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# TNFRp75-dependent immune regulation of alveolar macrophages and neutrophils during early *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG infection

Avril Walters,<sup>1</sup> Roanne Keeton,<sup>1</sup>  
Antoinette Labuschagné,<sup>1</sup>  
Nai-Jen Hsu<sup>1</sup> , and  
Muazzam Jacobs<sup>1,2,3</sup> 

<sup>1</sup>Division of Immunology, Department of Pathology and Institute of Infectious Disease and Molecular Medicine, Faculty of Health Sciences, University of Cape Town, Observatory, South Africa, <sup>2</sup>National Health Laboratory Service, Johannesburg, South Africa and <sup>3</sup>Immunology of Infectious Disease Research Unit, University of Cape Town, Observatory, South Africa

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Correspondence: Nai-Jen Hsu and Muazzam Jacobs, Division of Immunology, Department of Pathology, Faculty of Health Sciences, Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Observatory 7925, South Africa.  
Emails: nai-jen.hsu@uct.ac.za (N.J.H.); muazzam.jacobs@uct.ac.za (M.J.)

## Summary

TNF signalling through TNFRp55 and TNFRp75, and receptor shedding is important for immune activation and regulation. TNFRp75 deficiency leads to improved control of *Mycobacterium tuberculosis* (*M. tuberculosis*) infection, but the effects of early innate immune events in this process are unclear. We investigated the role of TNFRp75 on cell activation and apoptosis of alveolar macrophages and neutrophils during *M. tuberculosis* and *M. bovis* BCG infection. We found increased microbicidal activity against *M. tuberculosis* occurred independently of IFN $\gamma$  and NO generation, and displayed an inverse correlation with alveolar macrophages (AMs) apoptosis. Both *M. tuberculosis* and *M. bovis* BCG induced higher expression of MHC-II in TNFRp75<sup>-/-</sup> AMs; however, *M. bovis* BCG infection did not alter AM apoptosis in the absence of TNFRp75. Pulmonary concentrations of CCL2, CCL3 and IL-1 $\beta$  were increased in TNFRp75<sup>-/-</sup> mice during *M. bovis* BCG infection, but had no effect on neutrophil responses. Thus, TNFRp75-dependent regulation of mycobacterial replication is virulence dependent and occurs independently of early alveolar macrophage apoptosis and neutrophil responses.

**Keywords:** TNF; macrophage; neutrophil; TNF receptor; apoptosis; *Mycobacterium tuberculosis*; BCG.

## INTRODUCTION

Tuberculosis (TB) is the leading cause of mortality worldwide from a single infectious agent, *Mycobacterium tuberculosis* (*M. tuberculosis*), and is primarily spread by the aerosol route of infection.<sup>1</sup> Upon inhalation, *M. tuberculosis* can infect different subsets of mononuclear phagocytes in the lung, such as macrophages, neutrophils and dendritic cells (DCs). Although host macrophages are predominantly the cells targeted for initial infection,

studies suggest that macrophages of different lineages, for example alveolar macrophages (AMs), interstitial and infiltrating macrophages, contribute differently to immune function and disease control.<sup>2–4</sup> Following bacilli uptake, these macrophages are capable of producing reactive nitrogen species and reactive oxygen species (ROS), which generate a hostile environment to kill intracellular pathogens.<sup>5–7</sup> In addition, apoptosis of infected macrophages plays an important role in host defence, by preventing *M. tuberculosis* dissemination.<sup>8–10</sup> However,

Abbreviations: AM, alveolar macrophage; BALF, bronchoalveolar lavage fluid; BCG, Bacillus Calmette-Guerin; BMDM, bone marrow-derived macrophage; CCL, CC chemokine ligand; CFU, colony-forming unit; DC, dendritic cell; GFP, green fluorescent protein; IL, interleukin; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; MHC, major histocompatibility complex; MOI, multiplicity of infection; *M. bovis*, *Mycobacterium bovis*; *M. tuberculosis*, *Mycobacterium tuberculosis*; NF $\kappa$ B, nuclear factor kappa B; OADC, oleic acid-albumin-dextrose-catalase; RIPK, receptor interacting protein kinase; TB, tuberculosis; TNF, tumour necrosis factor; TRADD, TNF receptor-associated protein with death domain; TRAF, TNF receptor-associated factor; WT, wild type

intracellular mycobacteria have developed the ability to subvert macrophage protective responses by producing antioxidants, interrupting phagosome maturation and reducing major histocompatibility complex class II (MHC-II) antigen presentation.<sup>11–14</sup>

Tumour necrosis factor (TNF) is a well-characterized cytokine known to have pleiotropic functions in cellular communication, immunity and different modes of cell death.<sup>15,16</sup> TNF is expressed as a transmembrane protein that can be cleaved into a soluble form. Both molecular forms can bind to either TNFRp55 (TNFR1, CD120a) or TNFRp75 (TNFR2, CD120b) through which it elicits different receptor signalling outcomes. TNFRp55 is expressed on all cell types and has a cytoplasmic 'death domain', which recruits receptor-interacting protein kinase (RIPK) and TNF receptor-associated protein with death domain (TRADD) that activate downstream pro-inflammatory signalling pathways or alternatively, facilitate signalling leading to cell death via apoptosis and necroptosis. In contrast, TNFRp75 lacks the cytoplasmic death domain and mainly exerts pro-survival effects. However, both TNFRs bind TNF receptor associating factors (TRAFs) and cellular inhibitor of apoptosis proteins (cIAP1 and cIAP2) that ultimately activate the transcription factor nuclear factor of kappa B (NFκB),<sup>16–18</sup> suggesting crosstalk between TNFRp55 and TNFRp75 in tightly regulated signalling cascades. In many cases, immune cells express both TNFRp55 and TNFRp75 that makes it difficult to dissect the TNF-mediated signalling cascades and to understand how both receptors are regulated.

Importantly, TNF signalling mediates inflammatory responses and contributes to host protection as well as immune-mediated pathology during *M. tuberculosis* infection.<sup>19</sup> In humans, primates and mice, TNF plays a critical role in containment of chronic and latent *M. tuberculosis* infection.<sup>20–24</sup> While TNF contributes to adaptive Th1 immunity to control chronic infection, TNF synthesis by macrophages and neutrophils is critical for early protection against *M. tuberculosis* demonstrated in the MLys-Cre TNF murine model.<sup>20</sup> In addition, macrophage and neutrophil expression of TNFRp55 is similarly important as MLys-Cre TNFRp55-deficient mice rapidly succumb to *M. tuberculosis* infection.<sup>25</sup>

Unlike the constitutive expression of TNFRp55, TNFRp75 expression is limited to endothelial cells, immune cells and certain central nervous system cell types.<sup>26,27</sup> Most of the previous studies investigating TNFRp75 functions focus on non-communicable diseases and autoimmunity that demonstrated therapeutic benefits in diseases such as cancer and rheumatoid arthritis.<sup>28,29</sup> Early in vitro studies have found that *M. tuberculosis* exploits TNFRp75 shedding to reduce TNF-mediated apoptosis for self-preservation<sup>8,10</sup>. In mice, we have identified the regulatory role of TNFRp75 in host protection

against *M. tuberculosis* through shedding and binding to TNF. We have also shown that TNFRp75 deficiency improves mouse survival and is associated with increased control of bacterial growth during acute and chronic stages of infection.<sup>30</sup> It is known that rapid initiation of effective localized pulmonary immune responses can ultimately improve disease outcome<sup>31–33</sup>; therefore, in the present study we sought to define TNFRp75-dependent immune regulation as part of the host defence during the earliest events of *M. tuberculosis* infection. As the initial responder cells to mycobacterial insult that may be functionally important in early TNFRp75-mediated immune responses, AMs and neutrophils were of particular interest to our study. Through aerosol transmission, we infected TNFRp75<sup>−/−</sup> mice with fluorescent *M. tuberculosis* and used multi-parametric flow cytometry to characterize localized AM activation and apoptosis in the alveolar space of the lung. Considering TNF-mediated apoptosis of infected AMs is dependent on mycobacteria virulence,<sup>8–10</sup> we also assessed the contribution of TNFRp75 to regulate innate immune responses at the onset of *M. bovis* BCG infection. Therefore, our study reveals the effects of TNFRp75 deficiency on host macrophages and neutrophils during early innate responses against mycobacteria.

## METHODS

### Mice

Mice used in this study were housed at the Research Animal Facility of the University of Cape Town under specific pathogen-free conditions. Both male and female mice between 6 to 12 weeks of age were used. C57BL/6 mice were used as wild-type (WT) control mice, and TNFRp75<sup>−/−</sup> (*Tnfrsf1b*<sup>−/−</sup>) mice were obtained from The Jackson Laboratory. All experiments were approved by the Research Animal Ethics Committee of the University of Cape Town (Reference Number: AEC 015/016) in accordance with the South African National Standard 10386 – *The care and use of animals for scientific purposes*.

