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IL-21 RECEPTOR SIGNALING IS ESSENTIAL FOR OPTIMAL CD4+ T-CELL FUNCTION AND CONTROL OF *Mycobacterium tuberculosis* INFECTION IN MICE

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Abstract

In the current study we determined the role of IL-21 receptor signaling in *Mycobacterium tuberculosis* (*Mtb*) infection, using IL-21 receptor knockout (IL-21R KO) mice. Fifty percent of *Mtb* H37Rv infected IL-21R KO mice died in six months compared to no deaths in infected wild type (WT) mice. *Mtb* infected IL-21R KO mice had enhanced bacterial burden and reduced infiltration of antigen specific T-cells in lungs compared to *Mtb* infected WT mice. Antigen specific T-cells from the lungs of *Mtb* infected IL-21R KO mice had increased expression of T-cell inhibitory receptors, reduced expression of chemokine receptors, proliferated less and produced less IFN- γ, compared to antigen specific T cells from the lungs of *Mtb* infected WT mice. T cells from *Mtb* infected IL-21R KO mice were unable to induce optimal macrophage responses to *Mtb*. This may be due to a decrease in the antigen specific T cell population. We also found that IL-21R signaling is associated with reduced expression of a transcriptional factor Eomesodermin (Eomes) and enhanced functional capacity of antigen specific T-cells of *Mtb* infected mice. The sum of our findings suggests that IL-21 receptor signaling is essential for the optimal control of *Mtb* infection.

INTRODUCTION

Mycobacterium tuberculosis (*Mtb*) infects one-third of the world's population and causes almost 1.2 million deaths per year (1). Approximately 90% of infected persons have protective immunity and contain infection, but 10% of infected individuals develop primary tuberculosis soon after infection or reactivation tuberculosis many years later (1). The factors responsible for development of active tuberculosis are not known.

T cells play a crucial role in protective immunity against *Mtb*, in part through production of IFN-γ by antigen specific T cells (2). Even though a sustained T-cell response against *Mtb*

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infection is necessary, limited information is available about the expansion and maintenance of the antigen specific T cells during *Mtb* infection (3, 4). The T cell receptor and costimulatory signals initiate proliferation of naïve T cells, but for the expansion of antigen specific T cells and their effector functions specific cytokine signals are essential (5–7). Members of the common γ-chain (γc) cytokine family, including IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 are critically involved in deciding transcriptional profiles of effector T cells and the development of antigen-specific T cells (8–10). These cytokines regulate T-cell functions through transcription factors like T-bet, Eomesodermin (Eomes), Bcl-6 and Blimp-1 (11). Both T-bet and Eomes are critical to regulate the expression of IFN-γ, perforin, granzyme B and effector T cells responses (8, 12).

IL-21 belongs to the common γ-chain (γc) cytokine family, is highly expressed by Th cell lineages and signals via a heterodimeric receptor complex comprised of the specific IL-21R subunit and the common receptor γ-chain (13). IL-21 is produced by natural killer T (NKT) cells and various CD4+ T cell subsets including Th17 cells and follicular helper T (Tfh) cells during viral infections (14). IL-21 stimulates the function of multiple lymphocyte subsets, including Th17 cells, follicular helper cells, B cells, NK cells, and CD8+ T cells (13). IL-21 promotes CD8+ T cell responses against tumors (13) and is required for the clearance of chronic viral infections in animal models (15–17).

In human *Mtb* infection, NKT cells produce IL-21 at the site of disease (18) and circulating IL-21 levels are lower in active tuberculosis (TB) patients (19). In a mouse model of *Mtb* infection, memory-like NK cells contribute to vaccine-induced protective immune responses against *Mtb* infection and IL-21 mediates the development and expansion of memory-like NK cells (20). IL-21 also enhances immunogenicity of a DNA vaccine containing Ag85A (21) but is dispensable for protective Th17 recall responses (22) and is not absolutely necessary for protective immunity against TB (23). The function of IL-21 can be compensated for by other common γ-chain (γc) cytokine family members and it is important to determine the role of IL-21 receptor signaling in *Mtb* infection. Recent studies found that IL-21 produced by CD4+ T cells promotes CD8+ T cell expansion and effector functions and IL-21 is essential for the optimal control of *Mtb* infection in mice (24).

In the current study, using IL-21 receptor knockout (IL-21R KO) mice, we further determined whether IL-21 receptor signaling has any effect on antigen specific CD4+ T-cell responses against *Mtb* infection. We found that IL-21 receptor signaling is associated with optimal antigen specific CD4+ T cell effector function and essential for the optimal control of *Mtb* infection in mice.

MATERIALS AND METHODS

Animals

All animal studies were performed on specific-pathogen-free 8-week-old female C57BL/6, C57BL/6NJ (6NJ) and IL-21R knockout (IL-21R KO) mice. The Institutional Animal Care and Use Committee of the University of Texas Health Science Center at Tyler approved the studies. Animal procedures involving the care and use of mice were in accordance with the guidelines of NIH / OLAW (Office of Laboratory Animal Welfare).

Aerosol infection of mice with Mtb H37Rv

Mice were infected with H37Rv using an aerosol exposure chamber, as described previously (25).

Lung cell preparation

Lungs were harvested from WT and IL-21R KO mice, and single cell suspensions were prepared at the indicated time points after *Mtb* challenge. The total number of viable cells in the lungs was determined by trypan blue exclusion method. For flow cytometry experiments, we gated on total lung CD45+ cells (leukocytes) and measured various cell populations like CD45+CD4+ or CD45+CD8+ cells.

Abs and other reagents

For flow cytometry, we used FITC anti-T-bet, PE anti-CD8, PE/Cy7 anti-Eomes, APC anti-CD4, APC anti-IFN-γ, APC anti-CD160, APC anti-2B4, APC anti-PD1, APC anti-CXCR5, APC anti-CXCR3, APC anti-CCR7, APC anti-IL12Rβ2, FITC anti-CD3, PE anti-CD11b, APC-anti-MHC II, FITC-anti-CD80, FITC-anti-CD86 (all from BioLegend). We used γ-irradiated *Mtb* H37Rv for *in vitro* stimulation assays (BEI Resources).

Detection of ESAT-$6_{4–17}$ specific T cells

PE-labeled MHC class II tetramers (I-Ab) containing the stimulatory residues 4 to 17 (QQWNFAGIEAAASA) of the early secreted antigenic target 6 kD (ESAT-6) of *Mtb* were obtained from the National Institutes of Health Tetramer Core Facility (Emory University Vaccine Center). For Ag-specific responses and intracellular cytokine staining, cells were incubated with ESAT-$6_{4–17aa}$ MHC class II multimer (1:50 dilution) at 37°C 5% CO_2 for 60 min in media, washed three times and cultured with ESAT-$6_{4–17aa}$ peptide in the presence of monensin. After 5h, cells were surface stained for CD3, CD4, and CD8 at room temperature for 15 min in PBS 0.5% BSA 20% mouse serum, washed, and then fixed with 2% paraformaldehyde (Sigma-Aldrich) for 1 h. After fixation, cells were permeabilized, then washed with PBS 0.5% BSA 0.2% saponin. Cells were stained for intracellular IFN-γ (clone XMG-6.1) by incubating with anti-IFN-γ Abs in staining medium for 15 min at room temperature. Cells were washed and resuspended in 1% paraformaldehyde. Data were collected on a FACS CALIBUR and analyzed using FlowJo software 8.6.3 (Tree Star) (26).

