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Eicosanoid pathways regulate adaptive immunity to *Mycobacterium tuberculosis*

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Abstract

The fate of infected macrophages has an essential role in protection against *Mycobacterium tuberculosis* by regulating innate and adaptive immunity. *M. tuberculosis* exploits cell necrosis to exit from macrophages and spread. In contrast, apoptosis, which is characterized by an intact plasma membrane, is an innate mechanism that results in lower bacterial viability. Virulent *M. tuberculosis* inhibits apoptosis and promotes necrotic cell death by inhibiting production of prostaglandin E₂. Here we show that by activating the 5-lipoxygenase pathway, *M. tuberculosis* not only inhibited apoptosis but also prevented cross-presentation of its antigens by dendritic cells, which impeded the initiation of T cell immunity. Our results explain why T cell priming in response to *M. tuberculosis* is delayed and emphasize the importance of early immunity.

Mycobacterium tuberculosis is one of the most important bacterial pathogens¹. The ability of *M. tuberculosis* to persist in people with apparently normal immune systems indicates that *M. tuberculosis* has developed strategies to avoid, evade and even subvert innate and adaptive immunity². Although macrophages excel at destroying the biological particles they engulf by phagocytosis, which include dead cells and bacteria, *M. tuberculosis* has evolved into a parasite of the intracellular milieu of macrophages, where it not only survives but also replicates in the naturally hostile environment of the phagosome^{3,4}. Intracellular pathogens such as *M. tuberculosis* have developed complex and multifactorial mechanisms by which to evade host defenses. One important strategy used by intracellular pathogens is subversion of the host cell's death pathways⁵. Several intracellular pathogens, including *Leishmania*, *Coxiella*, *Salmonella*, *Chlamydia* and *Yersinia*, induce host-cell apoptosis as a way of minimizing the inflammatory response and avoid detection^{6–8}. In some of these cases, the induction of host-cell necrosis, sometimes by pyroptosis, leads to lower bacterial viability and is beneficial to the host^{9,10}. Other acute bacterial pathogens, such as *Pseudomonas*, *Neisseria* and *Streptococcus*, are cleared by host-cell apoptosis, and these pathogens evade innate immunity by inhibiting apoptosis¹¹. Virulent strains of *M. tuberculosis* induce necrosis of both human and mouse macrophages, whereas attenuated *M. tuberculosis* strains or other nonpathogenic mycobacterial species generally do not^{12–14}. Virulent *M. tuberculosis* actively inhibits the induction of macrophage apoptosis¹⁵, consistent with the identification of *M. tuberculosis* mutants that induce apoptosis instead of necrosis^{16,17}. In

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addition, there is accumulating evidence that apoptosis, whether induced by the pathogen itself^{18,19}, pharmacologically²⁰ or by cytotoxic lymphocytes^{21,22}, results in lower viability of *M. tuberculosis*.

One strategy used by *M. tuberculosis* to avoid apoptosis is the subversion of host eicosanoid biosynthetic pathways. The attenuated *M. tuberculosis* strain H37Ra induces the production of prostaglandin E₂ (PGE₂), which protects the mitochondrial inner membrane and induces the repair of plasma membrane microdisruptions inflicted by the pathogen^{12,13}. These events protect the host macrophages against necrosis and instead promote apoptosis. In contrast, intracellular infection with the virulent H37Rv strain of *M. tuberculosis* induces the production of lipoxin A₄ (LXA₄), which inhibits cyclooxygenase 2 production and PGE₂ biosynthesis^{12,13}. In a PGE₂-poor microenvironment, macrophages cannot prevent the mitochondrial damage or repair the plasma membrane disruptions caused by *M. tuberculosis*, and this leads to necrosis^{12,13}. Virulent *M. tuberculosis* in pre-necrotic macrophages continues to replicate and to spread to uninfected macrophages after the cells are lysed. Thus, the balance of PGE₂ and LXA₄ regulates the relative amounts of apoptosis and necrosis after *M. tuberculosis* infection, with important functional consequences for innate control of the intracellular infection. The essential role of these host lipid pathways in innate immunity has been confirmed by genetic analysis of the susceptibility of zebrafish to *Mycobacterium marinum*²³.

The cross-talk between innate immunity and adaptive immunity in response to intracellular infection is essential, particularly in the case of pathogens that infect macrophages. This is certainly true for immunity to *M. tuberculosis*, which requires complex synchronization and cooperation between the various arms of the immune system. The regulation of cell death during *M. tuberculosis* infection should be considered in this context because as well as limiting bacterial replication, apoptotic macrophages seem to provide an important bridge to adaptive immunity. Dendritic cells (DCs) can acquire viral or bacterial antigens through the uptake of apoptotic vesicles derived from infected macrophages^{24–26}. Cross-presentation, initially described in the context of viral infection²⁶, seems to occur during *M. tuberculosis* infection and to be important for priming of CD8⁺ T cell responses. For example, the secA2 mutant of *M. tuberculosis*, which induces more apoptosis of infected macrophages *in vitro* and *in vivo*, is associated with enhanced antigen-specific CD8⁺ T cell immunity, presumably as a consequence of more cross-priming^{16,27}. Furthermore, purified apoptotic vesicles derived from bacillus Calmette-Guérin (BCG)-infected macrophages that are free of any viable bacteria can transfer mycobacterial antigens to human DCs, which can cross-present the antigens to human CD8⁺ and CD1-restricted T cells²⁸. Similarly, vaccination of mice with purified apoptotic vesicles is sufficient to stimulate T cell immunity against the mycobacterial antigen cargo contained in the vesicles and to elicit protective immunity to challenge with virulent *M. tuberculosis*²⁹.

