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14. ABSTRACT

This is an annual report of the 3rd grant year. PI and 2 Research Associates moved to the current Institute in 2003 from East Carolina University. The project was re-started in December 2004 with approval of no-cost extension until 5/23/2008. We established that our chitin particle preparations induce phagocytic macrophages to produce cytokines (IL-12, IL-18 and TNFα) that down-regulate allergic immune responses. We also found that administration of chitin particles resulted in less likely induce the production of IL-10 and prostaglandin E2 (PGE2). Both IL-10 and PGE2 enhance allergic responses. In this grant year, we have completed the study defining the mechanisms underlying that chitin particles block IL-10 production and PGE2 biosynthesis in macrophages.

15. SUBJECT TERMS
Childhood asthma, N-acetyl-D-glucosamine polymer, IL-12, GATA-3, T-bet, macrophages, airway hyperreactivity

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INTRODUCTION:
This annual report includes a brief summary of the 3rd year research and related activities supported by DAMD17-03-1-0004.

This project funded by DOD DAMD17-03-1-0004 was initiated at East Carolina University on February 24, 2003. PI and two research associates moved to the current institute, Florida Atlantic University (FAU), on September 30, 2003. The transfer of grantee institute was approved on December 1, 2004. Since then, the project has re-started with no-cost extension until May 23, 2008. Two additional postdoctoral research associates, Shoutaro Tsuji, Ph.D. and Makiko Y. Tsuji, DVM, Ph.D. join the laboratory and conduct this project starting in April 2005.

BODY:
Task 1: To determine if oral administration of 1 – 4 μm particles of chitin will down-regulate airway hyperreactivity (AHR) and GATA-3 levels as a measure of Th2 responses, and enhance T-bet levels as a measure of Th1 responses in the lungs of mice that are sensitized with ragweed allergens.
a. Establish the effects of dose response of chitin particles (Months 1 – 4).
b. Establish therapeutic/prophylactic effects of chitin (Months 4 – 9).
c. Determine duration of chitin treatments (Months 8 - 12).

We established that 1 – 10 μm chitin preparations possess reliable Th1 adjuvant activities. An abstract, "A novel oral agent activates macrophages and downregulates Th2 allergic responses," by Munim A, Nishiyama A, Tsuji S, Tsuji M, Myrvik QN, and Shibata Y, is submitted for ATS meeting at San Diego on May 2006. A new pulmonary specialist, Dr. Tsutomu Shinohara, M.D., will join my lab in May 2006. We will continue to define the mechanisms, the dose-responses, and the therapeutic/prophylactic effects of chitin treatment in the following years. A summary of the findings is below.

The prevalence and incidence of asthma is on the rise in USA and worldwide; newer strategies are sought for reducing, the morbidity burden on the patient as well as society's health care costs. Chitin is a naturally occurring N-acetyl-D-glucosamine polymer and a member of new class of Th1 adjuvant that stimulates IL-12 production by macrophages *in vitro*. This study demonstrates that chitin down-regulates the allergic response in a murine model of allergic asthma. METHODS: Ragweed-sensitized BALB/c mice were treated orally with saline or 1- 10 μm chitin particles (8 mg/day for 3 days before and 13 days after ragweed allergen immunization, 7 mice per group). The mice were challenged with ragweed allergens intratracheally on day 11. Three days after the challenge dose, serum IgE levels and lung eosinophil numbers were quantified. Th2 responses were further explored by measuring cytokine production by spleen cells isolated from the ragweed-immunized mice (controls) and cultured in the presence of ragweed and/or chitin for 3 days. RESULTS: The ragweed-sensitized mice treated with saline showed high levels of serum IgE and lung eosinophils, and splenocytes from these animals produced IL-4, IL-5, and IL-10 in responding to ragweed in vitro. Chitin treatment resulted in a significant reduction of these Th2 parameters ($p<0.01$). CONCLUSIONS: Collectively, these results indicate that chitin, which induces innate Th1 immune responses, down-regulates Th2-facilitated IgE production and lung eosinophilia in the allergic mouse. Oral administration of chitin therefore represents a potentially effective treatment for IgE-mediated allergic diseases, including childhood asthma.

Task 2: To determine if the effects of 1 – 4 μm particles of chitin on endogenous IL-12- or IFNγ- mediated down-regulation of airway allergic responses will be greater than those of HK-BCG, ODN-CpG or an equal number of particles of 1 – 10 μm chitin.

a. Establish comparative studies on the effects of 1 – 4 μm chitin, 1 – 10 μm chitin, HK-BCG and ODN-CpG (Months 12 - 24).

b. Study if endogenous IL-12 or IFNγ is required for the chitin-induced down-regulation of GATA-3-mediated allergic responses (Months 24 – 40).

We found that splenic PGE_2-M∅ down-regulate the activity of HK-BCG as a Th1 adjuvant. Two manuscripts are either published or going to be published in the Journal of Leukocyte Biology. A summary of the findings is below.

Hosts infected with low doses of mycobacteria develop Th1 immunity, but at relatively higher doses, a switch to Th2 immunity occurs. PGE_2 is a proposed mediator of the Th1-to-Th2 shift of immune responses and mycobacterial products induce PGE_2-releasing macrophages (PGE_2-M∅) in the mouse spleen in a dose-dependent manner. Splenic PGE_2-M∅ from Balb/c mice given 0.01 or 1 mg heat-killed (HK) *Mycobacterium bovis* BCG i.p. were characterized by the *ex vivo* release of PGE_2 (>10 ng/10^6 cells), cytokine production and expression of cyclooxygenase (COX)-1, COX-2, cytosolic PGE synthase (cPGES) and microsomal COX-1. Day 14 after the treatment, mice treated with 1 mg, but not 0.01 mg, BCG had increased levels of PGHS-2^+ PGE_2-M∅, total serum IgE and serum IgG1 antibodies (Th2 responses) against HSP65 and PPD. Cultures of spleen cells isolated from these mice expressed IL-4 and IL-10 in recall responses. Treatment of mice receiving 1 mg BCG with NS-398 (a COX-2 inhibitor, 10 mg/kg i.p. daily) resulted in enhanced IFNγ production with reduced IL-4 and IL-10 production in recall responses. This treatment also resulted in decreased total serum IgE levels. Treatment of C57Bl/6 mice with HK-BCG (0.5 mg dose) also induced a mixture of Th1 and Th2 responses, although IFNγ production was markedly increased and IL-4 was decreased compared to Balb/c mice. Thus, our results indicate that, by 14 days following treatment of mice with high doses of HK-BCG, splenic PGE_2-M∅ formation is associated with a COX-2 dependent shift from Th1-to-Th2 immune responses.

Task 3: To determine if M∅ will phagocytose more particles and produce more IL-12 in response to 1 – 4 μm chitin compared to 1 – 10 μm chitin.

a. Determine if M∅ treated with 1 – 4 μm chitin particles phagocytose more particles than when treated with an equivalent number of 1 – 10 μm chitin particles (Months 41 – 44).

b. Determine if 1 – 4 μm chitin particles induce more production of IL-12 than an equivalent number of 1 – 10 μm chitin particles (Months 45 – 48).

Cellular events of phagocytosis that are associated with chitin-induced production of anti- and pro-inflammatory cytokines were characterized. A manuscript, "Phagocytosis of *N*-acetyl-D-glucosamine particles, a Th1 adjuvant, results in MAPK activation and TNF-α, but not IL-10, production," was accepted by Cellular Immunology. A summary of these findings is below.

A practical Th1 adjuvant should induce Th1 cytokines (IL-12, IL-18 and TNF-α) but not the Th2 cytokine IL-10, an inhibitor of Th1 responses. In this study, phagocytosis of *N*-acetyl-D-glucosamine polymer (chitin) particles by RAW264.7 macrophage-like cells resulted in phosphorylation of MAPK (p38, Erk1/2 and JNK) and production of relatively high levels of TNF-α and COX-2 with increased PGE_2 release. Similar results were observed in response to bacterial Th1 adjuvants (oligonucleotides with CpG motifs and mycobacterial components) and endotoxin. However, these bacterial components also induced a large amount of IL-10. Chitin particles, in contrast, induced only minimal levels of IL-10, although the production of high levels of PGE_2 and TNFα and the activation of MAPK's are potentially positive signals for IL-10 production. Thus, our results indicate that in macrophages chitin particles act as a unique Th1 adjuvant without inducing increased production of IL-10.

KEY RESEARCH ACCOMPLISHMENTS:

1. Establishment of cellular mechanisms underlying phagocytosis of chitin particles and production of anti-inflammatory cytokines by macrophages.
2. Establishment of the chitin Th1 adjuvant which does not induce IL-10 production.
3. Finding a mechanism underlying chitin particles down-regulating the activities of COX-2/PGE2 biosynthesis.
4. Establishment of characterization of splenic PGE_2-releasing macrophages which induce a Th1-to-Th2 shift of immune responses.

REPORTABLE OUTCOMES:

Paper Published

Shibata Y, A Nishiyama, H Ohata, J Gabbard, QN Myrvik, RA Henriksen. Differential effects of IL-10 on prostaglandin H synthase-2 expression and prostaglandin E_2 biosynthesis between spleen and bone marrow macrophages. *J. Leuko. Biol. 77:544-551, 2005.* Tasks 2 and 3 (Appendix I).

Shibata Y, Henriksen RA, Honda I, Nakamura RM, Myrvik QN. Splenic PGE2-releasing macrophages regulate Th1 and Th2 immune responses in mice treated with heat-killed BCG. *J Leukoc Biol 78, 1281-1290, 2005.* Task 2 (Appendix II).

Manuscript in press

Shibata Y. Radiosensitive macrophages and immune responses --- Prostaglandin E2-releasing macrophages induce a Th1-to-Th2 shift of immune responses in chronic inflammation --- *Radia. Res. (in press).* Task 2. (Appendix III).

Shibata Y, J Gabbard, M Yamashita Tsuji, S Tsuji, M Smith, A Nishiyama, RA Henriksen, QN Myrvik. Heat-killed BCG induces biphasic cyclooxygenase-2^+ splenic macrophage formation --- role of IL-10 and bone marrow precursors. *J Leukocyte Biol (in press).* Task 2 (Appendix IV).

Nishiyama, A, S Tsuji, M Yamashita Tsuji, RA Henriksen, QN Myrvik, Y Shibata. Phagocytosis of *N*-acetyl-D-glucosamine particles, a Th1 adjuvant, results in MAPK activation and TNF-α, but not IL-10, production. *Cellular Immunology* (in press). Tasks 1a, 3a and 3b (Appendix V).

Manuscript submitted or in preparation

Shibata, Y, P Vos, QN Myrvik. Neutrophils from BCG-immunized mice enhance innate immunity against lethal challenges of *Listeria monocytogenes*. Submitted for publication. Task 2.

Shibata Y, H Ohata, M Yamashita Tsuji, S Tsuji, JF Bradfiel, RA Hendrickson, A Nishiyama, QN Myrvik. Mechanism of BCG-induced high anti-HSP65 antibody formation and development of advanced atherosclerosis in apolipoprotein $E^{-/-}$ mice. Submitted for publication. Task 2.

Nishiyama A, MY Tsuji, S Tsuji, RA Henriksen, QN Myrvik, Y Shibata. Cellular Cholesterol Depletion Enhances Chitin Phagocytosis-Induced Macrophage Activation. Abstract will be presented at AAI Meeting at Boston in May 2006. Tasks 1 and 3.

Tsuji M Yamashita, A Nishiyama, QN Myrvik, RA Henriksen, S Tsuji, Y Shibata. Heat-killed BCG induces biphasic cyclooxygenase-2 $(COX\text{-}2)^+$ splenic macrophage formation – differential intracellular compartmentalization of COX-2 correlates with PGE_2 biosynthesis. Abstract will be presented at AAI Meeting at Boston in May 2006. Task 2.

Tsuji S, M Yamashita Tsuji, A Nishiyama, Y Shibata. Molecular structure of human and mouse interlectin-1 and comparison of binding to a mycobacterial galactofuranosyl residue.
Abstract will be presented at AAI Meeting at Boston in May 2006. Task 2.

Munim A, A Nishiyama, S Tsuji, M Tsuji, QN Myrvik, Y Shibata. A novel oral agent activates macrophages and down-regulates Th2 allergic responses. Abstract will be presented at American Thoracic Society Meeting at San Diego in May 2006. Task 1.

Presentations

"N-acetyl-β-D-glucosamine polymer particle-induced Th1 and Th2 cytokines production by murin macrophage-like RAW264.7 Cells are differentially regulated by p38, JNK, and Erk ½," by Akihito Nishiyama, Hiroyoshi Ohata, Jon Gabbard, Ruth Ann Henriksen, Quentin N. Myrvik, and Yoshimi Shibata, the 2005 Experimental Biology, 4/2 – 4/6/05, San Diego CA. Task 2a

"Biphasic expression of Cox-2 by splenic macrophages (M∅) in BCG-immunized mice," Yoshimi Shibata*, Hiroyoshi Ohata*, Akihito Nishiyama*, Jon Gabbard*, Quentin N. Myrvik‡, Ruth Ann Henriksen** the 2005 Experimental Biology, 4/2 – 4/6/05, San Diego CA. Task 2a

"Radiosensitive macrophages and immune responses --- Prostaglandin E2-releasing macrophages induce a Th1-to-Th2 shift of immune responses in chronic inflammation ---." International Symposium on Low-Dose Radiation Exposure and Bio-defense system (sponsored by Institute for Environmental Sciences), 9/28 – 9/30/05, Rokkasho, Aomori, Japan. Task 2a

EMPLOYMENT:

Two Postdoctoral Research Associates, Shoutaro Tsuji, Ph.D. and Makiko Y. Tsuji, DVM, Ph.D., joined the laboratory and conduct this project starting in April 2005. Tsutomu Shinohara, M.D., Visiting Associate Professor and a pulmonary specialist will join and perform animal studies in this project in May 2006.

CONCLUSIONS:

The neonates and young children are more susceptible than the young to infections and frequently develop asthmatic problems [1, 2]. The mechanism by which these populations become immunocompromised appears to be an altered regulation of immunity and not simple immune deficiency. It is likely that macrophages in these immunocompromised populations become hypofunctional with excessive production of PGE_2 and IL-10, both of which enhance allergic Th2 responses. Our studies clearly demonstrated that administration of chitin particles resulted in blocking the production of IL-10 and COX-2/PGE_2biosynthesis. In sharp contrast, bacterial Th1 adjuvants (heat-killed Mycobacteirum bovis BCG, CpG-ODN) and endotoxin (LPS) enhances both IL-10 production and PGE_2 release [3-5]. Thus chitin may be the most potent Th1 adjuvant presently available and is an attractive immunomodulator for allergic asthma.

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2. Blahnik, M.J., Ramanathan, R., Riley, C.R., Minoo, P. (2001) Lipopolysaccharide-induced tumor necrosis factor-alpha and IL-10 production by lung macrophages from preterm and term neonates. *Pediatr Res* **50**, 726-31.
3. Nishiyama, A., Tsuji, S., Tsuji, M.Y., Henriksen, R.A., Myrvik, Q.N., Shibata, Y. (2006) Phagocytosis of N-acetyl-D-glucosamine particles, a Th1 adjuvant, results in MAPK activation and TNF-α, but not IL-10, production. *Cellular Immunology* **in press**.
4. Shibata, Y., Henriksen, R.A., Honda, I., Nakamura, R.M., Myrvik, Q.N. (2005) Splenic PGE2-releasing macrophages regulate Th1 and Th2 immune responses in mice treated with heat-killed BCG. *J Leukoc Biol* **78**, 1281-90.
5. Shibata, Y., Gabbard, J., Tsuji, M.Y., Tsuji, S., Smith, M., Nishiyama, A., Henriksen, R.A., Myrvik, Q.N. (2006) Heat-killed BCG induces biphasic cyclooxygenase 2+ splenic macrophage formation --- role of IL-10 and bone marrow precursors. *J Leukoc Biol* **in press**.

APPENDICES:

Appendix I: Shibata Y, A Nishiyama, H Ohata, J Gabbard, QN Myrvik, RA Henriksen. Differential effects of IL-10 on prostaglandin H synthase-2 expression and prostaglandin E_2 biosynthesis between spleen and bone marrow macrophages. *J. Leuko. Biol. 77:544-551, 2005.*

Appendix II: Shibata Y, Henriksen RA, Honda I, Nakamura RM, Myrvik QN. Splenic PGE2-releasing macrophages regulate Th1 and Th2 immune responses in mice treated with heat-killed BCG. *J Leukoc Biol 78, 1281-1290, 2005.*

Appendix III: Shibata Y. Radiosensitive macrophages and immune responses --- Prostaglandin E2-releasing macrophages induce a Th1-to-Th2 shift of immune responses in chronic inflammation --- *Radia. Res. (in press).*

Appendix IV: Shibata Y, J Gabbard, M Yamashita Tsuji, S Tsuji, M Smith, A Nishiyama, RA Henriksen, QN Myrvik. Heat-killed BCG induces biphasic cyclooxygenase-2^+ splenic macrophage formation --- role of IL-10 and bone marrow precursors. *J Leukocyte Biol (in press).*

Appendix V: Nishiyama, A, S Tsuji, M Yamashita Tsuji, RA Henriksen, QN Myrvik, Y Shibata. Phagocytosis of *N*-acetyl-D-glucosamine particles, a Th1 adjuvant, results in MAPK activation and TNF-α, but not IL-10, production. *Cellular Immunology* (in press).

