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Bidirectional Effect of IFN-γ on Th17 Responses in Experimental Autoimmune Uveitis

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Abstract

Pro- and ant-inflammatory effects of IFN-γ have been repeatedly found in various immune responses, including cancer and autoimmune diseases. In a previous study we showed that the timing of treatment determines the effect of adenosine-based immunotherapy. In this study we examined the role of IFN-γ in pathogenic Th17 responses in experimental autoimmune uveitis (EAU). We observed that IFN-γ has a bidirectional effect on Th17 responses, when tested both *in vitro* and *in vivo*. Anti-IFN-γ antibody inhibits Th17 responses when applied in the initial phase of the immune response; however, it enhances the Th17 response if administered in a later phase of EAU. In the current study we showed that IFN-γ is an important immunomodulatory molecule in γδ T cell activation, as well as in Th17 responses. These results should advance our understanding of the regulation of Th17 responses in autoimmunity.

Keywords

IFN-gamma; experimental autoimmune uveitis; gamma delta T cell; Th17; autoimmunity

INTRODUCTION

IFN-γ production is a hallmark of the T helper (Th)1 response (1, 2). As a key player in cellular immunity, IFN-γ is capable of orchestrating numerous immune responses in infections and cancers. It immunomodulates antigen processing and presentation (3), increases leukocyte trafficking (4), induces anti-viral responses (5), boosts anti-microbial functions (6, 7) and affects cellular proliferation and apoptosis (8). Although the major source of IFN-γ in adaptive immune responses is T cells (9), various innate cells such as NK/NKT cells (2, 10), macrophages (11, 12), dendritic cells (13–15) and γδ T cells (16, 17) are capable of producing IFN-γ. Indeed, in the initiation stage of autoimmune diseases, innate cells such as NK cells are the main source of IFN-γ production (18), albeit transiently (2).

IFN-γ has either pro- or anti-inflammatory effects in various immune responses, including cancer and autoimmunity (18–22). The earlier finding that IFN-γ inhibits Th17 responses (23, 24) suggested that neutralization of IFN-γ would elicit stronger Th17 responses (25, 26). Because we previously found that adenosine-based immunotherapies are strictly "timing dependent" (27, 28), we wished to investigate whether immunomodulation by treatments other than adenosine or adenosine receptors also might depend on the timing of treatment. In our previous study, we found that treatment of mice with induced experimental autoimmune uveitis (EAU) with adenosine deaminase (ADA), an enzyme that degrades adenosine, inhibited Th17 pathogenic T cell responses and suppressed EAU (27). The inhibitory effect of ADA was restricted to the active stage of disease; but ADA was ineffective if administered during the quiescent disease stage (27). Likewise, treatment of EAU-induced mice with an antagonist specific for adenosine receptor A2AR only inhibited EAU if given during the active phase of intraocular inflammation (28). We investigated the effect of adding anti-IFN-γ antibody to *in vitro* responding T cells and EAU-induced mice at different time points to determine whether IFN-γ has a regulatory effect on Th17 responses, and whether a similar timing effect might be identified. Our results demonstrated that anti-IFN-γ antibody inhibited Th17 responses if provided during the initial phase of T cell activation (i.e., the early phase of EAU induction). However, the effect was reversed once T cell activation was initiated *in vitro* or when anti-IFN-γ antibody was administered to EAU-induced mice in a later phase of EAU induction.

Our previous studies showed that activation of γδ T cells closely correlated with augmented Th17 responses (29–32). Therefore, we also examined the effect of anti-IFN-γ treatment on γδ T cell activation. Anti-IFN-γ treatment during the early phase of EAU induction inhibited γδ T cell activation, whereas treatment during a later phase of EAU induction enhanced γδ T cell activation similar to the Th17 responses. Thus, in the current study we showed a "timing effect" for IFN-γ similar to that of adenosine-based immunotherapy in EAU (27, 33).

MATERIALS AND METHODS

Animals and Reagents

Female C57BL/6 (B6), TCR-$\delta^{-/-}$, and IFN-$\gamma^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA); 12- to 16-week-old mice were used in all studies. All mice were housed and maintained in the animal facilities of the University of California Los Angeles. All protocols in this study were approved by the Committee on the Ethics of Animal Experiments of University of California, Los Angeles (IACUC permit number: ARC#2014-029-03A), in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Recombinant murine IFN-γ, and IL-23 were purchased from R & D Systems (Minneapolis, MN, USA). FITC-, PE-, or allophycocyanin-conjugated Abs against mouse CD3, CD4, Foxp3, $\alpha\beta$ TCR, or $\gamma\delta$ TCR (GL3) and their isotype control Abs were purchased from Biolegend (San Diego, CA, USA). PE-conjugated anti-mouse IFN-γ (XMG1.2), IL-17 (TC11-18H10.1) Abs were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

EAU Induction and Anti-IFN-γ Treatment

EAU was induced in B6 mice by s.c. injection of 200 μl of emulsion containing 200 μg of human interphotoreceptor retinoid-binding protein (IRBP)$_{1-20}$ (Sigma-Aldrich, St. Louis, MO, USA) in CFA (Difco, Detroit, MI, USA) at six spots at the tail base and on the flank, and by i.p. injection with 300 ng of pertussis toxin. Mice were then examined three times a week until the end of the experiment (d 30 post-immunization).

For *in vivo* administration of IFN-γ, immunized B6 mice were randomly divided into three groups (n=6), one of which received an i.p. injection of anti-IFN-γ (100 μg/mouse) at d 0 (day of immunization and the second at d 8 post-immunization. The mice in the control group received PBS. At d 13 post-immunization (the time at which the highest T cell response is seen), responder T cells were purified from the spleen and draining lymph nodes stimulated *in vitro* with the immunizing peptide and APCs (irradiated spleen cells) under culture conditions that favor Th17 or Th1 autoreactive T cell expansion (medium containing 10 ng/ml of, respectively, IL-23 or IL-12) (24, 25) A schematic procedure of disease induction and examination of mice under investigation is shown in Scheme 1.

Adoptive Transfer Assay Testing Uveitogenic Activity of IRBP-Specific T Cells

IRBP-specific T cells were prepared as we previously described (29). Briefly, CD3$^+$ T cells from IRBP$_{1-20}$/CFA-immunized B6 mice were isolated 13 d postimmunization. Then 1×10^7 cells in 2 ml of RPMI medium in a 6-well plate (Costar) were stimulated with 20 μg/ml of IRBP$_{1-20}$ in the presence of 1×10^7 irradiated syngeneic spleen cells as APCs. After 2 d, the activated lymphoblasts were isolated by gradient centrifugation on Lymphoprep (Robbins Scientific, Mountain View, CA, USA). The pathogenic activity was determined after transfer of the IRBP-specific T cells to the naïve B6 recipient mice *via* i.p. injection (2×10^6/mouse).