### Mycobacteria

*Mycobacterium Bovis* Bacillus Calmette-Guerin-expressing green fluorescent protein (BCG-GFP) and *M. tuberculosis* (H37Rv-GFP and H37Rv) were cultured for 21 days in Millbrook 7H9 broth (Difco™, Becton, Dickinson and Company) containing 0.5% glycerol, 10% oleic acid-albumin-dextrose-catalase (OADC) (Becton, Dickinson and Company) and incubated at 37°C. An additional 50 µg/ml of hygromycin B (Roche Diagnostics) and kanamycin was added to the BCG-GFP and H37Rv-GFP broths, respectively. Aliquots were made and stored at −80°C. To determine the concentrations of the frozen stocks, 10-fold

serial dilutions were made after passing 30 x through a 29.5 G needle to eliminate clumping. Serial dilutions were plated on Middlebrook 7H10 agar (Difco™, Becton, Dickinson and Company) containing 10% OADC and antibiotics (50 µg/ml of hygromycin B or kanamycin), and incubated at 37°C for 21 days after which colony-forming units (CFUs) were enumerated.

### In vivo infection

For intranasal infection, anaesthetized mice were administered with 25 µl of inoculum into each nostril to give each mouse a total of  $1 \times 10^4$  CFU of mycobacteria. Aerosol infection was performed using a Glas-Col Inhalation Exposure System (Model A4224). The infection dose was verified by determining the bacterial burden of five mice 24 h post-infection. To determine pulmonary bacterial burdens, whole lungs were removed, homogenized in sterile PBS/0.04% Tween-80 and plated in 10-fold serial dilution on Middlebrook 7H10 agar with 10% OADC. Plates were incubated at 37°C for 19–21 days before CFUs were enumerated.

### Cells and infection

Primary bone marrow-derived macrophages (BMDMs) were cultivated from adult mouse femurs and maintained in RPMI medium (Sigma, St. Louis, MO) supplemented with 20% foetal calf serum (Gibco, Germany), 30% L929-conditioned medium, 10 mM L-glutamine (Gibco, Germany), 100 µg/ml Streptomycin and 100 U/ml Penicillin (Gibco, Germany) at 37°C with 5% CO<sub>2</sub>. After 8–10 days, confluent cells were seeded at a concentration of  $1 \times 10^5$  cells per well in a 96-well plate, and *M. tuberculosis* H37Rv were added at a multiplicity of infection (MOI) of 5:1 (bacillus to cell). To determine the ability of macrophages to control bacterial growth in vitro, BMDMs were pre-stimulated with or without 100 U/ml recombinant IFNγ for 24 h, then infected with H37Rv-GFP at 2:1 MOI for 4 h to 72 h. The amount of intracellular bacilli from the lysate of macrophages was determined by means of relative fluorescent unit (RFU) using a Modulus microplate fluorometer (Turner Biosystems Inc.). Culture supernatants were assayed for nitrite (NO<sub>2</sub><sup>-</sup>) concentrations using Griess reagent, and absorbance was recorded at 540 nm on SoftMax Pro (Molecular Devices Corporation).

For ex vivo experiments, the AMs were harvested from the bronchoalveolar lavage fluid (BALF) using fluorescently activated cell sorting (FACS, Becton Dickinson Aria flow cytometer). Neutrophils were isolated from the blood of naïve mice using magnetic cell sorting (Miltenyi Biotec). The cytospin slides of sorted AMs and neutrophils were Giemsa stained and analysed using a Nikon 90i Eclipse microscope. The sorted cells were then seeded at  $5 \times 10^4$  cells per well and incubated with BCG-GFP or

H37Rv-GFP at a MOI of 5:1. Neutrophils were stimulated for 4 h, whereas AMs were stimulated for 24 h and analysed using flow cytometry.

### Flow cytometric analysis

The flow cytometry antibodies were purchased from BD Pharmingen unless otherwise stated. Non-specific binding to cells was blocked through incubation with αFcγRIII (1 mg/ml of rat α-mouse CD32/16c). BMDMs were stained with FITC-conjugated anti-TNFRp55 (clone 55R-170, Santa Cruz) and PE-conjugated anti-TNFRp75 (clone TR75-89, Santa Cruz). BAL cells ex vivo and in vivo were labelled with fluorochrome-conjugated antibodies: anti-CD11b (clone M1/70), anti-CD11c (clone HL3), anti-Ly6G (clone 1A8), anti-SiglecF (E50-2440), BV711-conjugated anti-I-A/I-E-PE (M5/14.15.2) and PE-conjugated anti-Annexin V. The antibodies were used at 2 µg/ml per  $10^6$  cells. After washing with PBS, the cells were fixed in 2% paraformaldehyde and then analysed on a FACS Calibur (BD) using Cell Quest software (BD) or a FACS LSRII (BD) flow cytometer using FACS Diva version 6.0 (BD). Further analysis was performed on FlowJo software.

### ELISA

Supernatants of BMDM cultures were used for ELISA to assess the soluble TNFRp55 and TNFRp75 concentrations. BALF from the in vivo experiments was used to assess the CCL2, CCL3, IL-β, TNF and TNFRp55 cytokine concentrations. All ELISA reagents were purchased from R&D Systems and used according to the manufacturer's instructions. The absorbance was read at 405 nm using a VERSAmix Microplate Reader (Molecular Devices Corporation), and data were analysed using SoftMax Pro (Molecular Devices Corporation).

### Statistical analysis

Statistical analysis was performed by the Student's *t*-test or one-way ANOVA test with multiple comparisons using GraphPad Prism software. The data are presented as the standard error mean (SEM). For all tests, a *P*-value of ≤0.05 was considered significant.

## RESULTS

### TNFRp75 deficiency reduces TNFRp55 shedding in *Mycobacterium tuberculosis*-infected BMDM

Macrophages are considered the primary target cells of *M. tuberculosis*, which express and release both soluble TNFRp55 and TNFRp75 that regulate TNF-mediated activities including macrophage activation and survival in

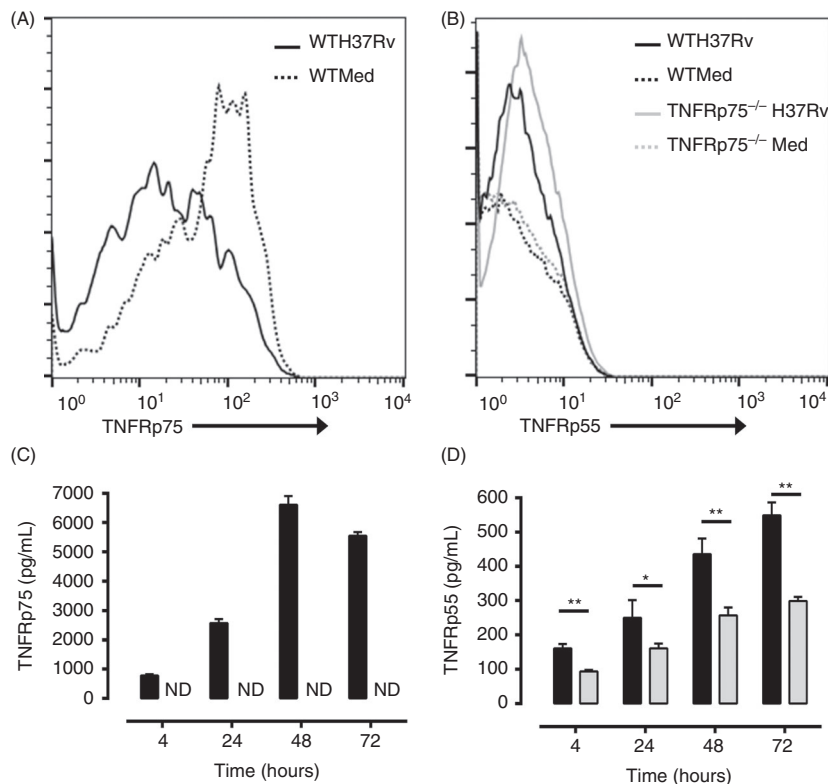
an autocrine fashion.<sup>8,16,34</sup> To study the effect of TNFRp75 in regulating macrophage functions, our initial experimental approach incorporated a bone marrow-derived macrophages (BMDM) culture system, which we and others have used extensively to study macrophage responses to *M. tuberculosis*.<sup>34,35</sup> We first confirmed constitutive expression of TNFRp75 in unstimulated WT BMDM (Figure 1A). Following 24 h of in vitro infection with H37Rv (MOI 5:1), TNFRp75 was subsequently released in WT BMDM cultures during *M. tuberculosis* infection (Figure 1A,C). As expected, no detectable soluble TNFRp75 was present in TNFRp75<sup>-/-</sup> BMDM cultures (Figure 1C).

