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Impact of Toll-Like Receptor 2 Deficiency on Immune Responses to Mycobacterial Antigens[▽][†]

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In the present study, we addressed the question of whether Toll-like receptor 2 (TLR2)-mediated innate immunity can contribute to the development of acquired immune responses. We immunized TLR2$^{-/-}$ and wild-type (WT) mice three times subcutaneously with the mycobacterial antigen (Ag19kDa) (a TLR2 ligand) or Ag85A (not a TLR2 ligand). One week after the last immunization, sera and spleens were collected. To evaluate cellular responses, we measured gamma interferon (IFN-γ) after *in vitro* restimulation of spleen cells with antigen alone or antigen-pulsed bone marrow-derived macrophages (BMM$_{Ag}$) or pulmonary macrophages (PuM$_{Ag}$). Antibody responses were comparable in the two mouse strains, but we observed differences in the cellular responses. Recall responses to Ag85A were similar in the two strains, but responses to Ag19kDa given alone or presented by BMM or PuM were lower in TLR2$^{-/-}$ than in WT mice. The largest differences in cellular responses were observed when Ag19kDa was presented by PuM. To understand this, we analyzed phenotypic and functional differences between BMM and PuM upon stimulation with various ligands. Generally, PuM had a lower response to the TLR2 ligand Pam$_3$Cys-Ser-(Lys)$_4$ trihydrochloride and to anti-CD40 than BMM, as measured by cytokine secretion and upregulation of costimulatory molecules. This might provide a partial explanation for the lower capacity of PuM when pulsed with Ag19kDa, also a TLR2 ligand. Altogether, our results revealed weaknesses in the T cell and antigen-presenting cell (APC) compartments of the Ag19kDa-immunized TLR2$^{-/-}$ mice but indicated that specific immune responses could be generated in the absence of TLR2 regardless of the characteristics of the antigen used.

Toll like receptors (TLRs) are pattern recognition receptors that recognize microbe/pathogen-associated molecular patterns, contribute to the activation of the innate responses (30), and are involved in the collaboration between the innate and the adaptive branches of the immune system (13, 24). Engagement of TLRs increases costimulatory molecule expression, enhances antigen (Ag) presentation, and induces proinflammatory cytokine production (24). TLRs are differentially expressed on hematopoietic and nonhematopoietic cells. Mononuclear phagocytes and dendritic cells express the widest TLR repertoires (21, 37). Moreover, in recent years, it has been shown that TLRs on human T cells act as costimulatory receptors and participate in the maintenance of T cell memory by enhancing proliferation and/or cytokine production by activated T cells (15, 17).

Mycobacterium tuberculosis contains many TLR ligands, among which TLR2 and TLR4 have been recognized as the most commonly involved in *M. tuberculosis*-mediated intracellular signaling (20, 32). The interaction of mycobacterial components with TLRs on macrophages may be a critical early step of macrophage activation and production of proinflammatory cytokines in a TLR-dependent manner. Mycobacterial cell wall components, such as the 19-kDa lipoprotein, the 26-kDa lipoprotein, and the lipoglycan lipoarabinomannan, bind to TLR2 and activate intracellular-signaling pathways, leading to the production of interleukin 12 (IL-12) and thus polarizing the immune system toward a Th1 type of response (4).

It has been suggested that TLR2 may be important for the control of mycobacterial infection. We have shown that deficiencies in TLR2 signaling increase susceptibility at an early stage of infection in the respiratory tract. This susceptibility was correlated with impaired proinflammatory responses measured as tumor necrosis factor (TNF) and gamma interferon (IFN-γ) production by lung mononuclear cells upon stimulation with various mycobacterial components (31). TNF is required for the control of mycobacterial growth and the formation of protective granulomas (10, 16), and IFN-γ is necessary for the activation of macrophages and generation of protective immunity (6, 35).

Vaccine antigens coupled with TLR agonists have been proven to be effective in increasing immune responses and protection (34, 38). In mouse models of tuberculosis (TB), vaccination with a mycobacterial fusion protein composed of a TLR2 agonist and early secretory antigenic target 6 (ESAT-6) exhibited enhanced T cell responses and protection (33).

A better understanding of the role of TLR2 in mycobacterial antigen recognition and presentation to T cells for the development of adaptive immune responses is needed for novel therapies and vaccines. In order to investigate these factors, we used two mycobacterial antigens, namely, the 19-kDa antigen (Ag19kDa), a known TLR2 agonist, and Ag85A, which is not

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considered to be a TLR2 agonist. Ag85A is one of the leading vaccine candidates already tested in preclinical and clinical studies and found to be promising for future TB vaccine development (11, 25). A number of studies (1, 2, 4, 19, 23) regarding the TLR2-mediated effects of Ag19kDa on phagocytes have been performed, but the role of Ag19kDa as an antigen has not been equally evaluated.

In this study, we sought to determine the contribution of TLR2-mediated innate immunity to the induction and maintenance of adaptive immune responses. A comparison between wild-type (WT) and TLR2$^{-/-}$ mice revealed that immunization with Ag85A or Ag19kDa induced antigen-specific humoral and cellular immune responses in both strains of mice. However, we found weaknesses in both the antigen-presenting cell (APC) and the T cell compartments of the TLR2$^{-/-}$ mice immunized with Ag19kDa, the TLR2 ligand. Recall responses to Ag85A were similar in the two strains, but responses to Ag19kDa given alone or presented by bone marrow-derived macrophages (BMM) or pulmonary macrophages (PuM) were lower in TLR2$^{-/-}$ mice than in WT mice. The largest differences were observed when Ag19kDa was presented by PuM. Due to the central role of PuM in the defense of the respiratory tract, we analyzed phenotypic and functional differences between BMM and PuM upon stimulation with various ligands. PuM were generally lower responders than BMM to some of the stimuli, both in the amount of cytokines produced and in upregulation of costimulatory molecules. The importance of these differences in APC/T cell collaboration is discussed.