EAU Evaluation

The mice were examined three times a week for 30 d post-immunization. The clinical signs of EAU were evaluated using fundoscopic examination. Fundoscopic grading of disease was performed using the scoring system described previously (34).

At 30 d post-immunization, the mice were euthanized, and the eyes were collected for histological examination. For histology, whole eyes were collected at the end of the experiment and prepared for histopathological evaluation. The eyes were immersed for 1 h in 4% phosphate-buffered glutaraldehyde, then transferred to 10% phosphate-buffered formaldehyde until they were processed. Fixed and dehydrated tissues were embedded in methacrylate, and 5 μM sections were cut through the pupillary-optic nerve plane and stained with H&E. The eyes were fixed overnight at 40C in Davison's solution and then processed as paraffin-embedded blocks.

T Cell Preparation

Responder $CD3^+$ T cells were purified from B6 mice immunized with the human $IRBP_{1-20}$ peptide (29, 31, 35). Nylon wool-enriched splenic T cells from naive or immunized mice were incubated sequentially for 10 min at 4°C with FITC-conjugated anti-mouse γδ TCR or αβ TCR Abs and for 15 min at 4°C with anti-FITC Microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The cells were then separated into bound and non-bound fractions on an autoMACS™ separator column (Miltenyi Biotec GmbH). To obtain a sufficient number of cells, we routinely pool the cells obtained from all six mice in the same group, before the T cells are further enriched using MACS column. The purity of the isolated cells, as determined by flow cytometric analysis using PE-conjugated Abs against αβ or γδ T cells, was >95%.

Assessment of Th1 and Th17 Polarized Responses

Responder $CD3^+$ T cells (3×10^6) were co-cultured for 48 h with $IRBP_{1-20}$ (10 μg/ml) and with irradiated spleen cells (2×10^6/well) as APCs in a 12-well plate under either Th17 polarized conditions (culture medium supplemented with 10 ng/ml of IL-23) or Th1 polarized conditions (culture medium supplemented with 10 ng/ml of IL-12). The responder αβ T cells were collected from $IRBP_{1-20}$-immunized B6 mice, on d 13 post-immunization. Forty-eight hours after stimulation, IL-17 and IFN-γ levels in the culture medium were measured using ELISA kits (R & D Systems) and the percentage of $IFN-\gamma^+$ and $IL-17^+$ T cells among the responder T cells was determined by intracellular staining after 5 d of culture, followed by FACS analysis, as described below (31, 36).

Immunofluorescence Flow Cytometry for Surface and Cytoplasmic Antigens

In vivo primed T cells were stimulated with the immunizing antigen and APCs for 5 d. The T cells were then separated using Ficoll gradient centrifugation and stimulated *in vitro* for 4 h with 50 ng/ml of PMA, 1 μg/ml of ionomycin, and 1 μg/ml of brefeldin A (all from Sigma). Aliquots of cells (2×105 cells) were incubated for 30 min at 4°C with anti-αβ or -γδ TCR antibodies, then fixed, permeabilized overnight with Cytofix/Cytoperm buffer (eBioscience, San Diego, CA, USA), and intracellularly stained with PE-conjugated

anti-mouse IFN-γ antibodies or FITC-labeled anti-mouse IL-17 antibodies. Data collection and analysis were performed on a FACScalibur flow cytometer using CellQuest software.

Cytokine Assays by ELISA

Cytokine (IL-17 and IFN-γ) levels in the culture medium were measured using ELISA kits (R & D) following manufacturer's instructions.

Statistical Analysis

The results in the figures are representative of one experiment, which was repeated 3–5 times. The statistical significance of differences between groups in a single experiment was initially analyzed by 2-way Student's t-tests, and if statistical significance was detected, the Student–Newman–Keuls *post-hoc* test was subsequently used. A P value of less than 0.05 was considered a statistically significant difference and marked with one *; when P<0.01, two ** were used.

RESULTS

Anti-IFN-γ Affects Th1 and Th17 Responses Differently *In Vitro*

To determine the effect of IFN-γ on autoreactive antigen specificT cell responses *in vitro*, $CD3^+$ T cells from the spleen and draining lymph nodes of B6 mice were isolated at the height of induced responses, 13 d after immunization with $IRBP_{1-20}$. They were stimulated with the immunizing antigen and irradiated splenic APCs under Th1- or Th17- polarized conditions, with/without anti-IFN-γ (d 0). As demonstrated in Figures 1A, B, anti-IFN-γ treated on day 0 (day of immunization) inhibited the response of Th17 cells to a much greater extent than it inhibited the response of Th1 cells; similarly, IL-17 production was decreased much more than production of IFN-γ (Figure 1E). The effect of anti-IFN-γ was reversed when the antibody was added 8 d post-stimulation (Figure 1C): with the Th17 cell and IL-17 responses enhanced. The effect on Th1 cells or IFN-γ production was minimal.

Anti-IFN-γ Affects Th1 and Th17 Responses Differently *In Vivo*

To determine whether anti-IFN-γ antibody would have a similar effect *in vivo*, two groups of B6 mice (n=6) were immunized with $IRBP_{1-20}$ with or without an i.p. injection of anti-IFN-γ (100μg/mouse) on the day of immunization (d 0). Thirteen days post-immunization the mice were sacrificed and Th1 and Th17 responses were assessed. Figure 2 shows that anti-IFN-γ antibody again significantly inhibited the Th17 response while inhibiting the Th1 response only slightly, similar to its effects seen *in vitro*.

