{b-/-}$ mice lacking $CD8^+$ and $CD4^+$ T cells with MOG35-55 peptide. Our experiments showed that MOG immunization-induced EAE only in C57BL/6 and H-$2K^{b-/-}$ mice, but not in $CD4^+$ T cell-deficient $Ia^{b-/-}$ mice, indicating that $CD4^+$ T cells are likely to play a critical role in MOG immunization-induced EAE in C57BL/6 mice. The apparent discrepancy between our findings and the previous report [62] may potentially result from genetic differences between different strains (DBA/1 and C57BL/6) of mice used in these two studies. DBA/1 mice are very sensitive to MOG immunization leading to EAE induction even in the absence of PT treatment, whereas C57BL/6 mice only develop MOG immunization-induced EAE, when mice are boosted with pertussis toxin, which greatly enhances $CD4^+$ T cell responses [62].

To further dissect (a) the potential relationship between the pathogenic $CD4^+$ Th17 and $CD8^+$ T cells in EAE and (b) to establish the extent of their influence on EAE pathogenesis, we generated two types of RORγt- and IL-17-expressing MOG-specific $CD4^+$ Th17 cells by cultivation of (a) naïve $CD4^+$ T cells derived from MOG-specific TCR transgenic mice and (b) primed MOG-specific $CD4^+$ T cells derived from EAE mice. We found that both MOG-specific $CD4^+$ Th17 cells were capable of stimulating MOG-specific $CD8^+$ CTL responses when transferred into C57BL/6 mice. To assess EAE induction by these MOG-specific $CD4^+$ Th17 cells or MOG-specific Th17-stimulated $CD8^+$ T cells, we transferred them into C57BL/6 mice or into H-$2K^{b-/-}$ mice with deficiency in the production of Th17-stimulated $CD8^+$ T cells. We have showed that the adoptively transferred $CD4^+$ Th17 cells, but not in vivo $CD4^+$ Th17-stimulated $CD8^+$ CTLs, are responsible for EAE initiation in C57BL/6 mice, indicating that $CD4^+$ Th17 cells play a crucial role in pathogenesis of EAE (Fig. 6). The failure of in vivo $CD4^+$ Th17-stimulated $CD8^+$ CTLs to trigger EAE may be due to their efficiency being lower than the efficiency of in vitro expanded MOG-specific $CD8^+$ T cells that were successful in EAE induction, when adoptively transferred into experimental mice [30–32]. Nevertheless, our observations indicate that $CD4^+$ Th17 cells, but not in vivo Th17-stimulated $CD8^+$ CTLs are likely to induce EAE under physiological conditions. Our data are also consistent with some recent reports showing that (a) IL-17A significantly contributes to the induction of EAE in immunized mice [63] and (b) adoptive transfer of MOG-specific Th17 cells induce EAE in C57BL/6 mice leading to the induction of EAE in wild-type C57BL/6 mice [22]. Increasing evidence suggests that Th17 cells mediate inflammatory responses through selective migration, accumulative retention at specific sites and secretion of inflammatory cytokines, such as IL-17 [64] inducing tissue inflammation, eventually leading to demyelination in the central nervous system [65–67].

EAE has long been considered the prototypic IFN-γ-secreting Th1-mediated autoimmune disease [68, 69]. Until some findings suggested a primary role for IL-17-secreting Th17 cells in this model [70, 71]. It has been shown that Th1 cells facilitate the entry of Th17 cells to the central nervous system during EAE [72]. Th1 and Th17 cells are shown to have different regulatory roles in inflammation of the brain and spinal cord [26] and EAE with different pathological phenotypes [73]. IFN-β was effective in reducing EAE symptoms induced by Th1 cells, but exacerbated disease induced by Th17 cells [74]. Therefore, this paradigm shift has sparked a rapid and remarkable change in emphasis in the search for disease-modifying drugs away from the Th1 pathway toward the Th17 pathway [75].

Conclusions

Taken together, our study shows that $CD4^+$ Th17 cells acquired pMHC I in the process of activation by DCs and became capable of stimulating OVA or MOG-specific $CD8^+$ CTL responses, when transferred into the mice. Our data also elucidate a distinct role of $CD4^+$ Th17 and Th17-stimulated $CD8^+$ T cells in autoimmune diseases, that T1D being directly mediated by Th17-stimulated $CD8^+$ cells, whereas EAE is likely to be triggered by $CD4^+$ Th17 cells. Therefore, this work may have great impact on the overall understanding of $CD4^+$ Th17 cells in the pathogenesis of autoimmune diseases.

Acknowledgments

This study was supported by Canadian Institute of Health Research (MOP 405674). Manjunatha Ankathatti Munegowda was supported by Dean's Scholarship of University of Saskatchewan.

References

1. Dong C. TH17 cells in development: an updated view of their molecular identity and genetic programming. Nat Rev Immunol. 2008; 8:337–48. [PubMed: 18408735]
2. Weaver CT, Harrington LE, Mangan PR, Gavrieli M, Murphy KM. Th17: an effector CD4 T cell lineage with regulatory T cell ties. Immunity. 2006; 24:677–88. [PubMed: 16782025]
3. Bettelli E, Korn T, Kuchroo VK. Th17: the third member of the effector T cell trilogy. Curr Opin Immunol. 2007; 19:652–7. [PubMed: 17766098]
4. Steinman L. A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. Nat Med. 2007; 13:139–45. [PubMed: 17290272]
5. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, et al. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell. 2006; 126:1121–33. [PubMed: 16990136]
6. Fukushima T, Zapata JM, Singha NC, Thomas M, Kress CL, Krajewska M, et al. Critical function for SIP, a ubiquitin E3 ligase component of the beta-catenin degradation pathway, for thymocyte development and G1 checkpoint. Immunity. 2006; 24:29–39. [PubMed: 16413921]
7. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity. 2006; 24:179–89. [PubMed: 16473830]
8. Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, et al. Transforming growth factor-beta induces development of the T(H)17 lineage. Nature. 2006; 441:231–4. [PubMed: 16648837]
9. Korn T, Bettelli E, Gao W, Awasthi A, Jager A, Strom TB, et al. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. Nature. 2007; 448:484–7. [PubMed: 17581588]
10. Zhou L, Ivanov II, Spolski R, Min R, Shenderov K, Egawa T, et al. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. Nat Immunol. 2007; 8:967–74. [PubMed: 17581537]
11. Peck A, Mellins ED. Plasticity of T-cell phenotype and function: the T helper type 17 example. Immunology. 2010; 129:147–53. [PubMed: 19922424]