Splenic PGE_2-releasing macrophages regulate Th1 and Th2 immune responses in mice treated with heat-killed BCG

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Abstract: Hosts infected with low doses of mycobacteria develop T helper cell type 1 (Th1) immunity, but at relatively higher doses, a switch to Th2 immunity occurs. Prostaglandin E_2 (PGE_2) is a proposed mediator of the Th1-to-Th2 shift of immune responses, and mycobacterial products induce PGE_2-releasing macrophages (PGE_2-M∅) in the mouse spleen in a dose-dependent manner. Splenic PGE_2-M∅ from Balb/c mice, given 0.01 or 1 mg heat-killed (HK) *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) intraperitoneally (i.p.), were characterized by the ex vivo release of PGE_2 (>10 ng/10^6 cells), cytokine production, and expression of PGG/H synthase (PGHS)-1, PGHS-2, cytosolic PGE synthase (PGES), and microsomal PGES-1. At Day 14 after the treatment, mice treated with 1 mg, but not 0.01 mg, BCG had increased levels of PGHS-2^+ PGE_2-M∅, total serum immunoglobulin E (IgE), and serum IgG1 antibodies (Th2 responses) against heat shock protein 65 and purified protein derivative. Cultures of spleen cells isolated from these mice expressed interleukin (IL)-4 and IL-10 in recall responses. Treatment of mice receiving 1 mg BCG with NS-398 (a PGHS-2 inhibitor, 10 mg/kg i.p., daily) resulted in enhanced interferon-γ (IFN-γ) production with reduced IL-4 and IL-10 production in recall responses. This treatment also resulted in decreased total serum IgE levels. Treatment of C57Bl/6 mice with HK-BCG (0.5 mg dose) also induced a mixture of Th1 and Th2 responses, although IFN-γ production was markedly increased, and IL-4 was decreased compared with Balb/c mice. Thus, our results indicate that by 14 days following treatment of mice with high doses of HK-BCG, splenic PGE_2-M∅ formation is associated with a PGHS-2-dependent shift from Th1-to-Th2 immune responses. *J. Leukoc. Biol.* 78: 1281–1290; 2005.

Key Words: *PGHS-2 (Cox-2) · cPGES · purified protein derivative · splenic macrophages · PGE_2 · Th1-to-Th2 shift*

INTRODUCTION

T helper cell type 1 (Th1) adjuvants play an important role in the development of protective immunity against intracellular infections such as tuberculosis. Previously, Power et al. [1] found that relatively low doses of live *Mycobacterium bovis* bacillus Calmette-Guerin (BCG), a vaccine strain for *Mycobacterium tuberculosis*, lead to a cell-mediated, Th1 response, and higher doses induce mixed cell-mediated immune and Th2-mediated humoral responses. The induction of antibodies usually leads to a chronic or progressive and fatal outcome in tuberculosis [2, 3]. The mechanism for such a dose-dependent immunological shift is unknown.

Complete Freund's adjuvant [CFA; heat-killed (HK)-*M. tuberculosis*] and HK-BCG in mineral oil have been widely used to establish animal models of autoimmune diseases, such as experimental autoimmune encephalomyelitis, neuritis, uveitis, thyroiditis, orchitis, and adjuvant arthritis [4, 5]. The adjuvants enhance Th1-mediated macrophage (M∅) activation and Th2-mediated antibody formation [6]. Pathogenic roles of Th1/Th2 responses appear to be varied among autoimmune disease models [5], which may be related to the range of HK-mycobacteria concentrations (0.05–0.5 mg) used in these animal models [5, 7].

Much attention has been directed to the role of mycobacterial heat shock protein 65 (HSP65), an immunodominant antigen. It has been proposed that a host Th1 response to HSP65 plays a protective role in mycobacterial infections [8]. However, Th1-to-Th2 shifts of immune responses against mycobacteria result in the formation of antibodies against HSP65, which does not have a decisive, protective role against infection [2]. Furthermore, as antibodies or T cells specific for bacterial HSP65 potentially cross-react with host HSPs, these immune responses may have pathogenic roles in autoimmune diseases [9]. Th1 cytokines, including interferon-γ (IFN-γ), promote or counteract pathogenesis, depending on the autoimmune disease [9]. Therefore, Th1-to-Th2 shifts of immune responses may be associated with the pathogenesis of various autoimmune diseases as well as chronic intracellular infections [10–12].

In previous studies, we have reported that normal, splenic M∅ stimulated ex vivo do not produce significant levels of

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prostaglandin E_2 (PGE_2), distinct from MØ originating from other tissues [13]. However, high doses of HK-BCG or related products such as HK-*Propionibacterium acnes* (*Corynebacterium parvum*) in vivo induce stable and sustained formation of PGE_2-releasing MØ (PGE_2-MØ) in the spleen [6, 14–16]. The mechanism for formation of these MØ is still unclear. Previous studies [15, 16] indicate that the formation of splenic PGE_2-MØ is dependent on radiosensitive bone marrow cells, which may supply precursors of splenic PGE_2-MØ. Alternatively, an inflammatory cytokine "milieu" may up-modulate PGE_2 biosynthesis directly by splenic MØ [17–19].

The spleen is the lymphoid tissue where PGE_2-MØ and immune lymphocytes interact in chronic inflammatory diseases. PGE_2 inhibits the production of Th1 cytokines, such as interleukin (IL)-2, IL-12, and IFN-γ [20]. In contrast, PGE_2, depending on stimulatory conditions, has no effect or enhances production of Th2 cytokines, such as IL-4, IL-5, and IL-10 [20, 21]. Therefore, we have examined the hypothesis that splenic PGE_2-MØ development in vivo following treatment with high doses of HK-BCG is time-, dose-, and PGG/H synthase (PGHS)-2-dependent and promotes a Th1-to-Th2 shift in specific immune responses. Because of described differences in immune responses between Balb/c and C57Bl/6 mice [18, 20], we have included results obtained with both strains.

MATERIALS AND METHODS

Mice

Nonpregnant female Balb/c and C57Bl/6 mice, 8–14 weeks old, were obtained from Harlan Laboratory (Indianapolis, IN). Mice were maintained in barrier-filtered cages under specific, pathogen-free conditions in the animal care facility at East Carolina University (Greenville, NC) or Florida Atlantic University (Boca Raton).

Preparations of HK-BCG

As described previously [6], cultured *M. bovis* BCG Tokyo 172 strain was washed, autoclaved, and lyophilized. The powder of HK-BCG was suspended in saline and dispersed by brief (10 s) sonication immediately prior to injection. These HK-BCG preparations contained undetectable levels of endotoxin (<0.03 EU/ml), as determined by the *Limulus* amebocyte lysate assay (Sigma Chemical Co., St. Louis, MO) [6]. HK-BCG was also suspended in mineral oil (M-5904, Sigma Chemical Co.) in some experiments.

Treatment of mice with HK-BCG

Groups of mice received 0.01, 0.1, 0.5, or 1 mg HK-BCG, intraperitoneally (i.p.), on Day 0. Controls received 0.2 ml saline. In some experiments, mice receiving BCG and controls were further treated i.p with 10 mg/kg NS-398 or nimesulide (Cayman Chemical, Ann Arbor, MI) daily, starting on Day 1. Control groups of mice received 0.5% ethanol in saline (0.2 ml/dose). Unless indicated, spleens and sera were harvested on Day 14. To compare the effects of HK-BCG suspended in saline or mineral oil, additional groups of mice were given HK-BCG in mineral oil i.p. Controls received 0.2 ml mineral oil.

Cytokine production in recall response of spleen cell cultures

Spleens from each group of mice were isolated, pooled, minced with scissors, digested with 50 U/ml collagenase D (C2139, Sigma Chemical Co.) in RPMI 1640 plus 10% fetal bovine serum (FBS), 37°C, for 60 min, and filtered through a 100-μm mesh. Single-cell suspensions were prepared by washing digested cells with RPMI 1640 containing 100 μg/ml DNase (DN-25, Sigma Chemical Co.). After washing with serum-free RPMI 1640, cell suspensions were applied to the top of a discontinuous Percoll gradient (35/60%). Following centrifugation, 800 *g*, 30 min, 22°C, cells at the Percoll interface were collected. Spleen cells were suspended in RPMI 1640 plus 10% FBS at 4×10^6 cells/ml and incubated with endotoxin-free mycobacterial HSP65 (Stressgen, Victoria, BC, Canada) or purified protein derivative (PPD; Japan BCG Laboratory, Tokyo) at 1 or 5 μg/ml, respectively, for 4 days. In some experiments, 10^{-6} M nimesulide was added to the cultures. After incubation, culture supernatants were collected, and IL-4, IL-10, and IFN-γ levels were measured by the respective enzyme-linked immunosorbent assay (ELISA; PharMingen, San Diego, CA).

PGE_2-MØ

Plastic adherent splenic MØ were isolated from spleen-cell suspensions prepared above [13]. Splenic MØ (2×10^6/ml) were cultured in serum-free RPMI-1640 medium with 10^{-6} M calcium ionophore A23187 (Sigma Chemical Co.), 1 μg/ml arachidonic acid (AA; Cayman Chemical), or 1 μg/ml bacterial endotoxin [lipopolysaccharide (LPS), Sigma Chemical Co.] for 2 h. In some experiments, splenic cells (2×10^6/ml) were cultured in RPMI-1640 medium plus 2% FBS with 5 μg/ml PPD or 1 μg/ml HSP65 for 2 days in the presence of the PGHS inhibitors nimesulide, indomethacin, or NS-398, all at 1 μM. PGE_2 levels in the culture supernatants were measured by competitive ELISA (Cayman Chemical).

Magnetic separation of F4/80-positive cells

Red cell-free spleen cells (10^8 cells) were stained with 5 μg/ml monoclonal antibody (mAb) F4/80 recognizing spleen MØ (Accurate Chemical and Scientific Corp., Westbury, NY), followed by addition of 200 μl magnetic microbead-conjugated goat anti-rat immunoglobulin G (IgG; 130-048-501, Miltenyi Biotec, Auburn, CA). F4/80- positive and -negative cells were isolated according to the company's instructions. The content of F4/80 cells, determined cytometrically, in positive and negative preparations, was 90% and less than 2%, respectively (data not shown).

PGE synthase (PGES) assay

PGES activity in cell lysates was measured as conversion of PGH_2 to PGE_2 [22]. Adherent, splenic MØ in 400 μl 10 mM Tris, pH 8.0, were disrupted by sonication using a Branson sonifier (10 s, three times at 1-min intervals). After centrifugation of the sonicates at $1700\times g$ for 10 min, 4°C, the supernatants were used as the source of enzyme activity. An aliquot of each lysate (10 μg protein) was incubated with 0.5 μg PGH_2 (Cayman Chemical) for 30 s at 24°C in 0.1 ml 0.1 M Tris, pH 8.0, containing 1 mM reduced L-glutathione (Sigma Chemical Co.) and 5 μg indomethacin. After terminating the reaction by addition of 100 mM $FeCl_2$, PGE_2 in the supernatants was quantified by competitive ELISA (Cayman Chemical). Protein concentrations were determined by bicinchoninic acid (BCA) assay (Pierce, Rockford, IL) using bovine serum albumin as standard.

Total serum IgE, antigen-specific IgG1 and IgG2a

Total serum IgE was determined by ELISA using purified mouse IgE κ isotype as the standard rat anti-mouse IgE mAb (clone R35-72) as capture antibody and biotinylated rat mAb detecting IgE (clone R25-92), as detection antibody (all reagents from PharMingen) [6]. Levels of PPD-specific IgG1/IgG2a and HSP65-specific IgG1 and IgG2a were measured by ELISA with 96-well plates coated overnight at 4°C with 0.5 μg PPD or 0.1 μg HSP65 per well in 100 μl 0.05 M sodium carbonate buffer, pH 9.6 [6]. Biotinylated rat mAb, detecting IgG1 and IgG2a, were clones A85-1 and R19-15, respectively (PharMingen).

Western blotting

Splenic MØ were prepared as described above, harvested, and washed three times with cold saline. Washed cells were resuspended in lysis buffer {50 mM Tris, pH 7.5, 150 mM NaCl, 1:500 Sigma protease inhibitor cocktail (P8340, Sigma Chemical Co.), 1% Nonidet P-40, and 1% sodium deoxycholate [13]}. Debris was eliminated by centrifugation (5 min, $1000\times g$). Protein concentration in the lysate was measured using BCA as described above. Equal amounts of protein were loaded onto sodium dodecyl sulfate-polyacrylamide minigels and separated by electrophoresis (200 V for 45 min). Proteins were then

transferred to a polyvinylidene difluoride (Sigma Chemical Co.) membrane, which was blocked with 5% nonfat dry milk and incubated with antibody [anti-PGHS-1, 1:1000; anti-PGHS-2, 1:4000; anticytosolic PGES (anti-cPGES), 1:1000; antimicrosomal PGES-1 (anti-mPGES-1), 1:1000, all from Cayman Chemical] in 5% nonfat dry milk overnight at 4°C. Following incubation with peroxidase-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA), proteins were detected by enhanced chemiluminescence (Amersham, Piscataway, NJ) following the manufacturer's instructions [13].

Statistics

Data were analyzed by one-way ANOVA. For cell culture studies, tissues isolated from at least four mice were pooled unless indicated; cells were cultured in at least triplicate in each group. $P < 0.05$ was considered statistically significant.

RESULTS

HK-BCG induces splenic PGE_2-M∅

Splenic M∅ isolated from normal mice show minimum levels of PGE_2 release [6, 13, 15, 16]. To characterize splenic PGE_2-M∅ formation, Balb/c mice were treated i.p. with 1 mg HK-BCG in saline. When splenic M∅ were isolated 1 and 3 days later and stimulated in vitro with A23187 for 2 h, PGE_2 release was not different from control cells obtained from untreated animals (**Fig. 1A**). Significantly higher PGE_2 release was observed 7 and 14 days after treatment with 1 mg HK-BCG. Similar experiments with C57Bl/6 mice treated with 0.5 mg HK-BCG showed that production of PGE_2 persists for at least 21 days. Because of the different treatment conditions, PGE_2 levels in the two strains of mice cannot be compared directly, although the results suggest that production of PGE_2 by C57Bl/6 M∅ is lower (Fig. 1, A and B). Further, AA, but not LPS, also elicited PGE_2 release from Balb/c spleen cells (Fig. 1A).

To examine the dose-response effect for HK-BCG treatment, mice were given increasing doses of HK-BCG in saline, and PGE_2-M∅ activities were determined 14 days after treatment. Figure 1B shows that HK-BCG-induced PGE_2-M∅ formation was dose-dependent. There was no increase in PGE_2 release on Day 14 for Balb/c or C57Bl/6 mice treated with 0.01 mg HK-BCG (Fig. 1B). Treatment with HK-BCG suspended in mineral oil also resulted in PGE_2-M∅ formation in a dose-dependent manner, similar to that seen with HK-BCG in saline (Fig. 1B).

To determine whether recall responses in vitro elicit PGE_2 release, spleen cells isolated from mice treated with HK-BCG in saline were stimulated with the mycobacterial antigens, PPD or HSP65. As shown in Figure 1C, these antigens induced spleen cells to release PGE_2 in both strains of mice, dependent on the HK-BCG dose and suggesting that interaction between antigen-specific lymphocytes and splenic M∅ triggers PGE_2 biosynthesis. Furthermore, in Balb/c mice treated with 1 mg HK-BCG in saline or mineral oil, PGE_2 biosynthesis was inhibited by NS-398, nimesulide, or indomethacin, consistent with mediation of PGE_2 synthesis by PGHS-2 in splenic PGE_2-M∅ (Fig. 1D).

Our results clearly indicate that HK-BCG, in a dose-dependent manner, induces splenic PGE_2-M∅ formation within 7–14 days. Inhibition by the PGHS-2 selective inhibitors nimesulide and NS398 implies a dependence on PGHS-2 for PGE_2 synthesis. There is no difference in the magnitude of PGE_2-M∅ formation in response to HK-BCG suspended in saline or in mineral oil. Therefore, HK-BCG suspended in saline was used to further characterize PGE_2-M∅ and determine whether these cells contribute to the Th1-to-Th2 shift of immune responses.