The Effect of Anti-IFN-γ on EAU Development and the Th17 Response Depends on Timing

To determine whether the timing effect observed *in vitro* also applied *in vivo*, groups of B6 mice (n=6) were immunized with $IRBP_{1-20}$/CFA. Because preliminary testing revealed that results differed when anti-IFN-γ was injected between d 0 to 3 vs d 6 to 8 post immunization, two time points were selected for subsequent studies. Those mice injected on the day of immunization (d0) were designated as "early-treated", and those injected on day 8 after immunization were designated as "late-treated". As shown in Figures 3A,

B, the number of IL-17$^+$ cells among the responder T cells in early-treated mice was significantly decreased (from 9.5% in the control group to 3.3% in the treated group), whereas this number was significantly increased in late-treated mice (from 9.5% in the control group to 11.6% in the treated group) (Figure 3A, upper panels). In contrast, numbers of IFN-γ^+ T cells changed little between early- and late-treated mice (Figure 3A, lower panels). Cytokine production (Figure 3C) confirmed intracellular staining in that mice in the late group produced more IL-17, whereas those in the early group produced less IL-17. *In vivo* administration of anti-IFN-γ had a stronger inhibitory effect on IFN-γ production of responder T cells, when compared with *in vitro* tests (Figure 1), regardless of whether the antibody was injected early or late. The difference between these results and the intracellular staining suggests that Th1 responses are modestly inhibited in treated mice.

IL-17$^+$ T Cells Isolated From Late IFN-γ Treated Recipients Are More Pathogenic

To determine whether pathogenic IRBP-specific IL-17$^+$ T cell responses were enhanced in mice that received late treatment with anti-IFN-γ we performed adoptive transfer tests. IL-17$^+$ T cells from IRBP-immunized mice administered anti-IFN-γ on d 8 were compared to PBS injected mice (i.e. the control) on d8. 2×10^6 IRBP-specific IL-17$^+$ cells were adoptively transferred to naive B6 mice by i.p. injection after 2 d of *in vitro* stimulation with the immunizing antigen and splenic APCs, under Th17-polarizing conditions. Adoptively transferred Th17 cells from late anti-IFN-γ treated mice induced significantly more severe EAU in recipient mice when compared to controls (Figure 3D). The total number of IL-17$^+$ T cells was also significantly greater in anti-IFN-γ treated mice (data not shown).

Similar Timing Effect Exists in $\gamma\delta$ T Cell Responses in Anti-IFN-γ Treated Mice

We have previously shown that $\gamma\delta$ T cells regulate Th17 responses (29, 31), whereby activation of $\gamma\delta$ T cells augmented Th17 reactivity (30–32). Therefore, we thought it important to examine the state of $\gamma\delta$ activation after anti-IFN-γ treatment. In early anti-IFN-γ treated mice, the frequency of $\gamma\delta$ T cells among total CD3$^+$ T cells was significantly reduced (Figures 4A, B), suggesting that IFN-γ is required for early-stage $\gamma\delta$ cell proliferation. Perhaps more importantly, the ratio between CD44$^+$ and CD44$^-$ $\gamma\delta$ T cells was greatly decreased (Figures 4C, D), indicating that neutralizing IFN-γ at early stages disease had an inhibitory effect on $\gamma\delta$ activation *in vivo*. By contrast, the number of $\gamma\delta$ T cells were significantly increased among total CD3$^+$ T cells in mice that received late anti-IFN-γ treatment (Figure 5A) compared to early anti-IFN-γ treated or untreated B6 mice. Moreover, the number of IL-17$^+$ among total $\gamma\delta$ T cells was significantly higher in mice that received late anti-IFN-γ treatment (Figure 5B).

$\gamma\delta$ T Cells Separated From Late Anti-IFN-γ Treated Mice Enhance Th17 Activity *In Vitro*

To further investigate if the enhanced $\gamma\delta$ activation in mice with late anti-IFN-γ treatment contributed to augmented Th17 responses, we compared the *in vitro* Th17 response of TCR-$\delta^{-/-}$ responder T cells with or without the addition of $\gamma\delta$ T cells from early or late treated anti-IFN-γ mice (30–32). Figure 5C shows that the Th17 response of the TCR-$\delta^{-/-}$ responder T cells were significantly higher after adding $\gamma\delta$ T cells (5% of total CD3$^+$ cells) isolated from late anti-IFN-γ treated mice (right panel) compared to those without added

γδ T cells (left panel) or with added γδ T cells isolated from early anti-IFN-γ treated mice (middle panel).

Augmented Th17 Responses in IFN-γ⁻/⁻ Mice Are Contributed by Multiple Aberrant Immune Responses

Finally, we compared the immune response between IFN-γ-deficient and wt-B6 mice, as a further means to better understand the role of IFN-γ in pathogenic Th17 responses. Figure 6A shows that a greater number of responder T cells expressed IL-17 in IFN-γ⁻/⁻ mice compared to B6 mice (Figure 6A) when CD3⁺ T cells were stimulated with the immunizing antigen and APCs for 5 d; additionally, IFN-γ⁻/⁻ T cells produced significantly higher amounts of IL-17 (Figure 6B). The number of γδ T cells among the CD3⁺ splenic cells did not differ significantly between unimmunized IFN-γ⁻/⁻ and B6 mice; however, the total number of γδ T cells, as well as numbers of activated γδ T cells (IL-17⁺), increased greatly among the IFN-γ⁻/⁻ mice after immunization (Figures 6C, D).

We also compared antigen-specific T cell responses between CD3⁺ T cells isolated from IFN-γ⁻/⁻ and wt-B6 mice, respectively. The results of crisscross tests, in which the T cells derived from immunized IFN-γ⁻/⁻ or B6 mice were stimulated either by splenic APCs of irradiated B6 mice or IFN-γ ⁻/⁻ mice (Figure 6E) revealed that IFN-γ⁻/⁻ T cells produced significantly greater amounts of IL-17 when compared to B6 T cells. In addition, IFN-γ⁻/⁻ splenic APCs had stronger stimulating activity compared to B6 splenic APCs, suggesting that APC function is also augmented in IFN-γ⁻/⁻ mice.

When Foxp3⁺ T cell responses of wt-B6 and IFN-γ⁻/⁻ mice were compared, we found that the number of Foxp3⁺ T cells was significantly higher among CD3⁺ T cells in IFN-γ⁻/⁻ mice (Figure 7A). However, the EAU-inducing activity of IRBP-specific T cells isolated from IFN-γ⁻/⁻ mice and from B6 mice was compared after a 2-d *in vitro* stimulation with the immunizing antigen and splenic APCs by adoptive transfer to naïve B6 mice. IRBP-specific T cells isolated from immunized IFN-γ⁻/⁻ mice had significantly stronger EAU-inducing activity compared to those from immunized B6 mice (Figures 7B, C). These results suggest that augmented Th17 responses in IFN-γ-deficient mice are associated with multiple aberrant immune cell functions or that the increased number of Th17 cells in IFN-γ⁻/⁻ exceeded the regulatory capacity of Tregs.