Release of soluble TNFRp55 has been described to regulate macrophage activation and apoptosis during mycobacterial infection.<sup>34,36</sup> We have previously found that TNFRp75 deficiency is associated with the release of less TNFRp55 from DCs during *M. tuberculosis* infection.<sup>30</sup> To gain insight into the regulatory relationship between TNFRp75 and TNFRp55 in macrophages during *M. tuberculosis* infection, we analysed TNFRp55 expression in TNFRp75<sup>-/-</sup> macrophages subsequent to *M. tuberculosis* exposure. Flow cytometric analysis revealed

the upregulation of TNFRp55 surface expression on both *M. tuberculosis*-infected WT and TNFRp75<sup>-/-</sup> BMDMs (Figure 1B). Consistent with our previous observation in DCs, the release of soluble TNFRp55 was significantly less in TNFRp75<sup>-/-</sup> BMDM cultures compared to WT BMDM from 4 to 72 h post-infection (Figure 1D). The data therefore support a regulatory relationship between TNFRp55 and TNFRp75 expressed on macrophages in response to *M. tuberculosis* infection.

### TNFRp75 deficiency enhances microbicidal activity of BMDM independent of IFN $\gamma$ and NO

Next, to evaluate the effects of TNFRp75 on the efficiency of macrophages to phagocytose and control bacterial growth, WT and TNFRp75<sup>-/-</sup> BMDM were infected with H37Rv-GFP (MOI 2:1) for 4–72 h. The number of H37Rv-GFP bacilli was significantly higher in the TNFRp75<sup>-/-</sup> BMDM compared to WT BMDM at 4 h post-infection (Figure 2A) indicating a higher potential for rapid phagocytosis in the absence of TNFRp75. Notably, TNFRp75<sup>-/-</sup> BMDM displayed a higher rate of mycobacterial killing over the first 24 h with a 60%



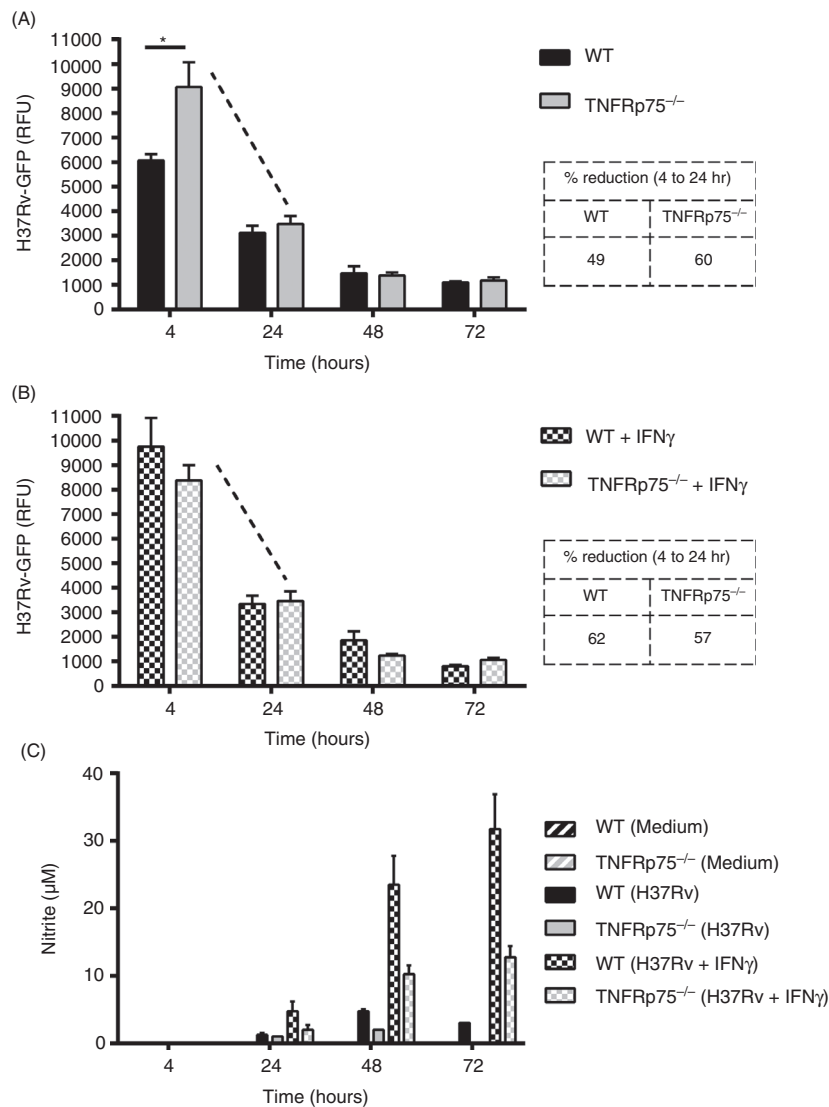
**Figure 1.** *Mycobacterium tuberculosis* infection induces TNFR shedding in BMDM. BMDMs were cultured from WT (black bar) and TNFRp75<sup>-/-</sup> mice (grey bar). Following 24 h of *M. tuberculosis* H37Rv (MOI 5:1) in vitro infection, flow cytometry analysis of surface expression of (A) TNFRp75 and (B) TNFRp55 in WT and TNFRp75<sup>-/-</sup> BMDM, and culture supernatants were analysed by ELISA for (C) TNFRp75 and (D) TNFRp55. Experiments were performed twice. The data shown as representative data are expressed as mean  $\pm$  SEM. Statistical significance (\* $P$  < 0.05; \*\* $P$  < 0.01). (ND = not detectable)



reduction in bacilli levels compared to WT BMDM that reduced bacilli levels by 49% over the same period. The amount of intracellular bacilli was comparable in both strains from 24 h to 72 h post-infection (Figure 2A).

Studies have shown that IFN $\gamma$  contributes to the activation of macrophages and their microbicidal functions, and synergizes with TNF for optimal *M. tuberculosis* growth control.<sup>37,38</sup> We treated WT and TNFRp75<sup>-/-</sup> BMDM with IFN $\gamma$  24 h prior to infection and as a result found similar uptake of H37Rv-GFP by both strains at 4 h post-infection. Moreover, IFN $\gamma$  pre-stimulation

increased the rate of microbicidal activity of WT BMDM equivalent to that observed for TNFRp75<sup>-/-</sup> BMDM with the percentage of H37Rv-GFP reduction increasing from 49% (Figure 2A) to 62% (Figure 2B). In contrast, IFN $\gamma$  stimulation did not further increase the initial uptake of bacilli by TNFRp75<sup>-/-</sup> BMDM or the rate of bacilli killing measured by bacilli reduction (Figure 2A,B). As part of the microbicidal activity induced by IFN $\gamma$  and TNF, activated macrophages release nitric oxide (NO) to kill intracellular bacilli within phagosomes.<sup>38</sup> To evaluate the functional impact of TNFRp75 on activated macrophages,



**Figure 2.** TNFRp75 deficiency increases control of *Mycobacterium tuberculosis* growth in BMDM. WT (black bar) and TNFRp75<sup>-/-</sup> BMDM (grey bar) cultures were infected with *M. tuberculosis* H37Rv-GFP at MOI of 2:1. The intracellular bacillary load was determined by GFP-microplate assay. (A) Relative fluorescent unit (RFU) was measured from 4 to 72 h. Dash line (--) represents bacilli reduction over 24 h. (B) RFU was also measured in BMDM cultures treated with recombinant IFN $\gamma$  for 24 h prior to H37Rv-GFP infection. (C) The levels of nitrite were determined over 72 h in culture supernatants from both IFN $\gamma$  pre-treated and untreated BMDM cultures after *M. tuberculosis* infection. No nitrite was detectable in medium control. Experiments were performed twice. The data shown as representative data are expressed as mean  $\pm$  SEM. Statistical significance (\* $P$  < 0.05)

we measured the levels of NO in the supernatant of WT and TNFRp75<sup>-/-</sup> BMDM cultures infected with H37Rv-GFP. Interestingly, TNFRp75<sup>-/-</sup> BMDM responded to *M. tuberculosis* with lower NO production when compared to WT BMDM over 72 h in vitro infection (Figure 2C). Interestingly, TNFRp75 deficiency dysregulated NO production in infected macrophages but did not impair its ability to control bacterial growth (Figure 2A). Although increased NO production was seen with IFN $\gamma$  pre-stimulation, the levels of NO production were consistently lower in TNFRp75<sup>-/-</sup> BMDM compared to WT BMDM over the experimental period (Figure 2C). Interestingly, this impaired capability of NO production in TNFRp75<sup>-/-</sup> BMDM is in agreement with the described observations of reduced NO production in TNFRp75-deficient myeloid-derived suppressor cells.<sup>39</sup> This suggests an independent role of TNFRp75 in inflammatory and immune regulatory responses. Overall, exogenous IFN $\gamma$  pre-stimulation enhanced macrophage function in the presence of TNFRp75 expression but was redundant for TNFRp75-deficient macrophage-mediated functions to control *M. tuberculosis* infection.