Protein detection of PGHS-1, PGHS-2, mPGES-1, and cPGES in splenic PGE_2-M∅

PGE_2-M∅ metabolize endogenous AA to PGE_2 through the rate-limiting enzymes PGHS and PGES. Two major isoforms of PGHS convert AA to PGH_2: PGHS-1, a constitutive form, and PGHS-2, an inducible form. PGH_2 is subsequently converted to PGE_2 by cPGES and mPGES-1 [22, 23]. Murakami et al. [22] reported that mPGES-1 is a terminal enzyme of PGHS-2-mediated PGE_2 synthesis, and PGHS-2 and mPGES-1 are induced in various cells, including peritoneal M∅, by proinflammatory stimuli. Normal, splenic M∅ expressed PGHS-1, mPGES-1, and cPGES but not PGHS-2 (**Fig. 2**). This profile was unchanged in splenic M∅ isolated from Balb/c mice 14 days after receiving 0.01 mg HK-BCG in saline (data not shown). When Balb/c mice were treated with 1 mg HK-BCG, PGHS-2 was detected on Days 7 and 14 (Fig. 2). The levels of PGHS-1, mPGES-1, and cPGES remained similar to those in normal, splenic M∅ (Fig. 2). Therefore, increased PGHS-2 levels, but not mPGES-1 levels, were associated with increases in PGE_2 release by splenic PGE_2-M∅. Figure 2B shows that PGHS-2 was highly enriched in F4/80-positive M∅ on Day 14.

PGES activities

cPGES and membrane-bound, glutathione-dependent PGES (mPGES-1) have been shown to be terminal enzymes of PGHS-1- and PGHS-2-mediated PGE_2 biosynthesis, respectively [22, 24]. PGES activity assays were performed to determine whether changes following treatment with 1 mg HK-BCG accounted for the increase in PGE_2 production. As shown in **Figure 3**, PGES activity was not altered significantly in Balb/c mice treated with HK-BCG, indicating that increases in PGE_2 production do not result from changes in PGES activity.

Serum IgE, IgG1, and IgG2a

Endogenous Th1 and Th2 cytokines are isotype-switching signals for antigen-specific B cells, which are biased toward IgG2a and IgE/IgG1, respectively [25, 26]. Treatment of Balb/c mice with 1 mg HK-BCG resulted in increased serum levels of total IgE, HSP65-specific IgG1, and HSP65-specific IgG2a (**Fig. 4**). Similar levels of IgE were found in C57Bl/6 mice treated with 0.5 mg HK-BCG (see Fig. 6). In contrast, when treated with 0.01 mg HK-BCG, there was no increase in total serum IgE or IgG1 levels specific against HSP65 or PPD but a significant increase in HSP65- and PPD-specific IgG2a (Fig. 4). These results indicate that treatment with 1 mg HK-BCG produces a mixture of Th1 and Th2 responses against mycobacterial antigens including HSP65, whereas 0.01 mg HK-BCG produces Th1-dominant responses.

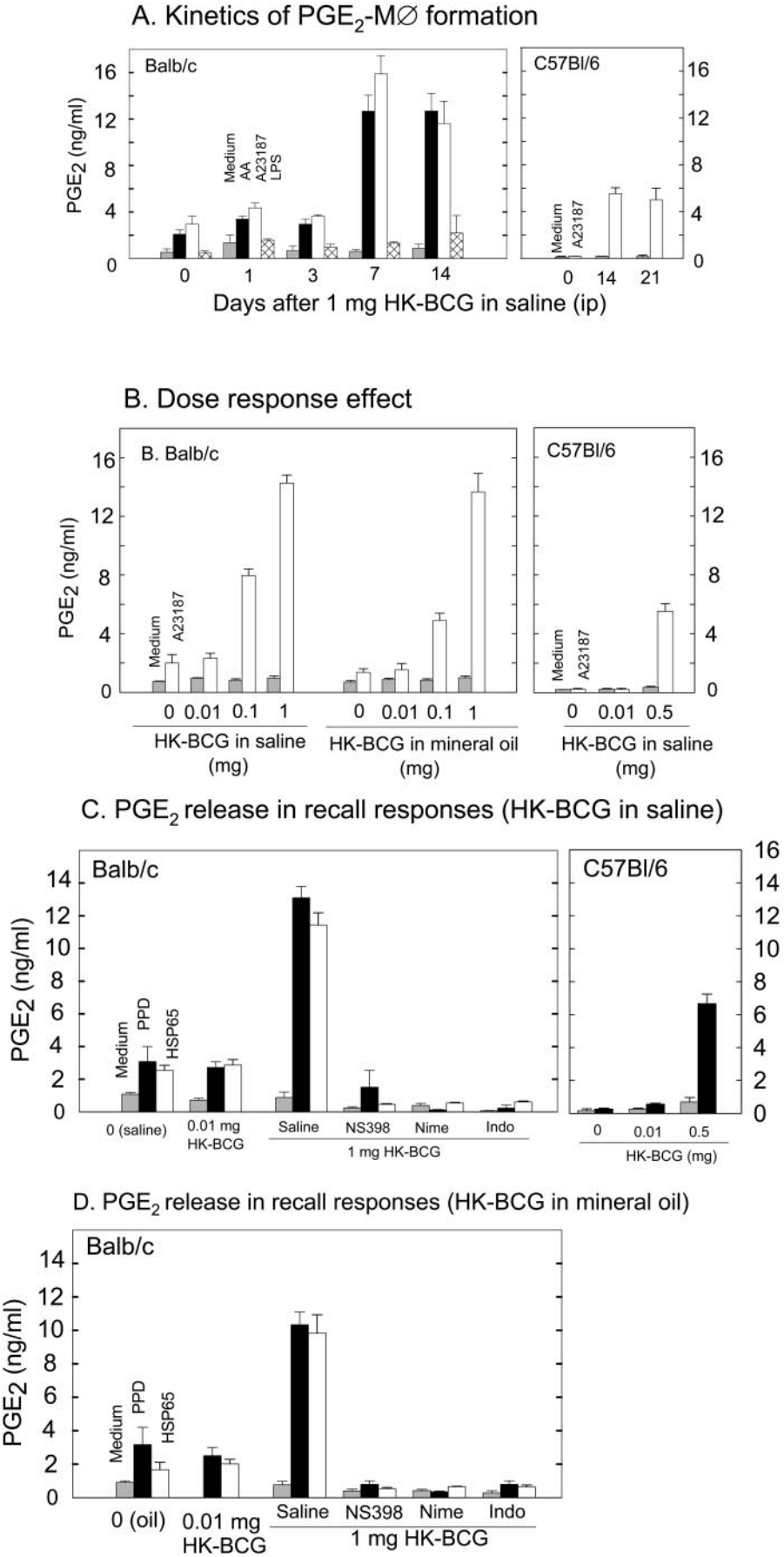

Fig. 1. HK-BCG induces splenic PGE_2-MØ formation. Groups of mice (four/group) received i.p. HK-BCG suspended in saline or mineral oil on Day 0. (A) Balb/c and C57Bl/6 mice were treated, respectively, with 1 mg and 0.5 mg HK-BCG in saline. Spleens were harvested on indicated day including Day 0 (untreated). Splenic MØ were isolated, pooled in each group, and incubated for 2 h in serum-free RPMI-1640 medium (10^6 MØ/ml) in the presence of 1 μg/ml AA, 1 μM A23187, 1 μg/ml LPS, or media alone (Medium). (B) Splenic MØ were isolated from mice 14 days after receiving HK-BCG suspended in saline or mineral oil at the indicated doses. PGE_2 release was elicited in vitro by 1 μM A23187 for 2 h. (C) Spleen cells were isolated from mice 14 days after receiving 1 mg HK-BCG in saline and incubated in RPMI 1640 plus 2% FBS (2×10^6 cells/ml) in the presence of mycobacterial antigens (5 μg/ml PPD or 1 μg/ml HSP65). In some groups, spleen cells were treated further with 1 μM NS-398, 1 μM nimesulide (Nime), 1 μM indomethacin (Indo), or saline (control) for 48 h. (D) Spleen cells were isolated from Balb/c mice, which received 1 mg HK-BCG in mineral oil. Recall responses and PGE_2 production were determined with methods similar to those in C. PGE_2 was measured by ELISA. Mean ± SD, n = 3. Each result represents a group of four mice from three experiments.

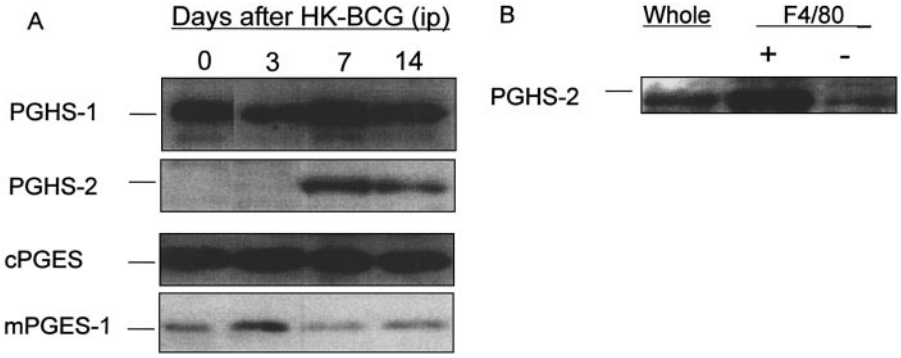

Fig. 2. PGHS-1, PGHS-2, cPGES, and mPGES-1 levels in HK-BCG-treated mice. (A) Balb/c mice received 1 mg HK-BCG in saline, i.p., on Day 0, and Days 0 (untreated), 3, 7, and 14 spleens were harvested. Lysates of splenic MØ isolated from each group of animals were analyzed by Western blotting. (B) F4/80-positive and -negative cells were isolated from Day 14 spleen cells as indicated in Materials and Methods. PGHS-2 expression in each fraction was determined by Western blotting. Each lane was loaded with 5 μg total protein. Results are representative of three separate experiments.

Splenic Th1 and Th2 immune responses against PPD and HSP65

To further characterize the mixed Th1 and Th2 response, levels of selected cytokines specific for Th1 or Th2 cells were measured in recall responses of cultured spleen cells. When cells from Balb/c mice receiving 0.01 mg HK-BCG were stimulated in vitro with 5 μg/ml PPD, significant amounts of IFN-γ (Th1 response) were detected compared with untreated mice (**Fig. 5**). These spleen cells also produced IL-10 (Th2 response, Fig. 5) but only minimal levels of IL-4 (data not shown). In contrast, spleen cells from animals receiving 1 mg HK-BCG produced less IFN-γ (Fig. 5) but increased amounts of IL-10 (Fig. 5) and IL-4 (**Fig. 6**). No IL-5 was detected (data not shown). It is interesting that HSP65 (1 μg/ml) stimulated IFN-γ production but not IL-4 or IL-10 production in recall responses (Fig. 5). When spleen cell cultures were treated with 1 μM nimesulide, a PGHS-2 inhibitor, IFN-γ production in recall responses was enhanced significantly. These results suggest that endogenous PGE_2 inhibits IFN-γ production, consistent with a shift from Th1-to-Th2 response in animals treated with 1 mg HK-BCG. However, the in vitro treatment with nimesulide only slightly reduced IL-10 production (Fig. 5A) and did not alter PPD-

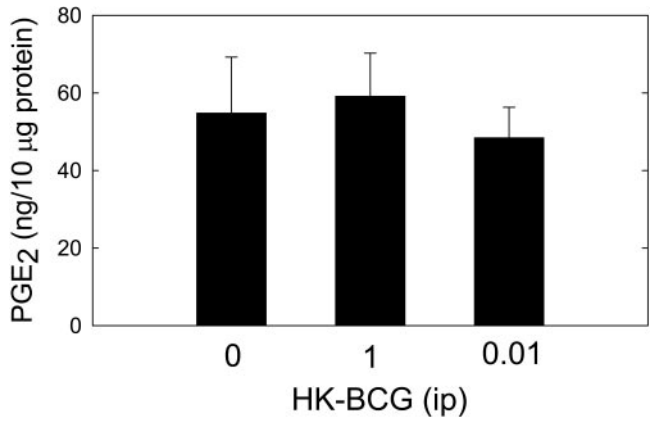

Fig. 3. PGES activities in splenic MØ isolated from BCG-treated mice. Groups of Balb/c mice (four/group) received 0 (0.2 ml saline), 0.01, or 1 mg HK-BCG i.p. in saline on Day 0; Day 14 spleens were harvested. Splenic MØ were isolated, pooled in each group, and sonicated. PGES activities in cell lysates were measured as described in Materials and Methods. PGE_2 was measured by ELISA. Mean ± SD, n = 3. In the absence of exogenous PGH_2, endogenous PGE_2 levels were ≤0.87 ng/10 μg protein.

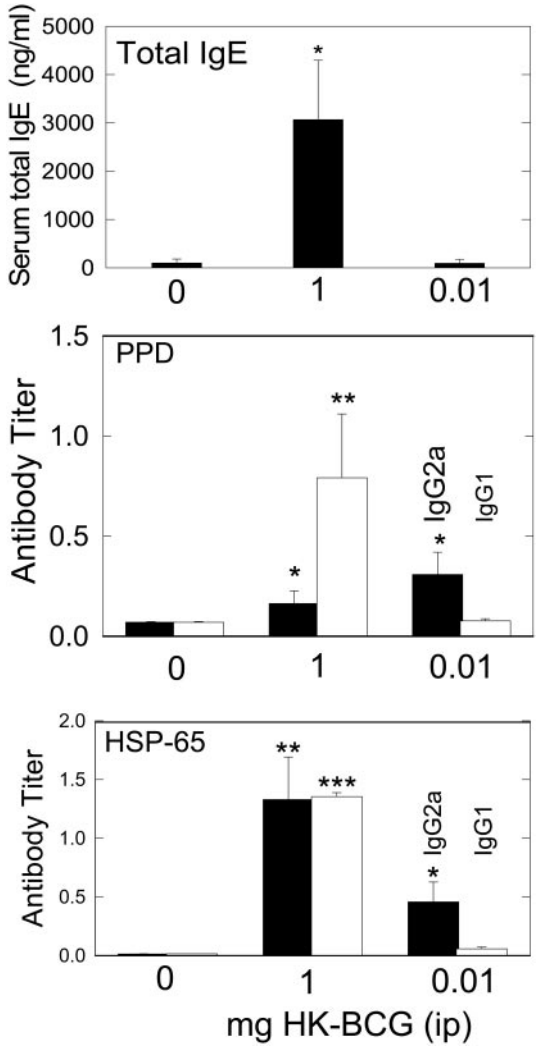

Fig. 4. Total serum IgE levels and mycobacterial antigen-specific IgG1 and IgG2a levels. Groups of Balb/c mice (five/group) received 0 (0.2 ml saline), 0.01, or 1 mg HK-BCG in saline on Day 0; Day 14 sera were harvested. Total IgE levels in the sera were measured by sandwich ELISA. Levels of PPD-specific IgG1 and IgG2a or HSP65-specific IgG1 and IgG2a in sera were measured as described in Materials and Methods. The sera were diluted 1/100 and 1/25 with saline for assays of antigen-specific IgG1 (open bars) and IgG2a (solid bars), respectively. Values are mean ± SD; n = 5. *, **, and ***, $P < 0.05$, $P < 0.01$, and $P < 0.005$, respectively, compared with the saline control group. Each result represents mice from two experiments.