12. Taplin CE, Barker JM. Autoantibodies in type 1 diabetes. Autoimmunity. 2008; 41:11–8. [PubMed: 18176860]
13. Di Lorenzo TP, Peakman M, Roep BO. Translational mini-review series on type 1 diabetes: systematic analysis of T cell epitopes in autoimmune diabetes. Clin Exp Immunol. 2007; 148:1–16. [PubMed: 17349009]
14. Kay TW, Parker JL, Stephens LA, Thomas HE, Allison J. RIP-beta 2-microglobulin transgene expression restores insulitis, but not diabetes, in beta 2-microglobulin null nonobese diabetic mice. J Immunol. 1996; 157:3688–93. [PubMed: 8871671]
15. Serreze DV, Gallichan WS, Snider DP, Croitoru K, Rosenthal KL, Leiter EH, et al. MHC class I-mediated antigen presentation and induction of CD8+ cytotoxic T-cell responses in autoimmune diabetes-prone NOD mice. Diabetes. 1996; 45:902–8. [PubMed: 8666141]
16. Wang B, Gonzalez A, Benoist C, Mathis D. The role of CD8+ T cells in the initiation of insulin-dependent diabetes mellitus. Eur J Immunol. 1996; 26:1762–9. [PubMed: 8765018]
17. Vukkadapu SS, Belli JM, Ishii K, Jegga AG, Hutton JJ, Aronow BJ, et al. Dynamic interaction between T cell-mediated beta-cell damage and beta-cell repair in the run up to autoimmune diabetes of the NOD mouse. Physiol Genomics. 2005; 21:201–11. [PubMed: 15671250]
18. Jain R, Tartar DM, Gregg RK, Divekar RD, Bell JJ, Lee HH, et al. Innocuous IFNgamma induced by adjuvant-free antigen restores normoglycemia in NOD mice through inhibition of IL-17 production. J Exp Med. 2008; 205:207–18. [PubMed: 18195074]
19. Sawcer S, Jones HB, Feakes R, Gray J, Smaldon N, Chataway J, et al. A genome screen in multiple sclerosis reveals susceptibility loci on chromosome 6p21 and 17q22. Nat Genet. 1996; 13:464–8. [PubMed: 8696343]
20. Ebers GC, Kukay K, Bulman DE, Sadovnick AD, Rice G, Anderson C, et al. A full genome search in multiple sclerosis. Nat Genet. 1996; 13:472–6. [PubMed: 8696345]
21. Haines JL, Ter-Minassian M, Bazyk A, Gusella JF, Kim DJ, Terwedow H, et al. A complete genomic screen for multiple sclerosis underscores a role for the major histocompatability complex. The Multiple Sclerosis Genetics Group. Nat Genet. 1996; 13:469–71. [PubMed: 8696344]
22. Yang Y, Weiner J, Liu Y, Smith AJ, Huss DJ, Winger R, et al. T-bet is essential for encephalitogenicity of both Th1 and Th17 cells. J Exp Med. 2009; 206:1549–64. [PubMed: 19546248]
23. Sutton C, Brereton C, Keogh B, Mills KH, Lavelle EC. A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. J Exp Med. 2006; 203:1685–91. [PubMed: 16818675]
24. Komiyama Y, Nakae S, Matsuki T, Nambu A, Ishigame H, Kakuta S, et al. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. J Immunol. 2006; 177:566–73. [PubMed: 16785554]
25. Bettelli E, Oukka M, Kuchroo VK. T(H)-17 cells in the circle of immunity and autoimmunity. Nat Immunol. 2007; 8:345–50. [PubMed: 17375096]
26. Stromnes IM, Cerretti LM, Liggitt D, Harris RA, Goverman JM. Differential regulation of central nervous system autoimmunity by T(H)1 and T(H)17 cells. Nat Med. 2008; 14:337–42. [PubMed: 18278054]
27. Tzartos JS, Friese MA, Craner MJ, Palace J, Newcombe J, Esiri MM, et al. Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. Am J Pathol. 2008; 172:146–55. [PubMed: 18156204]
28. Ford ML, Evavold BD. Specificity, magnitude, and kinetics of MOG-specific CD8+ T cell responses during experimental autoimmune encephalomyelitis. Eur J Immunol. 2005; 35:76–85. [PubMed: 15593305]
29. Friese MA, Fugger L. Autoreactive CD8+ T cells in multiple sclerosis: a new target for therapy? Brain. 2005; 128:1747–63. [PubMed: 15975943]
30. Sun D, Zhang Y, Wei B, Peiper SC, Shao H, Kaplan HJ. Encephalitogenic activity of truncated myelin oligodendrocyte glycoprotein (MOG) peptides and their recognition by CD8+ MOG-specific T cells on oligomeric MHC class I molecules. Int Immunol. 2003; 15:261–8. [PubMed: 12578856]