DISCUSSION

IFN-γ production is a hallmark of T helper (Th)1 responses (1, 2) and exerts diverse effects on immune responsiveness. It strengthens innate immunity *via* induction of antimicrobial factors and degradative pathways in other immune cells, such as macrophages; it also augments the antigen-processing and antigen-presenting ability of APCs, stimulates antibody production by B cells, induces the expression of cytokines and chemokines required for the recruitment of myeloid cells to the site of inflammation, and increases the expression of TLRs, NOS, and phagocyte oxidase by macrophages (38). Previous studies demonstrated that the effect of IFN-γ on immune responses could be either pro- (19, 39–42) or anti-inflammatory (23, 24, 43, 44). Elucidation of the bidirectional functions of

IFN-γ should increase our understanding of the regulated Th17 responses and their IFN-γ mediated immunomodulation.

Early studies of IFN-γ revealed it to be a key pathogenic molecule in human multiple sclerosis (MS) and in animal models, i.e., experimental autoimmune encephalitis (EAE) (39, 40). Clinical studies showed that MS patients treated with IFN-γ exhibited exacerbated symptoms (42), whereas MS patients treated with antibodies against IFN-γ exhibited reduced clinical symptoms (19). However, a protective effect of IFN-γ has also been demonstrated. Mice deficient in the IFN-γ gene showed an increased incidence of EAE, with earlier disease onset and more severe symptoms (45, 46). Injection of neutralizing antibodies to IFN-γ exacerbated both actively and passively induced EAE (43, 44). Injection of IFN-γ to EAE-prone mice reduced the severity of disease symptoms and mortality (43, 47). A similar protective effect of IFN-γ was also found in other autoimmune diseases, including collagen-induced arthritis, EAU, autoimmune nephritis, and myocarditis (43, 45, 48–52), underscoring the complex role of IFN-γ in disease pathogenesis. Disease stage-specific effects of IFN-γ were also observed. For example, administration of IFN-γ to EAE mice during the inductive period led to disease exacerbation, while a similar treatment during the effector phase was protective (18–22, 53).

Although it has been well established that both Th1 and Th17 autoreactive T cells are pathogenic in various autoimmune diseases (24, 36, 54, 55), including autoimmune uveitis (56), it remained largely undetermined whether therapeutic treatments directed at Th1 responses would also be effective in treating Th17 mediated disease. Our current results demonstrated that IFN-γ is also an effective molecule modulating Th17 responses, although its effect is bidirectional.

The aims of our study were to determine the regulatory mechanisms of Th17 responses in autoimmune diseases. Based on our previous report that the protective effect of adenosine-based treatments is dependent on "timing" — ADA was protective when administered during the active phase of EAU but ineffective if administered prior to intraocular inflammation (27). Subsequently, we found that A2AR antagonist SCH58261 (SCH) effectively modulates aberrant Th17 responses in induced EAU. Likewise, timing of the treatment is important (28). We therefore questioned whether such a timing effect applied to other immunoregulatory events. Our results showed that the therapeutic effects differed greatly for Th1 and Th17 responses. Anti-IFN-γ treatment can effectively modulate Th17 responses, albeit in a biphasic fashion. In early phases of EAU, the disease is inhibited by neutralizing IFN-γ activity, whereas during active and ongoing phases of EAU, IFN-γ inhibited disease development by inhibiting Th17 effector T cell responses. Our studies raised an important issue is treating pathogenic T cell responses, particularly the Th17 responses, by showing that an immunological manipulation will be more effective if a "timing" factor has been taken into consideration.

Comparing various immune responses of IFN-γ$^{−/−}$ and B6 mice showed that γδ T cells are overly active in IFN-γ$^{−/−}$ mice, and that IRBP-specific T cells isolated from immunized IFN-γ$^{−/−}$ mice have stronger EAU-inducing activity. Moreover, splenic APCs separated from IFN-γ$^{−/−}$ mice have stronger T cell stimulating activity, indicating that augmented

Th17 responses seen in IFN-$\gamma^{-/-}$ mice are the result of altered immune modulators, leading to delayed onset of disease with later exacerbation. The enhancement or inhibition of EAU correlates with $\gamma\delta$ T cell activation. In previous studies we showed that a "timing effect" is a hallmark of adenosine-based immunotherapy. In the current study we showed that the immunomodulatory effect of IFN-γ also involves both enhancement and inhibition of the Th17 response in a "timing effect". Continued efforts to elucidate the mechanisms underlying the influence of IFN-γ on immune responsiveness eventually should enable us to achieve desired effects, and thus bring us closer to the therapeutic goal of IFN-γ-based treatment of diseases.

FUNDING

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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HIGHLIGHTS

- IFN-γ has a bidirectional effect on Th17 responses

- Anti-IFN-γ antibody inhibits early inflammatory responses but enhances later responses