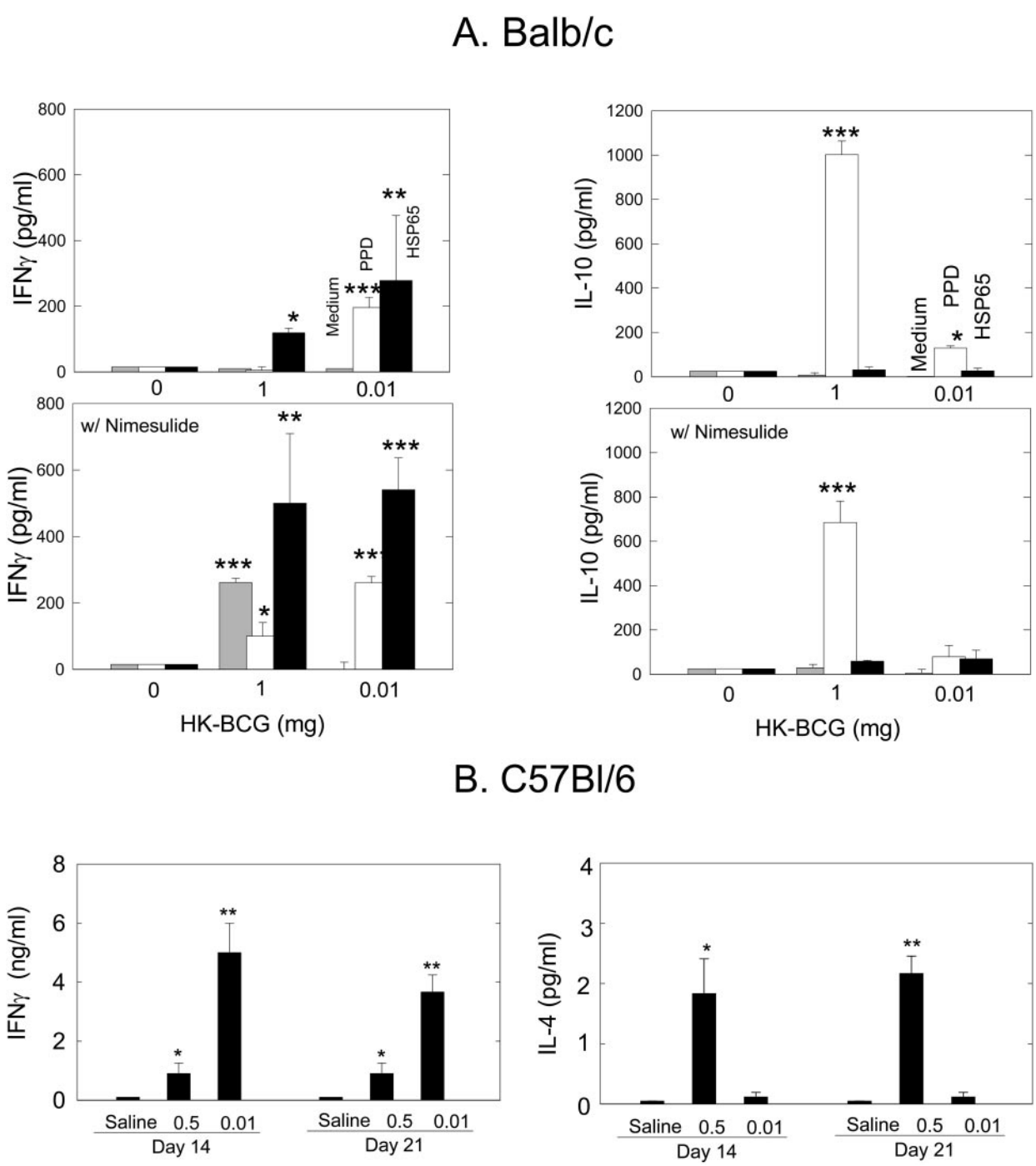

Fig. 5. Th1 and Th2 cytokine production in recall responses. (A) Groups of Balb/c mice (five/group) received 0 mg (0.2 ml saline), 0.01 mg, or 1 mg HK-BCG i.p. on Day 0; Day 14 spleen cells were isolated and stimulated in vitro with 5 μg/ml PPD or 1 μg/ml HSP65 for 4 days. In some cultures, 1 μM nimesulide was added. The levels of IFN-γ and IL-10 in the culture supernatants were measured by ELISA, as described in Materials and Methods. Values are mean ± SD from triplicate cultures. The data shown are for a group of five mice and are representative of two independent experiments. *, **, and ***, $P < 0.05$, $P < 0.01$, and $P < 0.005$, respectively, compared with the saline control group. (B) Groups of C57Bl/6 mice (four/group) received 0 (0.2 ml saline), 0.01, and 0.5 mg HK-BCG i.p. on Day 0; Days 14 and 21 spleens were isolated, and spleen cells were cultured in the presence of 5 μg/ml PPD for 4 days. The levels of IFN-γ and IL-4 in the supernatants were measured by ELISA. Mean ± SD, n = 3. * and **, $P < 0.05$, and $P < 0.01$, respectively, compared with the saline control group.

stimulated IL-4 production (data not shown), indicating that under these conditions, there is limited effect of PGE_2 on these Th2 cytokine responses.

Similar experiments with spleen cell cultures from C57Bl/6 mice stimulated with PPD revealed generally the same pattern of cytokine production (Fig. 5B). However, the levels of IFN-γ and IL-4 produced were much higher and lower, respectively, than those seen with spleen cells from Balb/c mice (Figs. 5 and 6). These responses were essentially unchanged when spleen cells were isolated 21 days following treatment with HK-BCG, as was also shown for PGE_2 production (Figs. 1 and 6). When spleen cells were depleted of $CD4^+$ cells by anti-CD4 antibody

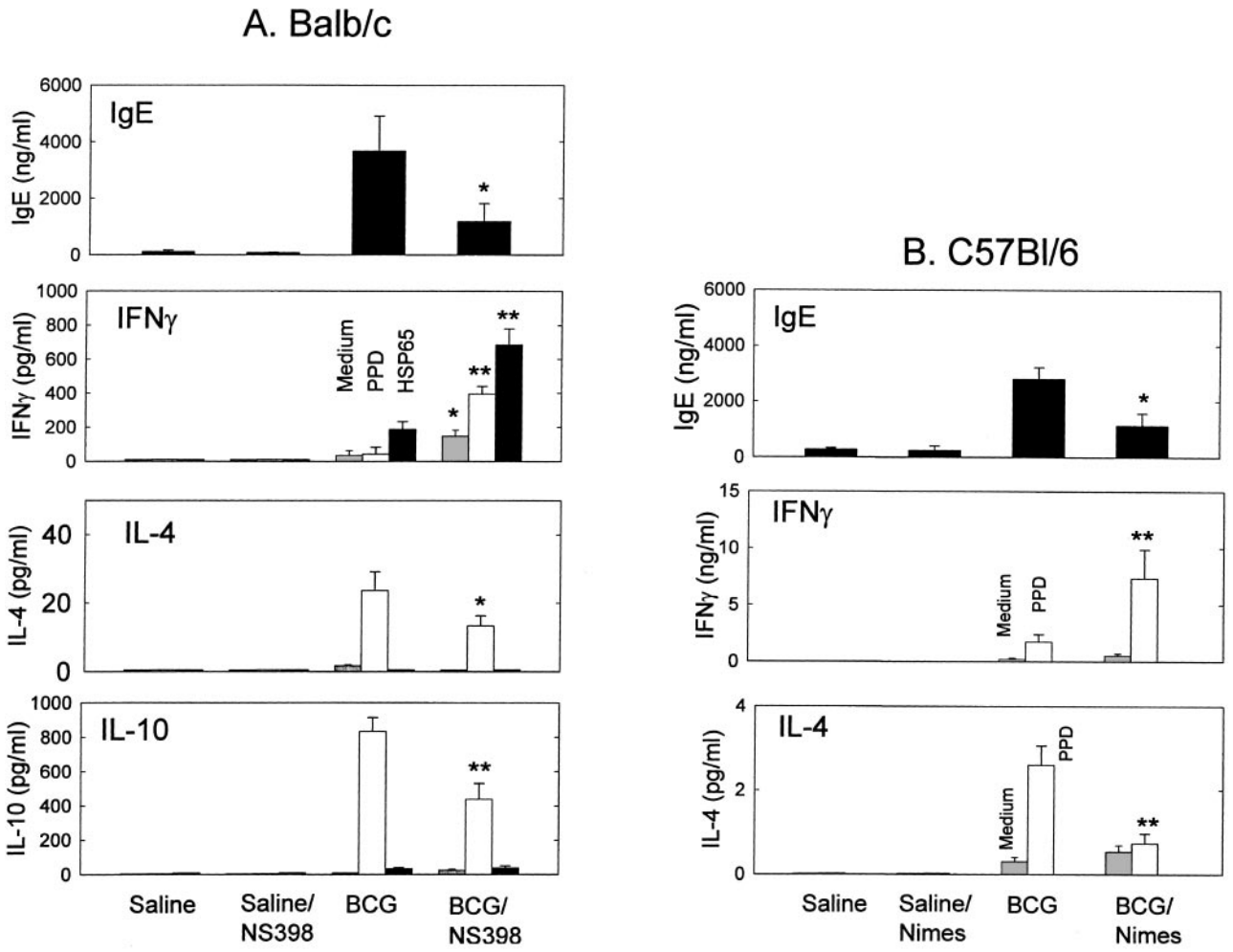

Fig. 6. PGHS-2 inhibitors (NS-398, nimesulide) in vivo inhibit Th2 cytokine production and decrease serum IgE levels but enhance IFN-γ production. (A) Groups of Balb/c mice (five/group) received 1 mg HK-BCG i.p. in saline on Day 0. Controls received 0.2 ml saline. BCG-treated mice and controls were treated i.p. daily starting on Day 1 with 10 mg/kg NS-398 or 0.5% ethanol in saline (0.2 ml). Day 14 sera and spleens were harvested. Total IgE in sera was measured by ELISA. Mean ± SD, n = 5. *, $P < 0.05$, compared with the BCG group. Spleen cells were cultured in the presence of 5 μg/ml PPD (open bars), 1 μg/ml HSP65 (solid bars), or medium alone (shaded bars) for 4 days; levels of IFN-γ, IL-4, and IL-10 in the culture supernatants were measured by ELISA. Mean ± SD, n = 3. * and **, $P < 0.01$, and $P < 0.005$, respectively, compared with the saline control group. (B) Groups of C57Bl/6 mice (four/group) received i.p. 0.5 mg HK-BCG on Day 0. Controls received 0.2 ml saline. Mice were treated i.p. daily starting on Day 1 with 10 mg/kg nimesulide or 0.5% ethanol in saline (0.2 ml). Day 14 sera and spleens were harvested, and spleen cells were cultured in the presence of 5 μg/ml PPD for 4 days. The levels of total IgE in sera and IL-4 and IFN-γ in the culture supernatants were measured by ELISA. Mean ± SD, n = 3. *, $P < 0.05$; **, $P < 0.01$, compared with saline treatment. Each result represents a group of four mice from two experiments.

(clone GK1.5) plus guinea pig serum, the remaining cells did not express detectable levels of IFN-γ, IL-10, or IL-4 in recall responses (data not shown), indicating that production of these cytokines was $CD4^+$ cell-dependent.

PGE_2 is known to inhibit production of IL-12, a Th1 cytokine inducing IFN-γ production [27]. However, in our studies, IL-12 was undetectable (<12 pg/ml) in the groups where IFN-γ levels were above baseline (data not shown). Despite the undetectable levels of IL-12 in the presence of nimesulide, where PGE_2 production is inhibited, IFN-γ levels are increased (Fig. 5). In our previous report [28], 1–10 μm chitin particles induced splenic MØ to produce IL-12, which was inversely dependent on the PGE_2 concentration. For example, when splenic MØ from normal C57Bl/6 and from high-dose HK-BCG groups were stimulated in vitro with 1–10 μm chitin particles at 100 μg/ml for 24 h, IL-12 levels were detected as 75 and <12 pg/ml/10^6 MØ, respectively. These results are consistent with earlier observations [20, 27, 28] that inhibition of IL-12 production by endogenous PGE_2 contributes to the reduction of Th1 responses.

In vivo effects of PGHS-2 inhibition in BCG-treated mice

To assess the effects of PGHS-2 activity on immune responses, Balb/c mice, which received 1 mg HK-BCG, were treated in vivo with NS-398. Recall responses of spleen cell cultures with PPD or HSP65 were determined 14 days after BCG treatment. Figure 6 shows that spleen cells isolated from animals treated with NS-398 in vivo and stimulated in vitro with PPD produced more IFN-γ but less IL-4 or IL-10 than those not treated with the PGHS-2 inhibitor. IL-4 and IL-10 were not detected in HSP65-stimulated recall responses, with or without NS-398.

Production of HSP65-stimulated IFN-γ was enhanced significantly by the treatment with NS-398 (Fig. 6). Finally, in vivo inhibition of PGHS-2 reduced total serum IgE levels (Fig. 6).

The pattern of response for total serum IgE in C57Bl/6 mice treated in vivo with NS-398 was similar to that for Balb/c mice. The IL-4 produced in PPD recall responses with cells from C57Bl/6 mice also followed a similar pattern to Balb/c mice, but the levels were reduced approximately tenfold (Fig. 6). Only relatively low levels (<100 pg/ml) of IL-10 were seen in C57Bl/6 mice (data not shown). The IFN-γ responses again followed a similar pattern to Balb/c mice, but the levels were increased markedly (Fig. 6). The results contrast with the recall responses for cells treated in vitro with a PGHS-2 inhibitor, described above. Following in vivo administration of a PGHS-2 inhibitor, the Th1-to-Th2 shift of splenic lymphocyte responses to mycobacterial antigens and serum IgE production develops in a PGHS-2-dependent manner.

DISCUSSION

These studies, as well as previous reports, clearly indicate that 5–21 days after treatment of mice with HK-BCG and HK-*C. parvum*, splenic PGE_2-MØ develop in a dose-dependent manner [6, 15, 16]. Splenic PGE_2-MØ express Fc receptors for IgG, phagocytic activity, and the surface marker F4/80. Calcium ionophore or AA but not LPS elicited PGE_2 release by splenic PGE_2-MØ. PGE_2 release also occurred when spleen cells from these BCG-treated mice were cultured and incubated in the presence of antigen (recall response; Fig. 1), suggesting that there is persistent, local release of PGE_2 in the spleen in the continued presence of antigen. Our results indicate that splenic PGE_2-MØ formation is associated with the induction of mixed Th1 and Th2 lymphocyte responses in a PGHS-2-dependent manner (Figs. 5 and 6).

Unlike MØ isolated from peritoneum, bone marrow, or blood, normal, splenic MØ exhibit only a relatively low level of PGE_2-release (<1 ng $PGE_2/10^6$ MØ) [13]. When activated in vitro by bacterial endotoxin and IFN-γ, splenic MØ produce a maximum of <2 ng/ml PGE_2 [13]. Additional factors must contribute to the enhanced PGE_2 release by splenic PGE_2-MØ isolated from mice 7–21 days after HK-BCG treatment. In mice depleted of bone marrow by ^{89}Sr, we previously found that splenic PGE_2-MØ are not formed following administration of HK-*C. parvum*, although these mice show increases in total splenic MØ and myeloid precursors [15, 16]. It is likely, therefore, that PGE_2-MØ precursors are generated in the radiosensitive bone marrow following HK-BCG treatment, then migrate, and localize in the spleen [16]. This process takes at least 5–7 days after administration of HK-BCG. Splenic PGE_2-MØ are located strategically to interact with lymphocytes and induce the shift of Th1-to-Th2 responses during mycobacterial infections. Once PGE_2-MØ are established, they persist for long periods [15], therefore prolonging the effect of PGE_2 on immune regulation. It should also be noted that splenic PGE_2-MØ formation is independent of circulating monocytes [15, 16]. Th1/Th2 cells that develop in the spleen eventually migrate to inflammatory sites [1].

Our results (Figs. 2 and 3) show that PGHS-2 is increased with increased PGE_2-MØ formation and is associated specifically with $F4/80^+$ cells. PGES activity and the mPGES-1 protein level are not increased in response to BCG treatment. In contrast, normal peritoneal MØ express high levels of mPGES-1 and PGHS-2 and release a large amount of PGE_2 [22, 24, 29] in responding to LPS in vitro. Therefore, the regulatory mechanisms PGHS-2 and PGE_2 synthesis in splenic PGE_2-MØ are distinct from peritoneal MØ. Although PGHS-2 may be necessary for the increased PGE_2 following BCG treatment, we have not demonstrated that this enzyme is sufficient or the rate-limiting factor. Secretory phospholipase A_2 type V is also known to be involved in prostaglandin production in splenic MØ, mast cells, and mesangial cells [19, 30], which makes this enzyme a possible candidate for regulation following BCG treatment. In addition, we have not investigated a possible role for the constitutively expressed PGES mPGES-2 [31]. Further analysis will be required to identify the required enzymes and rate-limiting factor(s) for PGE_2 synthesis in splenic PGE_2-MØ.

Balb/c mice, compared with C57Bl/6 mice, frequently show the induction of significant Th2 responses after infection or immunization, such as with BCG [32] and *Leishmania major* [21, 33]. Normal, splenic MØ isolated from Balb/c mice and treated in vitro with LPS produce more PGE_2 than those from C57Bl/6 mice [18] and have a greater sensitivity to the Th1-suppressive effect of PGE_2 [18, 19]. However, our studies indicate that C57Bl/6 mice express splenic PGE_2-MØ and a mixed Th1 and Th2 lymphocyte response against mycobacteria in responding to high-dose HK-BCG with a pattern similar to that for Balb/c mice. The remarkable differences between these two strains are the increased amount of IFN-γ and decreased amount of IL-4 produced.

Infections caused by *Chlamydia pneumoniae* and *Helicobacter pylori* as well as *Mycobacterium sp* have been implicated as co-risk factors in atherosclerosis [34, 35]. Seroepidemiological studies [36] indicate that patients with atherosclerosis express high antibody titers against mycobacterial HSP65, a prokaryotic HSP of the 60-kDa family (HSP60/65), indicating a Th2-mediated, humoral response against this antigen. Prokaryotic HSP60/65 are >97% homologous, whereas prokaryotic and human/mouse HSP60/65 have >70% amino acid sequence homology. By attacking stressed arteries that express endogenous HSP60, anti-HSP65 may contribute to atherosclerosis through antigenic mimicry [36]. Microbial HSP60/65 serves as an immunodominant antigen in protection from and in the pathogenesis of infectious diseases [34, 36–38]. The development of Th1 cells against HSP65 mediates resistance to *M. tuberculosis*, which constitutes a major vaccine strategy against tuberculosis [8]. However, in recurrent and chronic infections, anti-HSP65, which does not have a decisive, protective role against mycobacterial infections, may promote the further progression of atherosclerotic plaques [36, 39]. Similarly, Th1-to-Th2 shifts of the immune response may be associated with the pathogenesis of various chronic, immunologically mediated diseases.