31. Sun D, Whitaker JN, Huang Z, Liu D, Coleclough C, Wekerle H, et al. Myelin antigen-specific CD8+ T cells are encephalitogenic and produce severe disease in C57BL/6 mice. J Immunol. 2001; 166:7579–87. [PubMed: 11390514]
32. Huseby ES, Liggitt D, Brabb T, Schnabel B, Ohlen C, Goverman J. A pathogenic role for myelin-specific CD8(+) Tcells in a model for multiple sclerosis. J Exp Med. 2001; 194:669–76. [PubMed: 11535634]
33. Ahmed KA, Munegowda MA, Xie Y, Xiang J. Intercellular trogocytosis plays an important role in modulation of immune responses. Cell Mol Immunol. 2008; 5:261–9. [PubMed: 18761813]
34. Xiang J, Huang H, Liu Y. A new dynamic model of CD8+ T effector cell responses via CD4+ T helper-antigen-presenting cells. J Immunol. 2005; 174:7497–505. [PubMed: 15944248]
35. Umeshappa CS, Huang H, Xie Y, Wei Y, Mulligan SJ, Deng Y, et al. CD4+ Th-APC with acquired peptide/MHC class I and II complexes stimulate type 1 helper CD4+ and central memory CD8+ T cell responses. J Immunol. 2009; 182:193–206. [PubMed: 19109150]
36. Ye Z, Ahmed KA, Hao S, Zhang X, Xie Y, Munegowda MA, et al. Active CD4+ helper T cells directly stimulate CD8+ cytotoxic T lymphocyte responses in wild-type and MHC II gene knockout C57BL/6 mice and transgenic RIP-mOVA mice expressing islet beta-cell ovalbumin antigen leading to diabetes. Autoimmunity. 2008; 41:501–11. [PubMed: 18855194]
37. Ahmed KA, Xie Y, Zhang X, Xiang J. Acquired pMHC I complexes greatly enhance CD4(+) Th cell's stimulatory effect on CD8(+) T cell-mediated diabetes in transgenic RIP-mOVA mice. Cell Mol Immunol. 2008; 5:407–15. [PubMed: 19118506]
38. Kurts C, Heath WR, Kosaka H, Miller JF, Carbone FR. The peripheral deletion of autoreactive CD8+ T cells induced by cross-presentation of self-antigens involves signaling through CD95 (Fas, Apo-1). J Exp Med. 1998; 188:415–20. [PubMed: 9670055]
39. Chen Z, Dehm S, Bonham K, Kamencic H, Juurlink B, Zhang X, et al. DNA array and biological characterization of the impact of the maturation status of mouse dendritic cells on their phenotype and antitumor vaccination efficacy. Cell Immunol. 2001; 214:60–71. [PubMed: 11902830]
40. Xia D, Hao S, Xiang J. CD8+ cytotoxic T-APC stimulate central memory CD8+ T cell responses via acquired peptide-MHC class I complexes and CD80 costimulation, and IL-2 secretion. J Immunol. 2006; 177:2976–84. [PubMed: 16920933]
41. Xiang J, Moyana T. Cytotoxic CD4+ T cells associated with the expression of major histocompatibility complex class II antigen of mouse myeloma cells secreting interferon-gamma are cytolytic in vitro and tumoricidal in vivo. Cancer Gene Ther. 1998; 5:313–20. [PubMed: 9824051]
42. Sas S, Chan T, Sami A, El-Gayed A, Xiang J. Vaccination of fiber-modified adenovirus-transfected dendritic cells to express HER-2/neu stimulates efficient HER-2/neu-specific humoral and CTL responses and reduces breast carcinogenesis in transgenic mice. Cancer Gene Ther. 2008; 15:655–66. [PubMed: 18421311]
43. Stockinger B, Veldhoen M. Differentiation and function of Th17 T cells. Curr Opin Immunol. 2007; 19:281–6. [PubMed: 17433650]
44. Atkinson MA, Eisenbarth GS. Type 1 diabetes: new perspectives on disease pathogenesis and treatment. Lancet. 2001; 358:221–9. [PubMed: 11476858]
45. Haskins K, Portas M, Bradley B, Wegmann D, Lafferty K. T-lymphocyte clone specific for pancreatic islet antigen. Diabetes. 1988; 37:1444–8. [PubMed: 2458291]
46. Jones EY, Fugger L, Strominger JL, Siebold C. MHC class II proteins and disease: a structural perspective. Nat Rev Immunol. 2006; 6:271–82. [PubMed: 16557259]
47. Faustman DL, Davis M. The primacy of CD8 T lymphocytes in type 1 diabetes and implications for therapies. J Mol Med. 2009; 87:1173–8. [PubMed: 19693476]
48. Katz JD, Benoist C, Mathis D. T helper cell subsets in insulin-dependent diabetes. Science. 1995; 268:1185–8. [PubMed: 7761837]
49. Bending D, De La Peña H, Veldhoen M, Phillips JM, Catherine U, Stockinger B, et al. Highly purified Th17 cells from BDC2.5NOD mice convert into Th1-like cells in NOD/SCID recipient mice. J Clin Invest. 2009:119.