#### Enhanced microbicidal effects of TNFRp75<sup>-/-</sup> mice occurs independent of alveolar macrophage apoptosis during *Mycobacterium tuberculosis* infection

Macrophages of diverse lineages contribute differently to early immune responses,<sup>3,31,35</sup> particularly TNFRp75-mediated AM apoptosis which is considered beneficial to host defence.<sup>8,36</sup> In this study, we identified AMs as CD11b<sup>lo</sup>CD11c<sup>+</sup>SiglecF<sup>+</sup> cells,<sup>2,3</sup> which was confirmed microscopically by Giemsa staining (Figure 3A). TNFRp75<sup>-/-</sup> AMs and WT AMs from BALF of naïve mouse were sorted and infected ex vivo with H37Rv-GFP at a MOI of 5:1 for 24 h. We observed equivalent numbers of GFP<sup>+</sup> WT and TNFRp75<sup>-/-</sup> AMs in culture (Figure 3B), while the percentages of Annexin V<sup>+</sup> apoptotic AMs were also similar in both WT and TNFRp75<sup>-/-</sup> cultures (Figure 3C). Although the primary functions of AMs are not antigen presentation and activation of CD4<sup>+</sup> T cells, resident AMs express MHC-II molecules that can be interfered with by mycobacteria as an immune evasion strategy.<sup>12,40</sup> We analysed the surface expression of MHC-II in both WT and TNFRp75<sup>-/-</sup> AMs and flow cytometry analysis revealed similar expression levels in both strains (Figure 3D).

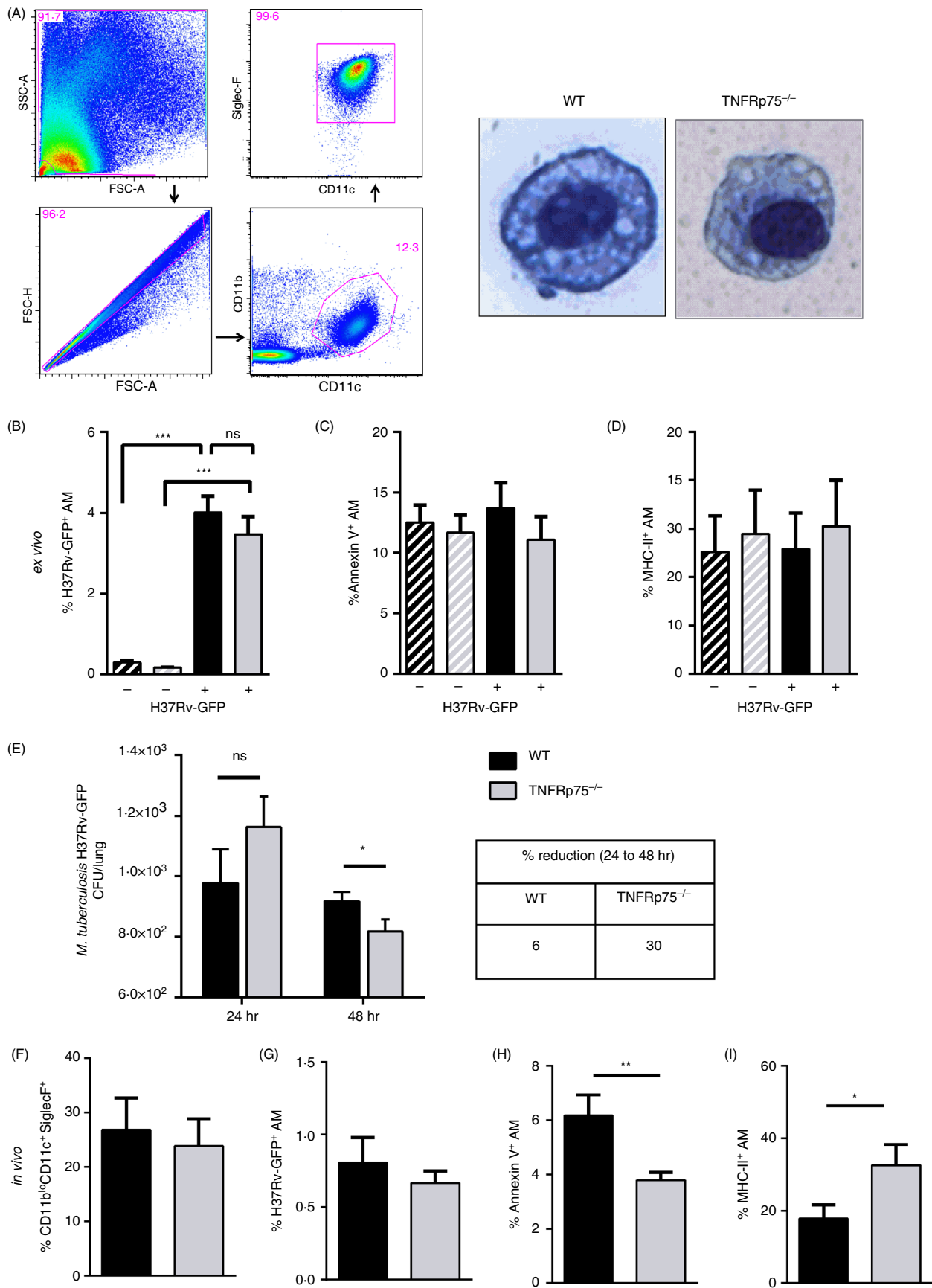
To determine whether our observations in culture had physiological relevance, we infected WT and TNFRp75<sup>-/-</sup> mice with  $1 \times 10^3$  CFU of *M. tuberculosis* H37Rv-GFP by aerosol inhalation and assessed the pulmonary bacterial burden at 24 h and 48 h post-infection. Enumeration of CFU revealed comparable mycobacterial uptake by WT and TNFRp75<sup>-/-</sup> mice after 24 h. A significantly lower bacterial burden was measured in TNFRp75<sup>-/-</sup> lungs

( $P < 0.05$ ; Figure 3E) following a 30% reduction after 48 h compared to 6% observed in WT mice. This suggested that TNFRp75 did not affect initial phagocytic events in the lung but may regulate intracellular bacterial replication. To explore the significance of TNFRp75 in AMs at the site of infection, BAL cells were collected from the H37Rv-GFP-infected mice and analysed by flow cytometry at 24 h post-infection. Measurement of the total number of AMs was similar between WT and TNFRp75<sup>-/-</sup> mice (Figure 3F). Since AMs are the primary phagocytes during initial events of *M. tuberculosis* infection,<sup>4</sup> the number of AMs containing GFP-positive bacilli was also analysed and found to be comparable in WT and TNFRp75<sup>-/-</sup> mice (Figure 3G). Shedding of TNFRp75 regulates TNF-dependent AM apoptosis,<sup>8</sup> and analysis of the number of apoptotic AMs showed it was significantly reduced in TNFRp75<sup>-/-</sup> mice ( $P < 0.01$ ; Figure 3H). We further observed that overall AM functionality was improved, as evident by the significantly higher number of MHC-II<sup>+</sup> AMs present in TNFRp75<sup>-/-</sup> mice ( $P < 0.05$ ; Figure 3I).

Thus, in accordance with our previous study,<sup>30</sup> the results show that TNFRp75 deficiency enhances early host protection during onset of *M. tuberculosis* infection. Deletion of TNFRp75 increases AM function but displayed an inverse correlation between microbicidal efficacy and apoptosis. Furthermore, the difference in observations from this in vivo challenge study (Figure 3C&D) compared to our ex vivo study findings (Figure 3H,I) indicates that the cellular environment contributes to the regulation of TNFRp75-facilitated apoptosis.