The microbiological and immunological data outlined above support the ‘detour’ model³⁰, in which apoptotic blebs derived from cells infected with intracellular pathogens facilitate cross-presentation of microbial antigens to CD8⁺ T cells. However, it is not known whether apoptosis of infected macrophages occurs *in vivo* and, if so, whether it has physiological importance. Because the supporting studies used apoptotic vesicles derived from BCG-infected macrophages^{28,29}, it remains unclear whether infection of macrophages with wild-type virulent *M. tuberculosis* would lead to apoptosis and enhanced T cell immunity or whether virulent *M. tuberculosis* prevents presentation of its antigens and inhibits initiation of adaptive immunity by other mechanisms. Finally, although immunization with purified apoptotic vesicles leads to cross-priming of antigen-specific T cells by DCs, it is not known whether the same mechanism occurs after infection with *M. tuberculosis in vivo*. Low-dose aerosol infection in mice is characterized by a delay in the initiation of T cell immunity^{31,32},

but it is unclear whether this delay is due to the inhibition of macrophage apoptosis by virulent *M. tuberculosis*. Although DCs are required to generate antigen-specific T cell responses³³, DCs can be directly infected by *M. tuberculosis*^{34,35}. *M. tuberculosis*-infected DCs in the lung traffic to the regional lymph node with kinetics that mirror those of T cell priming, consistent with the idea that *M. tuberculosis*-infected DCs prime *M. tuberculosis*-specific T cells directly. This indicates that there might be a priming pathway independent of the 'detour' pathway^{31,32,34}. Thus, the role of apoptosis and cross-priming in the generation of adaptive immunity during virulent *M. tuberculosis* infection remains unclear.

Macrophages from mice deficient in 5-lipoxygenase (*Alox5*^{-/-} mice), which cannot synthesize LXA₄, undergo more apoptosis after infection with virulent *M. tuberculosis*^{12,13}. Conversely, macrophages that lack prostaglandin E synthase (*Ptges*^{-/-} mice), which cannot produce PGE₂, undergo more necrosis after infection, even with avirulent *M. tuberculosis* strains. The greater resistance of *Alox5*^{-/-} mice³⁶ and the greater susceptibility of *Ptges*^{-/-} mice¹³ to infection with virulent *M. tuberculosis* led us to hypothesize that apoptosis and its regulation by eicosanoids are key determinants of innate and adaptive host resistance to infection. We have developed an adoptive-transfer model using *M. tuberculosis*-infected macrophages to determine whether inhibition of apoptosis by virulent *M. tuberculosis* leads to less cross-priming by DCs and to impaired T cell immunity. We find that by inhibiting apoptosis, virulent *M. tuberculosis* downregulated cross-presentation of *M. tuberculosis* antigens and impaired T cell responses.

RESULTS

Alox5^{-/-} mice have enhanced T cell responses

After being infected with *M. tuberculosis*, wild-type mice have lower expression of interleukin 12 (IL-12), interferon- γ (IFN- γ) and inducible NO synthase (iNOS) in their lungs than do *Alox5*^{-/-} mice, which suggests that lipoxins negatively regulate type 1 immunity to tuberculosis³⁶. To establish that the larger amounts of IL-12, IFN- γ and iNOS in *Alox5*^{-/-} mice infected with *M. tuberculosis* correlate with enhanced T cell immunity, we quantified the antigen-specific T cell response in wild-type, *Ptges*^{-/-} and *Alox5*^{-/-} mice after aerosol infection with virulent *M. tuberculosis*. At 2 weeks after infection, the CD8⁺ T cell response to the immunodominant antigen TB10.4 was greater in the draining lymph nodes of *Alox5*^{-/-} mice than in those of *Ptges*^{-/-} or wild-type mice (Fig. 1a). The frequency of TB10.4-specific CD8⁺ T cells was significantly higher (14%) in the lungs of *Alox5*^{-/-} mice infected with *M. tuberculosis* than in those of *Ptges*^{-/-} mice (4%) or wild-type (5%) mice (Fig. 1b). Moreover, splenic T cells from infected *Alox5*^{-/-} mice produced more IFN- γ after being stimulated with *M. tuberculosis* culture-filtrate proteins TB10.4(4–11) (a peptide of amino acids 4–11 of TB10.4, specific for CD8⁺ T cells) or Ag85B(241–256) (a peptide of amino acids 241–256 of Ag85B, specific for CD4⁺ T cells) than did cells from infected *Ptges*^{-/-} or wild-type mice (data not shown). Kinetic analysis of the TB10.4-specific CD8⁺ T cell response indicated that the response in the *Alox5*^{-/-} mice was earlier than that of wild-type or *Ptges*^{-/-} mice (Fig. 1c). At 5 weeks after infection, the *M. tuberculosis* antigen-specific CD8⁺ T cell response in the lung and spleen was similar in the various experimental groups, although the lung bacterial burden was significantly higher in *Ptges*^{-/-} mice and lower in *Alox5*^{-/-} mice than in wild-type mice ($P < 0.05$; Supplementary Fig. 1). This observation emphasizes the importance of early T cell immunity in protection against *M. tuberculosis* infection. Collectively, these data show that after *M. tuberculosis* infection, the CD4⁺ and CD8⁺ T cell response to *M. tuberculosis* was earlier and stronger in *Alox5*^{-/-} mice than in either wild-type mice or *Ptges*^{-/-} mice. Thus, LXA₄ negatively regulates T cell immunity to tuberculosis.

***Alox5*^{-/-} macrophages enhance CD8⁺ T cell responses**

To determine whether the enhanced T cell response in *Alox5*^{-/-} mice was due to an intrinsic property of *Alox5*^{-/-} T cells, we transferred splenic T cells from wild-type, *Ptges*^{-/-} or *Alox5*^{-/-} mice infected with *M. tuberculosis* into sublethally irradiated wild-type mice^{37,38}. At 1 d after T cell transfer, we infected the recipient mice with virulent *M. tuberculosis* by aerosol. Three weeks later, we detected no difference in colony-forming units (CFU) in the lungs (Supplementary Fig. 2) or spleens (data not shown) of the mice. Thus, the T cells of these mice had similar intrinsic capacity to protect against pulmonary infection with *M. tuberculosis*.

Because we found a greater T cell response early after infection in *Alox5*^{-/-} mice, we hypothesized that the initiation of T cell immunity differed in *Alox5*^{-/-} versus wild-type mice. To study the early events of T cell priming and to determine whether eicosanoids modulate initiation of T cell immunity, we developed an adoptive-transfer model using *M. tuberculosis*-infected macrophages. The model allows specific analysis of how lipid mediators produced by macrophages infected with *M. tuberculosis* affect T cell-mediated immunity independently of other eicosanoid-producing cell types.