Flow cytometry

Cells were surface stained as described previously (26) before acquisition using a FACS Calibur (BD Biosciences). In some experiments, intracellular staining for T-bet, Eomes, IFN-γ and isotype control was performed, according to the manufacturer's instructions. For T-bet, Eomes and IFN-γ analysis, we gated on CD4+ or CD8+ lymphocytes, and determined the percentages or the number of T cells expressing specific marker.

Real-time PCR for quantification of cytokine mRNA

Total RNA was extracted from macrophages or lung cells as described previously (26). RNA was reverse transcribed, and real-time PCR was performed using the Quantitect SYBR

Green PCR kit (Qiagen). All samples were normalized to the amount of β-actin/GAPDH transcript present in each sample.

Primers used in the study are mentioned in the supplemental Table I.

Carboxyfluorescein succinimidyl ester (CFSE) labeling

Mediastinal lymph node (MLN) cells were resuspended (5×10^6/ml) in 0.1% BSA in PBS. CFSE was added to a final concentration of 2 mM, and cells were incubated at 37°C for 15 min. Cells were washed once with 10% FCS in PBS, twice with 0.1% BSA in PBS, and resuspended in RPMI for subsequent stimulation.

In vitro stimulation

CFSE labeled cells were cultured for 3 d, with or without phorbol 12-myristate 13-acetate (PMA) + Ionomycin (500 ng/ml each). On day three, cell proliferation, inhibitory and chemokine receptor expression was determined by flow cytometry (BD Biosciences). Culture supernatants were collected to determine cytokine levels by ELISA (eBiosciences).

siRNA

Freshly isolated mouse MLN cells from *Mtb* infected IL-21R Knockout mice were transfected with siRNA for Eomes or control siRNA as described previously (26). After siRNA transfection, cells were washed and stimulated with γ-irradiated *Mtb* H37Rv or kept in medium alone as a control. On day three, cell proliferation of CFSE labeled cells, cytokine and transcription factor expression was determined by flow cytometry.

T-Cell isolation and purification

Effector T cells were isolated from the pooled spleen and MLN cells of WT C57BL/6NJ or IL-21R Knockout mice 3 months after infection with *Mtb* H37Rv. Briefly, lymphocytes were prepared from pooled cells by Ficoll density gradient. Pan T-cells were isolated from total lymphocytes by negative selection using LD columns (Miltenyi Biotech). The purity of CD3+ T-cells was > 95% as determined by flow cytometry.

Isolation of mouse peritoneal macrophages (PEMs) and infection with Mtb H37Rv

Mouse PEMs were isolated and infected with *Mtb* H37Rv at an MOI of 1:2.5 (one PEM and 2.5 *Mtb*) as described previously (26). Cells were washed to remove extracellular bacilli, and re-suspended in RPMI 1640 media for the subsequent culture with T lymphocytes.

Culturing of macrophages and T cells

Mtb infected PEMs were cultured with autologous effector T cells (1:5 ratio, one PEM and 5 T-cells) from WT or IL-21R KO mice for 5 days. Supernatants were collected to determine cytokine levels by ELISA. Bacterial load in the macrophages was enumerated by plating on 7H10 agar plates. Expression of MHC II, CD80 and CD86 was determined by flow cytometry.

Statistical analysis

Results are shown as the mean ± SE. Comparisons between groups were performed by a paired or unpaired t test, as appropriate. Mouse survival was compared using Kaplan- Meier Log-rank test.

RESULTS

IL-21R KO mice are susceptible to Mtb infection

To determine the role of IL-21R in *Mtb* infection, we infected wild type (WT) and IL-21R KO mice with *Mtb* H37Rv as mentioned in the methods section. As shown in Fig. 1, fifty percent of IL-21R KO mice died between 3 and 6 months ($p<0.05$) post *Mtb* infection compared to no deaths in the *Mtb* infected WT mice. Due to animal welfare reasons, we have not followed these mice for a longer period of time and this was a time to death study.

IL-21R is essential for optimal control of Mtb growth in mice

IL-21 expression was significantly upregulated in the lungs of *Mtb* infected WT mice compared to uninfected control mice lungs (Supplemental Fig. 1A). To determine the role of IL-21signaling in the control of *Mtb* growth, IL-21R KO and WT mice were infected with *Mtb* H37Rv by aerosol as mentioned in methods section. One, two and three months after infection, the bacterial burden in lungs, spleen, and liver was determined. There was a significant increase in the number of bacteria in the lungs of IL-21R KO mice after one month ($3.4 \pm 0.7 \times 10^6$ vs. $0.9 \pm 0.1 \times 10^6$ CFU, $p = 0.01$, Fig. 2A) and two months compared to WT mice ($3.2 \pm 0.1 \times 10^6$ vs. $0.8 \pm 0.06 \times 10^6$ CFU, $p = 0.01$, Fig. 2A). Ninety days after infection the bacterial burden in the lungs of IL-21R KO mice increased and it was one log higher compared to WT mice ($7.3 \pm 1.0 \times 10^6$ vs. $0.72 \pm 0.08 \times 10^6$ CFU, $p=0.0003$, Fig. 2A). In the spleen (Fig. 2B) and liver (Fig. 2C) a similar increase was seen in IL-21R KO mice compared to WT mice.

IL-21R is essential for optimal antigen specific T-cell responses during Mtb infection

We asked whether lack of IL-21R signaling had any effect on T-cell responses during *Mtb* infection. WT and IL-21R KO mice were infected with *Mtb* and after one and three months the number of CD4+ and CD8+ cells in the lungs of control and *Mtb* infected mice was determined. The gating strategy is shown in Supplemental Fig. 1B. Uninfected WT and IL-21R KO mice have similar numbers of CD45+ leucocytes (Supplemental Fig. 1C). As shown in Fig. 3A, one month after infection, there was a significant decrease in total CD4+ cells ($6.7 \pm 0.4 \times 10^5$ vs. $8.8 \pm 0.2 \times 10^5$ per 10^7 lung cells, $p=0.005$) and CD8+ cells ($3.2 \pm 0.6 \times 10^4$ vs. $7.4 \pm 0.7 \times 10^4$ per 10^7 lung cells, $p=0.007$) in the lungs of IL-21R KO mice compared to WT mice lungs. Three months after infection the number of CD4+ cells in the IL-21R KO mice lungs was also reduced compared to WT mice ($0.9 \pm 0.01 \times 10^5$ vs. $1.4 \pm 0.03 \times 10^5$ per 10^7 lung cells, $p=0.0001$, Fig. 3A). A Similar decrease in the number of total CD8+ cells was seen in *Mtb* infected IL-21R KO mice lungs compared to *Mtb* infected WT mice lungs ($4.2 \pm 0.1 \times 10^4$ vs. $5.6 \pm 0.1 \times 10^4$ per 10^7 lung cells, $p=0.0005$, Fig. 3A).