MATERIALS AND METHODS

Animals. The studies were performed using 10- to 12-week-old WT C57BL/6 (Scanbur AB, Sweden) mice and TLR2$^{-/-}$ mice with a C57BL/6 background. Breeding pairs of the TLR2$^{-/-}$ mice, which had been backcrossed at least six times to C57BL/6 mice, were obtained from Karolinska Institute, Sweden, with the permission of S. Akira (Osaka University, Japan), and kept at the Animal Department at the Arrhenius Laboratories, Stockholm University, Sweden. All experiments were done in accordance with the ethical guidelines of Stockholm University. The mice were supervised daily, and sentinel mice were used to assess and ensure pathogen-free conditions in the facility.

Antigens, stimulatory molecules, and adjuvants. Recombinant *M. tuberculosis* antigens Ag85A and Ag19kDa were provided by Lionex Diagnostics and Therapeutics GmbH, Braunschweig, Germany. The recombinant proteins without signal sequence and with N-terminal His tags were expressed in and purified from *Escherichia coli*. Endotoxin contents were routinely determined and were in the range of 10 to 30 IU/mg of protein. Lipopolysaccharide (LPS) from *E. coli* was obtained from Sigma, St. Louis, MO; Pam₃Cys-Ser-(Lys)₄ trihydrochloride (Pam3) was obtained from Enzo Life Sciences, Lausen, Swizerland; and monoclonal anti-CD40 antibodies (Ab) were made from precipitated supernatant from the 1C10 hybridoma cell line. The adjuvant, cholera toxin (CT), was obtained from Quadratech Ltd., Surrey, United Kingdom.

Mycobacteria. *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) (Pasteur strain), obtained from A. Williams, Health Protection Agency, Salisbury, United Kingdom, was grown in Middlebrook 7H9 broth (Difco, Sparks, MD) supplemented with albumin-dextrose-catalase, 0.5% glycerol, and 0.05% (vol/vol) Tween 80. BCG was collected at the logarithmic phase of growth (absorbance, 1.0, measured as the optical density at 650 nm [OD_{650}]). Aliquots were frozen in phosphate-buffered saline (PBS) with 10% glycerol and kept at −70°C. Three vials picked randomly from the stock were thawed and serially diluted in plating buffer (PBS with 0.05% Tween 80), and CFU were counted 2 to 3 weeks after plating on Middlebrook 7H11 agar (Karolinska Hospital, Solna, Sweden) with glycerol, oleic acid-albumin-dextrose-catalase, and the antibiotics polymyxin B and amphotericin B.

Immunizations. WT and TLR2$^{-/-}$ mice were immunized subcutaneously (s.c.) in the dorsal neck region three times at 2-week intervals with Ag85A (10 μg/

animal) or Ag19kDa (10 μg/animal) formulated with CT (1 μg/animal) as an adjuvant.

Sample collection. Sera and spleens were collected 7 days after the last immunization. The mice were bled from the tail vein, and sera were collected after centrifugation of coagulated blood. The mice were sacrificed, and the spleens were removed aseptically and placed in sterile PBS. Single-cell suspensions from the spleens were prepared by teasing the tissue with sterile forceps and were allowed to stand for 10 min to settle debris. The supernatants were removed gently, and the cells were washed and enumerated on a hemocytometer. Thereafter, the cells were cultured in complete Dulbecco's modified Eagle's medium (DMEM) or RPMI medium containing 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM sodium pyruvate (all from Invitrogen, Paisley, United Kingdom), and 0.05 M 2-mercaptoethanol (Sigma).

Generation of BMM and PuM. BMM were generated as previously described (28). Briefly, after mouse sacrifice, the femur and tibia of the hind legs were removed. The bone marrow cavities were flushed with cold sterile PBS, followed by washing and resuspension of the cells in complete DMEM or RPMI medium supplemented with 20% L929 cell conditioned medium (as a source of macrophage colony-stimulating factor). Bone marrow cells were plated in cell culture plates and incubated for 7 days at 37°C and 5% CO_2, replacing the medium every second day. Before use, BMM were washed and maintained in complete DMEM without L929 cell conditioned medium for 24 h before use as APCs.

PuM were prepared from C57BL/6 mice using the protocol of Corti et al. with some modifications (7). Dispase (Gibco-Invitrogen, Paisley, United Kingdom) was instilled into the lungs via the trachea, and the lungs were removed and incubated in a dispase-containing solution for 45 min at room temperature. The parenchymal tissue was carefully teased apart, and the cell suspension was treated with DNase I (Sigma). The cells were passed through 70- and 40-μm nylon cell strainers (BD Falcon). After treatment with red blood cell lysing buffer (Sigma), CD45$^+$ cells were isolated using magnetic associated cell sorting (MACS) and LC columns according to the manufacturer's protocol (Miltenyi   AQ: C Biotec, Bergisch Gladbach, Germany). After incubation for 48 h, the cells were washed to remove nonadherent cells and debris. On average, 98% of the cells were positive for the F4/80 macrophage marker, as determined by flow cytometry.

In vitro restimulation of splenocytes. (i) **Stimulation with antigen.** Splenocytes were plated (4 × 10⁵ cells/well) in 96-well flat-bottom plates (Costar, NY) and stimulated with Ag85A (10 μg/ml) or Ag19kDa (10 μg/ml) for 72 h at 37°C and 5% CO_2. The polyclonal T cell activator concanavalin A (ConA) (2 μg/ml) was used as a positive control. The culture supernatants were collected and stored at −20°C until further use.

(ii) **Stimulation with BMM$_{Ag}$ and PuM$_{Ag}$.** BMM and PuM were plated (4 × 10⁴ cells/well) in 96-well flat-bottom plates and pulsed with Ag85A or Ag19kDa (20 μg/ml) for 24 h, and the cell monolayers were washed three times to remove free antigen. Splenocytes were added to the antigen-pulsed BMM (BMM$_{Ag}$) and PuM (PuM$_{Ag}$) at a ratio of 10:1 and kept in culture for 72 h. The culture supernatants were then collected and stored at −80°C until further use.