We measured the migration of uninfected CD45.2⁺ macrophages in the airways and lung tissues at various time points after intratracheal adoptive transfer of these cells into CD45.1⁺ congenic mice. We detected the transferred macrophages for at least 7 d (Supplementary Fig. 3), which indicated that these cells can migrate into the lung tissue and persist. We next infected wild-type, *Ptges*^{-/-} and *Alox5*^{-/-} macrophages *in vitro* with *M. tuberculosis* using a low multiplicity of infection. We transferred the infected macrophages by the intratracheal route into wild-type recipient mice. We found that 17 d later, there were significantly more TB10.4-specific CD8⁺ T cells in both the thoracic draining lymph node and the lungs of mice that received *M. tuberculosis*-infected *Alox5*^{-/-} macrophages than in those of mice that received *M. tuberculosis*-infected wild-type or *Ptges*^{-/-} macrophages (Fig. 2a). Lymphocytes from the thoracic lymph nodes and lungs of mice that received *M. tuberculosis*-infected *Alox5*^{-/-} macrophages also secreted more IFN- γ after stimulation with *M. tuberculosis* antigens recognized by CD8⁺ T cells (TB10.4(4–11) and 32c(309–318)) or CD4⁺ T cells (Ag85B(241–256) and ESAT6(3–15); Fig. 2b). The enhanced T cell response after adoptive transfer of *Alox5*^{-/-} macrophages seemed to be the result of earlier initiation of the immune response, just as observed for intact *Alox5*^{-/-} mice (Fig. 1c). We detected the TB10.4-specific CD8⁺ T cell response earlier in the pulmonary draining lymph nodes and the lungs of the recipient mice after adoptive transfer of *M. tuberculosis*-infected *Alox5*^{-/-} macrophages than after wild-type macrophage transfer (Fig. 2c). Moreover, 4 weeks after adoptive transfer, the pulmonary bacterial burden was lower in wild-type mice that had received infected *Alox5*^{-/-} macrophages than in mice that had received infected wild-type or *Ptges*^{-/-} macrophages (Fig. 2d). At this time point, the number of TB10.4-specific CD8⁺ T cells in the lung and spleen was similar in the groups (Supplementary Fig. 4), which suggested that the early initiation of T cell immunity contributes substantially to protection against *M. tuberculosis*. The ability to recapitulate the early T cell response and lower pulmonary bacterial burden of *Alox5*^{-/-} mice produced solely by transfer of *M. tuberculosis*-infected *Alox5*^{-/-} macrophages into unmanipulated mice shows the important influence of eicosanoid production by infected macrophages on the regulation of T cell immunity and host resistance to pulmonary *M. tuberculosis* infection. Thus, the predominant production of PGE₂ by infected *Alox5*^{-/-} macrophages, which *in vitro* is the cause of more apoptosis, correlates with a greater T cell response during the early phase of infection *in vivo*.

***Alox5*^{-/-} alveolar macrophages enhance T cell responses**

We next investigated whether transfer of infected alveolar *Alox5*^{-/-} macrophages elicited an increased pulmonary T cell response. We obtained minimally manipulated infected alveolar macrophages by bronchoalveolar lavage 3 h after infection of wild-type or *Alox5*^{-/-} mice. We transferred the lavaged cells into naive wild-type mice via the intratracheal route. On day 17 after transfer of alveolar macrophages, we detected no TB10.4-specific CD8⁺ T cells in the lungs, pulmonary lymph nodes (PLNs) or spleens of mice that received infected wild-type alveolar macrophages (Fig. 3a). In contrast, we identified TB10.4-specific CD8⁺ T cells in all three tissues in mice that received infected *Alox5*^{-/-} alveolar macrophages. The percentage of CD8⁺ T cells specific for TB10.4(4–11) was significantly higher in the lungs and PLNs of mice that received infected *Alox5*^{-/-} alveolar macrophages than that in those of recipients of wild-type alveolar macrophages (Fig. 3b).

We measured the frequency of IFN- γ -secreting T cells in lymphocytes from lungs, PLNs and spleens of recipient mice stimulated with TB10.4(4–11) or 32c(309–318) (CD8⁺ epitopes) or with Ag85B(241–256) or ESAT6(3–15) (CD4⁺ epitopes). As noted after transfer of peritoneal macrophages (Fig. 2b), we detected a greater T cell response encompassing both CD4⁺ and CD8⁺ T cells after the transfer of infected *Alox5*^{-/-} alveolar macrophages than after the transfer of wild-type alveolar macrophages (Supplementary Fig. 5). Thus, similar to results obtained with thioglycollate-elicited peritoneal macrophages, the dysregulated production of prostanoids by alveolar macrophages is associated with the initiation of an earlier CD4⁺ and CD8⁺ T cell response.

***Alox5*^{-/-} macrophages enhance CD8⁺ T cell cross-priming**

We considered two potential mechanisms by which infected macrophages might affect T cell immunity: first, *Alox5*^{-/-} macrophages could directly prime naive T cells; and second, macrophage cell death could affect the cross-presentation of *M. tuberculosis* antigens by DCs. We first evaluated whether *Alox5*^{-/-} macrophages have a greater ability than other macrophages to stimulate *M. tuberculosis*-specific T cells *in vitro*. We pulsed wild-type, *Ptges*^{-/-} and *Alox5*^{-/-} macrophages with total *M. tuberculosis* antigens (*M. tuberculosis* sonicate), intact Ag85 protein or Ag85B(241–256) and found no difference in their ability to stimulate IL-2 production by the Ag85B-specific T cell hybridoma BB7³⁹ (Supplementary Fig. 6); this indicated that the intrinsic ability of these macrophages to process and present antigen was unaffected by PGE₂ synthase or 5-lipoxygenase.