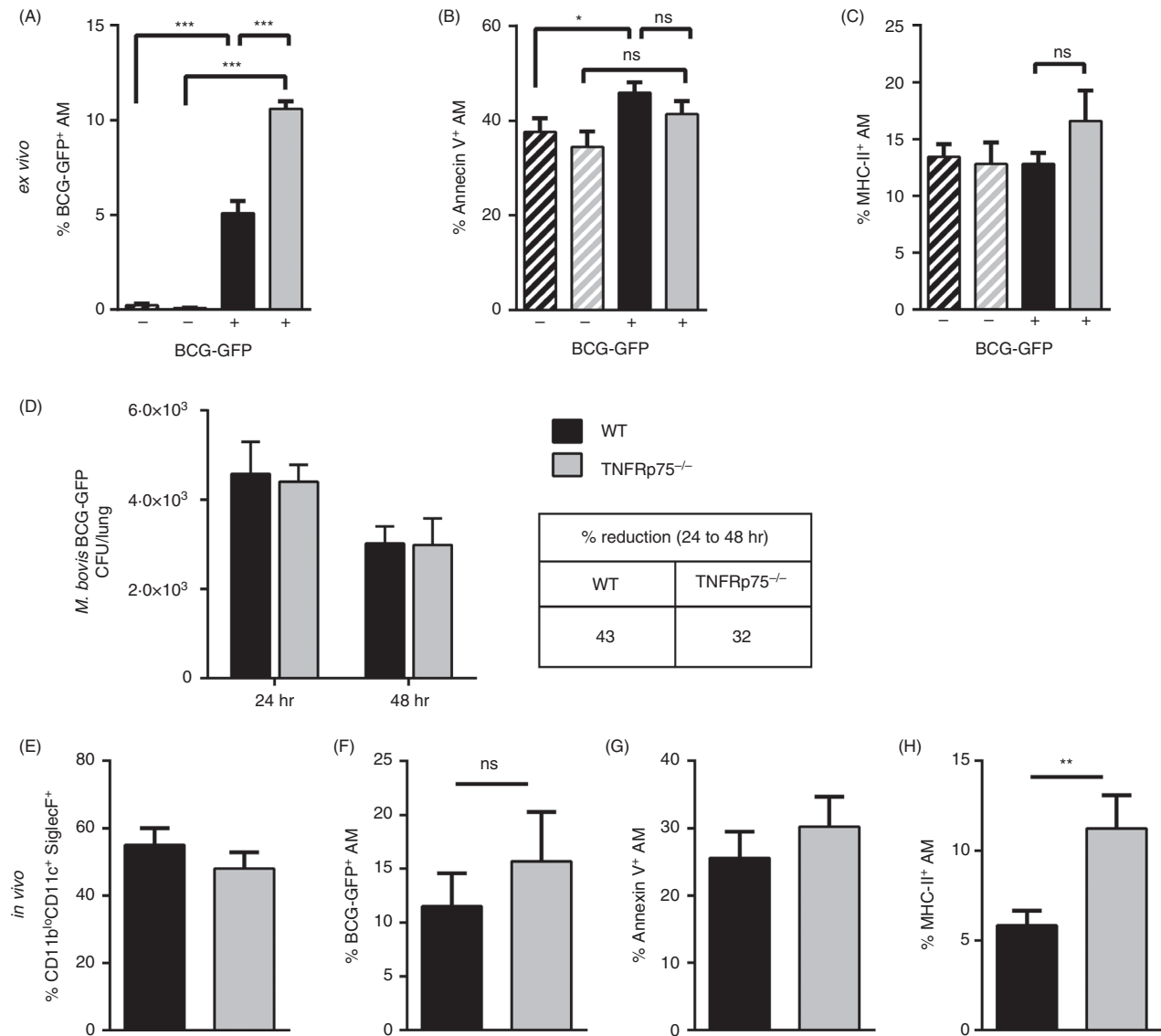
#### Alveolar macrophage apoptosis is unaltered in the absence of TNFRp75 during *M. bovis* BCG infection

Mycobacteria can induce shedding of TNFRp75 and induce apoptosis of alveolar macrophages in a virulence-dependent manner.<sup>8,36</sup> We have previously found that *M. bovis* BCG, unlike *M. tuberculosis*, is ineffective at inducing soluble TNFRp75 shedding that limits availability of bioactive TNF.<sup>30</sup> Here, we replicated experiments to investigate the effects of TNFRp75 deficiency in AMs using avirulent *M. bovis* BCG as the challenge agent. We wanted to determine whether there was a correlation between mycobacterial virulence and AM apoptosis in the absence of TNFRp75 expression. AM was sorted from the BALF of naïve WT and TNFRp75<sup>-/-</sup> mice and subsequently infected ex vivo with BCG-GFP at a MOI of 5:1 for 24 h. We measured a significantly higher percentage of GFP-positive TNFRp75<sup>-/-</sup> AMs compared to infected WT AMs ( $P < 0.001$ ; Figure 4A), indicating a permissiveness towards infection in the absence of TNFRp75, while similar percentages of Annexin V<sup>+</sup> and MHC-II<sup>+</sup> AMs were detected in the two strains (Figure 4B,C).





**Figure 3.** TNFRp75 deficiency protects early *Mycobacterium tuberculosis* infection and enhances alveolar macrophage response in vivo. (A) fluorescence-activated cell sorting of CD11b<sup>lo</sup>CD11c<sup>+</sup>SiglecF<sup>+</sup> cells from BALF of WT and TNFRp75<sup>-/-</sup> mouse. The sorted AMs were Giemsa stained. Ex vivo experiments were performed with *M. tuberculosis* H37Rv-GFP infection at a MOI of 5:1 and then analysed the composition of (B) GFP<sup>+</sup>, (C) Annexin V<sup>+</sup> and (D) MHC-II<sup>+</sup> AM by flow cytometry at 24 h post-infection. WT mice (black bar) and TNFRp75<sup>-/-</sup> mice (grey bar) were challenged by aerosol inhalation with  $1 \times 10^3$  CFU with *M. tuberculosis* H37Rv-GFP. (E) Bacilli burdens were enumerated in the lungs after 24- and 48-h infection. Flow cytometry analysis was performed to analyse the percentages of (F) CD11b<sup>lo</sup>CD11c<sup>+</sup>SiglecF<sup>+</sup> cells, as well as (G) GFP<sup>+</sup>, (H) Annexin V<sup>+</sup> and (I) MHC-II<sup>+</sup> AMs in the BALF of infected mice. The data shown are pooled from two experiments with six mice per group and are expressed as mean  $\pm$  SEM. Statistical significance (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; ns, not significant)



**Figure 4.** TNFRp75 deficiency enhances AM activation in vivo but does not affect disease progress. In ex vivo experiments, the AMs were sorted from BALF of WT (black bar) and TNFRp75<sup>-/-</sup> mouse (grey bar), and then infected with *M. bovis* BCG-GFP at a MOI of 5:1. The percentages of (A) GFP<sup>+</sup> (B) Annexin V<sup>+</sup> and (C) MHC-II<sup>+</sup> AMs were analysed by flow cytometry at 24 h post-infection. For in vivo studies, WT mice and TNFRp75<sup>-/-</sup> mice were challenged intranasally with  $1 \times 10^4$  CFU of *M. bovis* BCG-GFP. (D) Bacilli burdens were enumerated in the lungs after 24- and 48-h infection. Flow cytometry analysis was performed to analyse the percentages of (E) CD11b<sup>lo</sup>CD11c<sup>+</sup>SiglecF<sup>+</sup> cells, as well as (F) GFP<sup>+</sup>, (G) Annexin V<sup>+</sup> and (H) MHC-II<sup>+</sup> AMs in the BALF of infected mice. The data shown are pooled from at least two experiments with six mice per group and are expressed as mean  $\pm$  SEM. Statistical significance (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; ns, not significant)

To establish physiological relevance for outcomes observed in an avirulent challenge setting, WT and TNFRp75<sup>-/-</sup> mice were infected via intranasal inoculation with *M. bovis* BCG-GFP. We observed no difference in bacterial pulmonary burdens between WT and TNFRp75<sup>-/-</sup> mice after 24 h (Figure 4D), despite a tendency towards higher numbers of BCG-GFP<sup>+</sup> AMs recovered from the BALF of TNFRp75<sup>-/-</sup> mice (Figure 4F). Interestingly, we observed an increase of MHC-II<sup>+</sup> AMs present in TNFRp75<sup>-/-</sup> mice ( $P < 0.01$ ; Figure 4H), while comparable numbers of CD11b<sup>lo</sup>CD11c<sup>+</sup>SiglecF<sup>+</sup> cells and apoptotic AMs in WT and TNFRp75<sup>-/-</sup> mice were measured (Figure 4E,G).

Thus, in line with our previous study,<sup>41</sup> the results show that TNFRp75 is redundant to control *M. bovis* BCG infection. We found that AM responses including apoptosis are not altered during *M. bovis* BCG infection and highlight the influence of mycobacterial virulence on the effects of TNFRp75 to regulate AM responses.