Assessment of the macrophage phenotype. To assess possible differences between BMM and PuM, cells were cultured in the presence of LPS (10 μg/ml), Pam3 (1 μg/ml), and anti-CD40 (10 μg/ml). The expression of cell surface major histocompatibility complex class II (MHC II) and costimulatory molecules was determined 24 h poststimulation by using phycoerythrin (PE)-labeled antibodies to MHC II (I-A^d/I-E^d), CD40, CD80, and CD86 (BD-Bioscience Pharmingen, San Diego, CA) and allophycocyanin-labeled antibodies to F4/80 (AbD Serotec, Dusseldorf, Germany). All samples were analyzed on a Becton Dickinson FACScalibur, and the data were analyzed using CellQuestPro software (Becton Dickinson Immunocytometry Systems). A total of 10,000 events were acquired.

Cytokine ELISA. Commercially available kits for TNF, IL-6 (both from R&D Systems, Minneapolis, MN), IL-10, IL-12 (total), and IFN-γ (all from Mabtech, Stockholm, Sweden) were used to determine the cytokine levels in the culture supernatants according to the manufacturers' recommendations. The plates were developed using *p*-nitrophenyl phosphate (PNPP) (Sigma) for IL-12 and IFN-γ and tetramethylbenzidine substrate (Mabtech) for TNF, IL-6, and IL-10. The OD was measured in a multiscan enzyme-linked immunosorbent assay (ELISA) reader (Anthos Labtec Instruments, Salzburg, Austria) at 405 nm (IL-12 and IFN-γ) or 450 nm (TNF, IL-10, and IL-6).

Serum antibody detection. Antigen-specific antibodies in serum were analyzed by ELISA. ELISA plates (Costar, NY) were coated with either Ag85A (2 μg/ml) or Ag19kDa antigen (2 μg/ml) in carbonate buffer, pH 9.4, overnight at room temperature. The plates were washed three times, and the samples were applied to the wells in serial dilutions starting from 1:400 and incubated overnight at room temperature. Following incubation, the plates were washed and incubated for 2 h with alkaline phosphatase-labeled goat anti-mouse Ig isotypes (IgG, IgG1,

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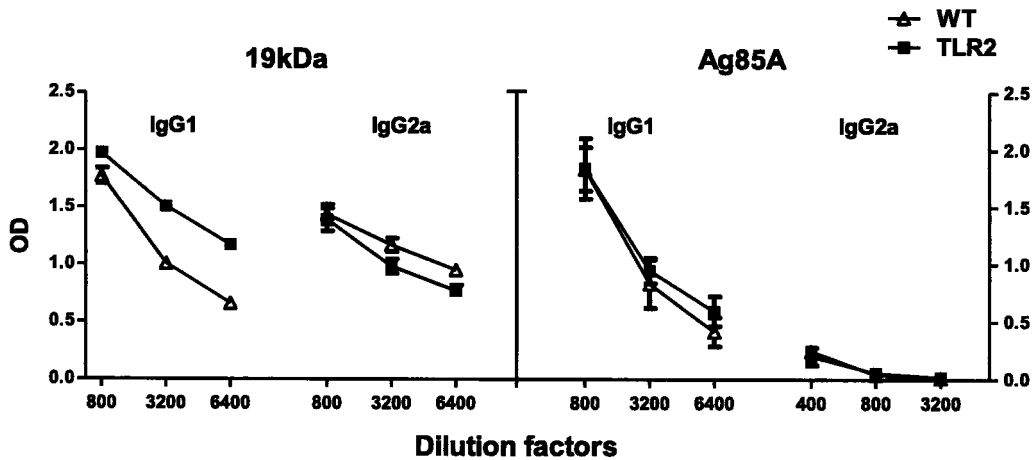| zii01111/zii9376d11g | springj | S=3 | 9/30/11 | Art: 5724-11 |

FIG. 1. Antigen-specific antibody responses. WT and TLR2$^{-/-}$ mice were immunized three times subcutaneously at 2-week intervals with 10 μg/animal of Ag19kDa or Ag85A formulated with cholera toxin (1 μg/animal) as an adjuvant. Serum samples were collected after the last immunization and analyzed for either Ag19kDa- or Ag85A-specific IgG1 and IgG2a levels as measured by ELISA. The data are expressed as OD versus the serum dilution factor ± the standard error of the mean from 3 or 4 mice per group from both mouse strains. A representative of two independent experiments is shown.

and IgG2a; Southern Biotechnology Associates, Birmingham, AL). The plates were developed using PNPP (Sigma) as a substrate, and the OD was read at 405 nm in a multiscan plate reader.

Statistical analysis. Comparison between the experimental groups was done by unpaired and paired Student's t tests. A P value of <0.05 was considered the level of significance. All data were analyzed using GraphPad InStat version 5.0 (GraphPad Software, San Diego, CA).

RESULTS

Antigen-specific antibody responses in WT and TLR2$^{-/-}$ mice. In order to reveal the importance of TLR2 in the development of acquired immune responses, we made a comparative evaluation between WT and TLR2$^{-/-}$ mice upon immunization with the mycobacterial antigen Ag19kDa or Ag85A. Animals were immunized s.c. three times at 2-week intervals, and serum samples were collected 1 week after the last immunization. Antigen-specific IgG, IgG1, and IgG2a Ab levels were measured by ELISA. Independent of the type of antigen used, the two mouse strains had comparable levels of specific IgG (data not shown) and IgG1 (Fig. 1). In contrast, high levels

of specific IgG2a were found only after immunization with Ag19kDa. In this case, also, the levels were comparable between the two mouse strains (Fig. 1). These results suggest that antibody production may not be dependent on TLR2 signaling.