To determine how infected *Alox5*^{-/-} macrophages elicit an enhanced T cell response, we evaluated the ability of *M. tuberculosis*-infected macrophages to prime CD8⁺ T cells. We used OT-I T cell antigen receptor-transgenic CD8⁺ T cells that were specific for the ovalbumin (OVA) epitope SIINFEKL and expressed the CD90.2 congenic T cell marker. We labeled these cells with the cytosolic dye CFSE (carboxyfluorescein diacetate succinimidylester) and transferred them into CD90.1⁺ congenic recipient mice. Then, 1 d later, we injected wild-type, *Ptges*^{-/-} or *Alox5*^{-/-} macrophages pulsed with SIINFEKL peptide and infected with *M. tuberculosis* into the footpads of wild-type mice. We analyzed T cells in the draining popliteal lymph nodes 4 d later. The proliferation and population expansion of OT-I T cells were significantly greater after the administration of *M. tuberculosis*-infected SIINFEKL-pulsed *Alox5*^{-/-} macrophages than after administration of similarly treated wild-type macrophages (Fig. 4a,b). After transfer of wild-type macrophages that had been pulsed with peptide and infected with *M. tuberculosis*, we detected only a small population of SIINFEKL-specific CD8⁺ T cells (~500 cells per lymph node). This was not substantially different from the number found in mice that received only OT-I cells, without macrophages. In contrast, we found an expanded population of OVA-specific T cells after transfer of *Alox5*^{-/-} macrophages that had been pulsed with SIINFEKL and

infected with *M. tuberculosis* ($\sim 1 \times 10^4$ cells per lymph node; 20-fold more than after transfer of wild-type macrophages treated similarly). We found no difference in OT-I T cell proliferation after the administration of wild-type or *Ptges*^{-/-} macrophages (data not shown).

Although the transfer of infected *Alox5*^{-/-} macrophages elicited a greater OT-I response than did the transfer of wild-type cells, we found no difference in OT-I T cell proliferation after the transfer of uninfected, SIINFEKL-pulsed *Alox5*^{-/-} or wild-type macrophages, with or without lipopolysaccharide (Supplementary Fig. 7). In addition, the transfer of uninfected, SIINFEKL-pulsed *Alox5*^{-/-} or wild-type macrophages that had been treated with the apoptosis-inducing agent staurosporine poorly stimulated OT-I proliferation (Supplementary Fig. 7). These data confirm that there is no intrinsic difference between *Alox5*^{-/-} and wild-type macrophages in their ability to stimulate T cell responses and instead support the idea that the greater apoptosis of *M. tuberculosis*-infected *Alox5*^{-/-} macrophages leads to more T cell priming than do wild-type or *Ptges*^{-/-} macrophages.

We next investigated whether the enhanced capacity of *Alox5*^{-/-} macrophages to prime antigen-specific T cells was due to direct presentation by the *M. tuberculosis*-infected *Alox5*^{-/-} macrophages or required cross-priming by DCs. We transferred infected macrophages and OT-I T cells into transgenic mice that expressed the diphtheria toxin receptor (DTR) fused to green fluorescent protein (GFP) under the control of the *CD11c* promoter (B6.FVB-Tg-Itgax-DTR/EGFP-57Lan/J, CD11c-DTR-transgenic mice). Administration of diphtheria toxin results in transient deletion of CD11c⁺ antigen-presenting cells (APCs; Supplementary Fig. 8). We adoptively transferred CFSE-labeled OT-I T cells into CD11c-DTR-transgenic mice and their non-transgenic littermates. After 24 h, we treated the mice with diphtheria toxin and transferred SIINFEKL-pulsed, *M. tuberculosis*-infected *Alox5*^{-/-} macrophages into them. The transfer of SIINFEKL-pulsed, infected *Alox5*^{-/-} macrophages into nontransgenic littermate controls treated with diphtheria toxin induced significant T cell population expansion in the popliteal lymph nodes 4 d after transfer, as described above (Fig. 4c). However, when we transferred SIINFEKL-pulsed, *M. tuberculosis*-infected *Alox5*^{-/-} macrophages into CD11c-DTR-transgenic mice treated with diphtheria toxin, we found only a small population of OT-I cells. This population was not significantly different from that in mice that had not received *Alox5*^{-/-} macrophages (Fig. 4c). Both the percentage and the total number of CD8⁺ T cells specific for SIINFEKL were much smaller after deletion of CD11c⁺ APCs (Fig. 4c), which indicated that the transferred *Alox5*^{-/-} macrophages were not sufficient to prime CD8⁺ T cells and that endogenous CD11c⁺ DCs were required.

To further investigate whether cross-priming was required for the response described above, we transferred CFSE-labeled OT-I T cells into mice deficient in TAP1, the transporter associated with antigen processing, or into mice deficient in β_2 -microglobulin, which lack mature major histocompatibility complex (MHC) class I molecules. In the absence of TAP1 or β_2 -microglobulin in recipient mice, OT-I T cell populations did not expand after transfer of SIINFEKL-pulsed, *M. tuberculosis*-infected *Alox5*^{-/-} macrophages. This observation shows that the population expansion of OT-I T cells after transfer of infected *Alox5*^{-/-} macrophages depended on the processing and presentation of SIINFEKL by the host-derived MHC class I pathway (Fig. 4d). Collectively, these data indicate that *M. tuberculosis*-infected *Alox5*^{-/-} macrophages enhance CD8⁺ T cell responses through DC-dependent cross-priming.

Macrophage apoptosis enhances T cell immunity

We next tested whether inhibition of apoptosis would reverse the greater T cell immunity seen after transfer of *M. tuberculosis*-infected *Alox5*^{-/-} macrophages. Infection of *Alox5*^{-/-}

macrophages with the virulent H37Rv strain of *M. tuberculosis* led to apoptosis, as reported before¹². The apoptosis of *M. tuberculosis*-infected *Alox5*^{-/-} macrophages was significantly inhibited *in vitro* by an inhibitor of caspase 8 and the extrinsic apoptotic pathway, by an inhibitor of caspase-9 and the intrinsic apoptotic pathway, and by a combination of both inhibitors (Fig. 5a).