50. Faustman D, Li XP, Lin HY, Fu YE, Eisenbarth G, Avruch J, et al. Linkage of faulty major histocompatibility complex class I to autoimmune diabetes. Science. 1991; 254:1756–61. [PubMed: 1763324]
51. Yan G, Fu Y, Faustman DL. Reduced expression of Tap1 and Lmp2 antigen-processing genes in the nonobese diabetic (NOD) mouse due to a mutation in their shared bidirectional promoter. J Immunol. 1997; 159:3068–80. [PubMed: 9300732]
52. Li F, Guo J, Fu Y, Yan G, Faustman D. Abnormal class I assembly and peptide presentation in the nonobese diabetic mouse. Proc Natl Acad Sci USA. 1994; 91:11128–32. [PubMed: 7972022]
53. Yan G, Shi L, Faustman D. Novel splicing of the human MHC-encoded peptide transporter confers unique properties. J Immunol. 1999; 162:852–9. [PubMed: 9916708]
54. Qu HQ, Lu Y, Marchand L, Bacot F, Frechette R, Tessier MC, et al. Genetic control of alternative splicing in the TAP2 gene: possible implication in the genetics of type 1 diabetes. Diabetes. 2007; 56:270–5. [PubMed: 17192492]
55. Christianson SW, Shultz LD, Leiter EH. Adoptive transfer of diabetes into immunodeficient NOD-scid/scid mice. Relative contributions of CD4+ and CD8+ T-cells from diabetic versus prediabetic NOD.NON-Thy-1a donors. Diabetes. 1993; 42:44–55. [PubMed: 8093606]
56. Wong FS, Karttunen J, Dumont C, Wen L, Visintin I, Pilip IM, et al. Identification of an MHC class I-restricted autoantigen in type 1 diabetes by screening an organ-specific cDNA library. Nat Med. 1999; 5:1026–31. [PubMed: 10470079]
57. Martin-Orozco N, Chung Y, Chang SH, Wang YH, Dong C. Th17 cells promote pancreatic inflammation but only induce diabetes efficiently in lymphopenic hosts after conversion into Th1 cells. Eur J Immunol. 2009; 39:216–24. [PubMed: 19130584]
58. Pinkse GG, Tysma OH, Bergen CA, Kester MG, Ossendorp F, van Veelen PA, et al. Autoreactive CD8 T cells associated with beta cell destruction in type 1 diabetes. Proc Natl Acad Sci USA. 2005; 102:18425–30. [PubMed: 16339897]
59. Russell JH, Ley TJ. Lymphocyte-mediated cytotoxicity. Annu Rev Immunol. 2002; 20:323–70. [PubMed: 11861606]
60. Bolitho P, Voskoboinik I, Trapani JA, Smyth MJ. Apoptosis induced by the lymphocyte effector molecule perforin. Curr Opin Immunol. 2007; 19:339–47. [PubMed: 17442557]
61. Merkler D, Oertle T, Buss A, Pinschewer DD, Schnell L, Bareyre FM, et al. Rapid induction of autoantibodies against Nogo-A and MOG in the absence of an encephalitogenic T cell response: implication for immunotherapeutic approaches in neurological diseases. FASEB J. 2003; 17:2275–7. [PubMed: 14563689]
62. Abdul-Majid KB, Wefer J, Stadelmann C, Stefferl A, Lassmann H, Olsson T, et al. Comparing the pathogenesis of experimental autoimmune encephalomyelitis in CD4−/− and CD8−/− DBA/1 mice defines qualitative roles of different T cell subsets. J Neuroimmunol. 2003; 141:10–9. [PubMed: 12965249]
63. Ishigame H, Kakuta S, Nagai T, Kadoki M, Nambu A, Komiyama Y, et al. Differential roles of interleukin-17A and -17 F in host defense against mucoepithelial bacterial infection and allergic responses. Immunity. 2009; 30:108–19. [PubMed: 19144317]
64. Kolls JK, Linden A. Interleukin-17 family members and inflammation. Immunity. 2004; 21:467–76. [PubMed: 15485625]
65. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol. 2005; 6:1133–41. [PubMed: 16200068]
66. Furuzawa-Carballeda J, Vargas-Rojas MI, Cabral AR. Autoimmune inflammation from the Th17 perspective. Autoimmun Rev. 2007; 6:169–75. [PubMed: 17289553]
67. Guo B, Chang EY, Cheng G. The type I IFN induction pathway constrains Th17-mediated autoimmune inflammation in mice. J Clin Invest. 2008; 118:1680–90. [PubMed: 18382764]
68. Ando DG, Clayton J, Kono D, Urban JL, Sercarz EE. Encephalitogenic T cells in the B10.PL model of experimental allergic encephalomyelitis (EAE) are of the Th-1 lymphokine subtype. Cell Immunol. 1989; 124:132–43. [PubMed: 2478300]

69. Wensky AK, Furtado GC, Marcondes MC, Chen S, Manfra D, Lira SA, et al. IFN-gamma determines distinct clinical outcomes in autoimmune encephalomyelitis. J Immunol. 2005; 174:1416–23. [PubMed: 15661899]

70. Cua DJ, Sherlock J, Chen Y, Murphy CA, Joyce B, Seymour B, et al. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. Nature. 2003; 421:744–8. [PubMed: 12610626]

71. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp Med. 2005; 201:233–40. [PubMed: 15657292]

72. O'Connor RA, Prendergast CT, Sabatos CA, Lau CW, Leech MD, Wraith DC, et al. Cutting edge: Th1 cells facilitate the entry of Th17 cells to the central nervous system during experimental autoimmune encephalomyelitis. J Immunol. 2008; 181:3750–4. [PubMed: 18768826]

73. Jager A, Dardalhon V, Sobel RA, Bettelli E, Kuchroo VK. Th1, Th17, and Th9 effector cells induce experimental autoimmune encephalomyelitis with different pathological phenotypes. J Immunol. 2009; 183:7169–77. [PubMed: 19890056]

74. Axtell RC, de Jong BA, Boniface K, van der Voort LF, Bhat R, De Sarno P, et al. T helper type 1 and 17 cells determine efficacy of interferon-beta in multiple sclerosis and experimental encephalomyelitis. Nat Med. 2010; 16:406–12. [PubMed: 20348925]

75. Kikly K, Liu L, Na S, Sedgwick JD. The IL-23/Th(17) axis: therapeutic targets for autoimmune inflammation. Curr Opin Immunol. 2006; 18:670–5. [PubMed: 17010592]