Antigen-specific T cell responses in WT and TLR2$^{-/-}$ mice. In order to investigate cellular immune responses, we collected spleen cells 1 week after the last immunization and restimulated them *in vitro* with the two antigens for 72 h. IFN-γ production was higher in the Ag19kDa-immunized group than in the Ag85A-immunized group (Fig. 2a and b), corroborating the differences in immunogenicity for the two antigens as observed in the antibody response. In contrast to the results observed in the humoral responses, there were differences in the responses of splenocytes from WT and TLR2$^{-/-}$ mice immunized with Ag19kDa but not in those immunized with Ag85A. Splenocytes from the TLR2$^{-/-}$ mice secreted significantly smaller amounts of IFN-γ upon restimulation with Ag19kDa (Fig. 2b). This suggests that cellular responses could be dependent on TLR2 signaling.
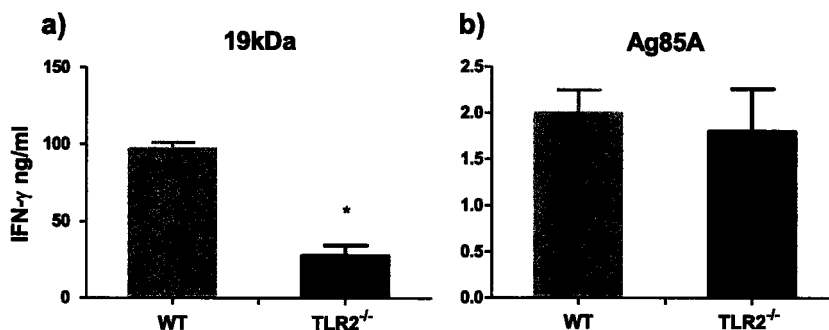

FIG. 2. Antigen-specific T cell responses. WT and TLR2$^{-/-}$ mice were immunized three times subcutaneously at 2-week intervals with 10 μg/animal of Ag19kDa (a) or Ag85A (b) formulated with cholera toxin (1 μg/animal) as an adjuvant. Spleens were collected 1 week after the last immunization. Single-cell suspensions were prepared as described in Materials and Methods and analyzed for the ability to produce IFN-γ upon *in vitro* stimulation with Ag19kDa or Ag85A (10 μg/ml) for 72 h. The data are expressed as means and standard errors of the mean from 3 or 4 mice per group. P values were calculated by comparing WT and TLR2$^{-/-}$ mice using an unpaired two-tailed Student t test; *, $P < 0.05$. A representative of two independent experiments is shown.
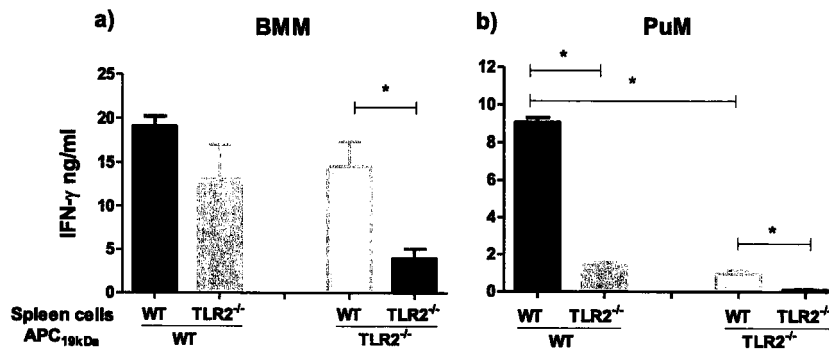
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FIG. 3. Antigen presentation by BMM and PuM. Shown are IFN-γ levels after *in vitro* restimulation of lymphocytes with BMM$_{19kDa}$ (a) and PuM$_{19kDa}$ (b). BMM and PuM were pulsed with Ag19kDa (20 μg/ml) as described in Materials and Methods. The cell monolayers were washed three times to remove free antigen from the wells. Mice were immunized as described in the legend to Fig. 1. One week after the last immunization, the mice were sacrificed and spleen cells were cocultured with BMM$_{19kDa}$ and PuM$_{19kDa}$ for 72 h. The supernatants were collected, and IFN-γ levels were measured by ELISA. The data are expressed as means and standard errors of the mean from 3 or 4 mice per group. P values were calculated by comparing two groups using an unpaired two-tailed Student t test; $*$, $P < 0.05$. A representative of two independent experiments is shown.

Antigen presentation by BMM from WT or TLR2$^{-/-}$ mice. The suboptimal IFN-γ production in the Ag19kDa-immunized TLR2$^{-/-}$ mice prompted us to investigate whether, in the absence of TLR2, (i) T cells could not be properly stimulated *in vivo* against the TLR2 ligand Ag19kDa or (ii) primed T cells from TLR2$^{-/-}$ mice could not be properly activated *in vitro*. To address these questions, BMM from WT and TLR2$^{-/-}$ mice were pulsed with the two antigens Ag19kDa and Ag85A (BMM$_{Ag}$) for 24 h. Free antigen was eliminated by thorough washing to avoid interference from APCs of the isogenic strains. Spleen cells from immunized mice were cultured together with WT BMM$_{Ag}$ and TLR2$^{-/-}$ BMM$_{Ag}$ to assess the functionality of primed T cells and APCs. IFN-γ levels were measured in the culture supernatants. This protocol was used for the two antigens and all combinations with splenocytes and APCs from the two mouse strains.

We did not observe major differences in IFN-γ production between the groups of animals immunized with Ag85A (see Fig. S1 in the supplemental material). For animals immunized with Ag19kDa, comparable levels of IFN-γ were produced by spleen cells from the WT and TLR2$^{-/-}$ mice when WT BMM$_{19kDa}$ were used, but BMM from TLR2$^{-/-}$ mice could induce large amounts of IFN-γ only in WT spleen cells. However, when both splenocytes and APCs were obtained from TLR2$^{-/-}$ mice, the IFN-γ levels were low. This suggests that there may be defects in the T cell and/or APC compartments of the TLR2$^{-/-}$ mice immunized with Ag19kDa but that these defects are observed only under suboptimal conditions (Fig. 3a).