To determine whether the reduced number of T cells in IL-21R KO mice also affects antigen specific responses three months after *Mtb* infection, we measured the frequency of

$(ESAT6)_{4–17}$/I-Ab MHC class II tetramer specific CD4+ cells. We found five fold less tetramer specific CD4+T-cells in the lungs of *Mtb* infected IL-21R KO mice compared to *Mtb* infected WT mice lungs (1150 ± 142.6 vs. 6400 ± 397.8, p<0.001, Fig. 3B). We also found 6.5 fold less CD4+IFN-γ+tetramer+ cells (173.0 ± 18.48 vs. 1200 ± 109.1, p<0.001, Fig. 3C,) in *Mtb* infected IL-21R KO mice lungs compared to *Mtb* infected WT mice lungs. Further we found increased frequency of CD4+tetramer+ cells that express inhibitory receptors CD160 (10.2 ± 1.4 % vs. 4.3 ± 0.5 %, p=0.006, Fig. 3D), 2B4 (69.7 ± 2.5 % vs. 47.0 ± 3.2 %, p=0.0001, Fig. 3D) and PD1 (37.0 ± 3.4 % vs. 13.4 ± 0.8 %, p=0.0006, Fig. 3D) and decreased frequency of CD4+tetramer+ cells that express chemokine receptors CXCR3 (33.4 ± 2.3 % vs. 80.2 ± 1.7 %, p=0.0001, Fig. 3E), CXCR5 (5.9 ± 1.3 % vs. 19.2 ± 1.5 %, p=0.0007, Fig. 3E) and CCR7 (9.2 ± 1.3 % vs. 20.7 ± 0.9 % p=0.0004, Fig. 3E) cells in the lungs of *Mtb* infected IL-21R KO mice compared to *Mtb* infected WT mice lungs. We found similar pattern of defective proliferation and enhanced expression of inhibitory receptors by PMA and ionomycin stimulated lung cells from IL-21R KO mice compared to uninfected WT mice (Supplemental Fig. 1D and E).

As shown in Supplemental Fig. 2A, the total number of IFN-γ+CD4+ and IFN-γ+CD8+ cells were significantly reduced in the lungs of IL-21R KO mice compared to WT mice three months after *Mtb* infection. The expression of IFN-γ, IL-17, IL-12, IL-1β, IL-27 and cytotoxic molecules like perforin and granzyme B were also reduced in the lungs of the above *Mtb* infected IL-21R KO mice compared to *Mtb* infected WT mice as determined by real time PCR (Supplemental Fig. 2B & 2C). In contrast, expression of TGF-β, IL-10, Type 1 interferons, TNF-α and transcription factor Eomes were significantly upregulated in the lungs of IL-21 R KO mice compared to WT mice (Supplemental Fig. 2C). We also found increased numbers (absolute) of CD4+ and CD8+ cells that express T cell inhibitory receptors like CD160, PD1, 2B4 and decreased numbers (absolute) of CXCR5, CXCR3, CCR7 and IL12Rβ2 cells in the lungs of IL-21R KO mice compared to WT mice three months after *Mtb* infection (Supplemental Fig. 2D & 2E).

Increased Eomes expression by T-cells of Mtb infected IL-21R KO mice

It is known that the transcription factor T-bet regulates IFN-γ production by T-cells in intracellular infections including *Mtb* (11, 27). Recent studies found that another transcription factor, Eomes, regulates IFN-γ production by T-cells in intracellular infections (28), but it has both positive and negative regulatory effects depending on the infection (29, 30). The role of Eomes in *Mtb* infection is not known. We next asked whether defective IL-21R signaling in *Mtb* infected mice has any effect on the expression of T-bet and Eomes. IL-21R KO and WT mice were infected with *Mtb* and after three months the number of CD4+ and CD8+ cells that express T-bet and Eomes in the lungs of control and *Mtb* infected mice was determined by flow cytometry. There is no significant difference in the number of CD4+ and CD8+ cells (Supplemental Fig.3A and 3B) that express T-bet between *Mtb* infected mice, but the number of Eomes+CD4+ cells and Eomes+CD8+ cells was significantly higher in *Mtb* infected IL-21R KO mice compared to WT *Mtb* infected mice (Supplemental Fig. 3A). Both Eomes and T-bet can influence memory T cell development (31). Therefore, we determined their expression in antigen specific T cells during *Mtb* infection. Three months after *Mtb* infection, we found a reduced frequency of T-bet

+ESAT6$_{4–17}$/I-Ab MHC class II tetramer positive CD4+ T cells (66.6 ± 2.4 % vs. 73.9 ± 1.8 %, p=0.04, Fig.4A) and increased frequency of Eomes+ ESAT6$_{4–17}$/I-Ab MHC class II tetramer positive CD4+ T cells (29.0 ± 2.2 % vs. 4.4 ± 0.7 %, p=0.0001, Fig. 4B) in the lungs of IL-21R KO mice compared to WT mice.

Eomes inhibits T-cell effector functions in Mtb infected mice

We next determined the role of Eomes on T-cell effector functions in *Mtb* infected mice. IL-21R knockout mice were infected with *Mtb* H37Rv by aerosol as mentioned in the methods section. Three months after infection, mediastinal lymph node cells were isolated and labeled with CFSE and cultured with γ-irradiated *Mtb* H37Rv (10 μg/ml) in the presence or absence of siRNA to Eomes or scrambled siRNA. After 72 hours, supernatants were collected and IFN-γ, TNF-α and IL-10 levels were determined by ELISA. Flow cytometry was performed on cells to determine the proliferation and expression of T-bet and Eomes. As shown in Fig. 5A, Eomes siRNA inhibited Eomes expression as determined by flow cytometry. Eomes siRNA enhanced γ-irradiated *Mtb* H37Rv induced CD4+ cell proliferation from 22 % to 36.5 % in CD4+ cells (p=0.009, Fig. 5B) and CD8+ cell proliferation from 4.5 % to 9.65 % (p=0.007, Fig. 5B). Eomes siRNA also significantly enhanced γ-irradiated *Mtb* H37Rv induced expression of CXCR3 and IL-12Rβ2 receptors on CD4+ and CD8+ T cells (Fig. 5C) and IFN-γ production (Fig. 5D). In contrast, Eomes siRNA significantly inhibited γ-irradiated *Mtb* H37Rv induced IL-10 production (Fig. 5D) and had no effect on γ-irradiated *Mtb* H37Rv induced TNF-α production (Fig. 5D). Eomes siRNA had no significant effect on the proliferation of PMA stimulated WT mice lung CD4+ T cells and IFN-γ and TNF-α production by lung cells. In contrast, Eomes siRNA significantly but marginally enhanced proliferation of CD8+ T cells and significantly inhibited IL-10 production by PMA stimulated WT mice lung cells (Supplemental Fig. 3D and 3E).

