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## A defect in the synthesis of Interferon- $\gamma$ by the T cells of Complement-C5 deficient mice leads to enhanced susceptibility for tuberculosis

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### Abstract

Interferon- $\gamma$  (IFN $\gamma$ ) plays a major role during host defense against *Mycobacterium tuberculosis* (*Mtb*). T cells produce IFN $\gamma$  in response to IL-12 and IL-18 secreted from *Mtb* infected macrophages. IFN $\gamma$  in turn, induces nitric oxide secretion in macrophages that kills *Mtb*. IFN $\gamma$  knock-out mice are thus hyper-susceptible to tuberculosis. We reported earlier that Complement C5 deficient (C5<sup>-/-</sup>) congenic mice are more susceptible to tuberculosis and showed reduced IL-12 synthesis in their macrophages. Using C5<sup>-/-</sup> congenic mice that carry a deletion in the C5 gene and the wild type C5<sup>+/+</sup> mice, we demonstrate here that, the C5<sup>-/-</sup> derived CD3<sup>+</sup> T cells, have an additional defect in the synthesis of IFN $\gamma$ . C5<sup>-/-</sup> T cells produced lower levels of IFN $\gamma$  upon stimulation by antigen presenting cells (APCs) infected with *Mtb* or when stimulated directly with a combination of IL-12 and IL-18. The latter was in part due to a reduced phosphorylation of STAT-4 following IL-12/IL-18 stimulation. Addition of C5a peptide to IL-12/IL-18 partially restored STAT4 phosphorylation and IFN $\gamma$  synthesis in C5<sup>-/-</sup> T cells indicating that IL-12/IL-18 mediated signaling within CD3<sup>+</sup> T cells involves C5a peptide. Finally, C5<sup>-/-</sup> T cells derived from *M.bovis* BCG or *Mtb* infected mice showed a reduced expression of T-bet (T-box expressed in T cells) transcription factor, which correlated well with a reduced T cell secretion of IFN $\gamma$ . Since T-bet mediated IFN $\gamma$  synthesis facilitates Th1 expansion, C5<sup>-/-</sup> mouse derived T cells appear to have an intrinsic defect in the production of IFN $\gamma$ , which is related to C5 deficiency and this may explain their increased susceptibility to infection with *Mtb* and BCG.

### Keywords

*Mycobacterium tuberculosis*; Complement; Interferon- $\gamma$ ; anaphylatoxin C5a; Mouse; IL-12 and IL-18

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## 1. Introduction

Tuberculosis is the leading cause of death in the world due to a single infectious agent and accounts for about 2 million deaths per year. One third of the world's population is latently infected with *Mycobacterium tuberculosis* (*Mtb*), and five to ten percent of these individuals reactivate to clinical disease. Immunity to tuberculosis is primarily T cell mediated and the use of various mouse models has enabled the characterization of multiple cytokines and cell populations in host defense [1,2]. A key mediator of ant-tuberculosis immunity is IFN $\gamma$ . Macrophages infected with *Mtb* release IL-12 and IL-18, that in turn, activate T cells to produce IFN $\gamma$  [3]. While *Mtb* can replicate within naïve macrophages, IFN $\gamma$  activates the inducible nitric oxide synthase in macrophages to produce nitric oxide and kill intracellular *Mtb* [4]. Thus, IFN $\gamma$  and IL-12 knockout mice are hyper susceptible to tuberculosis [2,5]. Furthermore, mice treated with inhibitors of NO synthesis are also more susceptible to tuberculosis [6]. IL-12 and IFN $\gamma$  are therefore major regulators of immunity to tuberculosis. In addition, interaction between *Mtb* infected macrophages and T cells results in the release of a variety of mediators such as IL-1 $\beta$ , IL-6, IL-10 and TNF- $\alpha$  [7]. These cytokines play various regulatory roles, culminating in either control or enhancement of *Mtb* growth within macrophages.

We previously reported that, C5 deficient A/J and congenic C5 deficient B/10 derived mice (C5<sup>-/-</sup>) are more susceptible to tuberculosis, compared to C5 intact, C57Bl/6 mice or B.10 derived C5 sufficient congenic mice (C5<sup>+/+</sup>) [8,9]. Lungs of infected C5<sup>-/-</sup> mice contain larger bacterial loads after aerosol or intravenous infections. Interestingly, other C5 deficient strains such as DBA/2 and SWR strains have also been reported to be hyper-susceptible to tuberculosis [10] [11]. C5 deficient A/J and C5<sup>-/-</sup> mice show poor granuloma formation needed for containment of the infection and instead show a pneumonitis. Macrophages secrete a C5 peptidase, which cleaves C5 into C5a and C5b [12]. The most potent anaphylatoxin of the complement system is C5a, the 14 kDa cleavage product of C5. We found that *Mtb* infected C5 sufficient (C5<sup>+/+</sup> and C57Bl/6) macrophages secrete and cleave C5 to C5a peptide [8]. Macrophages also express C5a receptor (C5aR) and we found that C5a-C5aR signaling along with stimulation of the TNF- $\alpha$  receptor regulates the production of IL-12 by macrophages through a feedback mechanism [8]. IL-12 is known to induce CD4 and CD8 T cells to secrete IFN $\gamma$  via a signal transducer and activator of transcription 4 (STAT4) dependent pathway [13]. We and Karp *et al.* reported that C5 deficient macrophages secrete reduced levels of IL-12 and that such macrophages were also deficient in the production of TNF- $\alpha$  and IL-1 $\beta$  [8,14].

Since IL-12 induced IFN $\gamma$  synthesis in T cells is well established, in our initial studies, we were surprised to note that reduced IL-12 mRNA of lungs of *Mtb* infected C5 deficient mice was not consistent with pulmonary expression of mRNA for IFN $\gamma$ . Indeed, mRNA messages for IFN $\gamma$  were comparable between the lungs of congenic C5 mice at certain time points after aerosol induced tuberculosis [9]. However, IFN $\gamma$  can be produced by a variety of immune cells including MHC-II restricted CD4 T cells, MHC-I restricted CD8 T cells, CD1d restricted NKT cells and even certain types of dendritic cells or macrophages [15]. Furthermore, IFN $\gamma$  producing T cells can have different effects depending upon the kinetics of expansion, and homing during the various phases of tuberculosis. CD4 T cells are thought to be effective against acute tuberculosis while CD8 T cells have been proposed to prevent reactivation of latent tuberculosis [16,17]. NKT cells have been reported to play an emerging protective role during latent stages of infection [18]. We therefore sought to characterize the ability of purified CD3<sup>+</sup> T cells to secrete IFN $\gamma$  during C5 deficiency using *in vitro* and *in vivo* models. We now demonstrate that C5<sup>-/-</sup> T cells show a defect in the production of IFN $\gamma$ , when activated with mycobacteria infected macrophages, recombinant proteins of IL-12 or IL-18 and during *in vivo* mycobacterial infection.