Antigen presentation by PuM from WT and TLR2$^{-/-}$ mice. Since TLR2 has been shown to be important in pulmonary mycobacterial infections (2, 22, 31), we asked whether the antigen-processing and -presenting capacities of macrophages enriched from the pulmonary tissue (PuM) were similar to the capacities of BMM. Spleen cells from WT and TLR2$^{-/-}$ mice immunized with Ag19kDa were cultured together with WT or TLR2$^{-/-}$ PuM pulsed with the same antigen (PuM$_{19kDa}$), and IFN-γ levels in the culture supernatants were measured. In contrast to BMM, the collaboration between PuM and splenocytes showed weaknesses in both the APC and the T cell compartments of the TLR2$^{-/-}$ immunized mice (Fig. 3b).

Differences between BMM and PuM upon stimulation. PuM are primary cells directly isolated from the lungs. Thus, they could have been influenced by the tissue environment and committed to a more restricted phenotype than BMM that are differentiated *in vitro* with no external influences other than the differentiation factor added to the cultures. To address this issue, we stimulated both cell types with various stimuli, namely, LPS and Pam3 (TLR4 and TLR2 ligands, respectively), as well as with anti-CD40, important in T cell-dependent responses. The kinetics were followed in cells cultured with the various stimuli for 4 and 24 h, and the supernatants were collected for cytokine determination by ELISA. For fluorescence-activated cell sorter (FACS) analysis of the expression/upregulation of costimulatory molecules, cells were cultured for 24 h and stained. The cytokines tested were TNF, IL-6, IL-12, and IL-10 as examples of pro- and anti-inflammatory cytokines, and as examples of costimulatory molecules, we looked at CD80, CD86, CD40, and MHC II, known to participate in antigen presentation to T cells (5).

Both types of cells responded to the various stimuli regarding levels of cytokine production and kinetics (4 h) (data not shown), indicating that PuM did not possess an anergic phenotype. However, PuM were generally lower responders than BMM to the TLR2 ligand Pam3 and to anti-CD40 (Fig. 4). Furthermore, for the upregulation of the costimulatory molecules CD86 and CD40, compared to BMM, PuM were lower responders to Pam3 and to anti-CD40, but not to LPS. The fact that Pam3 was not efficient in upregulating these important costimulatory molecules might be at least a partial explanation of the lower APC capacity of PuM when pulsed with the mycobacterial antigen Ag19kDa, also a TLR2 ligand. CD80 and MHC II were not upregulated following stimulation but were constitutively expressed to a greater extent in PuM than in BMM, possibly indicating a more mature phenotype (Fig. 5).

DISCUSSION

TLR2 has been suggested to be an important molecule in susceptibility to mycobacterial infection (8, 31). In the current study, we examined the role of TLR2 in the generation of
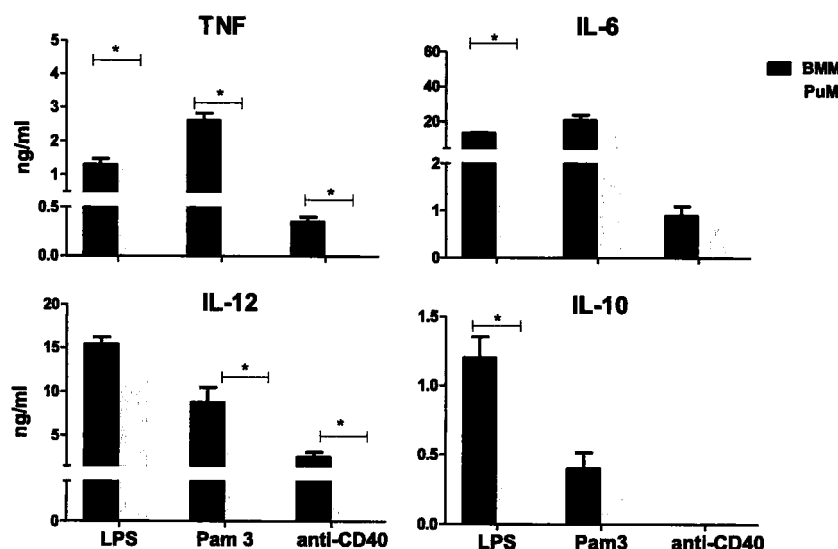
FIG. 4. Cytokine quantification on supernatants from BMM and PuM. BMM and PuM were stimulated for 24 h with LPS (10 µg/ml), Pam3 (1 µg/ml), or anti-CD40 (10 µg/ml) or left untreated. The supernatants were collected, and TNF, IL-6, IL-10, and IL-12 levels were measured using an ELISA. Values are expressed as means and standard errors of the mean of 3 or 4 independent experiments. P values were calculated by comparing BMM and PuM using an unpaired two-tailed Student t test; *, $P < 0.05$.

humoral immune responses, as well as in the recognition, processing, and presentation of mycobacterial antigens to T cells. For this, we compared the immune responses induced in WT and TLR2$^{-/-}$ mice upon immunization with Ag19kDa and Ag85A. We have shown that, in the absence of TLR2, mice were able to generate antigen-specific humoral immune responses independent of the type of antigen (TLR2 dependent or independent) used for immunizations. However, the cellular responses were suboptimal in TLR2$^{-/-}$ mice immunized with Ag19kDa. One explanation is that TLR2 may be more important for cellular responses or, alternatively, that TLR2 contributes more to other aspects of the humoral response, such as affinity maturation or the duration of the response, which were not considered in this work.

We demonstrated here that the suboptimal IFN-γ production in the Ag19kDa-immunized TLR2$^{-/-}$ mice was due to defects in both the T cell and the APC compartments. Antigen presentation by BMM revealed deficiencies in the response only when both the APCs and T cells were from TLR2$^{-/-}$ mice. On the other hand, upon antigen presentation by PuM, the deficiencies were more pronounced, since both the T cell and the APC compartments of the Ag19kDa TLR2$^{-/-}$ immunized mice were found to be compromised.