Effect of T cells from Mtb infected IL-21R KO mice on macrophage responses to Mtb

It is known that T cells enhance macrophage responses to inhibit *Mtb* growth. We determined the effect of T cells from *Mtb* infected IL-21R KO mice on macrophage responses to *Mtb*. Peritoneal macrophages (PEMs) were isolated from naïve mice and infected with H37Rv at an MOI of 1:2.5 (one PEM and 2.5 *Mtb*). After 2h, PEMs were washed to remove extracellular bacteria and then co-cultured with the pooled spleen and MLN T cells (1:5, one macrophage and five T-cells) isolated (three months after *Mtb* infection) from either WT or IL-21R KO *Mtb* infected mice. After 5 days, culture supernatants were collected to determine cytokine levels by ELISA, flow cytometry was performed to determine the expression of macrophage cell surface receptors and intracellular bacterial growth was determined as mentioned in the methods section. As shown in Fig. 6, *Mtb* infected 30.8 % of PEMs were MHCII+ (Fig. 6A) and the mean fluorescent intensity (MFI) of CD86 was 278.4 ± 17.3 (Fig. 6B) when the PEMs were cultured with T cells obtained from *Mtb* infected IL-21R KO mice, and the MHCII expression was 62.4% (Fig. 6A) and MFI of CD86 was 389.6 ± 27.3 (Fig. 6A) when *Mtb* infected PEMs were cultured with T cells from *Mtb* infected WT mice. *Mtb* infected PEMs produced 126.1 ± 9.9 pg/ml of IFN-α upon addition of T-cells from *Mtb* infected WT mice and when T-cells are from *Mtb* infected IL-21R KO mice this was 198.8 ± 12.8 pg/ml (p=0.01, Fig. 6C). A similar increase

in IL-10 levels was observed (2130 ± 132.5 pg/ml vs. 3032 ± 236.5 pg/ml, p=0.01, Fig. 6C). However, in contrast to IFN-α and IL-10 production, *Mtb* infected PEMs produced less IL-1β (59.9 ± 7.8 pg/ml vs. 37.3 ± 4.2 pg/ml, p=0.04, Fig. 6C) and IL-12p70 (1944 ± 157.4 pg/ml vs. 1327 ± 76.8 pg/ml, p=0.02, Fig. 6C) upon the addition of T-cells from *Mtb* infected IL-21R KO mice, but not with the addition of T-cells from *Mtb* infected WT mice. IL-21R KO mice T cells had no effect on TNF-α production (941.0 ± 31.2 pg/ml vs. 951.7 ± 107.2, p=ns, Fig. 6C). In 3 independent experiments, 5 day post-infection, CFU in PEMs were $4.3 \pm 0.2 \times 10^6$ CFU and this was reduced to $1.5 \pm 0.17 \times 10^6$ CFU (p=0.001, Fig. 6D) upon the addition of T-cells from *Mtb* infected WT mice and to $3.2 \pm 0.29 \times 10^6$ CFU (p=0.004, Fig. 6D) upon the addition of T-cells from IL-21R KO mice. The sum of these results suggest that T cells from *Mtb* infected IL-21R KO mice are unable to induce optimal macrophage responses to *Mtb*. This may be due to a decrease in the antigen specific T cell population.

Discussion

IL-21 is a Th17 cytokine mainly produced by follicular helper CD4 T cells and NKT cells. IL-21 was shown to play a crucial role in cancer, autoimmune, inflammatory diseases and viral infections (32). Most of the studies on the effects of IL-21 and IL-21R signaling were focused on B-cell memory, differentiation and plasma cell development (33). Although IL-21 is not required for CD4+ T cell development, it is essential for the functional differentiation of CD4+ T cell subsets (34). IL-21 signaling plays an important role in maintaining the sustained functionality of CD8 T cells, allowing for the control of chronic viral infections caused by the lymphocytic choriomeningitis virus (15–17). In *Mtb* infected mice, IL-21 signaling has an early striking effect on CD8+ T-cell responses and is essential for optimal host resistance (24). In the current study, we found IL-21 signaling has autocrine action on CD4+ T-cell responses in *Mtb* infected mice. We found that in *Mtb* infected IL-21R knockout mice there is a defective T cell response and higher bacterial burden and mortality (50% of mice died in 6 months, Fig. 1).

Our findings in the current study demonstrate that IL-21R signaling is required for the optimal proliferation of effector T cells, production of IFN-γ, IL-17, and expression of the cytolytic molecules perforin and granzyme B in *Mtb* infected mice. We found that there is an association between lack of IL-21R expression and increased expression of a transcription factor Eomes in activated T cells. The sum of our findings demonstrates that IL-21R signaling is essential to maintain T-cell effector function (Fig. 3), to stimulate optimal IFN-γ production (Fig. 3), reduce bacterial burden (Fig. 2) and mortality (Fig. 1) of *Mtb* infected mice.

In human *Mtb* infection, NKT cells from plural fluids of tuberculosis patients produce IL-21 to help B cells to secrete IgG and IgA (18), pulmonary tuberculosis patients have less circulating IL-21 levels (19) and IL-21 regulates *Mtb* specific IFN-γ+CD4+ T cell responses (35) suggesting a role for IL-21 in human *Mtb* infection. In a mouse model of *Mtb* infection, IL-21 enhances immunogenicity of a DNA vaccine containing Ag85A (21). In *Mtb* infected mice, IL-21 is required for the optimal generation of lymphoid structures in lungs and is not absolutely necessary for protective immunity against TB (22, 23). These studies were

performed using IL-21 knockout mice and it is possible that lack of IL-21 (soluble factor) may be compensated by other soluble mediators that can bind to IL-21 receptor to enhance signaling pathways. A recent study demonstrated that in *Mtb* infected mice, IL-21 signaling plays a crucial role in T cell responses during *Mtb* infection. In *Mtb* infected mice, IL-21 primes CD8+ T cells early and promotes the accumulation of T cells in the lung and enhances T cell cytokine production in *Mtb* infected mice (24). Our current study further demonstrates that IL-21 receptor signaling is involved in antigen specific CD4+ T-cell responses, is essential to maintain CD4+ T cell effector function and is important for the optimal control of *Mtb* growth and mortality of the *Mtb* infected mice.

We found that *Mtb* infected IL-21R knockout mice T cells (CD4+ and CD8+) express higher levels of inhibitory PD-1, CD160 and 2B4 receptors compared to *Mtb* infected WT mice. These findings are similar to previous findings that defective IL-21R signaling leads to increased expression of the inhibitory receptors TIM-3 and PD-1 (24, 36). The pattern of inhibitory-receptor co-expression and the number of receptors simultaneously expressed by the same T cell can substantially affect the severity of dysfunction (37). Our study for the first time demonstrates that IL-21R expression is an essential component for the optimal CD4+ T cell effector function during *Mtb* infection.