## 2. Results

### Differential IFN $\gamma$ response of T cells from C5 congenic mice to activation from Mtb infected macrophages

Mice were immunized with Mtb and purified CD3<sup>+</sup> T cells from spleens allowed to react *in vitro* with macrophages infected with Mtb. IFN $\gamma$  levels were determined and T cells analyzed by flow cytometry to correlate with the CD4 and CD8 phenotype. Fig.1A shows that, C5<sup>-/-</sup> T cells produce lower levels of IFN $\gamma$  compared to C5<sup>+/+</sup> T cells when primed from APCs, even when they were activated at comparable numbers. T cells overlaid on naïve macrophages secreted insignificant IFN $\gamma$ . Fig.1B illustrates that the reduced IFN $\gamma$  is likely due to reduced expansion of CD4<sup>+</sup>IFN $\gamma$  cells of C5<sup>-/-</sup> mice during coculture. Data on of three similar experiments are summarized in Fig.1C. CD8<sup>+</sup>IFN $\gamma$ <sup>+</sup> T cells showed less significant differences between C5<sup>-/-</sup> and C5<sup>+/+</sup> mice.

### C5<sup>+/+</sup> macrophages are able to compensate for the defect in IFN $\gamma$ secretion of C5<sup>-/-</sup> T cells

To determine if the reduced levels of IFN $\gamma$  in C5<sup>-/-</sup> T cells could be restored, C5<sup>+/+</sup> and C5<sup>-/-</sup> macrophages were infected separately with Mtb and allowed to cross-present antigens against both C5<sup>+/+</sup> and C5<sup>-/-</sup> T-cells. Interestingly, overlay of the C5<sup>-/-</sup> T cells on the C5<sup>+/+</sup> macrophages partially rescued the IFN $\gamma$  response (Fig.2A). Since IL-12 primes IFN $\gamma$  response in T cells, the levels of IL-12p70 in the supernatants were measured. ELISA confirmed that C5<sup>-/-</sup> macrophage-T cell cocultures contained reduced IL-12p70 compared those of C5<sup>+/+</sup> cocultures (Fig.2B).

### C5<sup>-/-</sup> T cells secrete less IFN $\gamma$ upon direct activation with IL-12 and IL-18

Since IL-12 was reduced in macrophage-T cell cocultures as shown above, the goal here was to determine if C5<sup>-/-</sup> T cells could be directly activated with IL-12 to produce sufficient IFN $\gamma$ . Initial studies with IL-12 alone were not that effective in inducing copious IFN $\gamma$  in T cells (not shown). Previous studies have shown that a combination of IL-12 plus IL-18 is most optimal for inducing IFN $\gamma$  via STAT4 activation [19]. Equal numbers of purified CD3<sup>+</sup> T cells were therefore activated *in vitro* with IL-12 or IL-18 or their combination and T cell supernatants were tested for IFN $\gamma$ . Fig.3A shows that, even after activation with IL-12 and IL-18, IFN $\gamma$  levels were significantly reduced in C5<sup>-/-</sup> T cells. This suggested that the defect in IFN $\gamma$  synthesis of C5<sup>-/-</sup> T cells is likely not because of a decreased signaling from macrophage mediated IL-12 signaling but, an intrinsic defect also exists in T cells.

Since IL-12 activates IFN $\gamma$  synthesis binding to the IL-12R1 $\beta$  and phosphorylating STAT-4, T cells were surface stained for IL-12R1 $\beta$ . Fig.3B indicates that C5<sup>-/-</sup> and C5<sup>+/+</sup> T cells had a comparable distribution of IL-12R1 $\beta$ . Next, T cells were activated with a combination of IL-12 and IL-18 in the presence or absence of 100 ng/mL of C5a peptide. The cell lysates were then examined for native STAT-4 protein and the phosphorylated protein in western blot studies. Fig.3C (bottom lane) shows the constitutive expression of STAT-4 where C5<sup>-/-</sup> and C5<sup>+/+</sup> T cells had comparable levels of STAT4. Addition of cytokines to T cells induced rapid phosphorylation of STAT4 (pSTAT4) in C5<sup>+/+</sup> T cells (top lane) and the band was denser when external C5a was added. However, C5<sup>-/-</sup> T cells did not show significant pSTAT4 bands with IL-12 /IL-18 and a less dense band was evident after addition of C5a peptide. Since T cells do not produce C5a peptide, we conclude from this experiment that, cytokines induce defective phosphorylation of STAT4 in C5<sup>-/-</sup> T cells, which can be partially restored with C5a. Murine T cells are thought to express C5a-R and we suggest that the externally added C5a can have an activation effect on C5<sup>-/-</sup> T cells.

Finally, replicate aliquots of treated T cells were also incubated for an additional 24 hr and supernatants tested for IFN $\gamma$  using ELISA. Addition of C5a to IL-12 and IL-18 enhanced

IFN $\gamma$  levels but these levels were still lower than those produced by C5<sup>+/+</sup> T cells (Fig. 3D). Addition of increasing amounts of C5a peptide did not result in a better phosphorylation or better yield of IFN $\gamma$  more than the levels illustrated in Fig.3C or 3D (not shown). These studies suggest that the defect in IFN $\gamma$  synthesis by C5<sup>-/-</sup> T cells is likely related to a defective intracellular signaling by IL-12 and IL-18 that can be partially corrected by addition of C5a peptide.

### **Defect in IFN $\gamma$ synthesis of C5<sup>-/-</sup>T cells is associated with a decreased expansion of T-box expressed in T cell (T-bet) transcription factor**

In mouse T cells, T-bet regulates IFN $\gamma$  synthesis through several pathways [20]. To determine if the defect in IFN $\gamma$  synthesis was related to T-bet expression and C5 deficiency, the following experiments were carried out.