#### TNFRp75 deficiency enhances neutrophil recruiting chemokines in response to *Mycobacterium bovis* BCG infection

While AMs phagocytose mycobacteria and limit intracellular bacterial growth, AMs also recruit other effector leukocytes and initiate inflammation by producing immune effector molecules,<sup>42</sup> such as monocyte chemo-attractant protein-1 (MCP-1, CCL2) and macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ , CCL3) known to recruit neutrophil and other leukocytes.<sup>43,44</sup> Pro-inflammatory cytokines, such as IL-1 $\beta$  and TNF, are not only known to control mycobacterial infection,<sup>19,45</sup> but also stimulate chemokine production by macrophages.<sup>46,47</sup> Therefore, to determine the effect of TNFRp75 to regulate inflammatory responses during avirulent challenge, we measured the concentrations of CCL2 and CCL3, as well as IL-1 $\beta$ , TNF and TNFRp55 in the BALF collected from BCG-GFP infected WT and TNFRp75<sup>-/-</sup> mice. We detected significantly increased ( $P < 0.05$ ) levels of CCL2 (Figure 5A) and CCL3 (Figure 5B) in the BALF of TNFRp75<sup>-/-</sup> lungs compared to WT mice following 24 h of infection. Further, significantly higher concentrations of pulmonary IL-1 $\beta$  were also measured in TNFRp75<sup>-/-</sup> mice ( $P < 0.05$ ; Figure 5C), whereas TNF concentrations (Figure 5D) were comparable. The regulation of TNF signalling between TNFRp75 and TNFRp55 is interdependent.<sup>16</sup> To determine whether TNFRp55 influences differences in chemokines expression observed between WT and TNFRp75-deficient strains, we measured soluble TNFRp55 concentrations in BALF of infected mice. Equivalent BALF concentrations (Figure 5E) indicated that increased chemokine expression occurred independently of soluble TNFRp55 regulation.

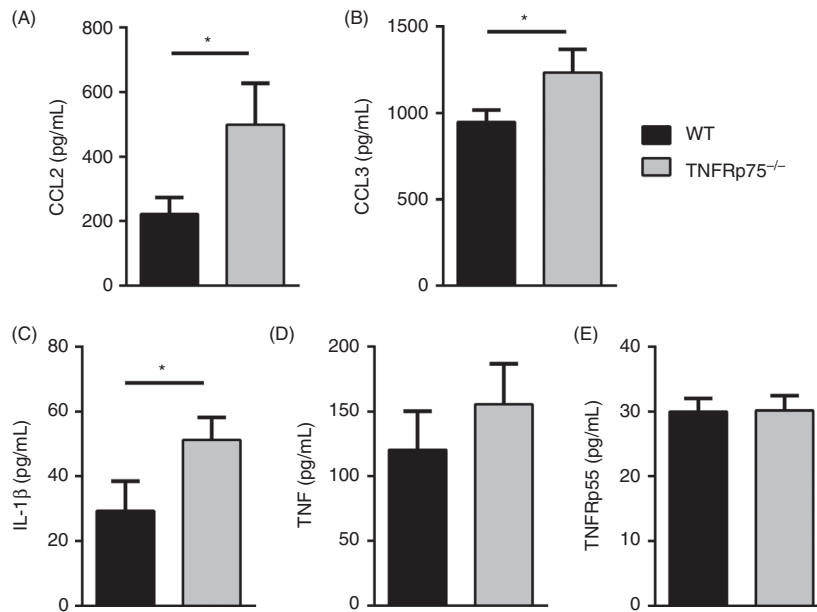
These data indicate that, in response to *M. bovis* BCG infection, TNFRp75 deficiency in the airways of BCG-infected lungs enhances the potential for preferential neutrophil recruitment through the upregulation of CCL2 and CCL3, as well as IL-1 $\beta$ , but not the production of TNF and TNFRp55.

#### TNFRp75 deficiency does not alter neutrophil responses to mycobacterial infection in vivo

Neutrophils are usually the first immune cell type to be recruited to the site of infection.<sup>48</sup> Since our data show TNFRp75 deficiency induced upregulation of neutrophil recruitment chemokines, we next examined the neutrophil influx following 24 h of intranasal BCG-GFP infection in WT and TNFRp75<sup>-/-</sup> mice. Neutrophils from BAL were identified as CD11b<sup>+</sup>Ly6G<sup>+</sup> via flow cytometric analysis and demonstrated with typical polysegmented nuclei by microscopic analysis (Figure 6A). We observed a tendency towards higher neutrophil recruitment in *M. bovis* BCG-GFP-infected TNFRp75<sup>-/-</sup> lungs compared to the WT strain (Figure 6B). Neutrophils can internalize, process and present antigen to T cells.<sup>49</sup> Measurement of the number of GFP<sup>+</sup> neutrophils (Figure 6C), the Annexin V<sup>+</sup> apoptotic neutrophils (Figure 6D) and MHC-II<sup>+</sup> neutrophils (Figure 6E) revealed it to be similar between the two strains. To consolidate what we observed in vivo, we sorted CD11b<sup>+</sup>Ly6G<sup>+</sup> cells from naïve mouse blood infected with BCG-GFP at a MOI of 5:1. The neutrophils are short-lived cells<sup>50</sup>; therefore, we performed flow cytometry analysis at 4 h post-infection and observed no difference in percentages of bacilli uptake (Figure 6F), apoptosis (Figure 6G) and MHC-II expression (Figure 6H) between WT and TNFRp75<sup>-/-</sup> neutrophils. Together, the data show that TNFRp75 deficiency does not alter the recruitment and functionality of neutrophils to the lungs during early *M. bovis* BCG infection.

## DISCUSSION

The establishment of early protection in *M. tuberculosis* infection, provided by innate immune cells, including resident alveolar and recruited macrophages, neutrophils and dendritic cells (DCs),<sup>2</sup> has beneficial effects on the eventual outcome of the disease.<sup>31</sup> Following infection, macrophages are the major cellular sources of inflammatory cytokines and chemokines to either enhance bacterial killing or activate inflammatory responses leading to granuloma formation. Among these, TNF-mediated host immunity to *M. tuberculosis* infection is well documented,<sup>19</sup> particularly its importance in granuloma formation and maintenance.<sup>20,22</sup> As the dominant receptor for TNF signalling, TNFRp55 is critical for host protection to *M. tuberculosis* infection.<sup>25,47</sup> Given the



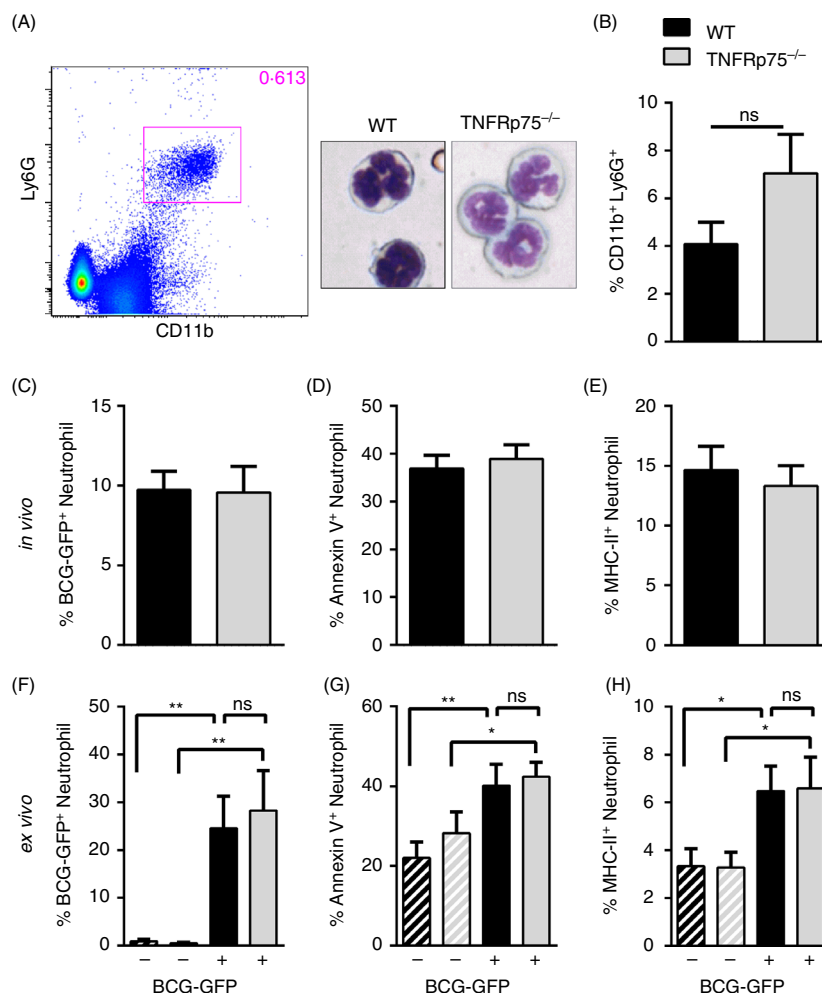
**Figure 5.** BCG infection increases chemokine production in TNFRp75-deficient mice. BALF from BCG-GFP-infected WT (closed bar) and TNFRp75<sup>-/-</sup> (grey bar) mice were analysed by ELISA for CCL2, CCL3, IL-1β, TNF and TNFRp55 at 24 h post-infection. The data are pool of two experiments with six mice per group. The data shown are expressed as mean ± SEM. Statistical significance (\**P* < 0.05) was determined by Student's *t*-test

therapeutic potential of TNFRp75 in cancer and autoimmunity,<sup>27,29,51</sup> the study on the immunological role of this receptor in infectious diseases is warranted. Previously, we have challenged TNFRp75<sup>-/-</sup> mice with *M. tuberculosis* and demonstrated better host survival associated with enhanced IL-12p40-dependent DC activation and overall Th1 immune responses.<sup>30</sup> The findings reported here demonstrates improved AM potential at initial onset of infection that leads to better host protection in TNFRp75<sup>-/-</sup> mice during *M. tuberculosis* challenge.