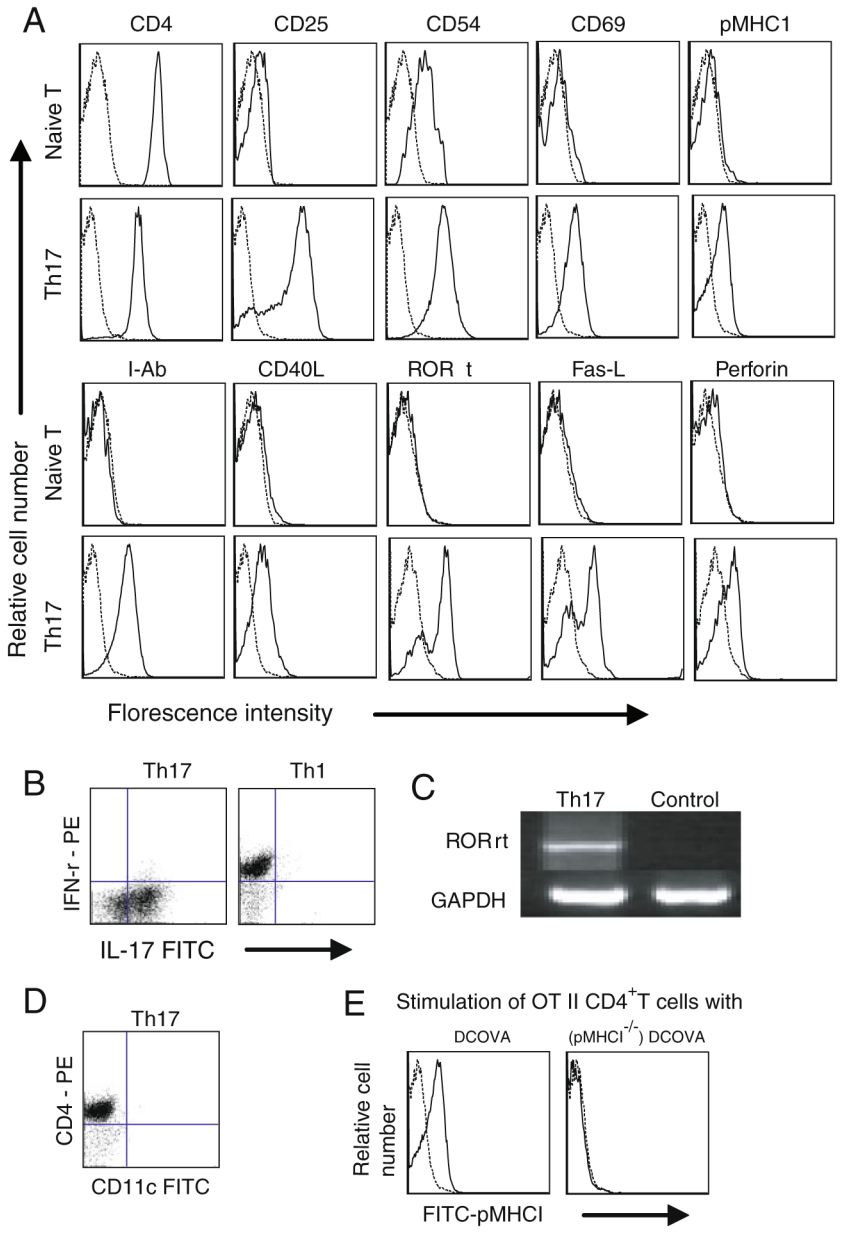

Fig. 1.

Phenotypic characterization of OVA-specific $CD4^+$ Th17 cells. **a** Naïve $CD4^+$ T cells and DC_{OVA}-activated $CD4^+$ Th17 cells derived from OT II mice were stained with a panel of biotin-conjugated Abs (*solid lines*) followed by staining with FITC-conjugated avidin and analyzed by flow cytometry. Irrelevant isotype-matched biotin-conjugated Abs were used as controls (*light dotted lines*). **b** In vitro DC_{OVA}-activated $CD4^+$ Th1 and Th17 cells were double-stained with FITC-anti-IL-17 Ab and PE-anti-IFN-γ Ab, and analyzed by flow cytometry. **c** RNA extracted from DC_{OVA}-activated $CD4^+$ Th17 and Con A-stimulated $CD4^+$ T (control) cells were analyzed by RT-PCR for expression of Th17 cell specific transcription factor ROR-γt. **d** Purified active $CD4^+$ Th17 cells were stained with PE-anti-CD4 and FITC-anti-CD11c Abs and analyzed by flow cytometry. **e** $CD4^+$ Th17 and ($K^{b-/-}$)Th17 cells were stained with FITC-anti-pMHC I antibody (*solid lines*) and irrelevant

isotype-matched antibody was used as control (*dotted lines*). One representative experiment of two experiments is shown

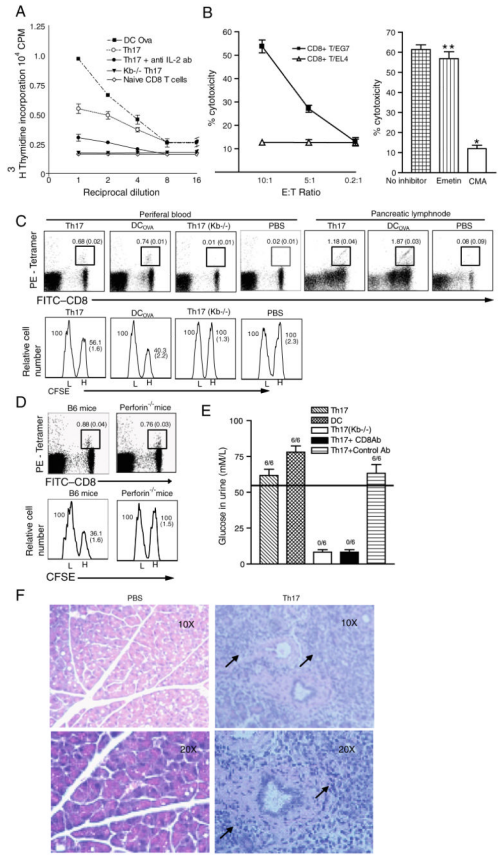

Fig. 2.

$CD4^+$ Th17 cells induced CTL leads to diabetes in transgenic RIP-mOVA mice. **a** In vitro $CD8^+$ T cell proliferation assay. Irradiated DC_{OVA}, $CD4^+$ Th17, $CD4^+$ Th17 with anti-IL-2 Ab and ($K^{b-/-}$)Th17 cells, and their twofold dilutions were co-cultured with naïve OTI $CD8^+$ T cells. After 2 days, the proliferative responses of $CD8^+$ T cells were determined by overnight ^3H-thymidine uptake assay. **b** In vitro cytotoxicity assay. Th17-activated OTI $CD8^+$ Tc1 cells were used as effector (E) cells and in another experiment, Th17-activated $CD8^+$ T cells with or without preincubation of concanamycin A (CMA, 1 μM) or emetin (5 μM) for 2 h were used as effector (E) cells, while ^{51}Cr-labeled EG7 and EL4 cells were used as target (T) cells in a chromium release assay. **c** In tetramer staining assay, the tail blood samples and pancreatic lymph node cells of transgenic RIP-mOVA mice adoptively transferred with $CD4^+$ Th17 cells, DC_{OVA}, ($K^{b-/-}$)$CD4^+$ Th17 cells, and PBS (controls) were stained with PE-H-$2K^b$/OVAI (PE-tetramer) and FITC-CD8 Ab (FITC-CD8), and then analyzed by flow cytometry. The *values in each panel* represent the percentage of tetramer-positive $CD8^+$ Tcells versus the total $CD8^+$ Tcell population. The *value in parenthesis* represents the standard deviation. In in vivo cytotoxicity assay, 16 h after target cell delivery, the residual OVAI-pulsed $CFSE^{high}$ and Mut1-pulsed $CFSE^{low}$ target cells remaining in the spleens of the above cohorts of mice were sorted and analyzed by flow cytometry. The *value in parenthesis* represents the standard deviation; (n=6, average±SD), *p<0.05 versus cohorts of mice adoptively transferred with DC_{OVA} (Student's t test). **d** In tetramer staining assay, the tail blood samples of wild-type C57BL/6 and perforin$^{-/-}$ mice adoptively transferred with $CD4^+$ Th17 cells were stained with PE-H-$2K^b$/OVAI (PE-tetramer) and FITC-CD8 Ab (FITC-CD8), and then analyzed by flow cytometry. In in vivo cytotoxicity assay, 16 h after target cell delivery, the residual OVAI-pulsed $CFSE^{high}$ and