#### ***In vitro* recall of T-bet response using T cells from BCG immunized mice**

CD3<sup>+</sup> splenic T cells were obtained from BCG infected mice and overlaid on BCG infected macrophages *in vitro*. 4 hours later, T cells were stained for intracellular expression of T-bet in CD4<sup>+</sup> T cells using fluorescent antibodies. In this *in vitro* model of macrophage activation, only immune T cells overlaid on BCG infected macrophages up-regulate T-bet but not those overlaid on naïve macrophages (Fig.4A-E). Data show that C5<sup>+/+</sup> T cells markedly up regulate T-bet expression upon activation from BCG infected macrophages, while C5<sup>-/-</sup> T cells show reduced T-bet. Fig.4F summarizes data from three experiments (p value, t test).

#### ***In vivo* expression of T-bet and IFN $\gamma$ response in *M.tuberculosis* infected mice**

To investigate whether the decreased expression of T-bet and IFN $\gamma$  occurred *in vivo*, C5 congenic mice were infected via aerosol with Mtb-Erdman and lungs were analyzed for T cell expression of T-bet and IFN $\gamma$  using flow cytometry on day 21 post-infection. This time point was chosen to avoid apoptosis of T cells that occurs around 4 weeks of infection. Organs were also harvested at the same time and processed for colony counts of Mtb. Fig. 5A shows that C5<sup>-/-</sup> mice show a better growth of Mtb in both spleens and lungs, consistent with our previously published observations [8,9]. Fig.5B shows data indicating absolute numbers of CD4<sup>+</sup>T-bet<sup>+</sup> IFN $\gamma$ <sup>+</sup> T cells calculated from flow cytometric analysis of cells stained from lungs and spleens. On day 21 post infection, C5<sup>-/-</sup> mouse lungs as well as spleens contained fewer CD4<sup>+</sup>T-bet<sup>+</sup> IFN $\gamma$ <sup>+</sup> T cells than C5<sup>+/+</sup> T cells. Thus, reduced T-bet expression of T cells in C5<sup>-/-</sup> mice correlated with an increase in the bacterial counts of Mtb in the organs of these mice. Data for T-bet among CD8 T cells were generally comparable between the C5<sup>-/-</sup> and C5<sup>+/+</sup> mice (not shown).

## **3: Discussion**

In our earlier studies, C5 deficient macrophages (C5<sup>-/-</sup>, A/J) were found to secrete lower levels of pro-inflammatory cytokines such as IL-12, IL-1 $\beta$ , IL-6, and TNF- $\alpha$  [8,9]. They were also deficient in chemokine secretion, which correlated with the reduced ability of these mice to form granulomas in the lungs after Mtb infection. Related studies indicated that, C5 deficient macrophages are also defective in killing intracellular Mtb due to reduced reactive oxygen species (ROS)-dependent mechanisms [21]. We anticipated defects in T cell function of C5 deficient mice since, IL-12 induces IFN $\gamma$ , IL-18 promotes helper T cell activation and IL-2 secretion [22]. In addition, TNF- $\alpha$  can regulate T cells through apoptosis [23]. However, preliminary studies on Mtb infected C5 congenic mice indicated comparable levels of IFN $\gamma$  mRNA in the lungs [9]. RT-PCR of mRNA messages in whole lungs measures global IFN $\gamma$  messages and may not identify defects in specific cell populations. In

this investigation, we used analyzed purified CD4 and CD8 T cells *in vitro* and *in vivo* for their ability to synthesize IFN $\gamma$ .

Purified C5<sup>-/-</sup> mouse T cells produced less IFN $\gamma$  in response to Mtb infected macrophages *in vitro* suggesting an IL-12 dependent defect. However, even priming with copious amounts of IL-12 and IL-18 showed a persisting defect in IFN $\gamma$  synthesis. IL-12 and IL-18 activate IFN $\gamma$  through phosphorylation of STAT4 and this step was found defective in C5<sup>-/-</sup> T cells [19]. Addition of C5a partially restored pSTAT4 activity and IFN $\gamma$  synthesis, implicating C5a as a potential regulator of IFN $\gamma$  synthesis of T cells. We therefore report a novel observation in this study. We noted that C5<sup>+/+</sup> macrophages were able to reconstitute the IFN $\gamma$  response of C5<sup>-/-</sup> T cells. While macrophages can release C5a to activate T cells, this remains a complex system where T cell activation may occur due to multiple mechanisms, obscuring intrinsic defects in IFN $\gamma$  secretion by CD3<sup>+</sup> T cells of C5<sup>-/-</sup> mice. One possibility is that C5<sup>-/-</sup> T cells may not have an initial C5aR expression, but may develop the capacity to express sufficient C5aR only over time. Long term or *in vivo* studies are perhaps required to answer this question. Finally, T-bet, a major transcription factor that regulates Th1 immunity through IFN $\gamma$  synthesis was also found reduced in mice with C5 deficiency. Reduced T-bet and IFN $\gamma$  levels of lungs during active tuberculosis correlated with enhanced growth of Mtb (Fig.5).

These observations suggest an important role of C5 during immune responses to tuberculosis in mice, although it is unclear at the moment, where C5a mechanistically fits into the regulation of IFN $\gamma$  synthesis. Although mycobacteria infected macrophages secrete IL-12 and IL-18 to prime for IFN $\gamma$ <sup>+</sup> secretion, IFN $\gamma$  synthesis in T cells involves several mechanisms that seem to cross-regulate each other. For example, IL-12 and IL-18 trigger IFN $\gamma$  synthesis through STAT4 phosphorylation [19]. The resulting IFN $\gamma$  promotes additional T-bet expression via activation of STAT-1 [20]. T-bet in turn, induces the expression of IL-12R $\beta$ 2 subunit required for T cells to respond to IL-12 [20]. IL-12 itself has been found to induce T-bet synthesis in murine T cells [24]. Thus, a complex feed-back regulatory mechanism appears to be in place between mycobacteria infected macrophages and Th1 type of T cells.

C5a peptide has been previously shown to affect the synthesis of many macrophage derived cytokines like IL-12, IL-6, IL-1 $\beta$  and TNF- $\alpha$  as well as several chemokines [8,9]. Many recent studies show that C5a plays an emerging role in the regulation and maturation of dendritic cells and activation as well as recruitment of T cells in mice [25] [26]. This study demonstrates that C5a can affect the IFN $\gamma$  secretion in T cells. Thus, C5a appears to affect both the innate arm of immunity involving antigen presenting cells like macrophages as well as the acquired arm of immunity mediated by T cells.