When *M. tuberculosis* enters the alveolar space, AMs act as host cells and initiate the first defence responses to combat intracellular bacilli. However, specific depletion of AMs improves bacterial clearance and subsequent protection from pulmonary tuberculosis.<sup>3,32</sup> This suggests that AM may provide a permissive niche for bacterial growth. AM apoptosis is considered a key mechanism to control mycobacterial replication and contributes significantly to host defence mechanisms. Previous in vitro studies have demonstrated that AM apoptosis is mediated by bioactive TNF, which can be attenuated by soluble TNFRp75 through *M. tuberculosis*-induced shedding.<sup>8</sup> In our earlier studies, we have also measured the levels of bioactive TNF in the BALF and shown that TNFRp75 shedding reduces bioavailability of TNF in *M. tuberculosis*-infected mice,<sup>34</sup> whereas deficiency of TNFRp75 increases bioavailability of TNF.<sup>30</sup> Therefore, we postulated that increased AM apoptosis to be a plausible mechanism to contribute

to enhanced pulmonary immune protection in TNFRp75<sup>-/-</sup> mice. Whereas annexin V staining revealed equivalent number of apoptotic AMs after ex vivo infection, on the contrary, in vivo animal infection studies showed less apoptotic AMs in the airway of *M. tuberculosis*-infected TNFRp75<sup>-/-</sup> mice, despite similar numbers of AMs recovered from both WT and TNFRp75<sup>-/-</sup> strains. This suggests that the effects of TNFRp75 on AMs in response to *M. tuberculosis* is dependent on the lung microenvironment rather than cell-intrinsic factors.

Alveolar macrophages reside in the alveolar space, a site of constant exposure to both harmless antigens and pathogenic micro-organisms; therefore, AM must have the plasticity to perform phagocytic functions without mounting excessive immune responses while rapidly reacting to pathogens in a pro-inflammatory manner. AMs in health express low level of MHC-II with a poor ability of antigen presentation and exhibit an anti-inflammatory phenotype to prevent undesirable inflammation in the lung.<sup>52</sup> However, AMs express high levels of integrin CD11c,<sup>40</sup> a phenotype similar to DC and serves as immune sentinels. Adoptive transfer studies have demonstrated the influence of the lung environment in upregulating CD11c expression and promoting DC-like features in macrophages.<sup>40</sup> Local immunoregulatory mechanisms are essential to tightly control AM activation when necessary, and studies with TNF have shown it as a key regulator in several transcription factor pathways that underlie immune responses to *M. tuberculosis*. In macrophages,

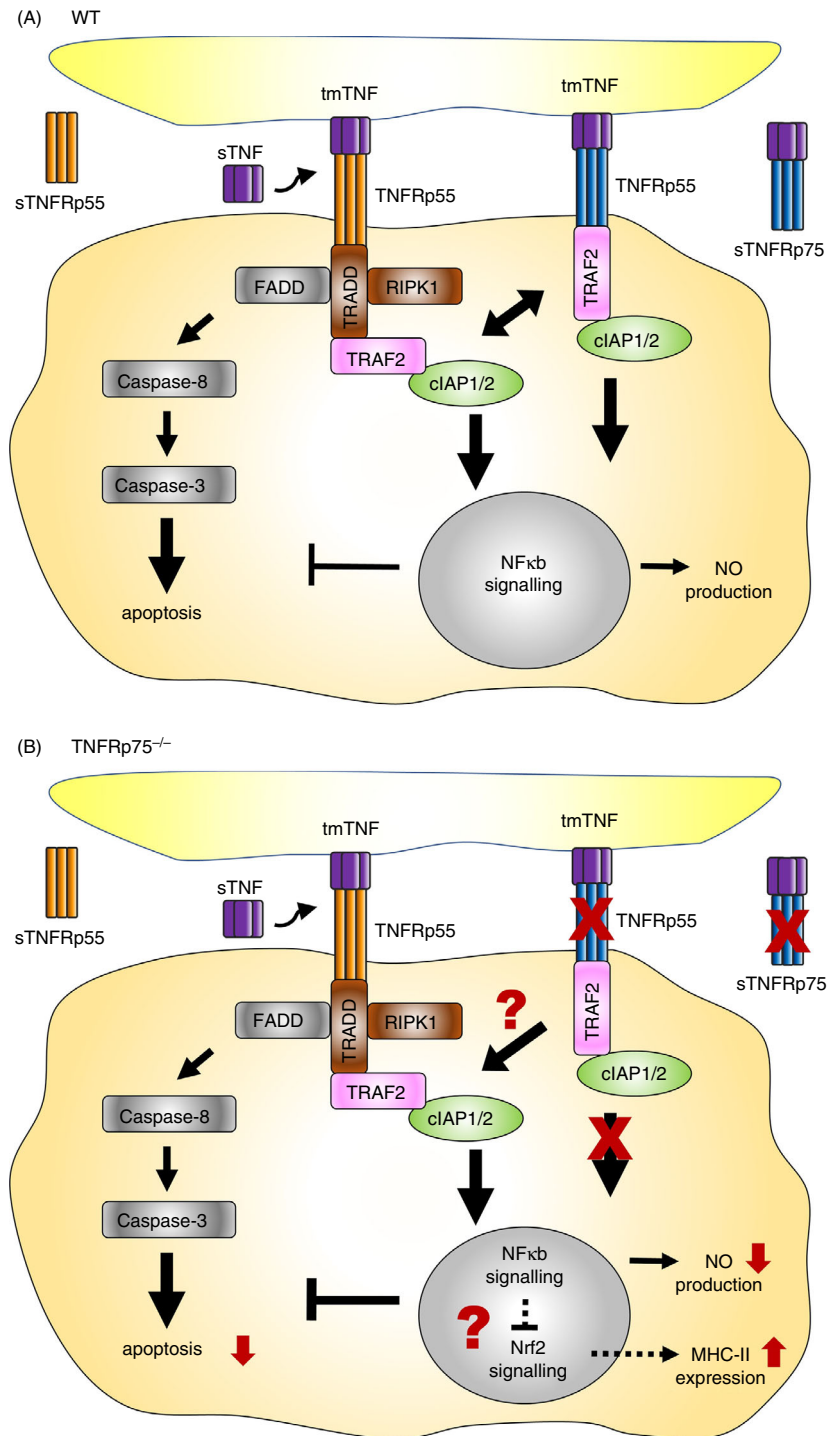


**Figure 6.** Neutrophil activity is not affected by mycobacterial virulence and TNFRp75 deficiency. (A) flow cytometry gating of CD11b<sup>lo</sup>CD11c<sup>+</sup>SiglecF<sup>+</sup> cells and microscopic examination of Giemsa stained neutrophils. For in vivo studies, WT mice (closed bar) and TNFRp75<sup>-/-</sup> mice (grey bar) were challenged intranasally with  $1 \times 10^4$  CFU of *M. bovis* BCG-GFP. After 24-h infection, flow cytometry analysis was performed to analyse the percentages of (B) CD11b<sup>+</sup>Ly6G<sup>+</sup> cells, as well as (C) GFP<sup>+</sup>, (D) Annexin V<sup>+</sup> and (E) MHC-II<sup>+</sup> neutrophils in the BALF of infected mice. In the ex vivo experiments, CD11b<sup>+</sup>Ly6G<sup>+</sup> neutrophils were sorted from naïve WT and TNFRp75<sup>-/-</sup> mouse blood, and infected with *M. bovis* BCG-GFP at a MOI of 5:1. The percentages of (F) GFP<sup>+</sup>, (G) Annexin V<sup>+</sup> and (H) MHC-II<sup>+</sup> AMs neutrophils were analysed by flow cytometry at 4 h post-infection. The data shown are pooled from at least two experiments with six mice per group and are expressed as mean  $\pm$  SEM. Statistical significance was determined by Student's *t*-test

TNF-mediated NF $\kappa$ B signalling induces cellular activation, inflammation and apoptosis,<sup>53</sup> whereas TNF-induced nuclear factor (erythroid-derived 2)-like 2 (Nrf2) signalling has antioxidative and anti-inflammatory response in TNFRp55-dependent manner.<sup>54</sup> Noteworthy, *M. tuberculosis* infection in cell-specific Nrf2 knockout mice has shown an increase of MHC-II expression in Nrf2-deficient AMs.<sup>35</sup> Since TNFRp55 and TNFRp75 compete for cytosolic components in signal transduction,<sup>16–18</sup> it is therefore plausible to infer from our findings that an upregulation of MHC-II expression in TNFRp75<sup>-/-</sup> AMs is attributed, in part, to the dysregulation of Nrf2 signalling (Figure 7). However, in contrary to the increased AM death in Nrf2-deficient mice,<sup>35</sup> we

observed a reduction of apoptotic TNFRp75<sup>-/-</sup> AMs in *M. tuberculosis*-infected mice, similar to the effects of NF $\kappa$ B activation to sustain macrophage viability.<sup>55</sup> Increasing evidence has demonstrated induction of Nrf2 to modulate TNF-mediated NF $\kappa$ B activation<sup>54</sup> that suggests a complex interplay and the possible balance of NF $\kappa$ B and Nrf2 pathways in the coordination of macrophage responses.<sup>56</sup> Therefore, future studies on the regulation of TNFR activity and intracellular molecular mechanisms controlling the TNF-induced NF $\kappa$ B and Nrf2 signalling in immune responses to *M. tuberculosis* infection are warranted.