- Effect of IFN-γ on γδ T cells is closely associated with an altered Th17 response
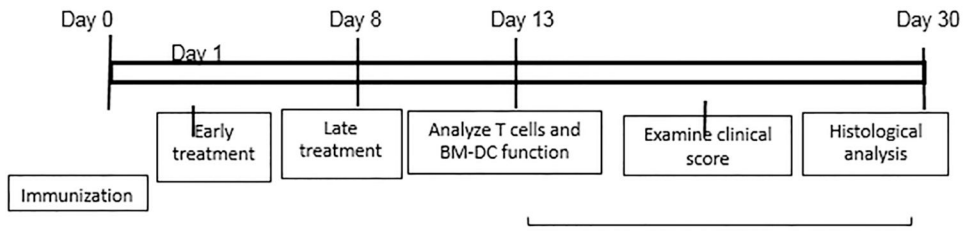
**SCHEME 1 |.**
Schematic experimental procedure.
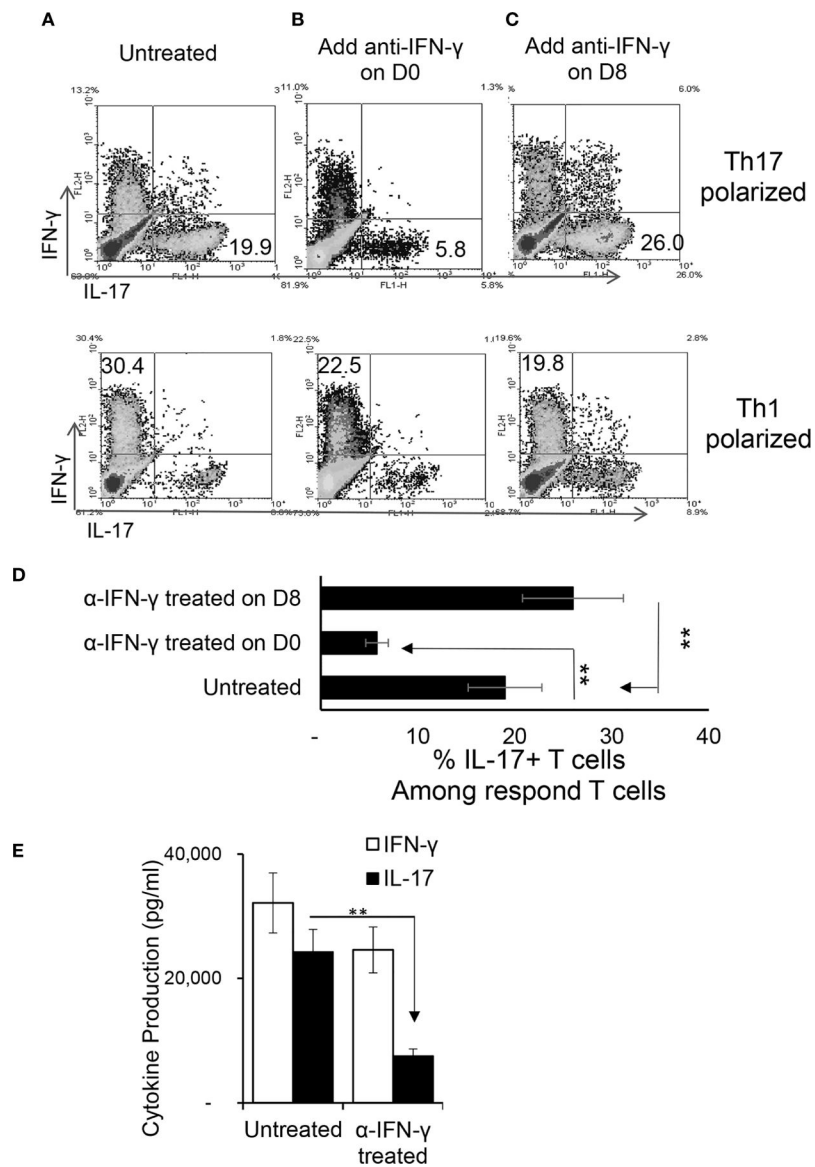
FIGURE 1 |.

Effect of anti-IFN-γ on *in vitro* Th1 and Th17 responses. Purified CD3$^+$ T cells were stimulated with the immunizing antigen and irradiated splenic APCs, under Th1- (culture medium containing IL-12) or Th17 (culture medium containing IL-23) polarized condition and in the absence or presence of anti-IFN-γ antibody (5 μg/ml). The abundance of IFN-γ$^+$ and IL-17$^+$ T cells among the responder T cells was estimated after intracellular staining with fluorescence labeled anti-IFN-γ or anti–IL-17 antibodies, 5 d after *in vitro* stimulation. Compare to untreated **(A)**, anti-IFN-γ inhibits T cell activation when added on d 0 **(B)** but enhances T cell activation if added on d 8 **(C)**. The results in **(A–C)** are from a single experiment and pooled results of three separate studies are shown in **(D)**. **p < 0.01. The IL-17 and IFN-γ levels in the culture medium were measured by ELISA after anti-IFN-γ Ab added on d0 **(E)**. The data are pooled from three independent experiments. **p < 0.01.
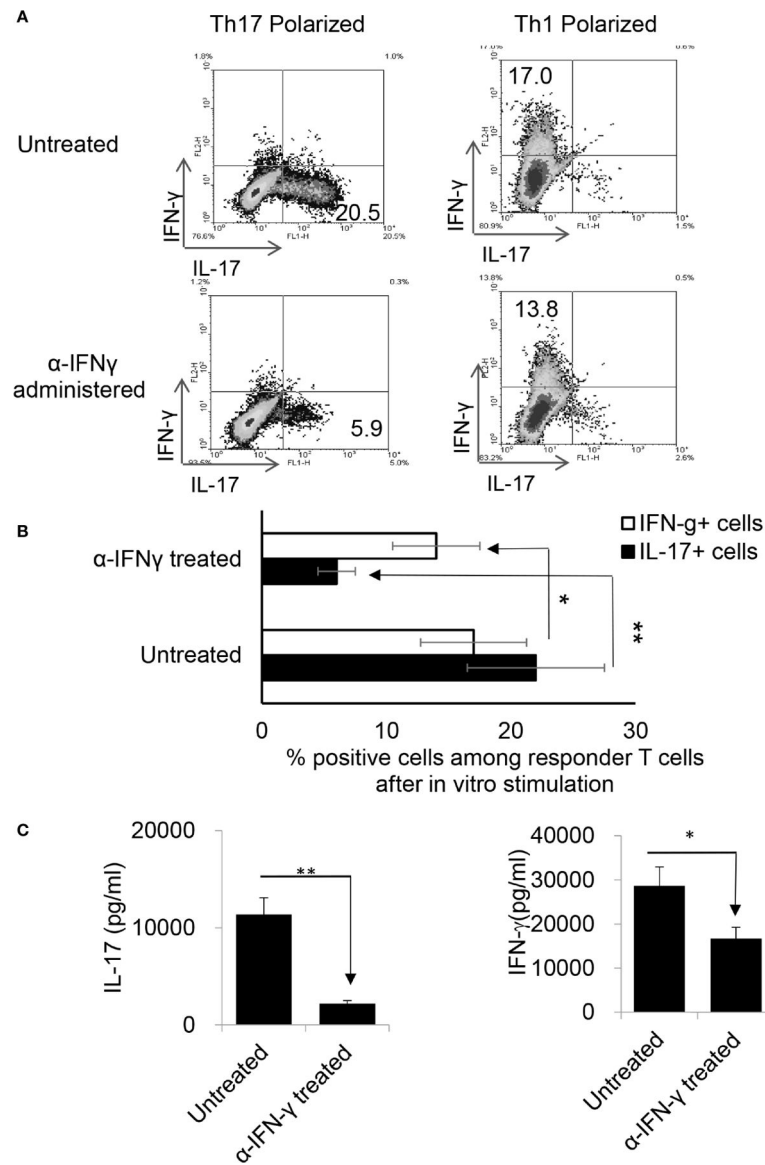
FIGURE 2 |.