## 4. Materials and Methods

### Mice and Bacteria

Four to eight week old B10.D2-H2<sup>d</sup> H2-T18<sup>c</sup>Hc<sup>1</sup>/C5<sup>+/+</sup>J (C5<sup>+/+</sup>) and B10.D2-H2<sup>d</sup> H2-T18<sup>c</sup>Hc<sup>0</sup>/C5<sup>-/-</sup>J (C5<sup>-/-</sup>) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed under specific pathogen free conditions in accordance with IACUC approved procedures. Stock strains of Mtb (H37Rv, ATCC 27294) and BCG (Pasteur, ATCC 35734) were cultured in 7H9 broth to early log phase and organisms were washed and suspended in sterile saline before sonication at 4 watts for 30 sec to disrupt clumps. They were matched to McFarland#1 and used for infections. Aliquots were plated for determination of CFU counts.



## Cytokines and reagents

IL-12 and IL-18 were used at concentrations of 5 ng/ml and 50 ng/ml, respectively (Cell Sciences Inc. MA, USA). Human recombinant C5a peptide was used for *in vitro* complementation assays (Sigma Chemical Co. USA). It was tested by limulus lysate technique and found to contain endotoxin < 10 pg/mL. It was used a dose of 100 ng/mL.

## Mouse infections

a) *BCG or Mtb infections*: For *in vitro* antigen presentation assays, mice were immunized with  $10^6$  CFU of mycobacteria via *i.p.* injection once. At weekly intervals, CD3 purified T cells from spleens were overlaid on bone-marrow derived macrophages (BMs) infected with appropriate mycobacteria for intracellular expression of T-bet or IFN $\gamma$  using flow cytometry.  
 b) *Mtb infections*: Mice were infected via aerosol with 100 CFU of Mtb as published [8]. Mice were sacrificed for CFU counts of organisms in spleens and lungs. T cells were purified from lungs and spleens for cytometric analysis.

## Bone marrow-derived macrophages

Macrophages were obtained culturing the marrow derived cells after lysis in ACK Buffer for 5 minutes in Iscoves' modification of DMEM (IDMEM) supplemented with 10% heat inactivated FBS, penicillin and gentamycin, and 10 ng/mL GM-CSF. After 7-10 days, non-adherent cells were removed and macrophages incubated in GM-CSF free medium for 1 day before use in infections and overlay experiments. Macrophages were F4/80+/MOMA1+/CD11c- as evaluated by flow cytometry.

## Isolation of T cells from spleens and lungs

T cells from naive or mycobacteria immunized or infected mice were purified from mouse organs using CD3 beads from Miltenyi Inc, CA, USA. Spleens were teased in ACK lysis buffer and washed before T cell purification. PBS lavaged lungs from mice were minced and incubated with calcium and magnesium free HBSS, pH 7.2 containing EDTA (1 mM), dextrose, 100 U per ml collagenase (Type VII) and 50  $\mu$ g/mL elastase (Sigma Chemical Co, USA) at 37°C and 5% CO $_2$  for 45 minutes on a shaker. The suspension was then passed through a cell strainer with 45  $\mu$ m pore size and single cells lysed in ACK buffer and washed. Single cell suspensions were then used to purify T cells using CD3 beads.

## *In vitro* activation assays

Macrophages were infected with BCG at a MOI of 1:1 and incubated for 4 hours at 37°C and 5% CO $_2$ . Macrophage monolayers were then washed 3 times with PBS to remove non-phagocytosed bacteria. T cells were then overlaid for activation using  $5 \times 10^6$  T cells per  $1 \times 10^6$  macrophages of the monolayer. T cells were stained for IFN $\gamma$  and T-bet at time intervals as below. Supernatants were measured for IFN $\gamma$  and IL-12p70 using an ELISA kit (R&D Systems).

## Flow Cytometry

T cells were suspended in Staining Buffer (PBS containing 0.1% BSA) and stained at 4°C using primary antibodies, isotype controls conjugated to fluorochrome, or primary unconjugated antibodies followed by a fluorochrome conjugated secondary antibody. The intracellular staining was performed as follows. T cells harvested from co-cultures were treated for 4 hours with Golgi Stop to block protein secretion from cells (BD Biosciences). Fc Receptor Block (CD16/32, Caltag Laboratories) was added to cells and incubated on ice for 15 minutes. The appropriate surface antibodies were added to each sample (CD4 FITC, clone RM4-5, Caltag Laboratories; CD8 $\alpha$  PerCP, 53-6.7, BD Biosciences) and incubated for 30 minutes in the dark. The cells were washed and fixed with 2% paraformaldehyde for 30

minutes, followed by addition of antibodies for intracellular targets (IFN $\gamma$  FITC, clone XMG1.2, Caltag Laboratories; T-bet unconjugated, clone 4B10, Santa Cruz Biotechnology) diluted according to manufacturers' recommendations in a perm buffer containing 0.1% Triton-X 100, 0.1% Saponin, 0.01% Digitonin in PBS for 18 hours at 4°C. The samples were washed in Staining Buffer and resuspended in 2% paraformaldehyde or were incubated for 30 minutes with the PE conjugated secondary antibody (anti-mouse IgG1 PE, clone A85-1, BD Biosciences) cells were analyzed using a BD FacSort and Cellquest software (BD Biosciences).

### Western blot

CD3 purified T cells were incubated in the presence of a mix of IL-12 and IL-18 with or without 100 ng/mL C5a peptide and after 15 min, pelleted and suspended in SDS lysis buffer. The proteins were estimated and lysates separated in 10% SDS gels and transferred to PVDF membranes. Lysates from naive T cells were first blotted to localize non phosphorylated STAT4 with an antibody that labeled a single band around 87 kDa. A cytokine activated T cell lysate blot was then labeled with anti STAT4, stripped and labeled with a phospho-STAT4 antibody (both antibodies were from Santa Cruz Biotechnology, CA, USA). Blots were developed using a chemiluminescence kit (ECL, Amersham Inc.). Experiments were repeated three times. Phospho-blot required 5 times more protein load than the unactivated T cell lysates. pSTAT4 also migrated at a higher mass.