T cells become activated upon antigen presentation by APCs. Formation of an immunological synapse, an area of contact between a T cell and an APC, is important for sustained signaling in T cells (9). Antigen presentation to T cells is a stepwise process where the first signal is delivered through the binding of T cell receptor (TCR) to the peptide-MHC complexes on the APCs. The subsequent signals delivered to accomplish activation of T cells include costimulatory signals generated as a result of interaction between molecules on the APCs and the T cell surface. A certain minimum level of signaling through the immunological synapse is possibly required to stimulate the specific T cells. Above that level, no

differences may be observed, as in the case of strong WT BMM presentation possibly compensating for weaker TLR2$^{-/-}$ primed T cells and the reverse. However, when a weaker APC was used as the PuM, the level of signaling was decreased to suboptimal levels, and deficiencies could be observed.

The lower capacity of PuM than BMM as APCs could be due to commitment to a more restricted phenotype in the lung tissue. This could include lower capacity for response to stimuli, lower phagocytic capacity, or lower processing capacity. To analyze this issue, we looked at cytokine production and the expression of costimulatory molecules upon stimulation. PuM responded relatively well by producing cytokines, but the profile was different from that of BMM. In general, PuM responded to a lower degree than BMM to the TLR2 ligand Pam3 and to anti-CD40. Also, for the upregulation of the costimulatory molecules CD86 and CD40, compared to BMM, PuM were lower responders to Pam3 and to anti-CD40 than to LPS. It is known that CD86 is an important costimulatory molecule for the priming and activation of naive and memory T cells, respectively (14). The fact that important costimulatory molecules were not properly upregulated upon stimulation with the TLR2 ligand may explain why PuM were less effective than BMM as APCs, since this is based on the response to Ag19kDa, which is a TLR2 ligand. However, PuM were able to secrete levels of IL-6 similar to those secreted by BMM after Pam3 stimulation, which suggests that the above-mentioned reduced response of PuM could be ascribed more to differences in maturation levels of PuM and BMM than to deficiencies in TLR2 signaling. Environmental factors could contribute to modulation of the response to TLR2 ligands. As reported by Sato et al. (29) the lung collectin surfactant protein A (SP-A) significantly attenuated zymosan-induced TNF secretion in RAW264.7 cells and alveolar macrophages in a concentration-dependent manner. It is intriguing that PuM were low responders to TLR2 and anti-CD40, but at present we do not
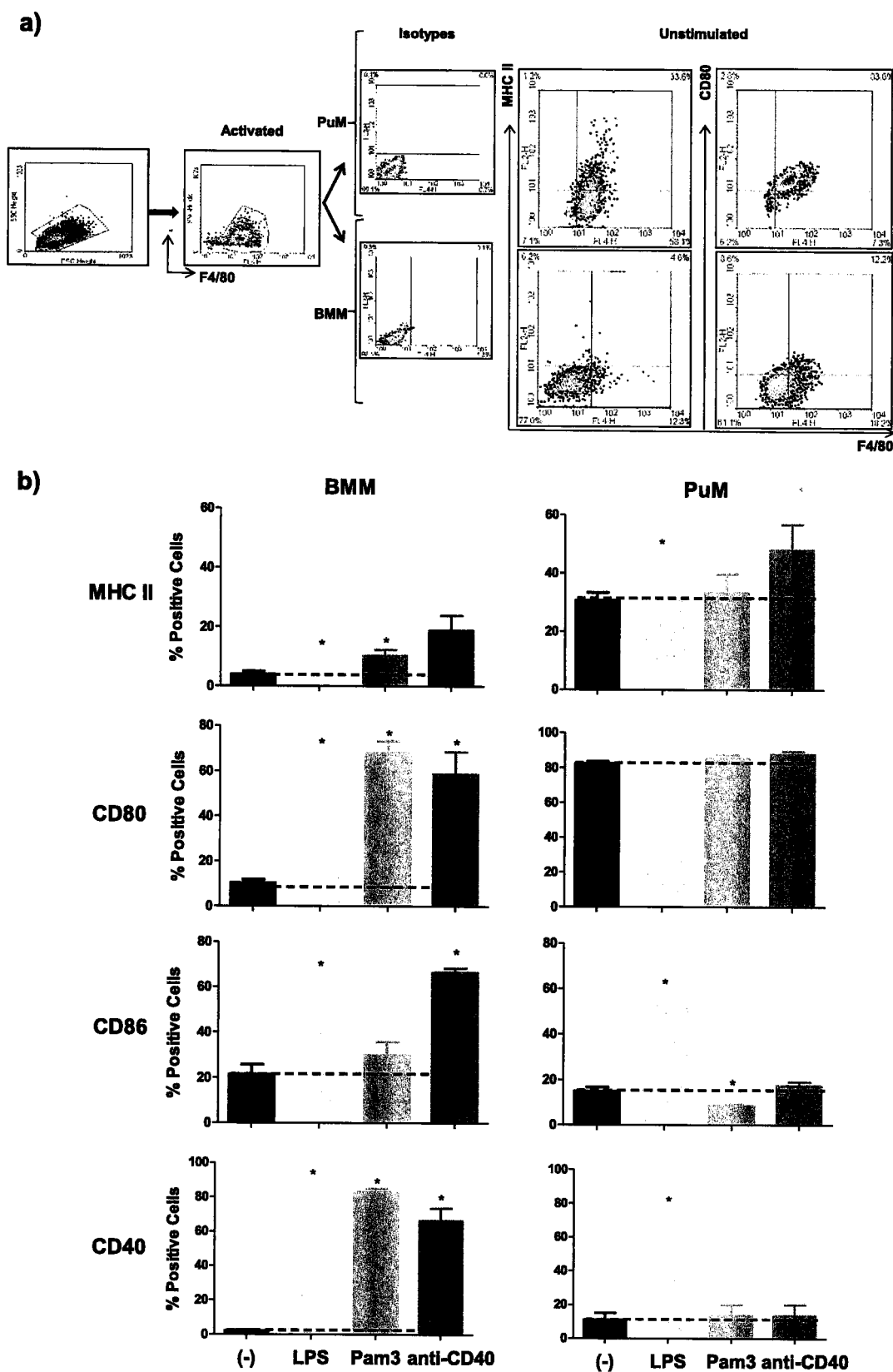
FIG. 5. Gating strategy and analysis of cell surface molecules. BMM and PuM were cultured for 24 h in the presence of LPS (10 μg/ml), Pam3 (1 μg/ml), or anti-CD40 (10 μg/ml) or left untreated. Cells were harvested, and the proportions of the cells expressing the indicated surface molecules were analyzed by flow cytometry. (a) Representative dot plots showing percentages of events positive for MHC II and CD80 versus F4/80 in the respective quadrants. (b) Percentages of BMM and PuM expressing MHC II, CD86, CD80, and CD40. The values are means and standard errors of the mean of 3 or 4 independent experiments. P values were calculated by comparing unstimulated and stimulated samples using a paired two-tailed Student t test; *, $P < 0.05$.

