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 (Ag85KDa) (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 (BMMΦ) or pulmonal macrophages (PMMΦs). 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 Ag85KDa given alone or presented by BMM or PMW were lower in TLR2−/− than in WT mice. The largest differences in cellular responses were observed when Ag85KDa was presented by PMW. To understand this, we analyzed phenotypic and functional differences between BMM and PMW upon stimulation with various ligands. Generally, PMW had a lower response to the TLR2 ligand PAMCys-Sec-(Lys)3, tritylchlorodrile 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 PMW when pulsed with Ag85KDa, also a TLR2 ligand. Altogether, our results revealed weaknesses in the T cell and antigen-presenting cell (APC) compartments of the Ag85KDa-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 (15, 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. Mono-nuclear phagocytes and dendritic cells express the widest TLR repertoire (21, 27). 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).

Listeria monocytogenes 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 lipoparabinomannan, 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 (Ag9kDa), a known TLR2 agonist, and Ag85A, which is not...
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 Ag85A on phagocytes have been performed, but the role of Ag85A 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 Ag85Ka 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 Ag85A, the TLR2 ligand. Recall responses to Ag85A were similar in the two strains, but responses to Ag85A given alone or presented by bone marrow-derived macrophages (BMM) or pulmonary macrophages (PM) were lower in TLR2–/– mice than in WT mice. The largest differences were observed when Ag85Ka was presented by PM. Based on the central role of PM in the defense of the respiratory tract, we analyzed phenotypic and functional differences between BMM and PM upon stimulation with various ligands. PM 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 importances 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 (Jackson, Bar Harbor, ME) and TLR2–/– mice with the 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 facility at the Arthritis Laboratory, 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, stimulants, molecules, and reagents. Recombinant M. tuberculosis H37Rv Ag85A (Ag85A) and Ag85Ka (Ag85K) were provided by Liesa Diagnosics and Therapeutics GmbH, Braunschweig, Germany. The recombinant protein without signal sequence and with N-terminal His tags were expressed in and purified from Saccharomyces cerevisiae. Endotoxin contents were routinely determined and were in the range of 10 to 30 EU/mg of protein. Lipopolysaccharide (LPS) from E. coli was obtained from Sigma, St. Louis, MO, Pam2Cys-Ser-Lys (Pam3CSK4), trioleylcardiolipin (PtdC) was obtained from Eza Life Sciences, Luzern, Switzerland, and monoclonal anti-CD40 antibodies (Ab) were made from precipitated supernatant from the IC10 hybridoma cell line. The adjuvant, cholera toxin (CT), was obtained from Sigma Chemical Co. 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) with antibiotics with amikacin-dextrose-catalase, 0.5% glycerol, and 0.3% (v/v) Tween 80. BCG was collected at the logarithmic phase of growth (absorbance, 1.0), measured as the optical density at 650 nm (OD650). 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 CFUs were counted 2 to 3 weeks after plating on Middlebrook 7H11 agar (Karolinska Hospital, Solna, Sweden) with glycerol, oleic acid-dextrose-catalase, and the antibiotics polymyxin B and ampicillin 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) or Ag85Ka (10 μg/mouse) formulated with CT (1 μg/mouse) 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. Three after, 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% 2-mercaptoethanol (Sigma).

Generation of BMM and PM. BMM were generated as previously described (26). 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 flushing and resuspension of the cells in complete DMEM or RPMI medium supplemented with 5% horse serum. Bone marrow cells were plated in cell culture plates and incubated for 7 days at 37°C and 5% CO2, replacing the medium every second day. RPMI medium with 10% fetal bovine serum (FBS) was used to maintain the cells in DMEM without 1292 cell conditioned medium for 24 h before use as APCs. PM were prepared from C57BL/6 mice using the protocol of Curt et al. with some modifications (27). 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 flushing and resuspension of the cells in complete DMEM or RPMI medium supplemented with 5% horse serum. Bone marrow cells were plated in cell culture plates and incubated for 7 days at 37°C and 5% CO2, replacing the medium every second day. RPMI medium with 10% fetal bovine serum (FBS) was used to maintain the cells in DMEM without 1292 cell conditioned medium for 24 h before use as APCs.

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In vivo restimulation of splenocytes. (i) Stimulation with antigen. Splenocytes were plated (4 × 106 cells/well) in 96-well flat-bottom plates (Costar, NY) and stimulated with Ag85A (10 μg/ml) or Ag85Ka (10 μg/ml) for 72 h at 37°C and 5% CO2. The polymeric 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 BMMAg85A and PMAg85A. BMM and PM were plated (4 × 106 cells/well) in 96-well flat-bottom plates and plated with Ag85A or Ag85Ka (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 (BMMAg85A) and PM (PMAg85A) at a ratio of 1:10 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 PM, cells were cultured in the presence of LPS (10 μg/ml), Pam3CSK4 (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 (anti-CD11b-PE, CD40, CD80, and CD86 (BD-Bioscence Pharmingen, San Diego, CA) and allophycocyanin-labeled antibodies to P440 (AbD Serotec, Düsseldorf, Germany). All samples were analyzed on a Becton Dickinson FACScalibur, and the data were analyzed using CellQuestPro software (Becton Dickinson Immunocytometry System). 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-12, IL-10 (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-α, IL-6, and IL-10. The OD was measured in a multiscan enzyme-linked immunosorbent assay (ELISA) reader (Anthos 2020; Anthos Laboratory, Austria) at 450 nm (IL-12 and IFN-γ) or 450 nm (TNF-α, IL-10, and IL-6).

Serum antibody detection. Antibodies-specific antibodies in serum were analyzed by ELISA. ELISA plates were coated with Ag85A or Ag85Ka antigen (2 μg/ml) or Ag85Ka antigen (2 μg/ml) or Ag85Ka 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 100 μl of dilutions ranging from 1:1000 and incubated overnight at room temperature. Following incubation, the plates were washed and incubated for 2 h with alkaline phosphatase-labeled goat anti-mouse IgG (1:5000)
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 microtiter plate reader.

Statistical analysis. Comparisons between the experimental groups were done by unpaired and paired Student’s t-test. 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’s 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 AgL9kDa-immunized TLR2−/− mice prompted us to investigate whether, in the absence of TLR2, (i) T cells could not be properly stimulated in vitro against the TLR2 ligand AgL9kDa 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 AgL9kDa and Ag85A (BMMAgL and BMMAg) 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 BMMAg and TLR2−/− BMMAg, 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 spleenocytes 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 AgL9kDa, comparable levels of IFN-γ were produced by spleen cells from the WT and TLR2−/− mice when WT BMMAgL9kDa were used, but BMM from TLR2−/− mice could induce large amounts of IFN-γ only in WT spleen cells. However, when both spleenocytes 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 AgL9kDa 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 primary 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 AgL9kDa were cultured together with WT or TLR2−/− PuM pulsed with the same antigen (PuMAgL9kDa), 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 4 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 CD80 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 AgL9kDa, 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...
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^{-/-} mouse 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 probably 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 CD6 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 collects 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 CD86 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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