Anti-IFN-γ inhibited Th17 responses *in vivo.* Anti-IFN-γ on d0 (the day of immunization) preferentially inhibited Th17 cells. Two groups of B6 mice (n = 6) were immunized with IRBP$_{1-20}$/CFA; one was injected with anti-IFN-γ (100cμg/mouse) *via* i.p. and the group received PBS. Thirteen days post-immunization, mice were sacrificed and the number of Th1 and Th17 cells among responder T cells were assessed 5 d after *in vitro* stimulation after intracellular staining with anti-IL-17 and anti-IFN-γ antibody. The results in **(A)** are from a single experiment and pooled results of three separate studies are shown in **(B)**. Responder T cells of B6 mice administered with anti-IFN-γ produced significantly decreased levels of IL-17. The IL-17 and IFN-γ levels in the culture supernatants were measured by ELISA after stimulation of responder T cells with the immunizing antigen and splenic APCs for 2 (d) The data are pooled from three independent experiments. *P < 0.05 and **P < 0.01.
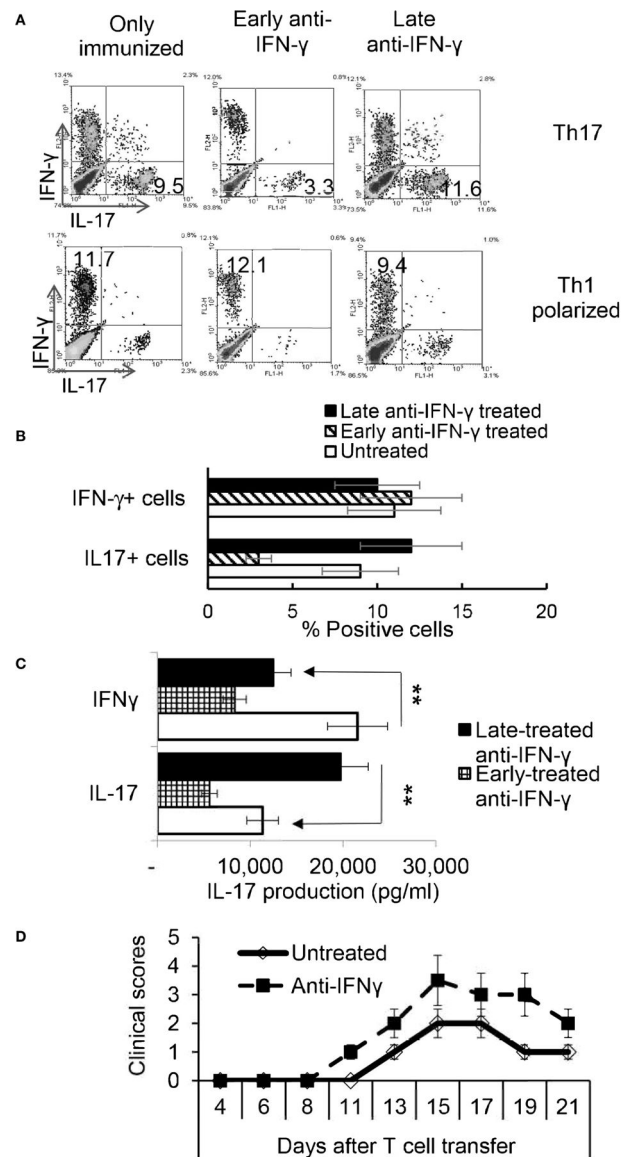
FIGURE 3 |.

Effect of anti-IFN-γ administration is "timing dependent". Th17 responses were significantly decreased in mice early treated with anti-IFN-γ but were enhanced in mice late treated with anti-IFN-γ. Groups of B6 mice (n=6) immunized with IRBP1–20/CFA received anti-IFN-γ on d0 or d8. Thirteen days post immunization CD3+ responder T cells were stimulated *in vitro* with the immunizing peptide and APCs, under Th1- or Th17-polarized conditions. 5 d after *in vitro* stimulation the number of IFN-γ+ and IL-17+ T cells was estimated after intracellular staining. The results from **(A)** are from a single experiment and pooled results of three separate studies are shown in **(B).** **P < 0.01. B) Cytokine production by Th17 cells was enhanced by late anti-IFN-γ treatment. The IL-17 and IFN-γ levels in the culture medium were measured by ELISA after stimulation of responder T cells with the immunizing antigen and splenic APCs for 2 d **(C).** **P < 0.01. IRBP-specific T cells were separated from IRBP-immunized mice with or without an
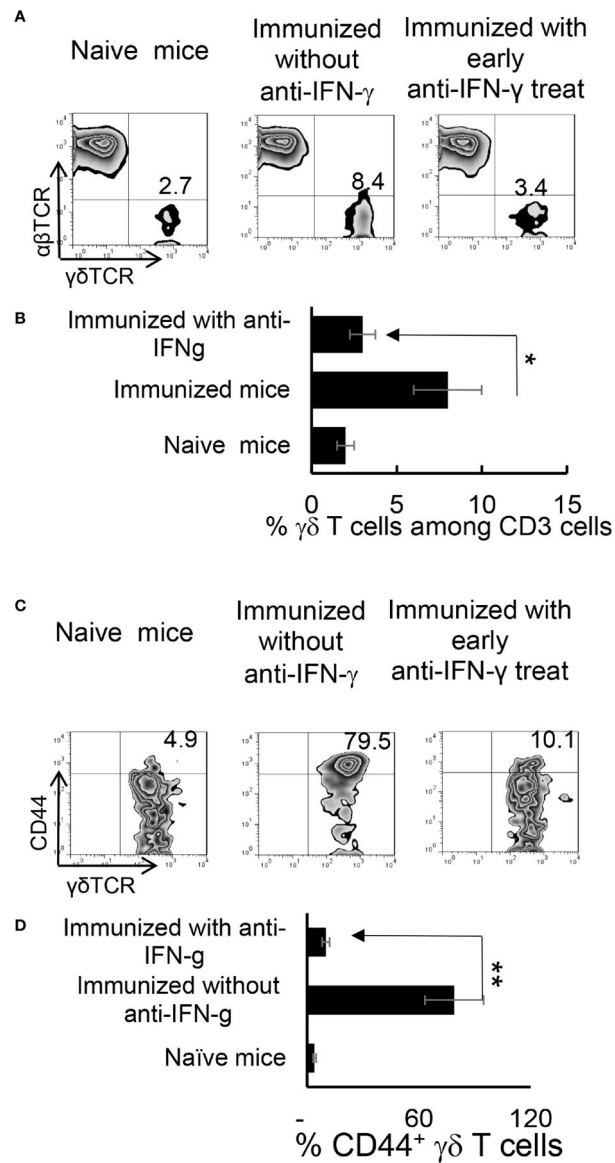
anti-IFN-γ administration on day 13 of immunization. After 2 d *in vitro* stimulation with the immunizing antigen and splenic APCs, under Th17-polarizing conditions, the activated T cells were adoptively transferred to naive B6 mice (2×10^6/mouse) *via* i.p. injection and clinical expression of EAU was scored **(D).**

FIGURE 4 |.