Mut1-pulsed $CFSE^{low}$ target cells remaining in the spleens of the above cohorts of mice were sorted and analyzed by flow cytometry. The *value in parenthesis* represents the standard deviation; (n=6, average±SD), *p<0.05 versus cohorts of perforin$^{-/-}$ mice (Student's t test). **e** Urine test for diabetes. Glucose levels in urine samples from transgenic RIP-mOVA mice adoptively transferred with irradiated $CD4^+$ Th17 cells, DC_{OVA}, ($K^{b-/-}$)$CD4^+$ Th17 cells, and PBS (controls). The cutoff line of urine glucose concentration for diabetes is shown. **f** Hematoxylin and eosin-stained sections from Th17- and PBS-injected mice at higher magnification showing extensive cellular infiltration in Th17-injected mice compared to control. Magnifications, ×10 and ×20. One representative experiment of two in the above different experiments is shown

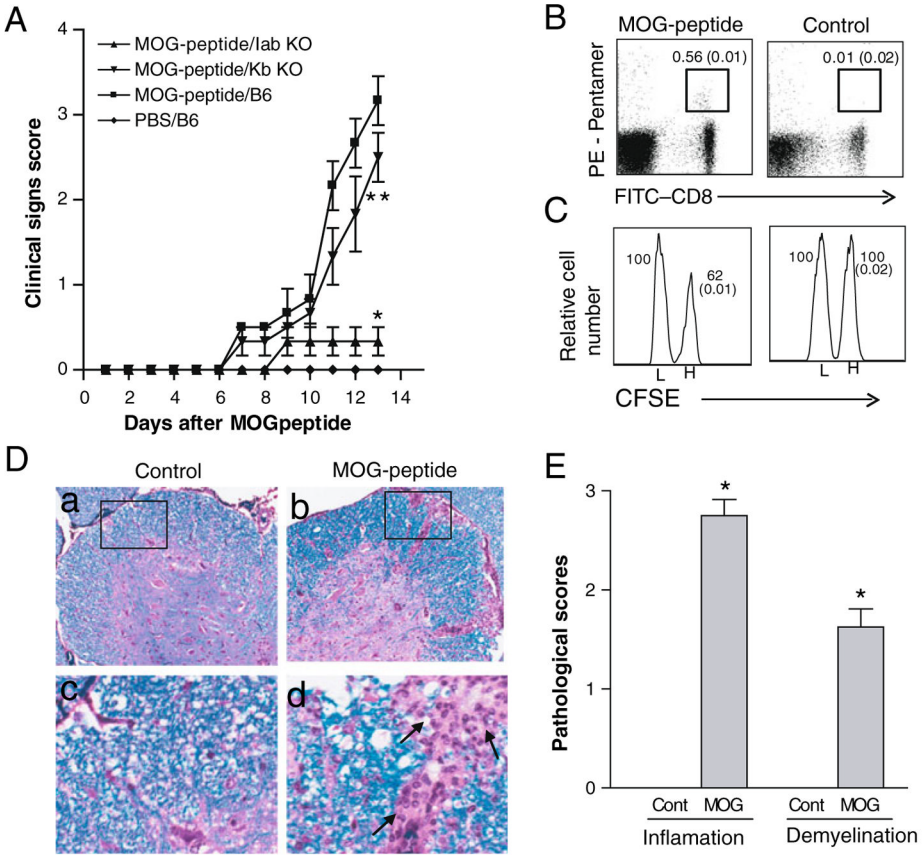

Fig. 3.

MOG peptide immunization stimulate MOG-specific CTL responses and induce EAE. **a** Wild-type C57BL/6 and $CD4^+$ T cell- or $CD8^+$ T cell-deficient Ia^b and $H\text{-}2K^b$ mice were immunized with $MOG_{35\text{-}55}$ + CFA. C57BL/6 mice immunized with CFA only were used as control. Clinical EAE was scored according to 0–5 scale. The difference between C57BL/6 and $CD4^+$ T cell-depleted mice (*two asterisks*) or $CD8^+$ T cell-depleted C57BL/6 mice (*single*) is very significant ($p<0.01$) or significant ($p<0.05$; Mann–Whitney *U* test). **b** The tail blood samples of mice immunized with MOG peptide or OVAI peptide (control) were stained with PE-$H\text{-}2D^b$/MOGI pentamer (PE-pentamer) and FITC-anti-CD8 Ab (FITC-CD8), and then analyzed by flow cytometry. The *value in each panel* represents the percentage of pentamer-positive $CD8^+$ T cells versus the total $CD8^+$ T cell population. **c** In in vivo cytotoxicity assay, 16 h after target cell delivery, the residual MOGI-pulsed $CFSE^{high}$ and Mut1-pulsed $CFSE^{low}$ target cells remaining in the spleens of the above immunized mice were sorted and analyzed by flow cytometry. The *value in parenthesis* represents the standard deviation. **d** Photographs of sections of spinal cords derived from mice with EAE; tissue sections were stained with Luxol fast blue along with H&E counterstaining. Control mice (*a* and *c*) and MOG-immunized mice (*b* and *d*). Magnifications, ×5 (*a* and *b*) and ×20 (*c* and *d*). Inflammatory infiltration and demyelination are shown with *arrows*. **e** Mean scores of inflammation and demyelination±SD. *$p<0.01$ versus cohorts of the control groups (Student's *t* test). One representative experiment of three in the above experiments is shown