It is not known how IL-21R signaling maintains CD4+ T cell effector function. Recent studies found IL-21 inhibits the expansion of immunosuppressive Foxp3+ regulatory CD4 T cells (38), which are known to inhibit effector T cell function in viral infection (39). IL-21R KO and WT mice infected with *Mtb* have a similar number of Foxp3+ regulatory CD4 T cells in their lungs and spleens (Supplemental Fig. 3C) suggesting that an increased number of Foxp3+ cells may not be responsible for altered T cell function in *Mtb* infected IL-2R KO mice.

We found that the lack of IL-21R signaling enhances the expression of a transcriptional factor Eomes. T-bet is a known transcription factor involved in T cell mediated immune responses against *Mtb* (26) and infections with other intracellular pathogens (40). T-bet and Eomes are related transcription factors that show some expressional overlap, but their functional roles are not entirely reciprocal. T-bet and Eomes bind to the promoters of IFN-γ, perforin, and granzyme B and induce gene expression which is essential for optimal cytotoxic lymphocyte differentiation (41). Recently it was reported that the balance between T-bet and Eomes is vital for the maintenance of effector T cells in both HIV and CMV infection models (42). T-bet and Eomes can physically interact to form a complex in helper T cells. The properties of the interaction between T-bet and Eomes could create a scenario in which T-bet is able to control Eomes activity when the balance of the two proteins favors T-bet expression. We found that T cells from *Mtb* infected IL-21R knockout mice express significantly higher levels of Eomes compared to *Mtb* infected WT mice T cells. Eomes siRNA significantly enhanced the expression of CXCR3 and IL-12Rβ and enhanced CD4+ T cell effector functions of *Mtb* infected IL-21 knockout mice. Our study for the first time demonstrates that IL-21 and IL-21R signaling regulates the T-bet and Eomes ratio to favor optimal Th1 cytokine responses during *Mtb* infection. These studies may be applicable to other transcription factors that regulate Th1 cytokine responses to intracellular pathogen infections.

In conclusion, our studies demonstrate that the lack of IL-21 receptor signaling leads to enhanced expression of inhibitory receptors by CD4+ T cells, defective antigen specific T-cell responses and unrestricted growth of *Mtb* and mortality of the infected mice during the first six months of *Mtb* infection. Further delineation of the mechanisms through which IL-21R signaling regulates optimal Th1 responses during *Mtb* infection will facilitate development of therapies and also vaccines against *Mtb* and other intracellular pathogens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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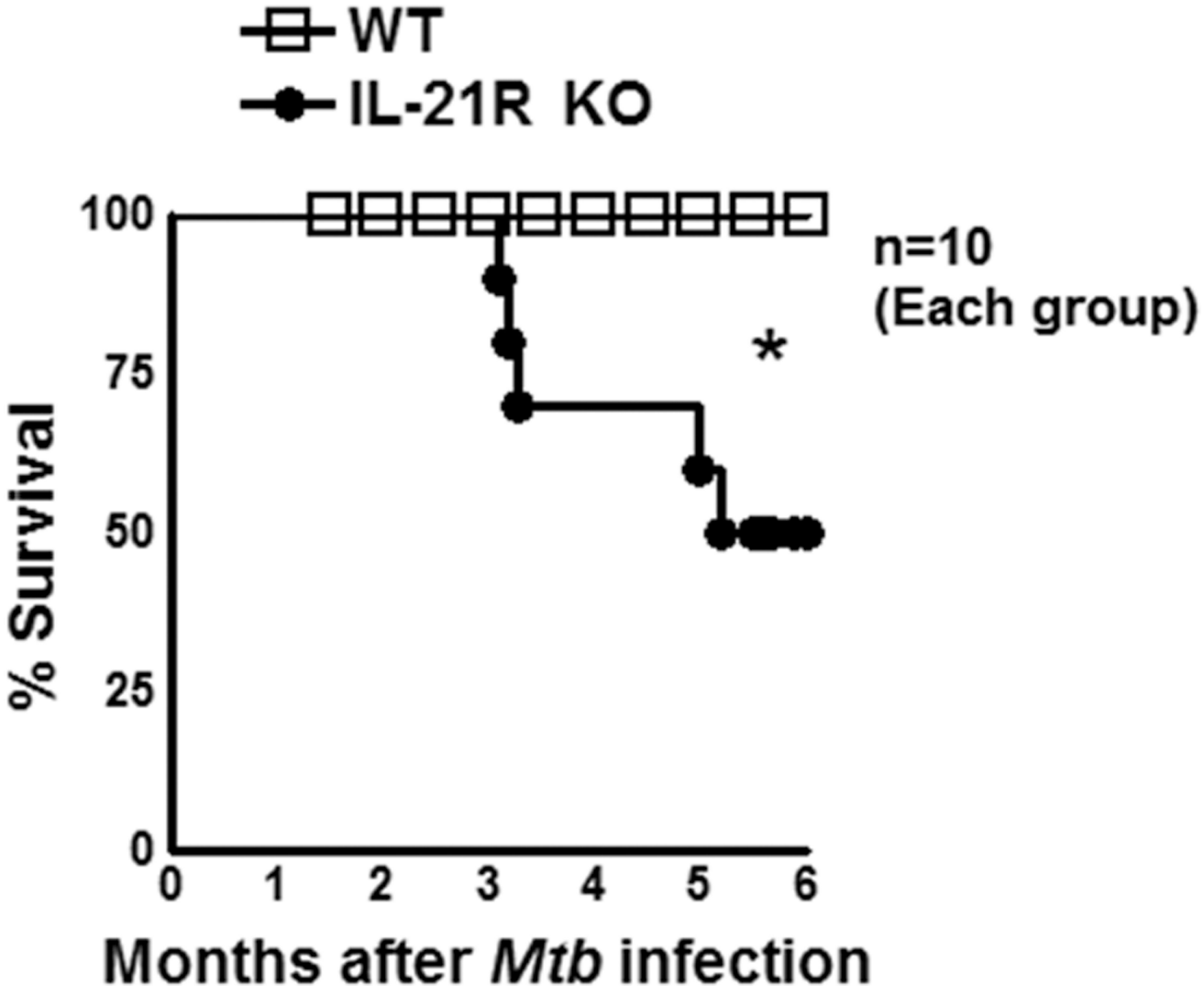

Fig. 1. IL-21R KO mice are susceptible to *Mtb* infection

Wild type (WT) and IL-21R knockout (KO) mice (both C57BL/6NJ background) were infected with 50–100 CFU of *Mtb* H37Rv by aerosol. Survival was determined. Data are representative of two independent experiments. Ten mice per group were used in each independent experiment. In the other independent experiment, 50% of *Mtb* infected IL-21R KO mice died in the first six months. Survival curves were compared using log rank test ($P < 0.05$). Mean values, p-values and SEs are shown. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