We postulated that DC uptake and cross-presentation of the antigen cargo contained in apoptotic vesicles derived from *M. tuberculosis*-infected *Alox5*^{-/-} macrophages augments the T cell response *in vivo*. To test this possibility, we treated *M. tuberculosis*-infected *Alox5*^{-/-} macrophages with the inhibitors of caspase-8 and caspase-9 described above or with an inactive caspase-inhibitor control and subsequently transferred the cells by the intratracheal route into the lungs of uninfected wild-type mice. As expected, the transfer of *M. tuberculosis*-infected *Alox5*^{-/-} macrophages treated with the negative control peptide augmented the TB10.4-specific CD8⁺ T cell response, as described above (Fig. 5b). In contrast, the transfer of *M. tuberculosis*-infected *Alox5*^{-/-} macrophages treated with inhibitors of caspase-8 and caspase-9 did not induce early population expansion of TB10.4-specific CD8⁺ T cells in the lung (Fig. 5b). Thus, apoptosis of *M. tuberculosis*-infected macrophages is required for the early initiation of T cell immunity after pulmonary infection with *M. tuberculosis*.

DISCUSSION

Pulmonary macrophages are the primary hosts for the replication of *M. tuberculosis*. The ability of *M. tuberculosis* to inhibit macrophage antimicrobial defenses makes it a successful pathogen. The induction of apoptosis, including formation of the apoptotic envelope, is inhibited in macrophages infected with virulent *M. tuberculosis*^{14,40,41}. PGE₂ can prevent necrosis of *M. tuberculosis*-infected macrophages by protecting against mitochondrial inner membrane instability and promoting the repair of plasma membrane damage, two independent insults induced by virulent *M. tuberculosis*^{12,13}. By preventing necrosis and increasing apoptosis of *M. tuberculosis*-infected macrophages, PGE₂ enhances innate immune control of the initial infection both *in vitro* and *in vivo*¹². Here we have used an adoptive-transfer model in which macrophages were infected *in vitro* with *M. tuberculosis* and then transferred into recipient mice to address whether apoptotic macrophages contribute to the initiation of adaptive immunity. We found that the host lipid mediator PGE₂ was required for the induction of apoptosis in *M. tuberculosis*-infected macrophages, which in turn promoted T cell priming *in vivo*. An enhanced antigen-specific CD8⁺ T cell response required presentation of apoptotic macrophage-derived antigens by the 'detour' pathway, as defined by the TAP-1-dependent and MHC class I-restricted cross-presentation by DCs. In addition to CD8⁺ T cell responses, CD4⁺ T cell responses were also considerably enhanced after the transfer of proapoptotic *M. tuberculosis*-infected macrophages. We have shown that the adoptive transfer of proapoptotic *M. tuberculosis*-infected macrophages led to an early and robust antigen-specific T cell response that correlated with considerable pulmonary protection. Thus, virulent *M. tuberculosis* inhibits PGE₂ production and prevents macrophage apoptosis, which impairs both innate and adaptive immunity to *M. tuberculosis*.

One way that *M. tuberculosis* evades host defense mechanisms is by delaying the initiation of T cell priming^{31,32}. There is a longer delay between *M. tuberculosis* infection and the initiation of adaptive immunity than for other intracellular pathogens, including *Salmonella enteritica*⁴², *Listeria monocytogenes*⁴³, *Leishmania major*⁴⁴ and the influenza virus⁴⁵. In people infected with tuberculosis, the development of adaptive immunity requires 5–6 weeks (ref. 46); in mice, the earliest T cell response is detected 8–10 d after aerosol infection³¹. Virulent *M. tuberculosis* can disrupt the crosstalk between innate and adaptive immunity by altering macrophage and DC functions that are central for the initiation and

instruction of the immune response. The delayed dissemination of *M. tuberculosis* into the draining lymph nodes correlates with delayed initiation of T cell immunity in susceptible C3H mice³¹. Delayed onset of immunity has consequences for the control of bacterial replication. Transient depletion of DCs at the time of infection delays the initiation of T cell immunity by 7–10 d (ref. 32). Although the T cell response reaches normal levels within 3 weeks of infection, the mycobacterial burden remains higher than that in control, undepleted mice for at least 2 months³². Thus, even a transient delay in the initiation of adaptive immunity leads to long-term impairment of bacterial control. Given the important role of DCs in T cell priming and transport of *M. tuberculosis* from the lungs to the lymph nodes, it is likely that *M. tuberculosis* interrupts this process, either by delaying the acquisition of antigen by DCs (apoptosis inhibition) or by impairing DC trafficking (prevention of DC activation). The links between apoptotic macrophages, DC activation and early T cell immunity in the control of virulent *M. tuberculosis* infection require further elucidation.

Understanding how cellular death affects innate and adaptive immunity is important for elucidating disease pathogenesis. In non-infectious diseases such as autoimmunity, cell death through necrosis enhances T cell-mediated immunity to specific antigens because loss of plasma-membrane integrity and release of the cellular contents acts as an adjuvant. In contrast, apoptosis might prevent autoimmunity because apoptotic cells maintain the integrity of their cell membranes and are rapidly removed by phagocytes⁴⁷. During infection with *M. tuberculosis*, cellular necrosis and loss of plasma membrane integrity allow bacteria to infect other cells¹², whereas apoptosis of *M. tuberculosis*-infected cells enhances the innate control of intracellular bacterial replication as well as T cell-mediated immunity. Published studies have emphasized the importance of apoptosis in the cross-presentation of *M. tuberculosis* antigens by human DCs *in vitro* and the role of apoptotic vesicles purified from BCG-infected macrophages *in vivo*^{16,29}. However, the response of host macrophages to live *M. tuberculosis in vivo* has not been addressed.

We developed an infected macrophage adoptive-transfer model that allows study of how altering macrophage function in an otherwise normal host affects immunity to *M. tuberculosis*. Just as infection with proapoptotic bacterial mutants generates greater CD8⁺ T cell responses¹⁶, a propensity for the host macrophage to undergo apoptosis also enhanced T cell immunity and led to better protection. Enhanced T cell-mediated immunity was not restricted to CD8⁺ T cells. *M. tuberculosis*-specific CD4⁺ responses were also greater after the transfer of infected proapoptotic *Alox5*^{-/-} macrophages. Thus, whereas apoptosis directly increased CD8⁺ T cell responses by cross-presentation, it also enhanced MHC class II-restricted antigen presentation. Presumably, phagocytosis of apoptotic vesicles by DCs delivers their *M. tuberculosis* antigen cargo to the endocytic system, which intersects with the MHC class II processing pathway, ultimately increasing the priming of CD4⁺ T cells.