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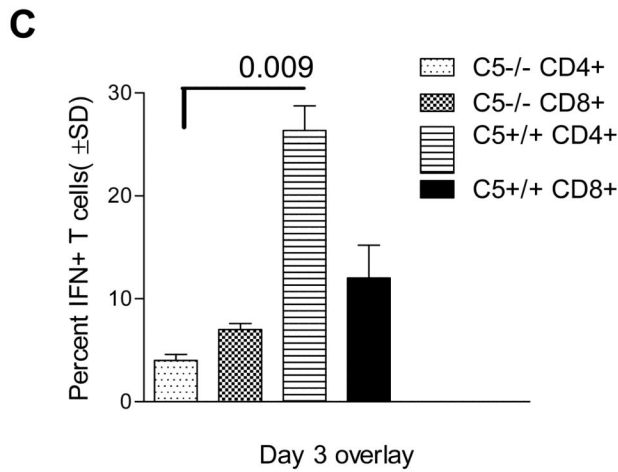
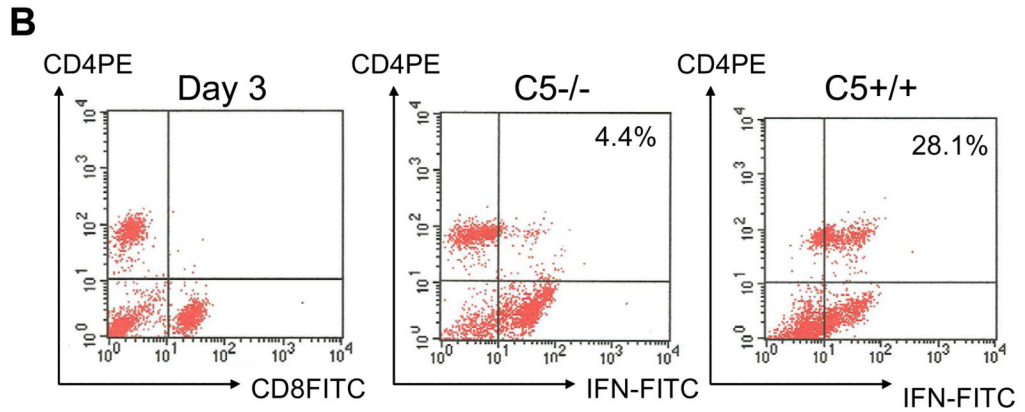
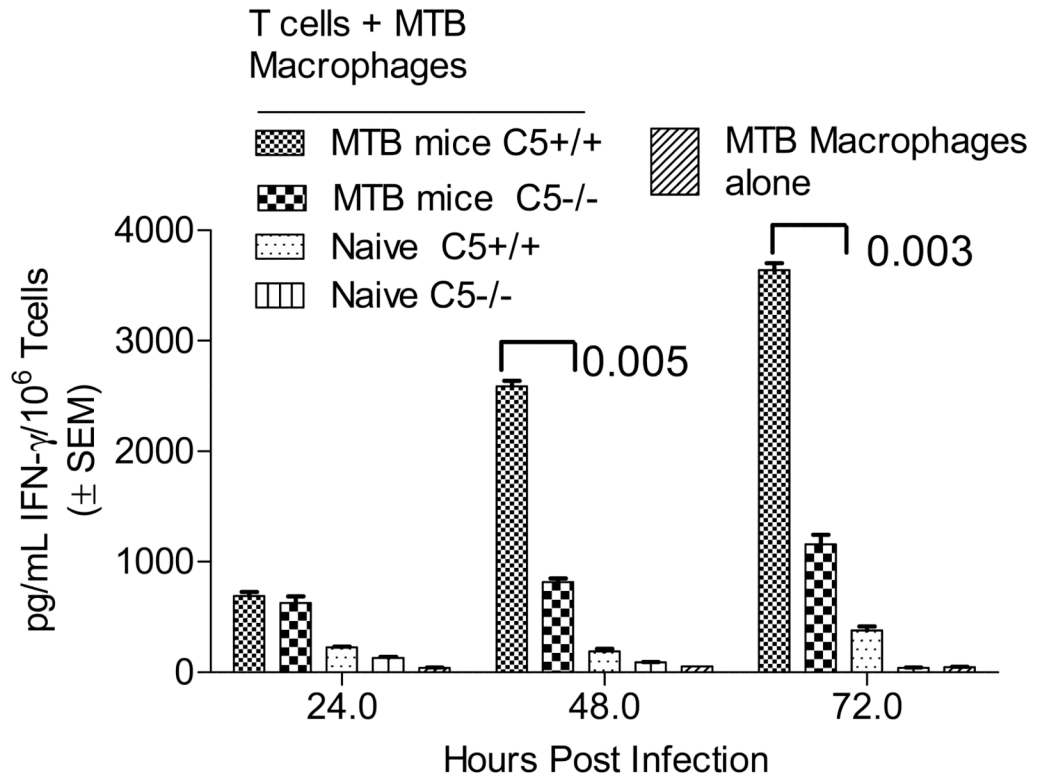
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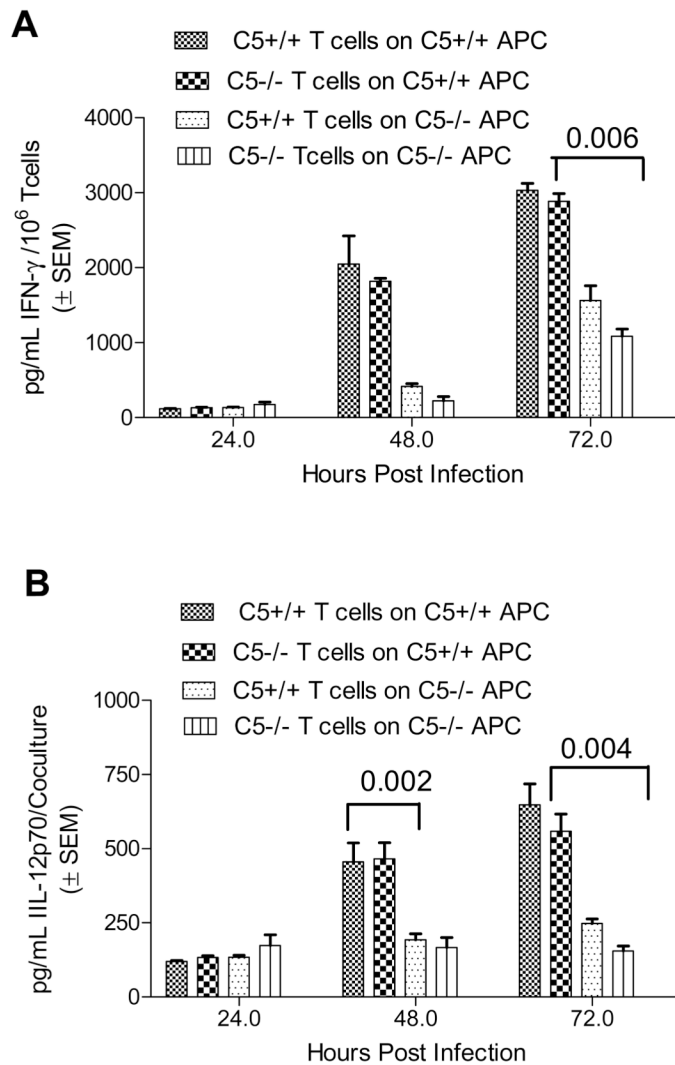
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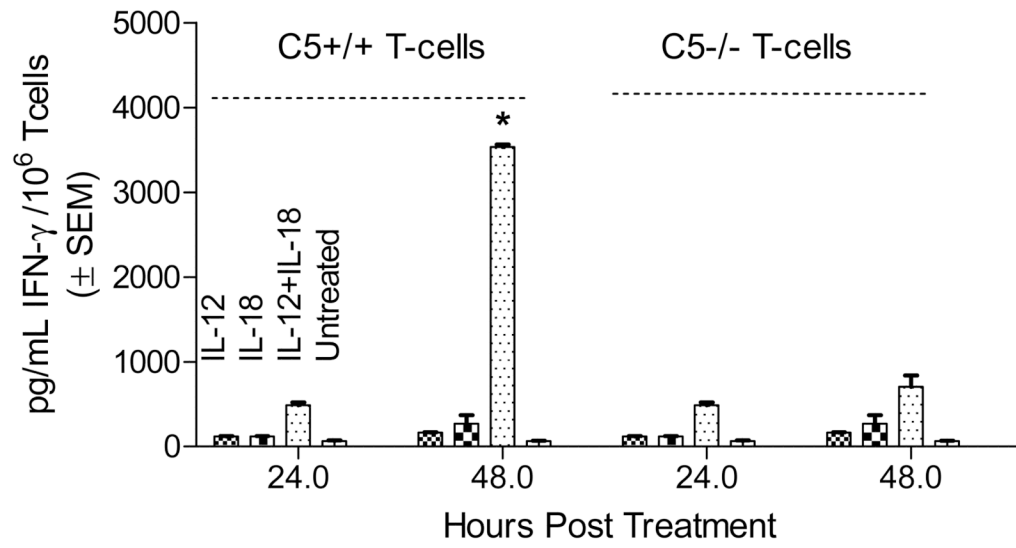
**Figure 1. Complement C5 sufficient B10.D2-H2<sup>d</sup> H2-T18<sup>c</sup>Hc<sup>1</sup>/C5<sup>+/+</sup>J (C5<sup>+/+</sup>) and deficient B10.D2-H2<sup>d</sup> H2-T18<sup>c</sup>Hc<sup>0</sup>/C5<sup>-/-</sup>J (C5<sup>-/-</sup>) mice derived T cells show a differential Interferon- $\gamma$  response to activation from macrophages**