Live BCG induces not only host resistance to *M. tuberculosis* but also tumoricidal activity and has been used widely for treatment of superficial bladder cancer [40, 41]. Live BCG also

induces a nonspecific M∅ microbicidal activity against *Listeria monocytogenes* [42, 43] and *Toxoplasma gondii* [44]. It has been reported that a low dose (0.01 mg) of HK-BCG suspended in saline induced a Th1 lymphocyte response against mycobacteria, but the effects were not sufficient to protect against mycobacterial infections, whereas immunization with HK-mycobacteria in mineral oil (CFA) was protective [45]. Although mineral oil provides additional adjuvant activity, our study indicates that mineral oil itself has no effect on the formation of splenic PGE_2-M∅.

In conclusion, previously, we found that development of splenic PGE_2-M∅ is dependent on radiosensitive bone marrow cells and that the PGE_2-M∅ are probably derived directly from the bone marrow [6, 15, 16]. Here, we have continued our investigation of the unique splenic PGE_2-M∅ population, which plays a novel, PGHS-2-dependent, immunoregulatory role in the Th1-to-Th2 shift of immune responses against mycobacterial antigens including HSP65. It is possible that PGE_2 as an antiapoptotic agent also promotes antigen-presenting activity of dendritic cells [46], whose maturation is induced directly by BCG, and may skew the immune response toward a Th2-like profile [47]. PGE_2 is known to increase regulatory T cell differentiation and function, which could contribute to the immune responses of BCG-treated mice [48, 49]. Finally, it remains to be elucidated whether similar mechanisms are involved in the pathogenesis of *Leishmania* infection, *Trichophyton* dermatophytosis, syphilitic infection, or human immunodeficiency virus infection progressing to AIDS [21, 50–53], where Th1-to-Th2 shifts of immune responses and splenic PGE_2-M∅ formation are frequently seen. Th1-to-Th2 shifts are also observed in neonatal, aged, tumor-bearing, diabetic, and hypercholesterolemic animals [14, 54–58].

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Splenic PGE_2-releasing macrophages regulate Th1 and Th2 immune responses in mice treated with heat-killed BCG

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Abstract: Hosts infected with low doses of mycobacteria develop T helper cell type 1 (Th1) immunity, but at relatively higher doses, a switch to Th2 immunity occurs. Prostaglandin E_2 (PGE_2) is a proposed mediator of the Th1-to-Th2 shift of immune responses, and mycobacterial products induce PGE_2-releasing macrophages (PGE_2-M∅) in the mouse spleen in a dose-dependent manner. Splenic PGE_2-M∅ from Balb/c mice, given 0.01 or 1 mg heat-killed (HK) *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) intraperitoneally (i.p.), were characterized by the ex vivo release of PGE_2 (>10 ng/10^6 cells), cytokine production, and expression of PGG/H synthase (PGHS)-1, PGHS-2, cytosolic PGE synthase (PGES), and microsomal PGES-1. At Day 14 after the treatment, mice treated with 1 mg, but not 0.01 mg, BCG had increased levels of PGHS-2^+ PGE_2-M∅, total serum immunoglobulin E (IgE), and serum IgG1 antibodies (Th2 responses) against heat shock protein 65 and purified protein derivative. Cultures of spleen cells isolated from these mice expressed interleukin (IL)-4 and IL-10 in recall responses. Treatment of mice receiving 1 mg BCG with NS-398 (a PGHS-2 inhibitor, 10 mg/kg i.p., daily) resulted in enhanced interferon-γ (IFN-γ) production with reduced IL-4 and IL-10 production in recall responses. This treatment also resulted in decreased total serum IgE levels. Treatment of C57Bl/6 mice with HK-BCG (0.5 mg dose) also induced a mixture of Th1 and Th2 responses, although IFN-γ production was markedly increased, and IL-4 was decreased compared with Balb/c mice. Thus, our results indicate that by 14 days following treatment of mice with high doses of HK-BCG, splenic PGE_2-M∅ formation is associated with a PGHS-2-dependent shift from Th1-to-Th2 immune responses. *J. Leukoc. Biol.* 78: 1281–1290; 2005.

Key Words: *PGHS-2 (Cox-2) · cPGES · purified protein derivative · splenic macrophages · PGE_2 · Th1-to-Th2 shift*

INTRODUCTION

T helper cell type 1 (Th1) adjuvants play an important role in the development of protective immunity against intracellular infections such as tuberculosis. Previously, Power et al. [1] found that relatively low doses of live *Mycobacterium bovis* bacillus Calmette-Guerin (BCG), a vaccine strain for *Mycobacterium tuberculosis*, lead to a cell-mediated, Th1 response, and higher doses induce mixed cell-mediated immune and Th2-mediated humoral responses. The induction of antibodies usually leads to a chronic or progressive and fatal outcome in tuberculosis [2, 3]. The mechanism for such a dose-dependent immunological shift is unknown.

Complete Freund's adjuvant [CFA; heat-killed (HK)-*M. tuberculosis*] and HK-BCG in mineral oil have been widely used to establish animal models of autoimmune diseases, such as experimental autoimmune encephalomyelitis, neuritis, uveitis, thyroiditis, orchitis, and adjuvant arthritis [4, 5]. The adjuvants enhance Th1-mediated macrophage (M∅) activation and Th2-mediated antibody formation [6]. Pathogenic roles of Th1/Th2 responses appear to be varied among autoimmune disease models [5], which may be related to the range of HK-mycobacteria concentrations (0.05–0.5 mg) used in these animal models [5, 7].

Much attention has been directed to the role of mycobacterial heat shock protein 65 (HSP65), an immunodominant antigen. It has been proposed that a host Th1 response to HSP65 plays a protective role in mycobacterial infections [8]. However, Th1-to-Th2 shifts of immune responses against mycobacteria result in the formation of antibodies against HSP65, which does not have a decisive, protective role against infection [2]. Furthermore, as antibodies or T cells specific for bacterial HSP65 potentially cross-react with host HSPs, these immune responses may have pathogenic roles in autoimmune diseases [9]. Th1 cytokines, including interferon-γ (IFN-γ), promote or counteract pathogenesis, depending on the autoimmune disease [9]. Therefore, Th1-to-Th2 shifts of immune responses may be associated with the pathogenesis of various autoimmune diseases as well as chronic intracellular infections [10–12].

In previous studies, we have reported that normal, splenic M∅ stimulated ex vivo do not produce significant levels of

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prostaglandin E_2 (PGE_2), distinct from MØ originating from other tissues [13]. However, high doses of HK-BCG or related products such as HK-*Propionibacterium acnes* (*Corynebacterium parvum*) in vivo induce stable and sustained formation of PGE_2-releasing MØ (PGE_2-MØ) in the spleen [6, 14–16]. The mechanism for formation of these MØ is still unclear. Previous studies [15, 16] indicate that the formation of splenic PGE_2-MØ is dependent on radiosensitive bone marrow cells, which may supply precursors of splenic PGE_2-MØ. Alternatively, an inflammatory cytokine "milieu" may up-modulate PGE_2 biosynthesis directly by splenic MØ [17–19].

The spleen is the lymphoid tissue where PGE_2-MØ and immune lymphocytes interact in chronic inflammatory diseases. PGE_2 inhibits the production of Th1 cytokines, such as interleukin (IL)-2, IL-12, and IFN-γ [20]. In contrast, PGE_2, depending on stimulatory conditions, has no effect or enhances production of Th2 cytokines, such as IL-4, IL-5, and IL-10 [20, 21]. Therefore, we have examined the hypothesis that splenic PGE_2-MØ development in vivo following treatment with high doses of HK-BCG is time-, dose-, and PGG/H synthase (PGHS)-2-dependent and promotes a Th1-to-Th2 shift in specific immune responses. Because of described differences in immune responses between Balb/c and C57Bl/6 mice [18, 20], we have included results obtained with both strains.

MATERIALS AND METHODS

Mice

Nonpregnant female Balb/c and C57Bl/6 mice, 8–14 weeks old, were obtained from Harlan Laboratory (Indianapolis, IN). Mice were maintained in barrier-filtered cages under specific, pathogen-free conditions in the animal care facility at East Carolina University (Greenville, NC) or Florida Atlantic University (Boca Raton).

Preparations of HK-BCG

As described previously [6], cultured *M. bovis* BCG Tokyo 172 strain was washed, autoclaved, and lyophilized. The powder of HK-BCG was suspended in saline and dispersed by brief (10 s) sonication immediately prior to injection. These HK-BCG preparations contained undetectable levels of endotoxin (<0.03 EU/ml), as determined by the *Limulus* amebocyte lysate assay (Sigma Chemical Co., St. Louis, MO) [6]. HK-BCG was also suspended in mineral oil (M-5904, Sigma Chemical Co.) in some experiments.

Treatment of mice with HK-BCG

Groups of mice received 0.01, 0.1, 0.5, or 1 mg HK-BCG, intraperitoneally (i.p.), on Day 0. Controls received 0.2 ml saline. In some experiments, mice receiving BCG and controls were further treated i.p with 10 mg/kg NS-398 or nimesulide (Cayman Chemical, Ann Arbor, MI) daily, starting on Day 1. Control groups of mice received 0.5% ethanol in saline (0.2 ml/dose). Unless indicated, spleens and sera were harvested on Day 14. To compare the effects of HK-BCG suspended in saline or mineral oil, additional groups of mice were given HK-BCG in mineral oil i.p. Controls received 0.2 ml mineral oil.

Cytokine production in recall response of spleen cell cultures

Spleens from each group of mice were isolated, pooled, minced with scissors, digested with 50 U/ml collagenase D (C2139, Sigma Chemical Co.) in RPMI 1640 plus 10% fetal bovine serum (FBS), 37°C, for 60 min, and filtered through a 100-μm mesh. Single-cell suspensions were prepared by washing digested cells with RPMI 1640 containing 100 μg/ml DNase (DN-25, Sigma Chemical Co.). After washing with serum-free RPMI 1640, cell suspensions were applied to the top of a discontinuous Percoll gradient (35/60%). Following centrifugation, 800 *g*, 30 min, 22°C, cells at the Percoll interface were collected. Spleen cells were suspended in RPMI 1640 plus 10% FBS at 4×10^6 cells/ml and incubated with endotoxin-free mycobacterial HSP65 (Stressgen, Victoria, BC, Canada) or purified protein derivative (PPD; Japan BCG Laboratory, Tokyo) at 1 or 5 μg/ml, respectively, for 4 days. In some experiments, 10^{-6} M nimesulide was added to the cultures. After incubation, culture supernatants were collected, and IL-4, IL-10, and IFN-γ levels were measured by the respective enzyme-linked immunosorbent assay (ELISA; PharMingen, San Diego, CA).

PGE_2-MØ

Plastic adherent splenic MØ were isolated from spleen-cell suspensions prepared above [13]. Splenic MØ (2×10^6/ml) were cultured in serum-free RPMI-1640 medium with 10^{-6} M calcium ionophore A23187 (Sigma Chemical Co.), 1 μg/ml arachidonic acid (AA; Cayman Chemical), or 1 μg/ml bacterial endotoxin [lipopolysaccharide (LPS), Sigma Chemical Co.] for 2 h. In some experiments, splenic cells (2×10^6/ml) were cultured in RPMI-1640 medium plus 2% FBS with 5 μg/ml PPD or 1 μg/ml HSP65 for 2 days in the presence of the PGHS inhibitors nimesulide, indomethacin, or NS-398, all at 1 μM. PGE_2 levels in the culture supernatants were measured by competitive ELISA (Cayman Chemical).

Magnetic separation of F4/80-positive cells

Red cell-free spleen cells (10^8 cells) were stained with 5 μg/ml monoclonal antibody (mAb) F4/80 recognizing spleen MØ (Accurate Chemical and Scientific Corp., Westbury, NY), followed by addition of 200 μl magnetic microbead-conjugated goat anti-rat immunoglobulin G (IgG; 130-048-501, Miltenyi Biotec, Auburn, CA). F4/80- positive and -negative cells were isolated according to the company's instructions. The content of F4/80 cells, determined cytometrically, in positive and negative preparations, was 90% and less than 2%, respectively (data not shown).

PGE synthase (PGES) assay

PGES activity in cell lysates was measured as conversion of PGH_2 to PGE_2 [22]. Adherent, splenic MØ in 400 μl 10 mM Tris, pH 8.0, were disrupted by sonication using a Branson sonifier (10 s, three times at 1-min intervals). After centrifugation of the sonicates at $1700\times g$ for 10 min, 4°C, the supernatants were used as the source of enzyme activity. An aliquot of each lysate (10 μg protein) was incubated with 0.5 μg PGH_2 (Cayman Chemical) for 30 s at 24°C in 0.1 ml 0.1 M Tris, pH 8.0, containing 1 mM reduced L-glutathione (Sigma Chemical Co.) and 5 μg indomethacin. After terminating the reaction by addition of 100 mM $FeCl_2$, PGE_2 in the supernatants was quantified by competitive ELISA (Cayman Chemical). Protein concentrations were determined by bicinchoninic acid (BCA) assay (Pierce, Rockford, IL) using bovine serum albumin as standard.

Total serum IgE, antigen-specific IgG1 and IgG2a

Total serum IgE was determined by ELISA using purified mouse IgE κ isotype as the standard rat anti-mouse IgE mAb (clone R35-72) as capture antibody and biotinylated rat mAb detecting IgE (clone R25-92), as detection antibody (all reagents from PharMingen) [6]. Levels of PPD-specific IgG1/IgG2a and HSP65-specific IgG1 and IgG2a were measured by ELISA with 96-well plates coated overnight at 4°C with 0.5 μg PPD or 0.1 μg HSP65 per well in 100 μl 0.05 M sodium carbonate buffer, pH 9.6 [6]. Biotinylated rat mAb, detecting IgG1 and IgG2a, were clones A85-1 and R19-15, respectively (PharMingen).

Western blotting

Splenic MØ were prepared as described above, harvested, and washed three times with cold saline. Washed cells were resuspended in lysis buffer {50 mM Tris, pH 7.5, 150 mM NaCl, 1:500 Sigma protease inhibitor cocktail (P8340, Sigma Chemical Co.), 1% Nonidet P-40, and 1% sodium deoxycholate [13]}. Debris was eliminated by centrifugation (5 min, $1000\times g$). Protein concentration in the lysate was measured using BCA as described above. Equal amounts of protein were loaded onto sodium dodecyl sulfate-polyacrylamide minigels and separated by electrophoresis (200 V for 45 min). Proteins were then

transferred to a polyvinylidene difluoride (Sigma Chemical Co.) membrane, which was blocked with 5% nonfat dry milk and incubated with antibody [anti-PGHS-1, 1:1000; anti-PGHS-2, 1:4000; anticytosolic PGES (anti-cPGES), 1:1000; antimicrosomal PGES-1 (anti-mPGES-1), 1:1000, all from Cayman Chemical] in 5% nonfat dry milk overnight at 4°C. Following incubation with peroxidase-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA), proteins were detected by enhanced chemiluminescence (Amersham, Piscataway, NJ) following the manufacturer's instructions [13].

Statistics

Data were analyzed by one-way ANOVA. For cell culture studies, tissues isolated from at least four mice were pooled unless indicated; cells were cultured in at least triplicate in each group. $P < 0.05$ was considered statistically significant.

RESULTS

HK-BCG induces splenic PGE_2-M∅

Splenic M∅ isolated from normal mice show minimum levels of PGE_2 release [6, 13, 15, 16]. To characterize splenic PGE_2-M∅ formation, Balb/c mice were treated i.p. with 1 mg HK-BCG in saline. When splenic M∅ were isolated 1 and 3 days later and stimulated in vitro with A23187 for 2 h, PGE_2 release was not different from control cells obtained from untreated animals (**Fig. 1A**). Significantly higher PGE_2 release was observed 7 and 14 days after treatment with 1 mg HK-BCG. Similar experiments with C57Bl/6 mice treated with 0.5 mg HK-BCG showed that production of PGE_2 persists for at least 21 days. Because of the different treatment conditions, PGE_2 levels in the two strains of mice cannot be compared directly, although the results suggest that production of PGE_2 by C57Bl/6 M∅ is lower (Fig. 1, A and B). Further, AA, but not LPS, also elicited PGE_2 release from Balb/c spleen cells (Fig. 1A).

To examine the dose-response effect for HK-BCG treatment, mice were given increasing doses of HK-BCG in saline, and PGE_2-M∅ activities were determined 14 days after treatment. Figure 1B shows that HK-BCG-induced PGE_2-M∅ formation was dose-dependent. There was no increase in PGE_2 release on Day 14 for Balb/c or C57Bl/6 mice treated with 0.01 mg HK-BCG (Fig. 1B). Treatment with HK-BCG suspended in mineral oil also resulted in PGE_2-M∅ formation in a dose-dependent manner, similar to that seen with HK-BCG in saline (Fig. 1B).

To determine whether recall responses in vitro elicit PGE_2 release, spleen cells isolated from mice treated with HK-BCG in saline were stimulated with the mycobacterial antigens, PPD or HSP65. As shown in Figure 1C, these antigens induced spleen cells to release PGE_2 in both strains of mice, dependent on the HK-BCG dose and suggesting that interaction between antigen-specific lymphocytes and splenic M∅ triggers PGE_2 biosynthesis. Furthermore, in Balb/c mice treated with 1 mg HK-BCG in saline or mineral oil, PGE_2 biosynthesis was inhibited by NS-398, nimesulide, or indomethacin, consistent with mediation of PGE_2 synthesis by PGHS-2 in splenic PGE_2-M∅ (Fig. 1D).