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have any explanation for the low responsiveness of PuM to these stimuli.

A perhaps trivial explanation for the lower APC ability of PuM is that BMM phagocytic capacity is 5 to 10 times higher than the capacity of PuM (data not shown). Obviously, the lower uptake would contribute to less cargo and less antigen to be processed and presented.

It has been shown that immunization with Ag19kDa does not lead to protective immune responses against *M. tuberculosis* (26), but it was important here to examine whether TLR2$^{-/-}$ mice, following immunization with protective mycobacterial antigens, such as Ag85A (36), could be protected against mycobacterial infection. Consistent with the findings reported previously by other groups, we also observed that immunization of WT or TLR2$^{-/-}$ mice with Ag19kDa was not protective against mycobacterial infection (data not shown). In contrast, WT and TLR2$^{-/-}$ mice immunized with Ag85A were protected to similar extents even when infection was done intranasally (see Fig. S2 in the supplemental material). This is interesting because, despite the observed deficiencies in response to TLR2 ligands, the TLR2$^{-/-}$ mice could be efficiently protected when vaccinated with other antigens.

In summary, we report here that TLR2$^{-/-}$ mice were deficient both in the T cell and in the APC compartments. Phagocytosis of bacteria and phagosome maturation have been found to be impaired in the absence of TLR signaling (3). We did not observe major differences in phagocytosis between TLR2$^{-/-}$ and WT macrophages (data not shown), but deficiencies in phagosome maturation may have contributed to the lower APC capacity in the TLR2$^{-/-}$ mice.

Our results are in agreement with those of Komai-Koma et al. (17), suggesting that TLR2 serves as a costimulatory receptor for antigen-specific T cell development and participates in the maintenance of T cell memory. It can be speculated that deficiencies in the T cell compartment could be due to reduced numbers of specific primed T cells or, alternatively, to indirect effects that may diminish the capacity of these T cells to respond to homeostatic stimuli (12, 18, 27). Although important, this type of speculation is beyond the scope of the current work.