A: *M.tuberculosis* immunized spleens of C5<sup>+/+</sup> and C5<sup>-/-</sup> mice were dissected and depleted of non-T-cells. Equal numbers of CD3<sup>+</sup> bead purified T-cells were layered on Mtb infected or naïve syngenic macrophages for 72 h. Supernatants were collected and analyzed for IFN $\gamma$  levels using sandwich ELISA. T-cells from C5<sup>+/+</sup> mice produce significantly more IFN $\gamma$  than their C5<sup>-/-</sup> counterparts (p values; *t* test, 3 experiments). B: T cells from the above experiment obtained on day 3, were stained for intracellular IFN $\gamma$  using antibody conjugates in combination with CD4 or CD8 surface phenotype and analyzed using BD Facsan and Cellquest software. Histograms illustrate that C5<sup>-/-</sup> T cells show a reduced expansion into CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup> T cells. C: Data of T cells stained for cytokines from one of three similar experiments are summarized.








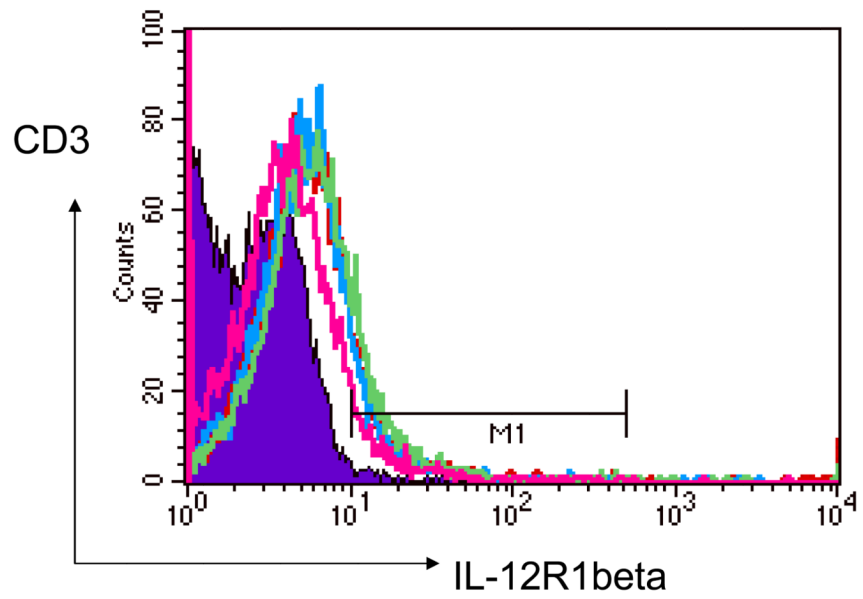
**Figure 2. C5<sup>+/+</sup> macrophages are able to compensate for the defect in C5<sup>-/-</sup> macrophages**  
 A: C5<sup>+/+</sup> and C5<sup>-/-</sup> macrophages were infected separately with Mtb and allowed to cross-activate C5<sup>-/-</sup> and C5<sup>+/+</sup> T-cells from Mtb immunized mice. IFN $\gamma$  response measured over time shows that C5<sup>+/+</sup> macrophages are able to induce IFN $\gamma$  in C5<sup>-/-</sup> T-cells that were reduced for this cytokine, when activated with C5<sup>-/-</sup> macrophages. Conversely, C5<sup>+/+</sup> T-cells primed with C5<sup>-/-</sup> macrophages show far reduced response when compared to their response primed with C5<sup>+/+</sup> macrophages (p values, t test; 2 experiments). B: Supernatants of macrophage-T cell cocultures contained reduced levels of IL-12p70 (pg/mL  $\pm$  SEM from duplicate experiments) when C5<sup>-/-</sup> APCs, which secrete less IL-12p70. Culture supernatants of experiment shown in panel A were titrated for IL-12p70 using a sandwich ELISA kit from R and D systems (p values; t test).