Our results clearly indicate that HK-BCG, in a dose-dependent manner, induces splenic PGE_2-M∅ formation within 7–14 days. Inhibition by the PGHS-2 selective inhibitors nimesulide and NS398 implies a dependence on PGHS-2 for PGE_2 synthesis. There is no difference in the magnitude of PGE_2-M∅ formation in response to HK-BCG suspended in saline or in mineral oil. Therefore, HK-BCG suspended in saline was used to further characterize PGE_2-M∅ and determine whether these cells contribute to the Th1-to-Th2 shift of immune responses.

Protein detection of PGHS-1, PGHS-2, mPGES-1, and cPGES in splenic PGE_2-M∅

PGE_2-M∅ metabolize endogenous AA to PGE_2 through the rate-limiting enzymes PGHS and PGES. Two major isoforms of PGHS convert AA to PGH_2: PGHS-1, a constitutive form, and PGHS-2, an inducible form. PGH_2 is subsequently converted to PGE_2 by cPGES and mPGES-1 [22, 23]. Murakami et al. [22] reported that mPGES-1 is a terminal enzyme of PGHS-2-mediated PGE_2 synthesis, and PGHS-2 and mPGES-1 are induced in various cells, including peritoneal M∅, by proinflammatory stimuli. Normal, splenic M∅ expressed PGHS-1, mPGES-1, and cPGES but not PGHS-2 (**Fig. 2**). This profile was unchanged in splenic M∅ isolated from Balb/c mice 14 days after receiving 0.01 mg HK-BCG in saline (data not shown). When Balb/c mice were treated with 1 mg HK-BCG, PGHS-2 was detected on Days 7 and 14 (Fig. 2). The levels of PGHS-1, mPGES-1, and cPGES remained similar to those in normal, splenic M∅ (Fig. 2). Therefore, increased PGHS-2 levels, but not mPGES-1 levels, were associated with increases in PGE_2 release by splenic PGE_2-M∅. Figure 2B shows that PGHS-2 was highly enriched in F4/80-positive M∅ on Day 14.

PGES activities

cPGES and membrane-bound, glutathione-dependent PGES (mPGES-1) have been shown to be terminal enzymes of PGHS-1- and PGHS-2-mediated PGE_2 biosynthesis, respectively [22, 24]. PGES activity assays were performed to determine whether changes following treatment with 1 mg HK-BCG accounted for the increase in PGE_2 production. As shown in **Figure 3**, PGES activity was not altered significantly in Balb/c mice treated with HK-BCG, indicating that increases in PGE_2 production do not result from changes in PGES activity.

Serum IgE, IgG1, and IgG2a

Endogenous Th1 and Th2 cytokines are isotype-switching signals for antigen-specific B cells, which are biased toward IgG2a and IgE/IgG1, respectively [25, 26]. Treatment of Balb/c mice with 1 mg HK-BCG resulted in increased serum levels of total IgE, HSP65-specific IgG1, and HSP65-specific IgG2a (**Fig. 4**). Similar levels of IgE were found in C57Bl/6 mice treated with 0.5 mg HK-BCG (see Fig. 6). In contrast, when treated with 0.01 mg HK-BCG, there was no increase in total serum IgE or IgG1 levels specific against HSP65 or PPD but a significant increase in HSP65- and PPD-specific IgG2a (Fig. 4). These results indicate that treatment with 1 mg HK-BCG produces a mixture of Th1 and Th2 responses against mycobacterial antigens including HSP65, whereas 0.01 mg HK-BCG produces Th1-dominant responses.

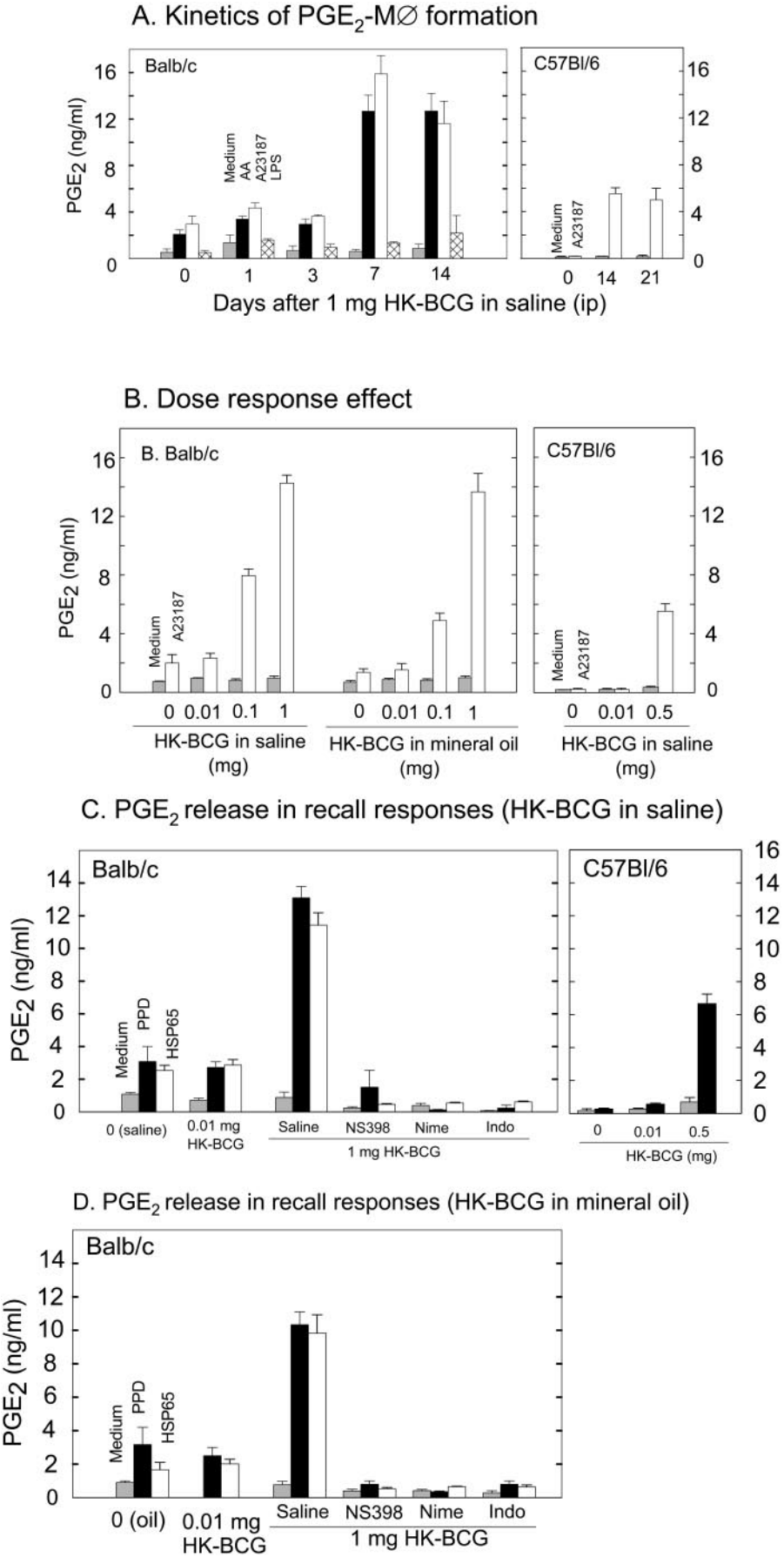

Fig. 1. HK-BCG induces splenic PGE_2-MØ formation. Groups of mice (four/group) received i.p. HK-BCG suspended in saline or mineral oil on Day 0. (A) Balb/c and C57Bl/6 mice were treated, respectively, with 1 mg and 0.5 mg HK-BCG in saline. Spleens were harvested on indicated day including Day 0 (untreated). Splenic MØ were isolated, pooled in each group, and incubated for 2 h in serum-free RPMI-1640 medium (10^6 MØ/ml) in the presence of 1 μg/ml AA, 1 μM A23187, 1 μg/ml LPS, or media alone (Medium). (B) Splenic MØ were isolated from mice 14 days after receiving HK-BCG suspended in saline or mineral oil at the indicated doses. PGE_2 release was elicited in vitro by 1 μM A23187 for 2 h. (C) Spleen cells were isolated from mice 14 days after receiving 1 mg HK-BCG in saline and incubated in RPMI 1640 plus 2% FBS (2×10^6 cells/ml) in the presence of mycobacterial antigens (5 μg/ml PPD or 1 μg/ml HSP65). In some groups, spleen cells were treated further with 1 μM NS-398, 1 μM nimesulide (Nime), 1 μM indomethacin (Indo), or saline (control) for 48 h. (D) Spleen cells were isolated from Balb/c mice, which received 1 mg HK-BCG in mineral oil. Recall responses and PGE_2 production were determined with methods similar to those in C. PGE_2 was measured by ELISA. Mean ± SD, n = 3. Each result represents a group of four mice from three experiments.

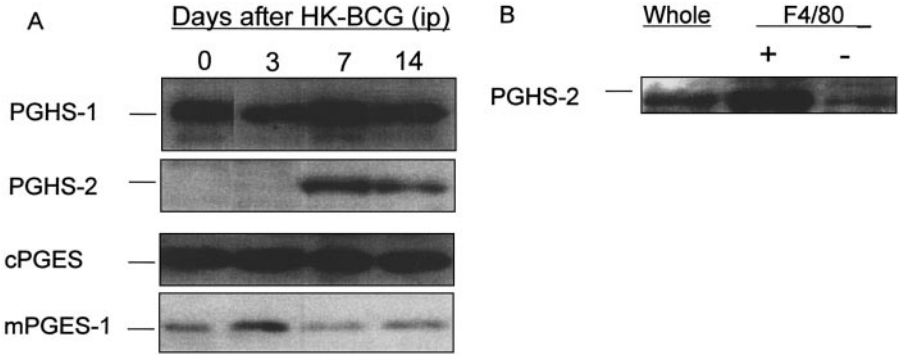

Fig. 2. PGHS-1, PGHS-2, cPGES, and mPGES-1 levels in HK-BCG-treated mice. (A) Balb/c mice received 1 mg HK-BCG in saline, i.p., on Day 0, and Days 0 (untreated), 3, 7, and 14 spleens were harvested. Lysates of splenic MØ isolated from each group of animals were analyzed by Western blotting. (B) F4/80-positive and -negative cells were isolated from Day 14 spleen cells as indicated in Materials and Methods. PGHS-2 expression in each fraction was determined by Western blotting. Each lane was loaded with 5 μg total protein. Results are representative of three separate experiments.

Splenic Th1 and Th2 immune responses against PPD and HSP65

To further characterize the mixed Th1 and Th2 response, levels of selected cytokines specific for Th1 or Th2 cells were measured in recall responses of cultured spleen cells. When cells from Balb/c mice receiving 0.01 mg HK-BCG were stimulated in vitro with 5 μg/ml PPD, significant amounts of IFN-γ (Th1 response) were detected compared with untreated mice (**Fig. 5**). These spleen cells also produced IL-10 (Th2 response, Fig. 5) but only minimal levels of IL-4 (data not shown). In contrast, spleen cells from animals receiving 1 mg HK-BCG produced less IFN-γ (Fig. 5) but increased amounts of IL-10 (Fig. 5) and IL-4 (**Fig. 6**). No IL-5 was detected (data not shown). It is interesting that HSP65 (1 μg/ml) stimulated IFN-γ production but not IL-4 or IL-10 production in recall responses (Fig. 5). When spleen cell cultures were treated with 1 μM nimesulide, a PGHS-2 inhibitor, IFN-γ production in recall responses was enhanced significantly. These results suggest that endogenous PGE_2 inhibits IFN-γ production, consistent with a shift from Th1-to-Th2 response in animals treated with 1 mg HK-BCG. However, the in vitro treatment with nimesulide only slightly reduced IL-10 production (Fig. 5A) and did not alter PPD-

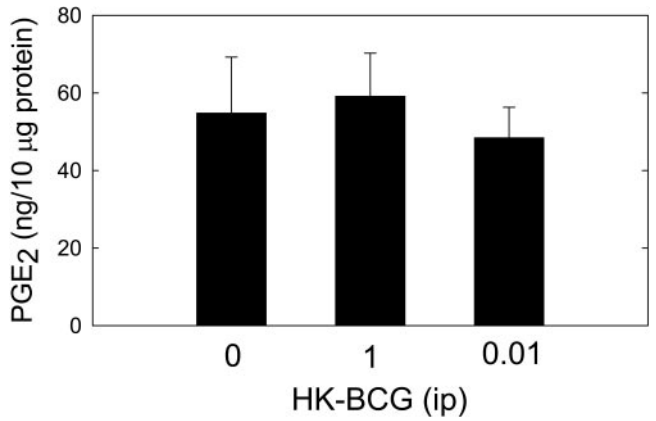

Fig. 3. PGES activities in splenic MØ isolated from BCG-treated mice. Groups of Balb/c mice (four/group) received 0 (0.2 ml saline), 0.01, or 1 mg HK-BCG i.p. in saline on Day 0; Day 14 spleens were harvested. Splenic MØ were isolated, pooled in each group, and sonicated. PGES activities in cell lysates were measured as described in Materials and Methods. PGE_2 was measured by ELISA. Mean ± SD, n = 3. In the absence of exogenous PGH_2, endogenous PGE_2 levels were ≤0.87 ng/10 μg protein.

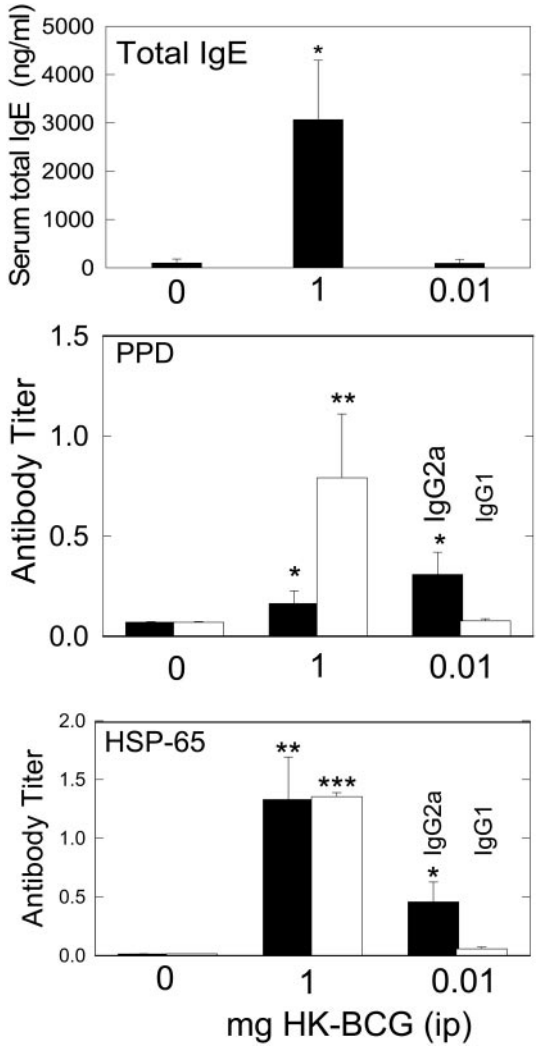

Fig. 4. Total serum IgE levels and mycobacterial antigen-specific IgG1 and IgG2a levels. Groups of Balb/c mice (five/group) received 0 (0.2 ml saline), 0.01, or 1 mg HK-BCG in saline on Day 0; Day 14 sera were harvested. Total IgE levels in the sera were measured by sandwich ELISA. Levels of PPD-specific IgG1 and IgG2a or HSP65-specific IgG1 and IgG2a in sera were measured as described in Materials and Methods. The sera were diluted 1/100 and 1/25 with saline for assays of antigen-specific IgG1 (open bars) and IgG2a (solid bars), respectively. Values are mean ± SD; n = 5. *, **, and ***, $P < 0.05$, $P < 0.01$, and $P < 0.005$, respectively, compared with the saline control group. Each result represents mice from two experiments.