Whereas DCs are required for priming mycobacterial specific T cell immunity³³, the demonstration that DCs are directly infected by *M. tuberculosis* and facilitate the local dissemination of *M. tuberculosis* to regional lymph nodes³⁴ suggests that *M. tuberculosis*-infected DCs might be crucial for T cell priming. Our finding that intratracheal transfer of alveolar or peritoneal *M. tuberculosis*-infected *Alox5*^{-/-} macrophages led to earlier initiation of CD4⁺ and CD8⁺ T cell immunity suggests that the transfer of antigen to uninfected DCs is sufficient for T cell priming. The thioglycollate-elicited peritoneal macrophages used in our study here were activated and recruited into the peritoneal space, just as most macrophages found in the lungs of infected mice were recruited from the periphery. Because peritoneal macrophages can directly prime CD8⁺ T cells *in vivo*⁴⁸, we determined whether *M. tuberculosis*-infected macrophages directly prime T cells or whether their propensity to undergo apoptosis leads to the entry of mycobacterial antigens into the detour pathway. We found that infected *Alox5*^{-/-} macrophages could not directly prime T

cells and instead that T cell priming required endogenous DCs. A limitation of the CD11c-DTR model is macrophage toxicity owing to ingestion of diphtheria toxin from dying DCs, which can mask macrophage priming of T cells⁴⁹. However, by using mice deficient in TAP-1 or β_2 -microglobulin as recipients, we have demonstrated the importance of the endogenous MHC class I processing and presentation pathway. Similar to results obtained with DC depletion, we did not observe OT-I T cell population expansion when we administered proapoptotic *M. tuberculosis*-infected *Alox5*^{-/-} macrophages to mice deficient in TAP-1 or β_2 -microglobulin. These experiments showed that CD8⁺ T cell priming required cross-presentation of antigens acquired by DCs from apoptotic, *M. tuberculosis*-infected macrophages through the detour pathway.

Finally, we speculate that the proinflammatory function of apoptosis during infection, in contrast to its anti-inflammatory propensity during physiological cell death, might be a consequence of Toll-like receptor ligands and other microbe-derived signals that are contained in the apoptotic vesicles. Our data indicate that *Alox5*^{-/-} macrophages are not intrinsically more stimulatory than are wild-type macrophages, as cells of both genotypes processed and presented antigen to T cells *in vitro* and *in vivo* with similar efficiency. Instead, we found that apoptosis under proinflammatory conditions led to more efficient cross-priming. These results are in keeping with the idea that apoptotic cells do not stimulate primary immune responses unless there are additional danger signals, such as infection, that lead to inflammation by triggering Toll-like receptor or other pattern-recognition receptor signaling.

Despite the worldwide application of BCG vaccination and other anti-*M. tuberculosis* interventions, *M. tuberculosis* remains one of the most successful human pathogens. Eight to ten million new cases of active tuberculosis occur each year due in large part to the large reservoir of asymptomatic people chronically infected with *M. tuberculosis*¹. Estimates suggest that up to a third of the world's population is latently infected with *M. tuberculosis*, which indicates that *M. tuberculosis* can persist over the long term in humans¹. An understanding of the strategies that *M. tuberculosis* uses to evade host immunity is essential if an effective vaccine is to be developed⁵⁰. The work reported here has demonstrated that by inhibiting PGE₂ production and apoptosis, *M. tuberculosis* impairs T cell immunity. Moreover, we have established a direct link between apoptosis of *M. tuberculosis*-infected macrophages and T cell cross-priming by DCs *in vivo*. This improved mechanistic understanding might help in optimizing vaccination.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/natureimmunology/>.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Inhibition by 5-lipoxygenase of the early initiation of an immune response after *M. tuberculosis* infection. **(a,b)** Frequency of TB10.4(4–11)-specific CD8⁺ T cells in the thoracic draining lymph nodes **(a)** and lungs **(b)** of *Ptges*^{-/-}, wild-type (WT) and *Alox5*^{-/-} mice 2 weeks after *M. tuberculosis* infection. Numbers adjacent to outlined areas indicate percent CD8⁺ T cells stained with the TB10.4(4–11)-loaded H-2K^b tetramer (H-2K^b–TB10.4(4–11)). Data are representative of two independent experiments (mean of four to five mice per group). **(c)** Kinetics of the pulmonary TB10.4-specific CD8⁺ T cell response in *Ptges*^{-/-}, wild-type and *Alox5*^{-/-} mice. **P* < 0.05 (one-way analysis of variance (ANOVA)). Data are representative of two experiments (mean ± s.e.m. of four mice per group).

Figure 2.

Transfer of *M. tuberculosis*-infected *Alox5*^{-/-} macrophages into wild-type mice recapitulates the phenotype of intact *Alox5*^{-/-} mice. **(a)** Frequency of TB10.4(4-11)-specific CD8⁺ T cells in the thoracic draining lymph nodes (top) and lungs (bottom) 17 d after intratracheal transfer of *M. tuberculosis* H37Rv-infected *Alox5*^{-/-}, *Ptges*^{-/-} or wild-type macrophages into wild-type mice. Numbers adjacent to outlined areas indicate percent CD8⁺ T cells stained with H-2K^b-TB10.4(4-11). Data are representative of four independent experiments (mean and s.e.m. of 16 total mice per group). **(b)** Enzyme-linked immunospot assay of IFN- γ production showing the frequency of *M. tuberculosis*-specific CD4⁺ and CD8⁺ T cells 17 d after intratracheal transfer of *M. tuberculosis* H37Rv-infected *Ptges*^{-/-}, wild-type or *Alox5*^{-/-} macrophages into wild-type mice; splenic T cells from the wild-type recipient mice were cultured with APCs and Ag85B(241-256), ESAT6(3-15), TB10.4(4-11) or 32c(309-318). Data are representative of three experiments. **(c)** Kinetics of PLN and lung TB10.4-specific CD8⁺ T cell responses in recipient mice after transfer of infected *Ptges*^{-/-}, wild-type or *Alox5*^{-/-} macrophages. SFC, spot-forming cells. Data are representative of two experiments (mean $\pm$ s.e.m. of four mice per group). **(d)** Bacterial burden in the lung 28 d after intratracheal transfer of *M. tuberculosis* H37Rv-infected *Alox5*^{-/-}, *Ptges*^{-/-} or wild-type macrophages into wild-type mice. Each symbol represents an individual mouse; small horizontal bars indicate the mean. Data are from two independent experiments ($n = 4$ mice per group). NS, not significant; * $P < 0.05$, compared with wild-type (one-way ANOVA).