A

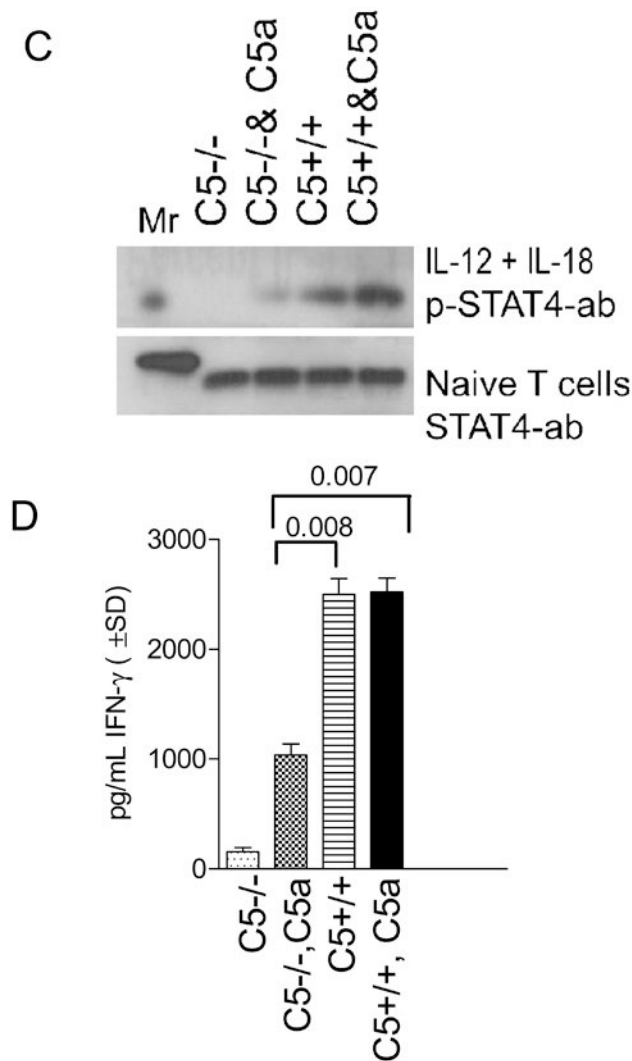


B

Key	Name	Parameter	Gate
	Rat IgG2a isotype		
	C5-/- untreated		
	C5-/- IL-12+IL-18		
	C5+/+ untreated		
	C5+/+ IL-12+IL-18		