Not all macrophages respond equally to *M. tuberculosis* infection. Following phagocytosis of *M. tuberculosis* by



**Figure 7.** Schematic diagram of proposed mechanisms affected by TNFR75 deficiency in alveolar macrophages during early *Mycobacterium tuberculosis* infection. (A) In the WT macrophages, TNF is expressed as a transmembrane molecule (tmTNF) and released to a soluble protein (sTNF). Both TNF forms are biologically active that can bind to either TNFRp55 or TNFRp75 leading to the canonical NFκB pathway or the apoptotic pathway. (B) In the TNFRp75<sup>-/-</sup> macrophages, there is no TNFRp75 to either modulate TNF activity or to deplete the cytosolic pool of adaptor protein TRAF2. It is therefore possible that exclusive stimulation of TNFRp55 mediated by TRAF2 aggregation consequently increases NFκB activation

AMs, the initiation of inflammatory responses is associated with recruitment of macrophages. Selective depletion of macrophage subsets by clodronate liposomes

demonstrates the differential significance of interstitial and recruited macrophages from AMs in response to *M. tuberculosis*.<sup>3,32,57</sup> Further transcriptional profiling revealed

distinct gene expression, which confirms their functional differences.<sup>3</sup> In our study, *in vitro* infection of BMDMs has demonstrated that the effects of TNFRp75 on macrophages in response to *M. tuberculosis* can be cell intrinsic, such that TNFRp75-deficient BMDM is more efficient independent of extrinsic IFN $\gamma$  activation. Also, the finding of reduced TNFRp55 shedding in TNFRp75<sup>-/-</sup> macrophages alludes to the complexity of TNF-TNFRs signalling mechanisms within macrophages. At the molecular level, the complex interplay between TNFRp55 and TNFRp75 signalling mechanisms has been identified.<sup>16</sup> Generally, TNF induces NF $\kappa$ B activation mainly through the TNFRp55 signalling complex consisting of TRADD, TRAF2 and cIAP, whereas the molecular mechanism of TNFRp55-induced caspase-mediated apoptosis is acting through FADD, which is indirectly linked to TRADD, and therefore, TRAF2-mediated recruitment of cIAP to TNFRp55 is a regulated process to selectively induce apoptosis. Although the formation of the TNFRp75 signalling complex lacks TRADD, it also recruits TRAF2 that reveals TNF receptors crosstalk and overlapping functions. TNFRp75 competes for the cytoplasmic TRAF2-cIAP complex and subsequently modulates TNFRp55-induced activation of the canonical NF $\kappa$ B pathway or the apoptotic pathway<sup>16,18,58</sup>; hence, the affinity of TRAF2 binding to either TNFR signalling complexes regulates the balance between cell survival and cell death. Although it is unclear from our study how TNFRp75 deficiency affects these cytoplasmic adaptor proteins and the downstream signalling events in *M. tuberculosis* infected mice, others have shown an inhibition of TRAF2 degradation in the TNFRp75<sup>-/-</sup> macrophage following TNF stimulation.<sup>59</sup> Therefore, we speculated in our TNFRp75<sup>-/-</sup> mouse model that the potential increase of cytosolic TRAF2 enhances TNFRp55 signalling and its subsequent NF $\kappa$ B-mediated anti-apoptotic function during *M. tuberculosis* infection (Figure 7).

Previous studies have found that mycobacterial virulence is an important factor for AM apoptosis; virulent strains reduce AM apoptosis compared to the less virulent strains.<sup>9,10</sup> One of the mechanisms driving these differences stemmed from bioactive TNF levels determined by TNF receptor shedding.<sup>8</sup> Our data further demonstrate that TNFRp75 does not affect AM apoptosis during low virulence BCG infection. This is in line with our previous reports where we demonstrated similar bioactive TNF levels in cultured WT and TNFRp75<sup>-/-</sup> dendritic cells due to low levels of TNF receptor shedding in response to low virulence BCG infection of DCs.<sup>30</sup> Further, *in vivo* BCG-GFP infection observations in TNFRp75<sup>-/-</sup> mice from this study are supported by our earlier work, which demonstrated that TNFRp75 is dispensable for protection against BCG infection with similar control of bacilli replication, granuloma formation and survival as WT mice.<sup>41</sup>

Increasing evidence suggests that inflammasomes, a group of multimeric protein complexes containing NLRP3 that activate IL-1 $\beta$  proteolytically in the caspase-1-dependent manner, play important roles in host immunity against mycobacteria.<sup>60</sup> Similar to TNF, IL-1 is a potent inflammatory cytokine implicated in host protection to *M. tuberculosis* infection; mice deficient in IL-1 $\beta$  or its receptor IL-1RI develop high bacterial burdens and mortality.<sup>61,62</sup> By using TNFRp55<sup>-/-</sup> and TNFRp75<sup>-/-</sup> peritoneal macrophages, others have shown that IL-1 $\beta$ -mediated antimicrobial effects involve caspase-3-induced apoptosis, which depends on TNFRp55 and, in part, TNFRp75. Interestingly, this antimicrobial effect of IL-1 $\beta$  is not due to apoptosis but rather mediated through efferocytosis<sup>63</sup>; engulfment of *M. tuberculosis*-infected apoptotic cells by uninfected neighbouring cells restricts bacterial replication.<sup>64</sup> Studies showed that BCG was able to hinder inflammasome activation<sup>65,66</sup> that reduced the secretion of mature IL-1 $\beta$ . It was therefore interesting to observe elevated level of IL-1 $\beta$  in the BALF of BCG-infected TNFRp75<sup>-/-</sup> mice in this study. Another factor that has been implicated in IL-1 $\beta$  processing is caspase-8, which can be activated by cIAP1/2 depletion and subsequent RIPK1 kinase activation as a result of TNFRp55 and TNFRp75 stimulation.<sup>16,58</sup>

During bacterial infection, chemokine expression is of importance for recruiting cells from the periphery to the infected macrophages to form granulomas. Clinical studies have detected increased CCL2 and CCL5 (RANTES) in the BALF of active TB patients compared to healthy controls.<sup>43</sup> While CCL3 and CCL5 are capable of inhibiting intracellular bacterial growth in human AMs,<sup>67</sup> CCL2 participates in granuloma formation during *M. tuberculosis* infection despite its redundancy in overall protection.<sup>68,69</sup> In TNF-deficient mice, *M. tuberculosis* infection leads to a defective granulomatous response together with increased mRNA expression of CCL2 and CCL3.<sup>70</sup> Higher CCL2 and CCL3 secretion in TNFRp75<sup>-/-</sup> lung is believed to be one of the driving factors for the cellular recruitment to the lungs. However, disruption of the TNFRp75 gene did not alter AM or neutrophil accumulation in the lungs during initial events of infection. Interestingly, others have shown exacerbated pulmonary neutrophil influx in TNFRp75<sup>-/-</sup> mice challenged with *Micropolyspora faeni* antigens.<sup>71</sup> In BALF of CCL2-overexpressing mice, the numbers of AM are constitutively higher than the WT but did not increase in response to BCG infection. Although overexpression of CCL2 showed earlier neutrophil recruitment to the bronchoalveolar space, the overall neutrophil influx was only observed in the later phase of BCG infection.<sup>72</sup>

Therefore, our work provides evidence that TNFRp75 regulates early host protection during onset of infection. Although TNFRp75 is redundant in macrophage and neutrophil recruitment, we found TNFRp75 deficiency



had differential effects on the activation and cell death of alveolar macrophages during early *M. tuberculosis* and *M. bovis* BCG infection.

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## CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

## DATA AVAILABILITY STATEMENT

The corresponding authors can provide raw data of results on reasonable request.

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