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REFERENCES

1. **Aliprantis, A. O., et al.** 1999. Cell activation and apoptosis by bacterial lipoproteins through Toll-like receptor-2. Science **285:**736–739.
2. **Arko-Mensah, J., E. Julian, M. Singh, and C. Fernandez.** 2007. TLR2 but not TLR4 signalling is critically involved in the inhibition of IFN-gamma-induced killing of mycobacteria by murine macrophages. Scand. J. Immunol. **65:**148–157. doi:10.1111/j.1365-3083.2006.01888.x.
3. **Blander, J. M., and R. Medzhitov.** 2004. Regulation of phagosome maturation by signals from toll-like receptors. Science **304:**1014–1018. doi:10.1126/science.1096158.
4. **Brightbill, H. D., et al.** 1999. Host defense mechanisms triggered by microbial lipoproteins through Toll-like receptors. Science **285:**732–736.
5. **Clatza, A., L. C. Bonifaz, D. A. Vignali, and J. Moreno.** 2003. CD40-induced aggregation of MHC class II and CD80 on the cell surface leads to an early enhancement in antigen presentation. J. Immunol. **171:**6478–6487.
6. **Cooper, A. M., L. B. Adams, D. K. Dalton, R. Appelberg, and S. Ehlers.** 2002. IFN-gamma and NO in mycobacterial disease: new jobs for old hands. Trends Microbiol. **10:**221–226.
7. **Corti, M., A. R. Brody, and J. H. Harrison.** 1996. Isolation and primary culture of murine alveolar type II cells. Am. J. Respir. Cell Mol. Biol. **14:**309–315.
8. **Drennan, M. B., et al.** 2004. Toll-like receptor 2-deficient mice succumb to Mycobacterium tuberculosis infection. Am. J. Pathol. **164:**49–57. doi:10.1016/S0002-9440(10)63095-7.
9. **Dustin, M. L., S. Y. Tseng, R. Varma, and G. Campi.** 2006. T cell-dendritic cell immunological synapses. Curr. Opin. Immunol. **18:**512–516. doi:10.1016/j.coi.2006.05.017.
10. **Flynn, J. L., et al.** 1995. Tumor necrosis factor-alpha is required in the protective immune response against Mycobacterium tuberculosis in mice. Immunity **2:**561–572.
11. **Giri, P. K., and J. S. Schorey.** 2008. Exosomes derived from M. Bovis BCG infected macrophages activate antigen-specific CD4+ and CD8+ T cells in vitro and in vivo. PLoS One **3:**e2461. doi:10.1371/journal.pone.0002461.
12. **Goldrath, A. W., et al.** 2002. Cytokine requirements for acute and basal homeostatic proliferation of naive and memory CD8+ T cells. J. Exp. Med. **195:**1515–1522.
13. **Hou, B., B. Reizis, and A. L. DeFranco.** 2008. Toll-like receptors activate innate and adaptive immunity by using dendritic cell-intrinsic and -extrinsic mechanisms. Immunity **29:**272–282. doi:10.1016/j.immuni.2008.05.016.
14. **Jeannin, P., et al.** 2000. Soluble CD86 is a costimulatory molecule for human T lymphocytes. Immunity **13:**303–312.
15. **Kabelitz, D.** 2007. Expression and function of Toll-like receptors in T lymphocytes. Curr. Opin. Immunol. **19:**39–45. doi:10.1016/j.coi.2006.11.007.
16. **Kaneko, H., et al.** 1999. Role of tumor necrosis factor-alpha in Mycobacterium-induced granuloma formation in tumor necrosis factor-alpha-deficient mice. Lab. Invest. **79:**379–386.
17. **Komai-Koma, M., L. Jones, G. S. Ogg, D. Xu, and F. Y. Liew.** 2004. TLR2 is expressed on activated T cells as a costimulatory receptor. Proc. Natl. Acad. Sci. U. S. A. **101:**3029–3034. doi:10.1073/pnas.0400171101.
18. **Lazarevic, V., D. J. Yankura, S. J. DiVito, and J. L. Flynn.** 2005. Induction of Mycobacterium tuberculosis-specific primary and secondary T-cell responses in interleukin-15-deficient mice. Infect. Immun. **73:**2910–2922. doi:10.1128/IAI.73.5.2910-2922.2005.
19. **Liu, P. T., et al.** 2006. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. Science **311:**1770–1773. doi:10.1126/science.1123933.
20. **Means, T. K., et al.** 1999. Human Toll-like receptors mediate cellular activation by Mycobacterium tuberculosis. J. Immunol. **163:**3920–3927.
21. **Muzio, M., et al.** 2000. Differential expression and regulation of Toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. J. Immunol. **164:**5998–6004.
22. **Nicod, L. P.** 2007. Immunology of tuberculosis. Swiss Med. Wkly. **137:**357–362. doi:2007/25/smw-11499.
23. **Noss, E. H., et al.** 2001. Toll-like receptor 2-dependent inhibition of macrophage class II MHC expression and antigen processing by 19-kDa lipoprotein of Mycobacterium tuberculosis. J. Immunol. **167:**910–918.
24. **Pasare, C., and R. Medzhitov.** 2005. Toll-like receptors: linking innate and adaptive immunity. Adv. Exp. Med. Biol. **560:**11–18. doi:10.1007/0-387-24180-9_2.
25. **Pathan, A. A., et al.** 2007. Boosting BCG with recombinant modified vaccinia Ankara expressing antigen 85A: different boosting intervals and implications for efficacy trials. PLoS One **2:**e1052. doi:10.1371/journal.pone.0001052.
26. **Post, F. A., et al.** 2001. Mycobacterium tuberculosis 19-kilodalton lipoprotein inhibits Mycobacterium smegmatis-induced cytokine production by human macrophages in vitro. Infect. Immun. **69:**1433–1439. doi:10.1128/IAI.69.3.1433-1439.2001.
27. **Prlic, M., L. Lefrancois, and S. C. Jameson.** 2002. Multiple choices: regulation of memory CD8 T cell generation and homeostasis by interleukin (IL)-7 and IL-15. J. Exp. Med. **195:**F49–F52.
28. **Rothfuchs, A. G., et al.** 2001. IFN-alpha beta-dependent, IFN-gamma secretion by bone marrow-derived macrophages controls an intracellular bacterial infection. J. Immunol. **167:**6453–6461.
29. **Sato, M., et al.** 2003. Direct binding of Toll-like receptor 2 to zymosan, and zymosan-induced NF-kappa B activation and TNF-alpha secretion are downregulated by lung collectin surfactant protein A. J. Immunol. **171:**417–425.
30. **Takeda, K., and S. Akira.** 2005. Toll-like receptors in innate immunity. Int. Immunol. **17:**1–14. doi:10.1093/intimm/dxh186.
31. **Tjärnlund, A., E. Guirado, E. Julian, P. J. Cardona, and C. Fernandez.** 2006. Determinant role for Toll-like receptor signalling in acute mycobacterial infection in the respiratory tract. Microbes Infect. **8:**1790–1800. doi:10.1016/j.micinf.2006.02.017.
32. **Tsuji, S., et al.** 2000. Maturation of human dendritic cells by cell wall skeleton of Mycobacterium bovis bacillus Calmette-Guerin: involvement of toll-like receptors. Infect. Immun. **68:**6883–6890.
33. **Wang, B., et al.** 2007. A Toll-like receptor-2-directed fusion protein vaccine against tuberculosis. Clin. Vaccine Immunol. **14:**902–906. doi:10.1128/CDLI.00077-07.
34. **Weeratna, R. D., S. R. Makinen, M. J. McCluskie, and H. L. Davis.** 2005. TLR agonists as vaccine adjuvants: comparison of CpG ODN and Resiquimod (R-848). Vaccine **23:**5263–5270. doi:10.1016/j.vaccine.2005.06.024.
35. **Xing, Z., A. Zganiacz, and M. Santosuosso.** 2000. Role of IL-12 in macrophage activation during intracellular infection: IL-12 and mycobacteria syn-

ergistically release TNF-alpha and nitric oxide from macrophages via IFN-gamma induction. J. Leukoc. Biol. **68:**897–902.

36. **Yeremeev, V. V., et al.** 2000. The 19-kD antigen and protective immunity in a murine model of tuberculosis. Clin. Exp. Immunol. **120:**274–279.

37. **Zhang, F. X., et al.** 1999. Bacterial lipopolysaccharide activates nuclear factor-kappaB through interleukin-1 signaling mediators in cultured human dermal endothelial cells and mononuclear phagocytes. J. Biol. Chem. **274:**7611–7614.

38. **Zhang, W. W., and G. Matlashewski.** 2008. Immunization with a Toll-like receptor 7 and/or 8 agonist vaccine adjuvant increases protective immunity against Leishmania major in BALB/c mice. Infect. Immun. **76:**3777–3783. doi:10.1128/IAI.01527-07.

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