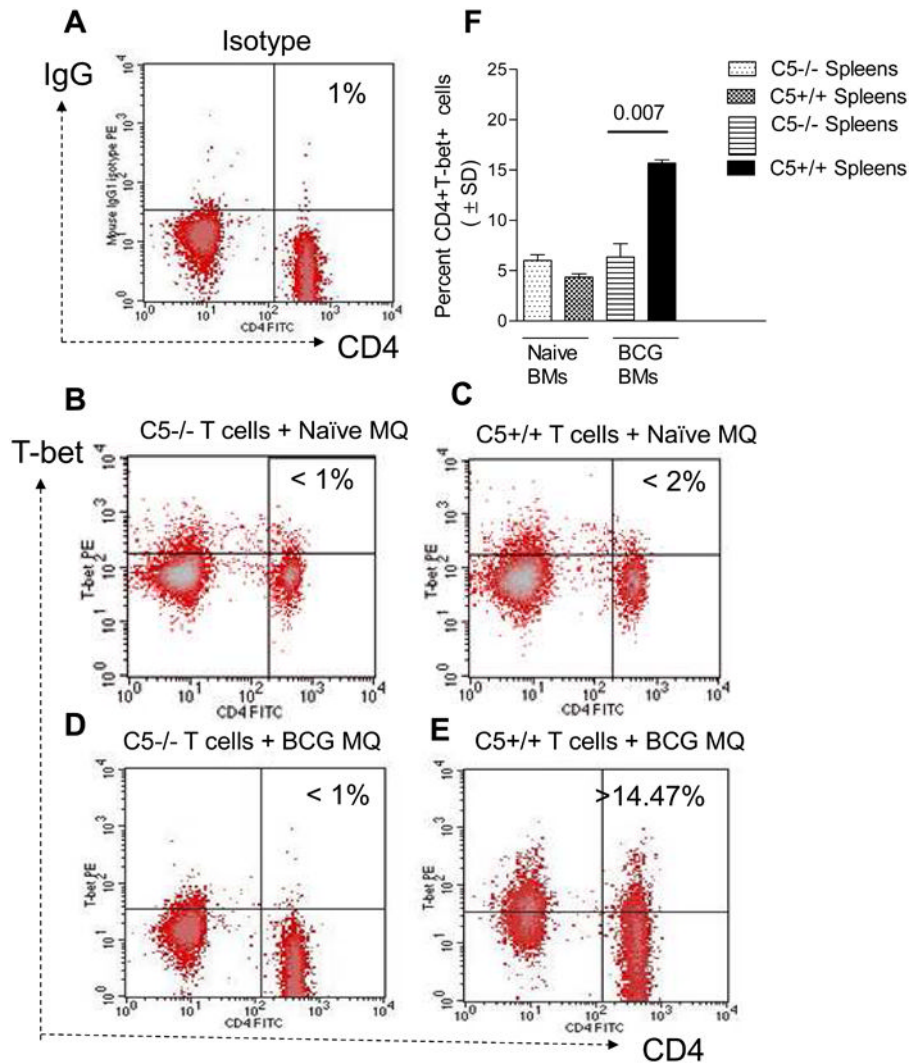




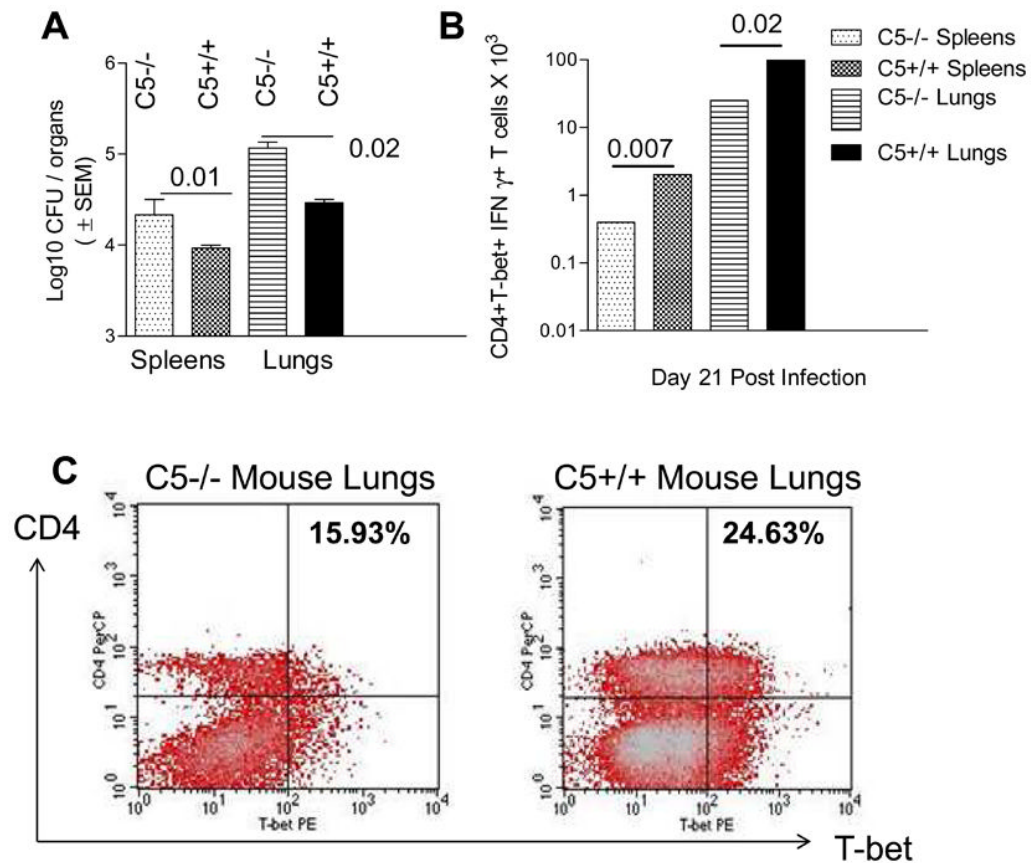


**Figure 3. C5<sup>-/-</sup> T cells have an intrinsic defect and secrete less IFN $\gamma$  upon *in vitro* activation with IL-12 and IL-18**

**A:** CD3 bead purified C5<sup>+/+</sup> and C5<sup>-/-</sup> T cells from naïve mice were activated *in vitro* with 5 ng/mL of IL-12 and 50 ng/mL of IL-18 and T cell supernatants tested for IFN $\gamma$  using sandwich ELISA. IFN $\gamma$  levels are shown at two time points. C5<sup>-/-</sup> T cells secrete reduced levels of IFN $\gamma$  compared to C5<sup>+/+</sup> T cells (\*  $p < 0.002$  vs. all other groups at the same time point; *t* test). **B:** Reduced IFN $\gamma$  response is not due to a reduced expression of IL-12R1 $\beta$ . T cells from the experiment above were stained for IL-12R1 $\beta$  using antibodies conjugated to FITC and analyzed in a BD FacsCan using Cellquest software. C5<sup>+/+</sup> and C5<sup>-/-</sup> T cells show a comparable expression of IL-12R1 $\beta$  when activated with cytokines IL-12 and IL-18. Both CD4 and CD8 T cells showed a similar pattern (not shown). **C:** CD3 bead purified T cells from C5<sup>+/+</sup> and C5<sup>-/-</sup> mice were tested naïve with anti-STAT4 antibody (bottom lane) or stimulated for 15 min with IL-12 and IL-18 in the presence or absence of C5a peptide and assayed for phosphorylated STAT4 (p-STAT4) using western blot. C5<sup>-/-</sup> T cells show a lack of phosphorylation at 15 min, which is partially restored after the addition of C5a peptide (100 ng/mL; pSTAT-4 migrates at a higher mass). **D:** Replicate aliquots of T cells activated as in panel C, were incubated for an additional 24 h and supernatants tested for IFN $\gamma$  using sandwich ELISA. Addition of C5a peptide to IL-12 and IL-18 enhances IFN $\gamma$  levels in C5<sup>-/-</sup> T cells but the levels are still lower than that in C5<sup>+/+</sup> T cells (*p* values, *t* test).



**Figure 4. *Mycobacterium bovis* BCG infection in C5<sup>-/-</sup> mice is associated with a decreased expression of T-box expressed in T cell (T-bet) transcription factor**  
 A-E: C5<sup>+/+</sup> and C5<sup>-/-</sup> mice were immunized with 10<sup>6</sup> CFU of BCG i.p. and spleens harvested 14 days later. CD3 purified T cells were overlaid on naïve macrophages or those infected with BCG and 4 hours later, T cells were stained for intracellular expression of T-bet in CD4 T cells using fluorescent antibodies and flow cytometry. A: T cells do not up regulate T-bet when overlaid on naïve BMs (panels B and C) and C5<sup>+/+</sup> but not C5<sup>-/-</sup> T cells show an up-regulation when cocultured with BCG infected BMs (panels D and E). F: Data generated from triplicate experiments (T cells from 3 mice each) show that C5<sup>-/-</sup> T cells from BCG immunized mice show a markedly reduced expansion of T-bet in CD4 T cells compared to C5<sup>+/+</sup> T cells from BCG immunized (p values, *t* test)



**Figure 5. Decreased expansion of IFN $\gamma$  positive T cells in C5<sup>-/-</sup> mice during experimental tuberculosis is associated with decreased T-bet and increased susceptibility to tuberculosis** Mice were infected via aerosol with 100 CFU of Mtb-Erdman and on day 21 post-infection, lungs and spleens harvested for colony counts of Mtb on 7H11 agar. A: Spleens and lungs of C5<sup>-/-</sup> mice contain larger number of organisms compared to C5<sup>+/+</sup> mice (n=3 mice per group, p values, ANOVA; 4 mice per group on day 21). B: Splenic and lung derived T cells from mice collected on day 21 post infection were stained for intracellular expression of T-bet and IFN $\gamma$  using flow cytometry and three colored antibodies. C5<sup>-/-</sup> T cells show markedly reduced IFN $\gamma$  and T-bet levels in CD4 T cell population compared to CD4 T cells from C5<sup>+/+</sup> mice (p values, *t* test). No significant differences in T-bet IFN $\gamma$  were found among CD8 T cells (not shown). C: Histograms illustrate the staining pattern for T-bet for T cells from lungs of Mtb infected C5<sup>-/-</sup> and C5<sup>+/+</sup> mice on day 21 post infection.