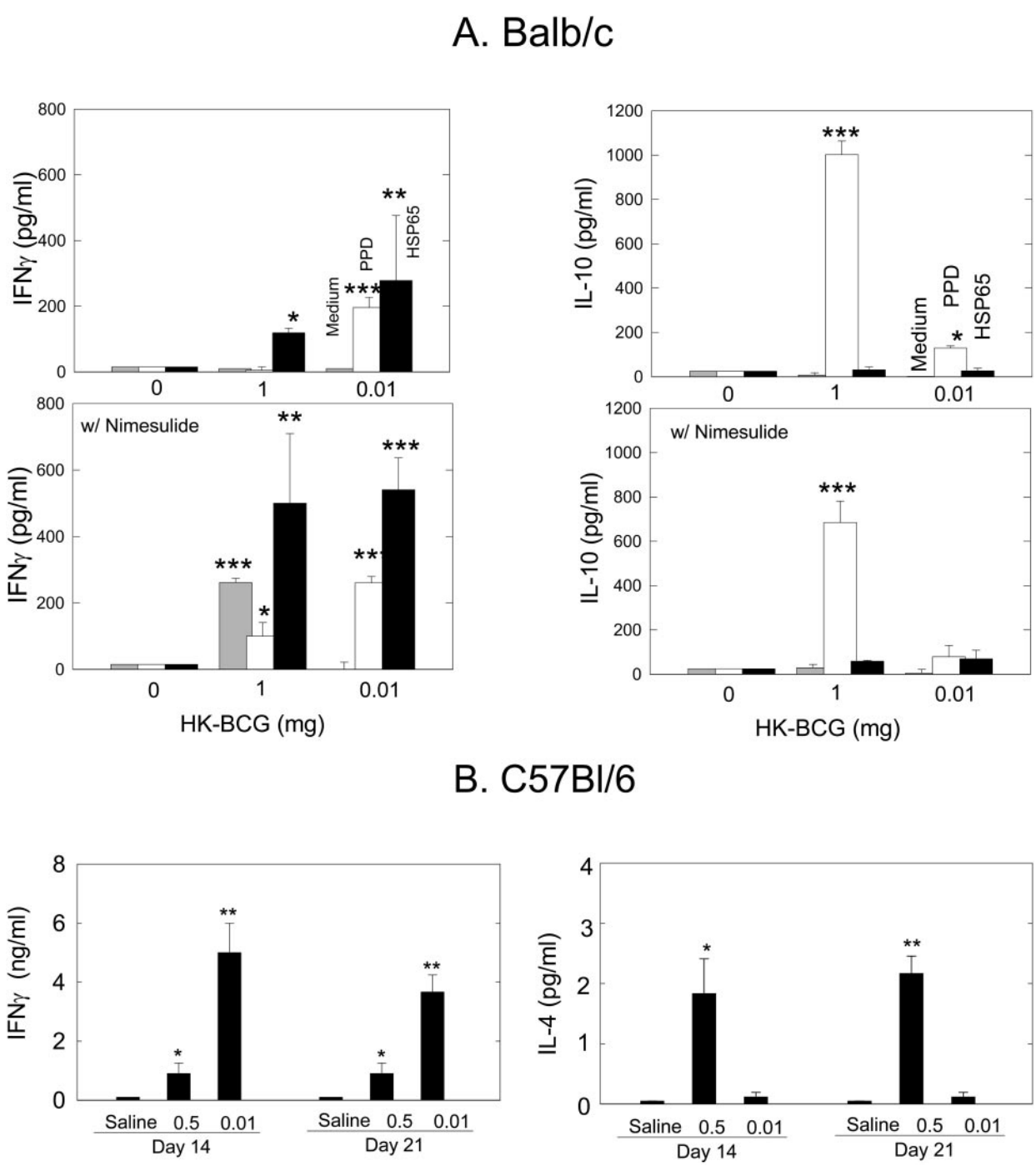

Fig. 5. Th1 and Th2 cytokine production in recall responses. (A) Groups of Balb/c mice (five/group) received 0 mg (0.2 ml saline), 0.01 mg, or 1 mg HK-BCG i.p. on Day 0; Day 14 spleen cells were isolated and stimulated in vitro with 5 μg/ml PPD or 1 μg/ml HSP65 for 4 days. In some cultures, 1 μM nimesulide was added. The levels of IFN-γ and IL-10 in the culture supernatants were measured by ELISA, as described in Materials and Methods. Values are mean ± SD from triplicate cultures. The data shown are for a group of five mice and are representative of two independent experiments. *, **, and ***, $P < 0.05$, $P < 0.01$, and $P < 0.005$, respectively, compared with the saline control group. (B) Groups of C57Bl/6 mice (four/group) received 0 (0.2 ml saline), 0.01, and 0.5 mg HK-BCG i.p. on Day 0; Days 14 and 21 spleens were isolated, and spleen cells were cultured in the presence of 5 μg/ml PPD for 4 days. The levels of IFN-γ and IL-4 in the supernatants were measured by ELISA. Mean ± SD, n = 3. * and **, $P < 0.05$, and $P < 0.01$, respectively, compared with the saline control group.

stimulated IL-4 production (data not shown), indicating that under these conditions, there is limited effect of PGE_2 on these Th2 cytokine responses.

Similar experiments with spleen cell cultures from C57Bl/6 mice stimulated with PPD revealed generally the same pattern of cytokine production (Fig. 5B). However, the levels of IFN-γ and IL-4 produced were much higher and lower, respectively, than those seen with spleen cells from Balb/c mice (Figs. 5 and 6). These responses were essentially unchanged when spleen cells were isolated 21 days following treatment with HK-BCG, as was also shown for PGE_2 production (Figs. 1 and 6). When spleen cells were depleted of $CD4^+$ cells by anti-CD4 antibody

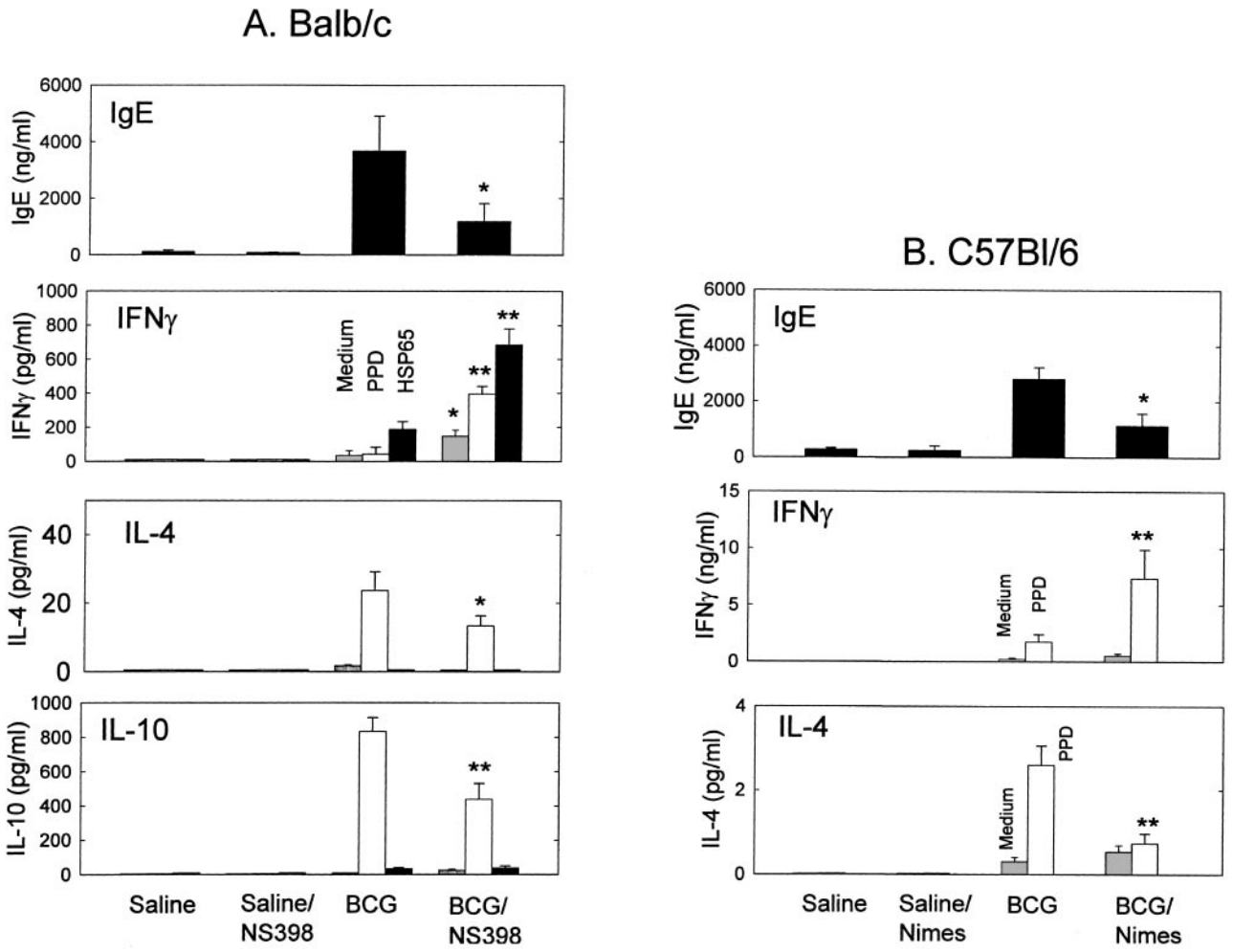

Fig. 6. PGHS-2 inhibitors (NS-398, nimesulide) in vivo inhibit Th2 cytokine production and decrease serum IgE levels but enhance IFN-γ production. (A) Groups of Balb/c mice (five/group) received 1 mg HK-BCG i.p. in saline on Day 0. Controls received 0.2 ml saline. BCG-treated mice and controls were treated i.p. daily starting on Day 1 with 10 mg/kg NS-398 or 0.5% ethanol in saline (0.2 ml). Day 14 sera and spleens were harvested. Total IgE in sera was measured by ELISA. Mean ± SD, n = 5. *, $P < 0.05$, compared with the BCG group. Spleen cells were cultured in the presence of 5 μg/ml PPD (open bars), 1 μg/ml HSP65 (solid bars), or medium alone (shaded bars) for 4 days; levels of IFN-γ, IL-4, and IL-10 in the culture supernatants were measured by ELISA. Mean ± SD, n = 3. * and **, $P < 0.01$, and $P < 0.005$, respectively, compared with the saline control group. (B) Groups of C57Bl/6 mice (four/group) received i.p. 0.5 mg HK-BCG on Day 0. Controls received 0.2 ml saline. Mice were treated i.p. daily starting on Day 1 with 10 mg/kg nimesulide or 0.5% ethanol in saline (0.2 ml). Day 14 sera and spleens were harvested, and spleen cells were cultured in the presence of 5 μg/ml PPD for 4 days. The levels of total IgE in sera and IL-4 and IFN-γ in the culture supernatants were measured by ELISA. Mean ± SD, n = 3. *, $P < 0.05$; **, $P < 0.01$, compared with saline treatment. Each result represents a group of four mice from two experiments.

(clone GK1.5) plus guinea pig serum, the remaining cells did not express detectable levels of IFN-γ, IL-10, or IL-4 in recall responses (data not shown), indicating that production of these cytokines was $CD4^+$ cell-dependent.

PGE_2 is known to inhibit production of IL-12, a Th1 cytokine inducing IFN-γ production [27]. However, in our studies, IL-12 was undetectable (<12 pg/ml) in the groups where IFN-γ levels were above baseline (data not shown). Despite the undetectable levels of IL-12 in the presence of nimesulide, where PGE_2 production is inhibited, IFN-γ levels are increased (Fig. 5). In our previous report [28], 1–10 μm chitin particles induced splenic MØ to produce IL-12, which was inversely dependent on the PGE_2 concentration. For example, when splenic MØ from normal C57Bl/6 and from high-dose HK-BCG groups were stimulated in vitro with 1–10 μm chitin particles at 100 μg/ml for 24 h, IL-12 levels were detected as 75 and <12 pg/ml/10^6 MØ, respectively. These results are consistent with earlier observations [20, 27, 28] that inhibition of IL-12 production by endogenous PGE_2 contributes to the reduction of Th1 responses.

In vivo effects of PGHS-2 inhibition in BCG-treated mice

To assess the effects of PGHS-2 activity on immune responses, Balb/c mice, which received 1 mg HK-BCG, were treated in vivo with NS-398. Recall responses of spleen cell cultures with PPD or HSP65 were determined 14 days after BCG treatment. Figure 6 shows that spleen cells isolated from animals treated with NS-398 in vivo and stimulated in vitro with PPD produced more IFN-γ but less IL-4 or IL-10 than those not treated with the PGHS-2 inhibitor. IL-4 and IL-10 were not detected in HSP65-stimulated recall responses, with or without NS-398.

Production of HSP65-stimulated IFN-γ was enhanced significantly by the treatment with NS-398 (Fig. 6). Finally, in vivo inhibition of PGHS-2 reduced total serum IgE levels (Fig. 6).

The pattern of response for total serum IgE in C57Bl/6 mice treated in vivo with NS-398 was similar to that for Balb/c mice. The IL-4 produced in PPD recall responses with cells from C57Bl/6 mice also followed a similar pattern to Balb/c mice, but the levels were reduced approximately tenfold (Fig. 6). Only relatively low levels (<100 pg/ml) of IL-10 were seen in C57Bl/6 mice (data not shown). The IFN-γ responses again followed a similar pattern to Balb/c mice, but the levels were increased markedly (Fig. 6). The results contrast with the recall responses for cells treated in vitro with a PGHS-2 inhibitor, described above. Following in vivo administration of a PGHS-2 inhibitor, the Th1-to-Th2 shift of splenic lymphocyte responses to mycobacterial antigens and serum IgE production develops in a PGHS-2-dependent manner.

DISCUSSION

These studies, as well as previous reports, clearly indicate that 5–21 days after treatment of mice with HK-BCG and HK-*C. parvum*, splenic PGE_2-MØ develop in a dose-dependent manner [6, 15, 16]. Splenic PGE_2-MØ express Fc receptors for IgG, phagocytic activity, and the surface marker F4/80. Calcium ionophore or AA but not LPS elicited PGE_2 release by splenic PGE_2-MØ. PGE_2 release also occurred when spleen cells from these BCG-treated mice were cultured and incubated in the presence of antigen (recall response; Fig. 1), suggesting that there is persistent, local release of PGE_2 in the spleen in the continued presence of antigen. Our results indicate that splenic PGE_2-MØ formation is associated with the induction of mixed Th1 and Th2 lymphocyte responses in a PGHS-2-dependent manner (Figs. 5 and 6).

Unlike MØ isolated from peritoneum, bone marrow, or blood, normal, splenic MØ exhibit only a relatively low level of PGE_2-release (<1 ng $PGE_2/10^6$ MØ) [13]. When activated in vitro by bacterial endotoxin and IFN-γ, splenic MØ produce a maximum of <2 ng/ml PGE_2 [13]. Additional factors must contribute to the enhanced PGE_2 release by splenic PGE_2-MØ isolated from mice 7–21 days after HK-BCG treatment. In mice depleted of bone marrow by ^{89}Sr, we previously found that splenic PGE_2-MØ are not formed following administration of HK-*C. parvum*, although these mice show increases in total splenic MØ and myeloid precursors [15, 16]. It is likely, therefore, that PGE_2-MØ precursors are generated in the radiosensitive bone marrow following HK-BCG treatment, then migrate, and localize in the spleen [16]. This process takes at least 5–7 days after administration of HK-BCG. Splenic PGE_2-MØ are located strategically to interact with lymphocytes and induce the shift of Th1-to-Th2 responses during mycobacterial infections. Once PGE_2-MØ are established, they persist for long periods [15], therefore prolonging the effect of PGE_2 on immune regulation. It should also be noted that splenic PGE_2-MØ formation is independent of circulating monocytes [15, 16]. Th1/Th2 cells that develop in the spleen eventually migrate to inflammatory sites [1].

Our results (Figs. 2 and 3) show that PGHS-2 is increased with increased PGE_2-MØ formation and is associated specifically with $F4/80^+$ cells. PGES activity and the mPGES-1 protein level are not increased in response to BCG treatment. In contrast, normal peritoneal MØ express high levels of mPGES-1 and PGHS-2 and release a large amount of PGE_2 [22, 24, 29] in responding to LPS in vitro. Therefore, the regulatory mechanisms PGHS-2 and PGE_2 synthesis in splenic PGE_2-MØ are distinct from peritoneal MØ. Although PGHS-2 may be necessary for the increased PGE_2 following BCG treatment, we have not demonstrated that this enzyme is sufficient or the rate-limiting factor. Secretory phospholipase A_2 type V is also known to be involved in prostaglandin production in splenic MØ, mast cells, and mesangial cells [19, 30], which makes this enzyme a possible candidate for regulation following BCG treatment. In addition, we have not investigated a possible role for the constitutively expressed PGES mPGES-2 [31]. Further analysis will be required to identify the required enzymes and rate-limiting factor(s) for PGE_2 synthesis in splenic PGE_2-MØ.

Balb/c mice, compared with C57Bl/6 mice, frequently show the induction of significant Th2 responses after infection or immunization, such as with BCG [32] and *Leishmania major* [21, 33]. Normal, splenic MØ isolated from Balb/c mice and treated in vitro with LPS produce more PGE_2 than those from C57Bl/6 mice [18] and have a greater sensitivity to the Th1-suppressive effect of PGE_2 [18, 19]. However, our studies indicate that C57Bl/6 mice express splenic PGE_2-MØ and a mixed Th1 and Th2 lymphocyte response against mycobacteria in responding to high-dose HK-BCG with a pattern similar to that for Balb/c mice. The remarkable differences between these two strains are the increased amount of IFN-γ and decreased amount of IL-4 produced.