Figure 3.

Transfer of infected *Alox5*^{-/-} alveolar macrophages generates pulmonary and systemic CD4⁺ and CD8⁺ T cell responses. **(a)** Frequency of TB10.4(4-11)-specific CD8⁺ T cells in the lungs, PLN and spleen 17 d after intratracheal transfer of alveolar macrophages (MΦ) from wild-type mice (left) or *Alox5*^{-/-} mice (right) infected by aerosol with *M. tuberculosis* (virulent Erdman strain). Numbers adjacent to outlined areas indicate percent CD8⁺ T cells stained with H-2K^b-TB10.4(4-11) or streptavidin-phycoerythrin alone (Control; bottom row). Data are from one experiment. **(b)** Frequency of H-2K^b-TB10.4(4-11)-stained CD8⁺ T cells in the lungs, PLN and spleens of mice treated as described in a (*n* = 5 per group). Staining with isotype-matched control antibody (Isotype) is shown for the wild-type group only. **P* < 0.05 (one-way ANOVA with the Bonferroni post-test). Data are from one experiment (mean and s.e.m.).

Figure 4.

CD8⁺ T cell activation induced by *M. tuberculosis*-infected *Alox5*^{-/-} macrophages requires cross-presentation by CD11c⁺ DCs. **(a,b)** CD8⁺ T cell proliferation **(a)** and population expansion of cells positive for the H-2K^b-SIINFEKL pentamer **(b)** 5 d after intravenous transfer of CFSE-labeled Thy-1.2⁺ splenic OT-I CD8⁺ T cells into Thy-1.1⁺ B6.PL mice, followed within 24 h by *M. tuberculosis* H37Rv-infected wild-type or *Alox5*^{-/-} macrophages pulsed with SIINFEKL. Numbers above bracketed lines **(a)** indicate percent CFSE⁻ cells (left) or CFSE⁺ cells (right); numbers adjacent to outlined areas **(b)** indicate percent CD8⁺ T cells stained with H-2K^b-SIINFEKL. **(c)** Frequency of SIINFEKL-specific CD8⁺ T cells in the draining lymph nodes of CD11c-DTR-transgenic mice (CD11c-DTR) and nontransgenic littermate control mice (Non-TG) given intravenous injection of OT-I CD8⁺ T cells, followed by subcutaneous transfer of *M. tuberculosis* H37Rv-infected, SIINFEKL-pulsed *Alox5*^{-/-} macrophages and treatment with diphtheria toxin. No Mφ (right), control mice that did not receive macrophages. Numbers adjacent to outlined areas indicate percent CD8⁺ T cells stained with H-2K^b-SIINFEKL after 5 d. Data are representative of three experiments (mean and s.e.m. of four to five mice per group). **(d)** OVA-specific OT-I CD8⁺ T cells in draining lymph nodes of β₂-microglobulin-deficient (*B2m*^{-/-}), TAP-1-deficient (*Tap1*^{-/-}) or Thy-1.1⁺ recipient mice 5 d after the transfer of CFSE-labeled Thy-1.2⁺ splenic OT-I CD8⁺ T cells and *M. tuberculosis* H37Rv-infected, SIINFEKL-pulsed wild-type or *Alox5*^{-/-} macrophages. Data are representative of two to three independent experiments (mean ± s.e.m. of three to five mice per group). **P* < 0.05 (one-way ANOVA).

Figure 5.

Caspase-dependent apoptosis of *M. tuberculosis*-infected *Alox5*^{-/-} macrophages is required for the early initiation of T cell immunity. **(a)** Apoptosis of *Alox5*^{-/-} macrophages infected with *M. tuberculosis* H37Rv (multiplicity of infection, ~2), assessed 3 d after treatment for 2 h with a negative control peptide (Negative control) or with an inhibitor of caspase-8 or caspase-9 or both (0.5 μ M each), presented relative to that of uninfected *Alox5*^{-/-} macrophages (Untreated). **P* < 0.05, compared with control group (one-way ANOVA with Dunnett's multiple-comparison test). Data are representative of three experiments (mean and s.e.m.). **(b)** Frequency of TB10.4(4-11)-specific CD8⁺ T cells in the lungs of mice 17 d after the intratracheal transfer of *M. tuberculosis* H37Rv-infected *Alox5*^{-/-} macrophages treated with inhibitors of caspase-8 and caspase-9 (Both inhibitors) or a negative control peptide (Negative control); results (right) are normalized by division of the absorbance by the absorbance of cultures of uninfected macrophages. Numbers adjacent to outlined areas indicate percent CD8⁺ T cells stained with H-2K^b-TB10.4(4-11). **P* < 0.05 (Mann-Whitney test). Data are representative of two independent experiments (mean and s.e.m. of four mice per group).

Supplementary Figure 1. The immune response is similar in *Ptges*^{-/-}, wild type, and *Alox5*^{-/-} mice five weeks after *Mtb* infection. The frequency of TB10.4(4-11)-specific CD8⁺ T cells was determined five weeks after aerosol *Mtb* infection (~100 CFU) in the lungs **(a)** and the spleens **(b)** of *Ptges*^{-/-}, wild type, and *Alox5*^{-/-} mice. The percentage of CD8⁺ T cells that stained with the H-2 K^b-TB10.4(4-11) tetramer is indicated in each representative FACS plot. Each bar is the mean $\pm$ SE, of 5 individual mice. **(c)** The pulmonary bacterial burden in *Ptges*^{-/-}, wild type, and *Alox5*^{-/-} mice (n=5/group) five weeks after infection with aerosolized *Mtb*. Results are representative of two independent experiments. *, *p* < 0.05 by a one-way ANOVA compared to wild type group, or †, *p* < 0.05 *Alox5*^{-/-} vs. *Ptges*^{-/-}.