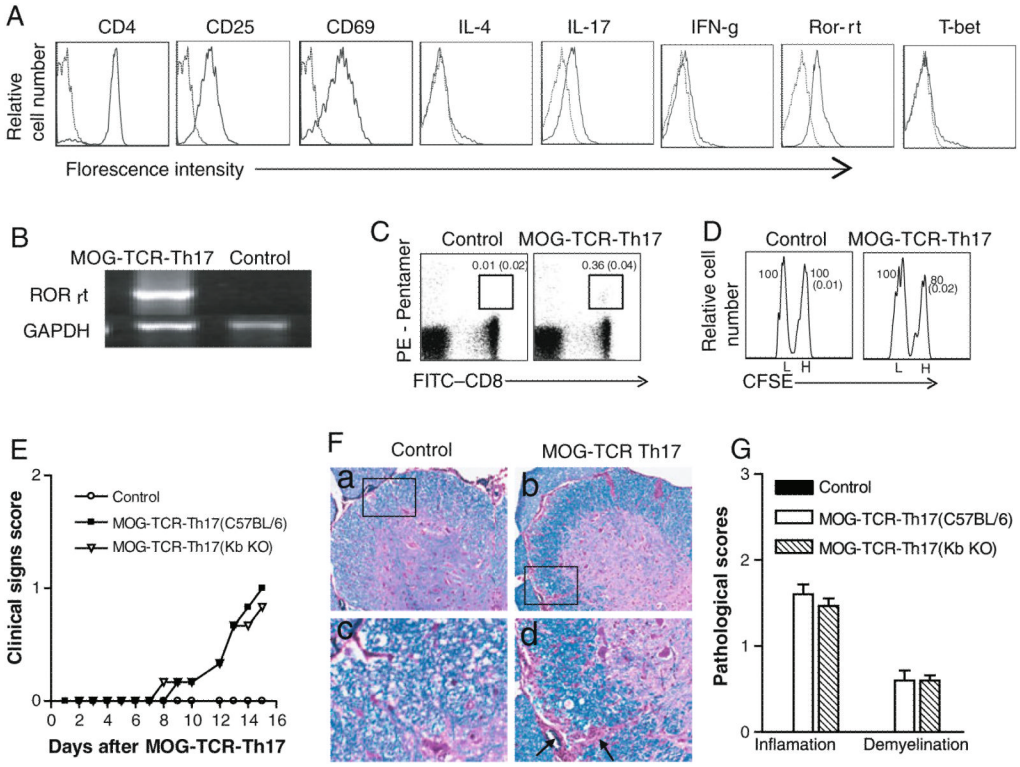

Fig. 4.

In vitro-activated MOG-specific $CD4^+$ MOG-TCR-Th17 cells stimulate MOG-specific $CD8^+$ CTL responses and induce EAE. **a** Phenotypic analysis of MOG-specific $CD4^+$ MOG-TCR-Th17 cells. MOG-specific $CD4^+$ MOG-TCR-Th17 cells derived from transgenic MOG-TCR mice were stained with a panel of biotin-conjugated Abs (*solid lines*) followed by staining with FITC-conjugated avidin and analyzed by flow cytometry. Irrelevant isotype-matched biotin-conjugated Abs were used as controls (*light dotted lines*). **b** RNA extracted from MOG-specific $CD4^+$ MOG-TCR-Th17 and Con A-stimulated $CD4^+$ T (*control*) cells were analyzed by RT-PCR for assessment of expression of RORγt. **c** Pentamer staining assay. The tail blood samples of mice adaptively transferred with $CD4^+$ MOG-TCR-Th17 cells or Con A-stimulated $CD4^+$ T (control) cells were stained with PE-H-$2D^b$/MOGI pentamer (PE-pentamer) and FITC-anti-CD8 Ab (FITC-CD8), and then analyzed by flow cytometry. The *value in each panel* represents the percentage of pentamer-positive $CD8^+$ T cells versus the total $CD8^+$ T cell population. The *value in parenthesis* represents the standard deviation. **d** In vivo cytotoxicity assay. Sixteen hours after target cell delivery, the residual MOGI-pulsed $CFSE^{high}$ and Mut1-pulsed $CFSE^{low}$ target cells remaining in the spleens of the above cohorts of mice were sorted and analyzed by flow cytometry. The *value in parenthesis* represents the standard deviation. **e** Wild-type C57BL/6 mice were adoptively transferred with MOG-specific MOG-TCR-Th17 cells or OVA-specific Th17 cells (control). The clinical EAE was scored according to 0–5 scale. **f** Photographs of sections of spinal cords derived from mice with EAE; tissue sections were stained with Luxol fast blue along with H&E counterstaining. Control mice (*a* and *c*) and MOG-immunized mice (*b* and *d*). Magnifications, ×5 (*a* and *b*) and ×20 (*c* and *d*). Inflammatory infiltration and demyelination are shown with *arrows*. **g** Mean scores of inflammation and demyelination±SD. *$p<0.01$ versus cohorts of the control groups (Student's *t* test). One representative experiment of three in the above experiments is shown