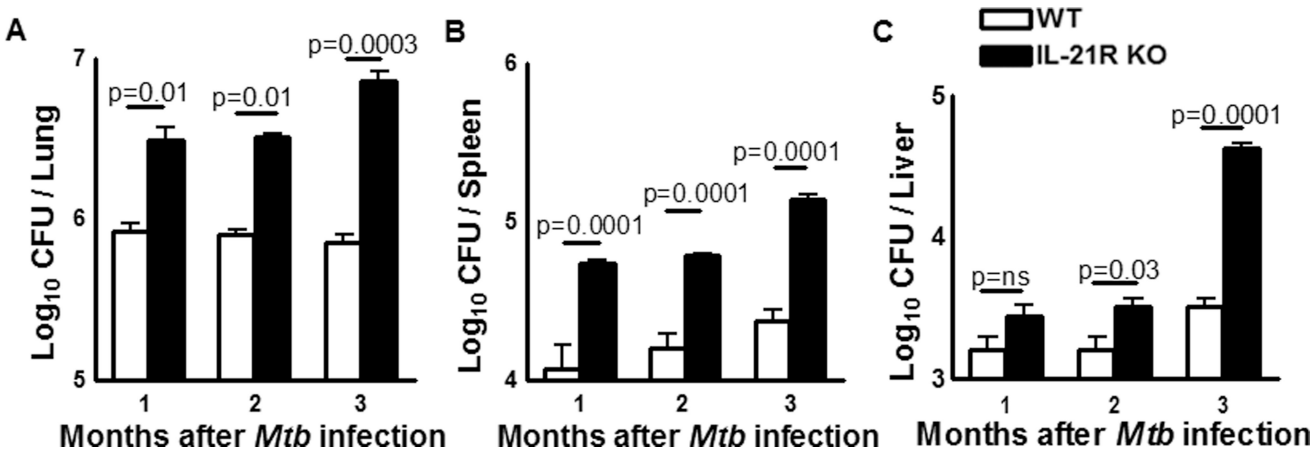

Fig. 2. IL-21R is essential for optimal control of *Mtb* growth in mice

A. WT and IL-21R KO mice (both C57BL/6NJ background) were infected with 50–100 CFU of H37Rv by aerosol. One, two and three months after infection, the bacterial burden in the lungs, spleen and liver was measured. Data are representative of two independent experiments. Five mice per group were used in each independent experiment. Mean values, p values and SEs are shown.

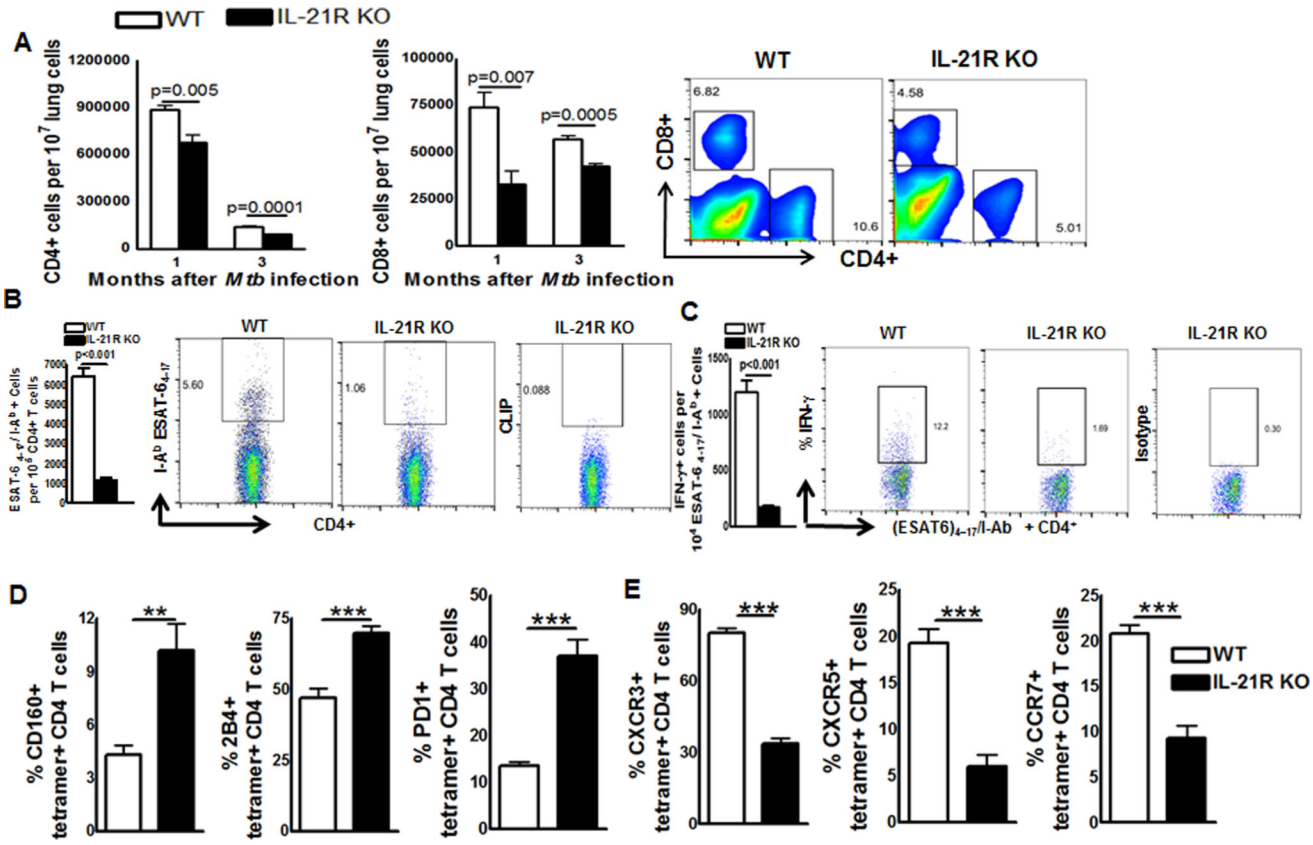

Fig. 3. IL-21 is essential for optimal T-cell responses during *Mtb* infection

A. WT and IL-21R KO mice (both C57BL/6NJ background) were infected with 50–100 CFU of H37Rv by aerosol. Three months after infection, lung cells were prepared as mentioned in methods section. Total lung CD45+ cells (leukocytes) were gated and various cell population were measured. The absolute numbers of cells was determined by calculating the percentage of gated cells multiplied by total lung cell number (excluded dead cells by Trypan blue dye exclusion). **A.** The number of CD4+ and CD8+ cells in the lungs was measured by flow cytometry (left) and representative plots of CD4 and CD8+ T-cells in the lung on day 90 post-infection are shown (right). **B.** Percentage of tetramer+ CD4 T cells in the lung (left) and representative plots of $IA^bESAT\text{-}6_{4–17}$ MHC class II tetramer staining gated on CD4 T cells in the lung on day 90 post-infection (right). **C.** Percentage of IFN-γ–producing tetramer+ lung CD4 T cells (left) and representative plots of IFN-γ staining gated on tetramer+ lung CD4 T cells (right). **D and E.** Percentage of CD160, 2B4, PD1, CXCR3, CXCR5 and CCR7 gated on $IA^bESAT\text{-}6_{4–17}$ tetramer+ lung CD4 T cells from WT and IL-21R KO mice. Data are representative of two independent experiments. Three mice per group were used in each independent experiment. Mean values, p values and SEs are shown. *p < 0.05, ** p < 0.01, *** p < 0.001.