Altered γδ T cell responses in anti-IFN-γ treated mice. γδ T cell activation and expansion was inhibited in early anti-IFN-γ treated mice. Freshly isolated CD3$^+$ cells from naïve and immunized B6 mice, with or without anti-IFN-γ administration (day 0), were stained with PE-anti-αβTCR and FITC-anti-γδTCR antibodies before they were subjected to FACS analysis **(A).** They were also dually stained with PE-anti-mouse CD44 and FITC-anti-γδTCR antibodies **(C).** A summary of multiple assays is shown in **(B, D).** *P < 0.05, **P < 0.01.

A

B

C

Th17 responses of TCR-δ$^{-/-}$ responder
T cells in the presence of:
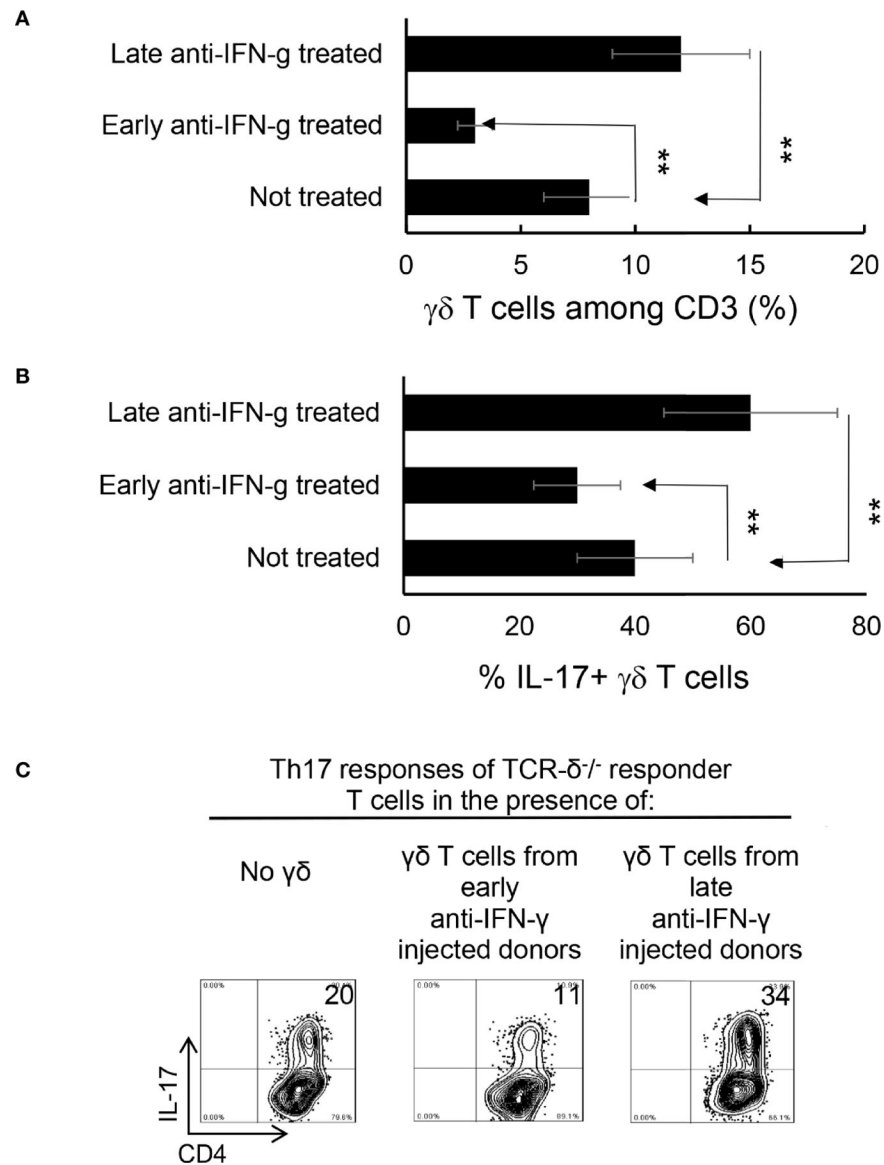
FIGURE 5 |.

γδ T cells isolated from late anti-IFN-γ treated mice have enhanced pro-Th17 activity.CD3^{+} responder T cells ($1\ 5 \times 10^{6}$/well) isolated from immunized TCR-δ$^{-/-}$ mice, with or without a prior anti-IFN-γ administration were stimulated with the immunizing antigen and irradiated splenic APCs, under Th17 polarized condition. Total γδ T cell numbers **(A)**, as well as proportional number of IL-17^{+} versus total γδ T cells **(B)** were compared. **P < 0.01. *In vitro* Th17 response of TCR-δ$^{-/-}$ responder T cells were assessed with or without an addition of γδ T cells isolated from early or late treated anti-IFN-γ mice **(C).** The number of IL-17^{+} T cells among the gated CD4^{+} responder T cells was estimated after intracellular staining with anti–IL-17 antibody, 5 d after *in vitro* stimulation.

Th17 Polarized

Th1 Polarized

A

Wt B6 mouse

IFNγ-/- mouse

Wt B6 mouse

IFNγ-/- mouse

αβTCR

IL-17

8.0

13

27

14

IFN-γ

αβTCR

14

0.5

B

IFNγ-/-

Wt B6

**

30,000 60,000 90,000
IL-17(pg/ml)

IFNγ-/-

Wt B6

30,000   60,000
IFN-γ(pg/ml)

C

Naïve

Naïve IFNγ-/-

Immunized B6

Immunized IFNγ-/-

IL-17

γδTCR

1.7

1.4

45

67

D

% IL-17+ γδ T cells

100
80
60
40
20
0

NS

**

Naïve | Immunized | Naïve | Immunized

B6 mice | IFN-γ-/- mice

E

IL-17 production (pg/ml)

50,000
40,000
30,000
20,000
10,000
-

**

**

B6 T | IFN-γ/- T | B6 T | IFN-γ/- T

B6 APCs | IFN-γ-/- APCs

FIGURE 6 |.