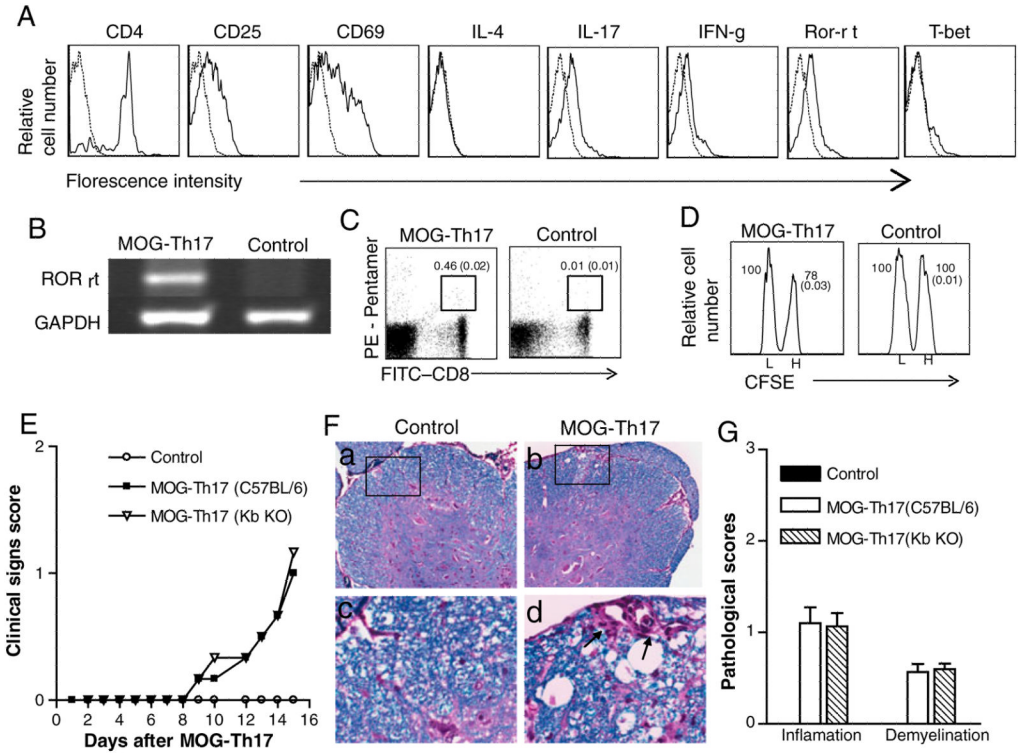

Fig. 5.

In vivo-generated MOG-specific $CD4^+$ Th17 cells stimulate MOG-specific $CD8^+$ CTL responses and induce EAE. **a** Phenotypic analysis of in vivo-generated MOG-specific $CD4^+$ Th17 cells. MOG-specific $CD4^+$ Th17 cells derived from MOG peptide-immunized mice with EAE and expanded in vitro by co-culturing with MOG peptide-pulsed splenocytes were stained with a panel of biotin-conjugated Abs (*solid lines*) followed by staining with FITC-conjugated avidin and analyzed by flow cytometry. Irrelevant isotype-matched biotin-conjugated Abs were used as controls (*light dotted lines*). **b** RNA extracted from MOG-specific $CD4^+$ Th17 and Con A-stimulated $CD4^+$ T (control) cells were analyzed by RT-PCR for assessment of expression of RORγt. **c** Pentamer staining assay. The tail blood samples of mice adoptively transferred with $CD4^+$ Th17 cells or Con A-stimulated $CD4^+$ T (control) cells were stained with PE-H-$2D^b$/MOGI pentamer (PE-pentamer) and FITC-anti-CD8 Ab (FITC-CD8), and then analyzed by flow cytometry. The *value in each panel* represents the percentage of pentamer-positive $CD8^+$ T cells versus the total $CD8^+$ T cell population. The *value in parenthesis* represents the standard deviation. **d** In vivo cytotoxicity assay. Sixteen hours after target cell delivery, the residual MOGI-pulsed $CFSE^{high}$ and Mut1-pulsed $CFSE^{low}$ target cells remaining in the spleens of the above cohorts of mice were sorted and analyzed by flow cytometry. The *value in parenthesis* represents the standard deviation. **e** Wild-type C57BL/6 mice were adoptively transferred with MOG-specific Th17 cells or OVA-specific Th17 cells (control). The clinical EAE was scored according to 0–5 scale. **f** Photographs of sections of spinal cords derived from mice with EAE; tissue sections were stained with Luxol fast blue along with H&E counterstaining. Control mice (*a* and *c*) and MOG-immunized mice (*b* and *d*). Magnifications, ×5 (*a* and *b*) and ×20 (*c* and *d*). Inflammatory infiltration and demyelination are shown with *arrows*. **g** Mean scores of inflammation and demyelination±SD. **p*<0.01 versus cohorts of the control groups (Student's *t* test). One representative experiment of three in the above experiments is shown

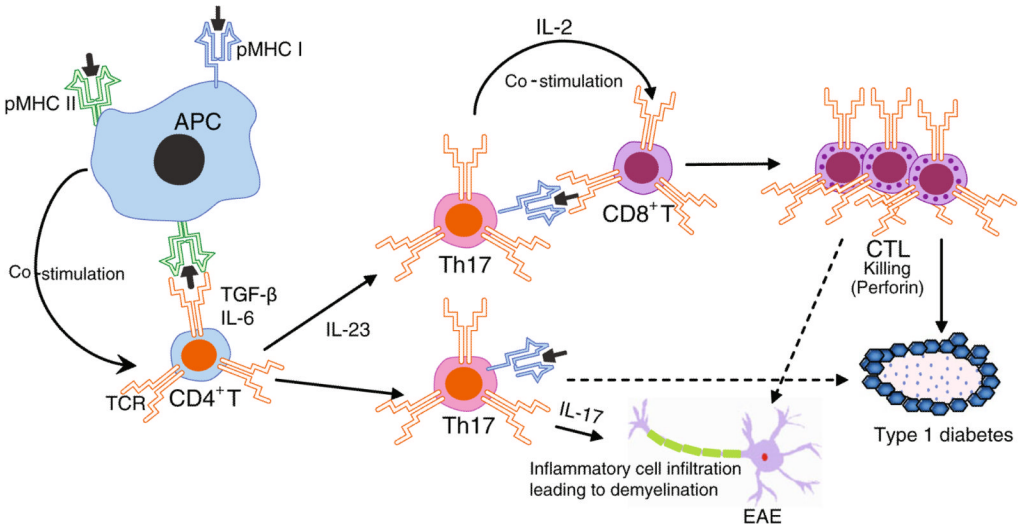

Fig. 6.

Distinct role of $CD4^+$ Th17- and Th17-stimulated $CD8^+$ CTL in pathogenesis of T1D and EAE. Both $CD4^+$ Th17 cells and Th17-stimulated $CD8^+$ CTLs are involved in pathogenesis of T1D and EAE. However, T1D is directly mediated by Th17-stimulated $CD8^+$ CTLs to destroy OVA-expressing pancreatic islets of RIP-mOVA mice via perforin-mediated cytotoxicity. On the contrary, $CD4^+$ Th17 cells play a major role in pathogenesis of EAE by Th17 cytokine-mediated tissue inflammation leading to demyelination in the central nervous system