Supplementary Figure 2. The intrinsic ability of T cells from *Ptges*^{-/-}, wild type, and *Alox5*^{-/-} mice to provide protection against pulmonary *Mtb* infection is similar. **(a)** Sublethally irradiated wild type mice were used as recipients for splenic T cells (CD3⁺) from *Mtb* infected *Ptges*^{-/-}, wild type, or *Alox5*^{-/-} mice. Recipient mice were infected with *Mtb* by the aerosol route within 24 hrs after transfer of purified T cells. Three weeks after infection, the bacterial burden in the lung and spleen were determined. Each point represents data from an individual mouse, and the bars represent the mean (n=4-5 mice per group). The differences in lung CFU between *Ptges*^{-/-} and wild type, or wild type and *Alox5*^{-/-} mice were not significant (as determined by a one-way ANOVA). **(b)** Control experiment done under the same conditions as **(a)**. Recipient mice were irradiated and purified splenic T cells from *Mtb* infected syngeneic mice were transferred intravenously (WT). These were compared to control mice that did not receive T cells (No Tx). These two groups were infected as above and analyzed after three weeks. The difference in lung CFU was very significant as determined using a t-test (*p* = 0.0002).

Supplementary Figure 3. Adoptively transferred macrophages traffic into the lung. Uninfected CD45.2⁺ macrophages were transferred into CD45.1 recipient mice via the

intratracheal route. The presence of donor CD45.2⁺ macrophages was evaluated in the air space (by bronchoalveolar lavage) and lung tissue (after collagenase digestion) 6h, 24h, 48h, and 7 days after adoptive transfer.

Supplementary Figure 4. The frequency of TB10.4(4-11) specific CD8⁺ T cells in the lungs and spleens of wild type mice is similar 28 days after the intratracheal transfer of H37Rv-infected *Ptges*^{-/-}, wild type, or *Alox5*^{-/-} macrophages. Representative of FACS plots showing the H-2 K^b-TB10.4(4-11) tetramer staining of CD8⁺ T cells from the lung (top row) or spleen (bottom row) of recipient mice 4 weeks after infected macrophages transfer. The frequency of TB10.4(4-11)-specific CD8⁺ T cells is indicated in each plot and in the bar graph. Bars, mean $\pm$ SE bar graph of 4 mice per group.

Supplementary Figure 5. The frequency of *Mtb*-specific CD4⁺ and CD8⁺ T cells following adoptive transfer of *Mtb* infected alveolar macrophages from wild-type or *Alox5*^{-/-} mice into naïve wild type mice. Seventeen days after the adoptive transfer of *Mtb* infected alveolar macrophages from wild type or *Alox5*^{-/-} mice into wild type recipient mice, the frequency of *Mtb*-specific CD4⁺ and CD8⁺ T cells was enumerated using an IFN- γ elispot to measure the response to Ag85B(247-256), ESAT6(3-15), TB10.4(4-11), or 32c(309-318) synthetic peptides in the lungs, pulmonary lymph nodes (PLN), and spleens. Mice that received *Alox5*^{-/-} alveolar macrophages had larger IFN- γ responses to ESAT6 (CD4⁺ T cell epitope) and TB10.4 (CD8⁺ T cell epitope) in the PLN and spleen, respectively, at this early time point ($p < 0.05$, t-test). Statistical analysis of the other elispot responses is difficult because many of the mice that received wild type alveolar macrophages had no detectable response, and the *Alox5*^{-/-} alveolar macrophages group showed significant variability in their responses. To compare the magnitude of the response, recipient mice were categorized as responders or non-responders based on detection of IFN- γ secreting T cells. When all the possible responses were analyzed in aggregate, it was clear that transfer of infected *Alox5*^{-/-} alveolar macrophages induced a better response than elicited by infected wild type alveolar macrophages ($p < 0.0001$ by Fischer exact test).

Supplementary Figure 6. *Ptges*^{-/-}, wild type, and *Alox5*^{-/-} macrophages have a similar ability to process and present antigen. *Ptges*^{-/-}, wild type, and *Alox5*^{-/-} macrophages were pulsed with different concentration of sonicated *Mtb*, *Mtb*-culture filtrate proteins, Ag85 protein, or Ag85B(241-256) peptide, for 6 hrs. BB7 hybridoma cells were added to macrophages and IL-2 production was measured in the supernatants 24 hrs later. The ratio of the macrophages to T cells was 1:1. Values represent mean $\pm$ SE of triplicate samples.

Supplementary Figure 7. Wild type and *Alox5*^{-/-} macrophages have a similar capacity to enhance OVA-antigen specific T cell response in the absence of *Mtb* infection. (a) CD8⁺ T cell proliferation 5 days after IV transfer of CFSE-labeled Thy1.2⁺ splenic OT-I CD8⁺ T cells into Thy1.1⁺ B6.PL mice followed within 24 hrs by uninfected wild type or *Alox5*^{-/-} macrophages pulsed with SIINFEKL. (b) Purified CD45.1⁺ OT-I CD8⁺ T cells were injected IV into CD45.2⁺ C57BL/6 mice ($n=2-5$ /group). Within 24 hours, uninfected wild type or *Alox5*^{-/-} macrophages cultured with SIINFEKL (pOVA) and treated with LPS (100 ng/ml) or staurosporine (1 μ M) for 6 hrs. OT-I cells were identified as CD45.1⁺CD8⁺V β 5⁺V α 2⁺ cells. The frequency of OT-I cells in each draining LN is shown. Bars represent mean $\pm$ SE.

Supplementary Figure 8. *In vivo* depletion of CD11c⁺ cells. Three different doses of diphtheria toxin (DT) (25, 50, 100 ng/mouse) were administered by the IP route to CD11c-DTR TG and non-TG littermate mice. The frequency of CD11c⁺ cells were measured in the spleens 24 hours later. The majority of CD11c⁺ cells were depleted following 100 ng of DT compared to their littermate non-tg controls.