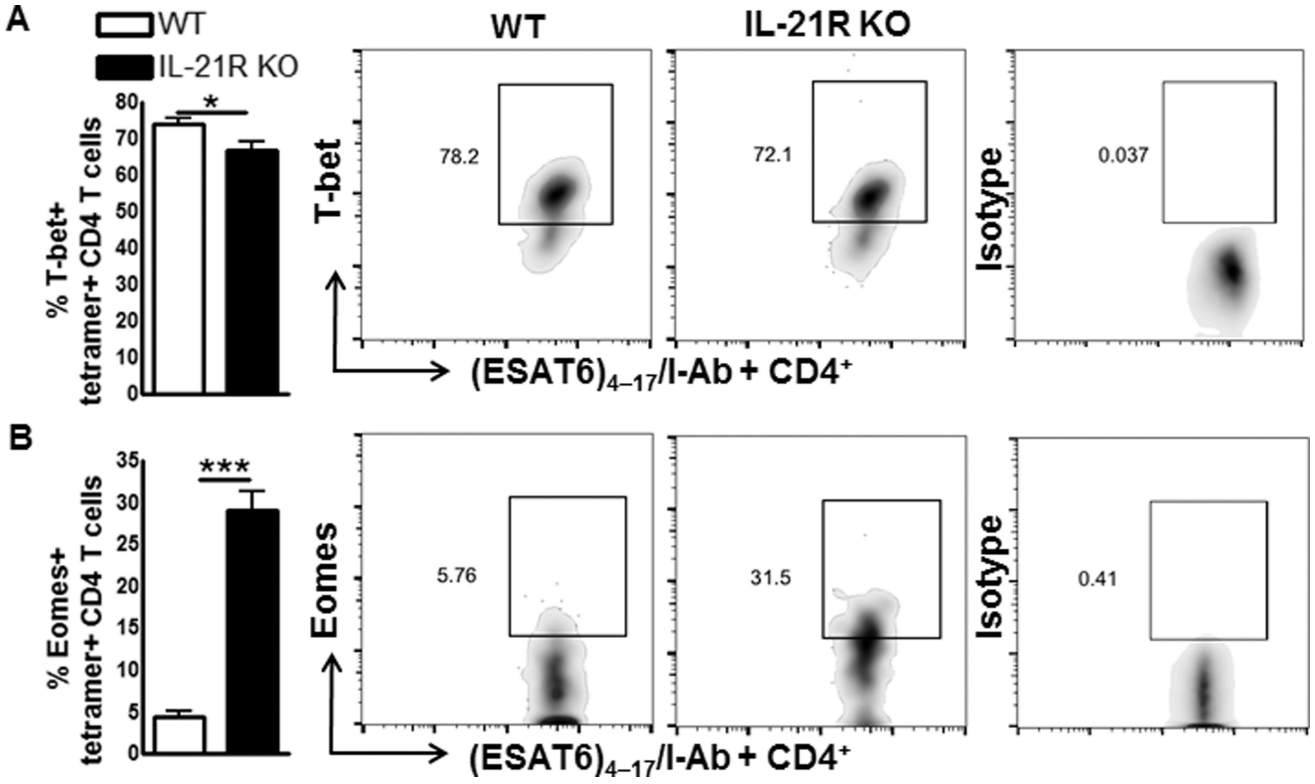

Fig. 4. Increased Eomes expression by tetramer+CD4+ T-cells in IL-21R KO mice

WT and IL-21R KO mice (both C57BL/6NJ background) were infected with 50–100 CFU of H37Rv by aerosol. **A and B.** Percentage of T-bet **(A)** and Eomes **(B)** expressing tetramer+ lung CD4 T cells (left). Representative plots of T-bet and Eomes expression gated on $IA^bESAT\text{-}6_{4–17}$ tetramer+ CD4 T cells from WT and IL-21R KO mice lungs on day 90 post-infection (right). Data are representative of three independent experiments. Three mice per group were used in each independent experiment. Mean values, p values and SEs are shown. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