Augmented Th17 responses in IRBP$_{1-20}$/CFA-immunized IFN-$\gamma^{-/-}$ mice. CD3$^+$ responder T cells were purified from IRBP$_{1-20}$/CFA-immunized B6 and IFN-$\gamma^{-/-}$ mice. They were stimulated *in vitro* with the immunizing peptide and APCs, under Th17 or Th1 polarized conditions as indicated. After 5 d of *in vitro* stimulation, Th1 and Th17 responses specific for the immunizing antigen were estimated by assessing IFN-γ^+ and IL-17$^+$ T cells intracellularly stained with fluorescence -labeled anti-IFN-γ or anti–IL-17 antibodies **(A).** Immunized IFN-γ–/– mice also produced increased IL-17 compared to B6 mice. Production of IFN-γ and IL-17 by the B6 or IFN-γ–/– responder T cells after 48 h antigen stimulation *in vitro* was assessed by ELISA. Data are pooled from three independent experiments are shown **(B).** **P < 0.01. Number of γδ T cells and IL-17 secretion from immunized IFN-$\gamma^{-/-}$ mice exceeded that of immunized wt B6 mice. CD3$^+$ T cells isolated from naïve, immunized B6 and immunized IFN-$\gamma^{-/-}$ mice were stained with anti-IL-17 and

anti-γδTCR, before FACS analysis **(C).** The data from three independent experiments is shown in **(D).** **P < 0.01. NS, not significant. Contribution of antigen-specific T cells and the effect of splenic APCs in augmented Th17 responses were examined. IFN-$\gamma^{-/-}$ and B6 mice responder T cells (1.5×10^6/well) from IFN-$\gamma^{-/-}$ or B6 mice were stimulated by B6 splenic APCs (**E**, left panels) or IFN-$\gamma^{-/-}$ splenic APCs (**E**, right panels). IL-17 in culture supernatants were assessed by ELISA harvested 48 h after *in vitro* stimulation. The data are pooled from three independent experiments. **P < 0.01.
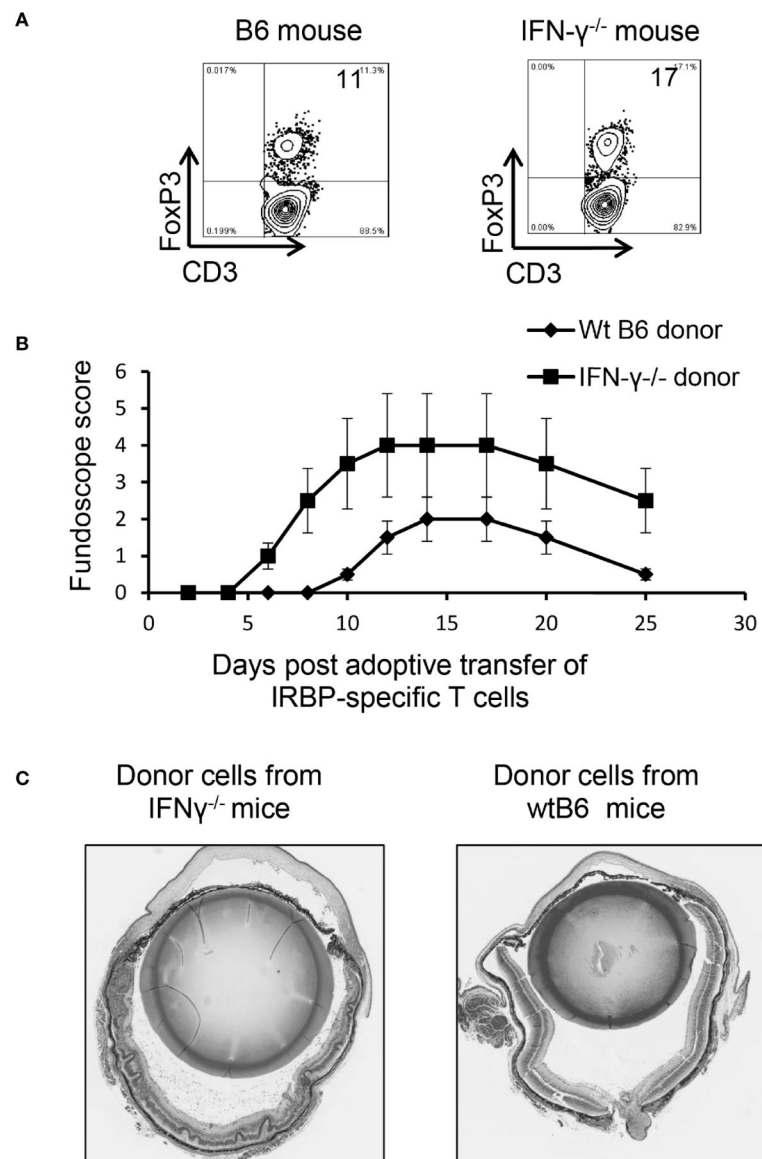
FIGURE 7 |.

Multiple aberrant immune responses in IFN-$\gamma^{-/-}$ mice. Foxp3$^+$ T cell expansion is increased in IFN-$\gamma^{-/-}$ mice. In a 24-well plate, the CD3$^+$ responder T cells (1.5×10^6/well) derived from B6 mice or IFN-$\gamma^{-/-}$ mice were cultured in medium containing a very low dose of IL-2 (1ng/ml) for 5 d, which preferentially favors Foxp3$^+$ T cell expansion (37). The percentage of Foxp3$^+$ T cells among $\alpha\beta$TCR$^+$ cells was determined by FACS analysis **(A).** Data are from one single experiment, which is representative of three independent experiments. IRBP-specific T cells isolated from immunized IFN-$\gamma^{-/-}$ mice have stronger pathogenic activity after adoptive transfer to naïve B6 recipients. IRBP-specific T cells were prepared from the responder T cells of IFN-$\gamma^{-/-}$ mice and B6 mice. After a 2-d *in vitro* stimulation with the immunizing antigen and splenic APCs. 2×10^6 cells were adoptively transferred to naïve B6 recipient mice *via* i.p. injection **(B).** Pathologic examination. H&E histologic sections from an eye in each group were obtained on day 30 post-immunization.

Severe vitritis and chorioretinal folds occur in IFN-$\gamma^{-/-}$ mice compared to minimal vitreous inflammation and a normal retina in wt-B6 mice **(C).**