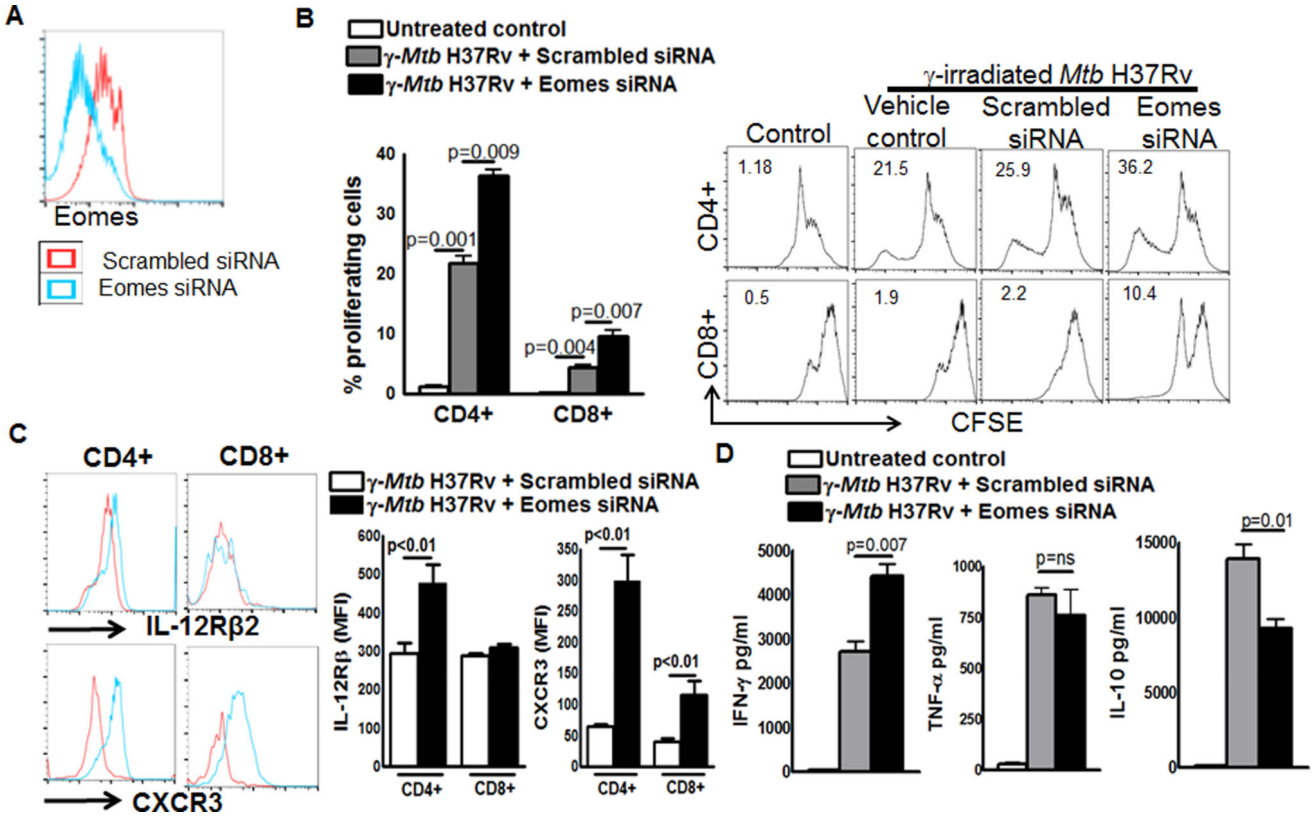

Fig. 5. Eomes inhibits T-cell effector functions in *Mtb* infected mice

IL-21R KO mice were infected with 50–100 CFU of H37Rv by aerosol. Three months after infection, mediastinal lymph node cells were isolated and labelled with CFSE and cultured with γ-irradiated *Mtb* H37Rv (10 μg/ml) in the presence or absence siRNA to Eomes or scrambled siRNA. After 72 hours, supernatants were collected and flow cytometry was performed on cells to determine the T cell proliferation. **A.** Histogram plot showing the expression of Eomes by T cells. **B.** T-cell proliferation. **C.** Expression of CXCR3 and IL-12Rβ2 by CD4+ and CD8+ cells. **D**. IFN-γ, TNF-α and IL-10 levels in culture supernatants as determined by ELISA. Data are representative of three independent experiments. Pooled cells from two mice were used in each independent experiment. Mean values, p values and SEs are shown.

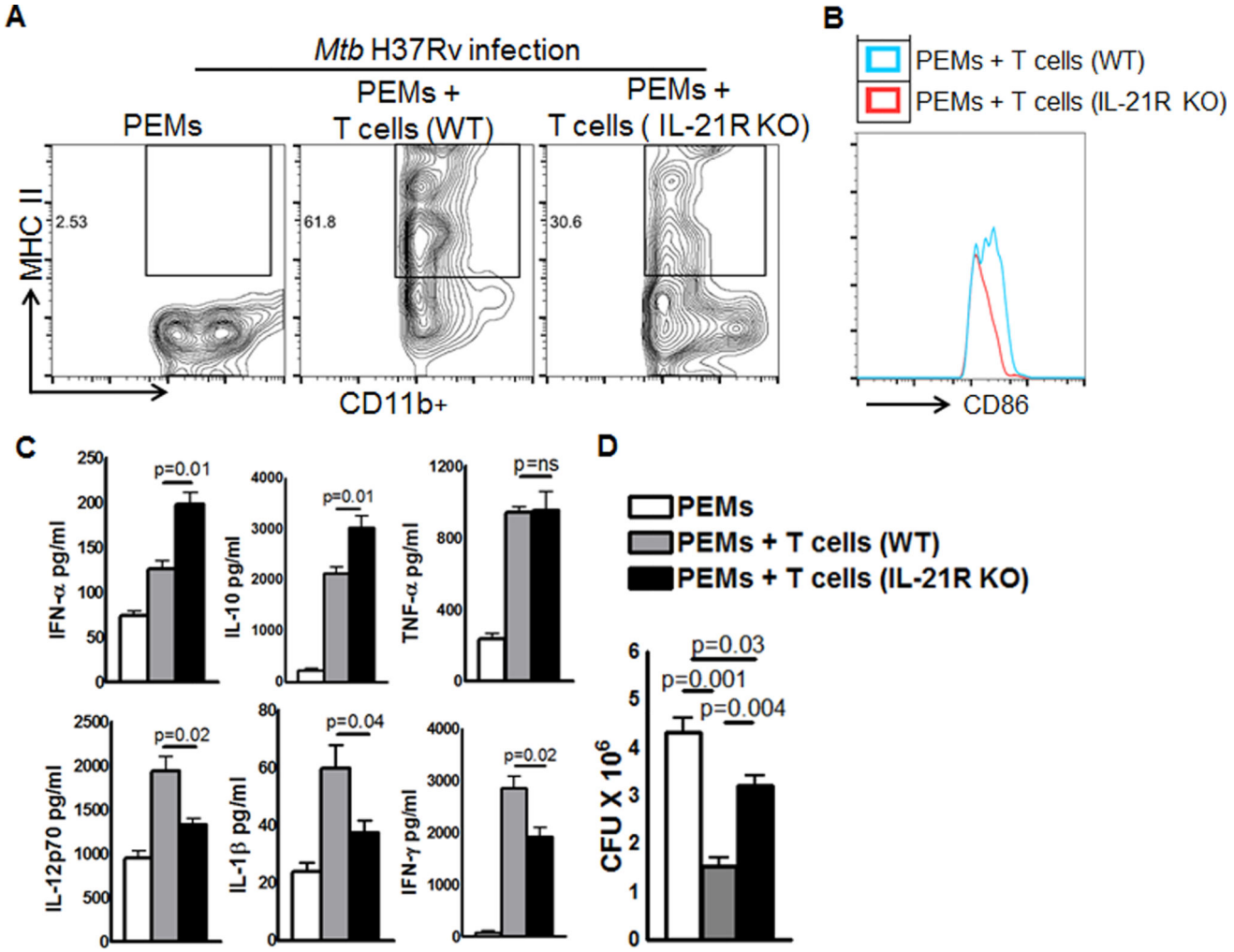

Fig. 6. Effect of T cells from *Mtb* infected IL-21R KO mice on macrophage responses to *Mtb*

WT and IL-21R KO mice were infected with 50–100 CFU of H37Rv by aerosol as mentioned in the methods section. Three months after infection, pooled spleen and MLN T-cells were isolated using magnetic selection as mentioned in the methods section. PEMs were isolated from naïve mice and infected with H37Rv at an MOI of 1:2.5 (one PEM to 2.5 *Mtb*). After 2 h, PEMs were washed to remove extracellular bacteria and cultured with T cells from *Mtb* infected WT or IL-21R KO mice (1:5, one macrophage and 5 T-cells). After 5 days, culture supernatants were collected to determine cytokine levels by ELISA, flow cytometry was performed on some cells to determine the expression of surface receptors and other cells were used to determine intracellular bacterial growth as mentioned in methods section. **A.** MHC class II and **B**. CD86 expression by *Mtb* infected PEM. **C.** IFN-α, IL-1β, IL-10, IL-12p70, IFN-γ and TNF-α levels as measured by ELISA. **D**. Bacterial burden of PEMs was measured by plating homogenates and counting CFU as mentioned in the methods section. Data are representative of three independent experiments. Two mice per group were used in each independent experiment. Mean values, p values and SEs are shown.