Infections caused by *Chlamydia pneumoniae* and *Helicobacter pylori* as well as *Mycobacterium sp* have been implicated as co-risk factors in atherosclerosis [34, 35]. Seroepidemiological studies [36] indicate that patients with atherosclerosis express high antibody titers against mycobacterial HSP65, a prokaryotic HSP of the 60-kDa family (HSP60/65), indicating a Th2-mediated, humoral response against this antigen. Prokaryotic HSP60/65 are >97% homologous, whereas prokaryotic and human/mouse HSP60/65 have >70% amino acid sequence homology. By attacking stressed arteries that express endogenous HSP60, anti-HSP65 may contribute to atherosclerosis through antigenic mimicry [36]. Microbial HSP60/65 serves as an immunodominant antigen in protection from and in the pathogenesis of infectious diseases [34, 36–38]. The development of Th1 cells against HSP65 mediates resistance to *M. tuberculosis*, which constitutes a major vaccine strategy against tuberculosis [8]. However, in recurrent and chronic infections, anti-HSP65, which does not have a decisive, protective role against mycobacterial infections, may promote the further progression of atherosclerotic plaques [36, 39]. Similarly, Th1-to-Th2 shifts of the immune response may be associated with the pathogenesis of various chronic, immunologically mediated diseases.

Live BCG induces not only host resistance to *M. tuberculosis* but also tumoricidal activity and has been used widely for treatment of superficial bladder cancer [40, 41]. Live BCG also

induces a nonspecific M∅ microbicidal activity against *Listeria monocytogenes* [42, 43] and *Toxoplasma gondii* [44]. It has been reported that a low dose (0.01 mg) of HK-BCG suspended in saline induced a Th1 lymphocyte response against mycobacteria, but the effects were not sufficient to protect against mycobacterial infections, whereas immunization with HK-mycobacteria in mineral oil (CFA) was protective [45]. Although mineral oil provides additional adjuvant activity, our study indicates that mineral oil itself has no effect on the formation of splenic PGE_2-M∅.

In conclusion, previously, we found that development of splenic PGE_2-M∅ is dependent on radiosensitive bone marrow cells and that the PGE_2-M∅ are probably derived directly from the bone marrow [6, 15, 16]. Here, we have continued our investigation of the unique splenic PGE_2-M∅ population, which plays a novel, PGHS-2-dependent, immunoregulatory role in the Th1-to-Th2 shift of immune responses against mycobacterial antigens including HSP65. It is possible that PGE_2 as an antiapoptotic agent also promotes antigen-presenting activity of dendritic cells [46], whose maturation is induced directly by BCG, and may skew the immune response toward a Th2-like profile [47]. PGE_2 is known to increase regulatory T cell differentiation and function, which could contribute to the immune responses of BCG-treated mice [48, 49]. Finally, it remains to be elucidated whether similar mechanisms are involved in the pathogenesis of *Leishmania* infection, *Trichophyton* dermatophytosis, syphilitic infection, or human immunodeficiency virus infection progressing to AIDS [21, 50–53], where Th1-to-Th2 shifts of immune responses and splenic PGE_2-M∅ formation are frequently seen. Th1-to-Th2 shifts are also observed in neonatal, aged, tumor-bearing, diabetic, and hypercholesterolemic animals [14, 54–58].

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Radiosensitive macrophages and immune responses
--- Prostaglandin E_2-releasing macrophages induce a Th1-to-Th2 shift of immune responses in chronic inflammation ---

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ABSTRACT

Mycobacterial infections or treatment of animals with mycobacterial components (heat-killed [HK] BCG) results in the dose-dependent formation of PGE_2-releasing COX-2^+ splenic macrophages (PGE_2-MØ) within 5 – 21 days. PGE_2 release from splenic PGE_2-MØ is increased 10 fold compared to normal splenic MØ. One effect of PGE_2 is the modulation of local immune responses including Th1-to-Th2 responses against mycobacterial antigens (PPD, HSP65). This immunological switch is reversed by administration of COX-2 inhibitors, NS-398 and nimuslide. Studies following bone marrow depletion by ^{89}Sr or transfusion of GFP donor cells indicate that following BCG treatment radiosensitive bone marrow cells supply precursors of splenic PGE_2-MØ, which migrate and localize to the spleen. Once these splenic PGE_2-MØ are established, they persist for long periods. These PGE_2-MØ are strategically located to induce shifts of Th1-to-Th2 responses in the spleen during mycobacterial infections and probably in other chronic inflammatory conditions.

INTRODUCTION

Mononuclear phagocytes (macrophages [MØ]) act as the first line of defense against invading microorganisms and have important immunoregulatory roles in acute and chronic inflammatory diseases. MØ are ontogenically and functionally heterogeneous populations. To study the responses and relationships of functionally identifiable MØ populations, we employed mice depleted of bone marrow by ^{89}Sr, a bone-seeking isotope (1,2). The treatment of mice with ^{89}Sr permits the identification of radiosensitive bone marrow-dependent and independent MØ populations, which are, for example, circulating monocytes and tissue MØ including peritoneal and alveolar MØ, respectively. Based on our studies, we discuss our results showing that splenic MØ releasing PGE_2 (PGE_2-MØ) (3,4), and derived from radiosensitive bone marrow, may contribute to the regulation of Th1/Th2 immune responses in chronic inflammatory diseases (5).

RESULTS

BCG induces splenic PGE_2-MØ.

Splenic MØ isolated from normal mice release only minimal levels of PGE_2 (6). To characterize splenic PGE_2-MØ formation, Balb/c mice were treated ip with 1 mg HK-BCG in saline or mineral oil. When splenic MØ were isolated 1 and 3 days later and stimulated *in vitro* with A23187 for 2 hrs, PGE_2 release was not different from control cells obtained from untreated animals (Figure 1). Significantly higher PGE_2 release was observed 7 and 14 days after treatment with 1 mg HK-BCG. Furthermore, AA, but not LPS, also elicited PGE_2 release. Similar results were obtained for C57Bl/6 mice as well for HK-BCG in mineral oil (5). Additional studies (1,5,6) indicate that splenic PGE_2-MØ formation is dependent on both radiosensitive bone marrow and HK-BCG dose, and is suppressed by endogenous IL-10 (Table 1).

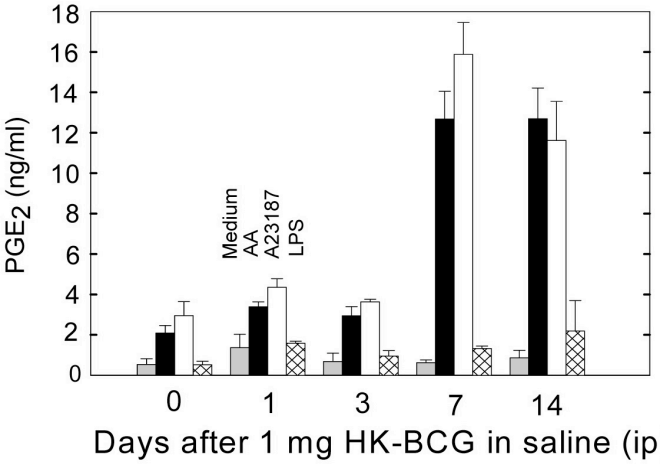

Figure 1. HK-BCG induces splenic PGE_2-MØ formation.
Groups of Balb/c mice (4 /group) received i.p. HK-BCG suspended in saline on day 0. Spleens were harvested on day indicated, including day 0 (untreated). Splenic MØ were isolated, pooled in each group and incubated for 2 hrs in serum-free RPMI 1640 medium (10^6 MØ/ml) in the presence of 1 g/ml AA, 1μM A23187, 1 g/ml LPS or media alone. PGE_2 was measured by ELISA. Mean SD, n=3.

PGHS-1, PGHS-2, mPGES-1, and cPGES in splenic PGE_2-MØ.

PGE_2-MØ metabolize endogenous AA to PGE_2 through the rate-limiting enzymes

Table 1. Biphasic COX-2$^+$ splenic MØ formation in BCG-treated mice.

	Day 1 COX-2$^+$ MØ	Day 14 COX-2$^+$ MØ
Surface antigen	F4/80	F4/80
PGE_2 release	<2x	5 – 10x
Bone marrow-dependent formation	No	Yes
Catalytic COX activity	No	Yes
COX-1, mPGES-1 and cPGES levels	Unchanged	Unchanged
IL-10-dependent COX-2 induction	No	Yes
Interaction with	?	Th1/Th2, DC, Treg

PGH synthase (COX) and PGE synthase (PGES). Two major isoforms, COX-1 and COX-2, convert AA to PGH_2. PGH_2 is subsequently converted to PGE_2 by cytosolic PGES (cPGES) and microsomal PGES-1 (mPGES-1). Normal splenic MØ expressed COX-1, mPGES-1 and cPGES but not COX-2 (Figure 2). When Balb/c mice were treated with 1 mg HK-BCG, COX-2 was detected on days 7 and 14. The levels of COX-1, mPGES-1 and cPGES remained similar to those in normal splenic MØ (Figure 2). Unexpectedly, day 1 MØ showed high levels of COX-2, in an IL-10-independent manner, without an increase in release of PGE_2 (Figure 1). Distinctions between day 1 COX-2$^+$ and day 14 COX-2$^+$ MØ are summarized in Table 1.

GFP-positive PGE_2-MØ in Spleen.

To explore the role of bone marrow-derived cells as precursors of splenic PGE_2-MØ, we used adoptive transfer of functional GFP-positive bone marrow cells into recipients. As shown in Figure 3, splenic PGE_2-MØ may be derived from GFP$^+$ bone marrow cells transfused on days 1 and 2 after HK-BCG immunization (3).

In vivo treatment of BCG-treated mice with COX-2 inhibitors prevents shift of Th1-to-Th2 responses.

When mice were treated with 1 mg HK-BCG, at 14 days PPD or HSP65 recall responses by spleen cell

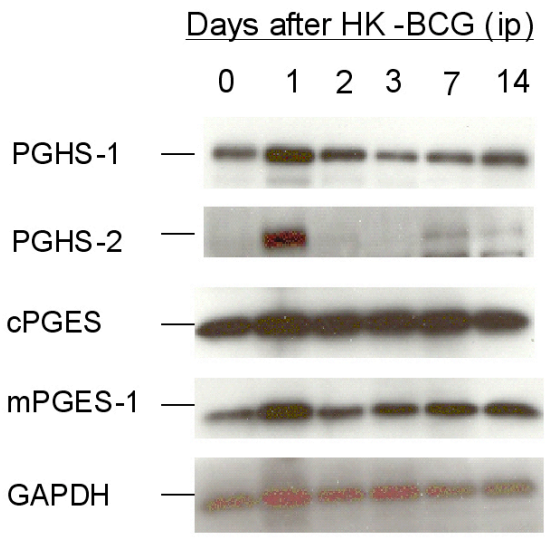

Figure 2. COX-1, COX-2, mPGES-1 and cPGES levels in HK-BCG-treated mice.
C57Bl/6 mice received 1 mg HK-BCG in saline, ip, on day 0. Day 0 (untreated), day 1, day 3, day 7 and day 14, spleens were harvested. Lysates of splenic MØ isolated from each group of animals were analyzed by Western blotting. Results are representative of three separate experiments.

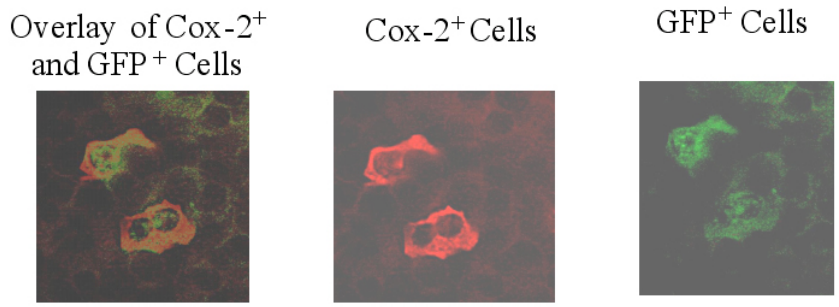

Figure 3. Immunofluorescent detection of spleen cells expressing both GFP and COX-2 in the HK-BCG-treated bone marrow chimera.
A GFP-bone marrow chimera was established in C57Bl/6 recipients that received bone marrow cells iv from GFP-donors. Both C57Bl/6 recipients and GFP-transgenic donors were immunized with 1 mg HK-BCG ip on day 0. On days 1 and 2, bone marrow cells were isolated from GFP-donors and transfused into C57Bl/6 recipients at 2 x 10^7 cells/dose/day. On Day 14, spleens were harvested from the C57Bl/6 recipients. To detect COX-2$^+$ cells immunohistologically, spleens were fixed, frozen and sectioned. The sections were stained with anti-COX-2 primary antibody followed by tetramethyl rhodamine (red)-conjugated second antibody. In the section with 100x magnification, GFP$^+$ spleen cells (green) were co-localized with COX-2$^+$ cells (red). Without HK-BCG immunization, there were no COX-2$^+$ cells or GFP$^+$ cells in the spleens of either donors or recipients (data not shown).

cultures showed less IFNγ (Th1 response) but more IL-4 and IL-10 (Th 2 responses) than for untreated mice. To assess the effects of COX-2 on immune responses, Balb/c mice receiving 1 mg HK-BCG were treated with the COX-2 inhibitor NS-398. Figure 4 shows that spleen cells isolated from animals treated with NS-398 produced more IFNγ but less IL-4 or IL-10 in a PPD recall response than those not treated with the COX-2 inhibitor. IL-4 and IL-10 production were not detected in HSP65-stimulated recall responses with or without NS-398, although production of IFN was significantly enhanced by the treatment with NS-398. Finally, total serum IgE levels were reduced by treatment with the COX-2 inhibitor. A similar pattern of COX-2-dependent Th1-to-Th2 shifts were also observed in HK-BCG-treated C57Bl/6 mice, although IFNγ levels were significantly higher and IL-4 levels were lower.

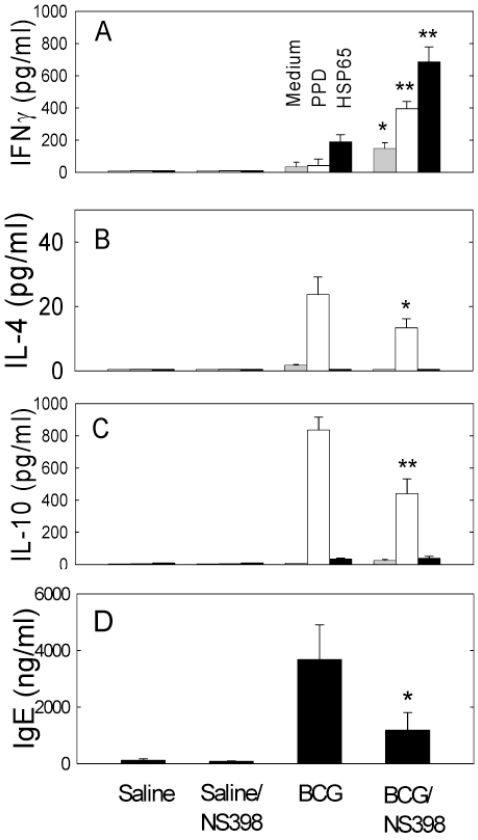

Figure 4. COX-2 inhibitor (NS-398) in vivo inhibits Th2 cytokine production and decreases serum IgE level, but enhances IFNγ production.

Groups of Balb/c mice (5/group) received 1 mg HK-BCG i.p. in saline on day 0. Controls received 0.2 ml saline. BCG-treated mice and controls were treated i.p. daily starting on day 1 with 10 mg/kg NS-398 or 0.5% ethanol in saline (0.2 ml). On day 14, sera and spleens were harvested. Total serum IgE was measured by ELISA. Mean SD, n=5. * $p < 0.05$ compared to the BCG group. Spleen cells were cultured in the presence of 5 μg/ml PPD (open bars), 1 μg/ml HSP65 (closed bars) or medium alone (gray bars) for 4 days; levels of cytokines IFNγ, IL-4 and IL-10 in the culture supernatants. Mean SD, n=3. * and **, $p < 0.01$ and $p < 0.005$, respectively, compared to the saline control group.

DISCUSSION

A unique PGE_2-MØ population has been characterized (1,2) and found to regulate the Th1-to-Th2 shift of immune responses against mycobacterial antigens including HSP65 (5). It is possible, in addition, that PGE_2 as an anti-apoptotic agent promotes antigen-presenting activity of dendritic cells (DC) (7). DC, whose maturation is directly induced by BCG, also may skew the immune response toward a Th2-like profile (8). PGE_2 also increases regulatory T cell differentiation and function that could contribute to the immune responses of BCG-treated mice (9). Finally, it remains to be elucidated whether similar splenic PGE_2-MØ-mediated mechanisms are involved in the pathogenesis of *Leishmania* infection, syphilitic infection, or HIV infection progressing to AIDS where Th1-to-Th2 shifts of immune responses are frequently seen (5). Such shifts are also observed in neonatal, aged tumor-bearing, diabetic and hypercholesterolemic animals (5).

Freund's complete adjuvant (HK-mycobacteria in mineral oil) has been widely used to establish animal models of autoimmune diseases, such as experimental autoimmune encephalomyelitis, neuritis, uveitis, thyroiditis, orchitis, and adjuvant arthritis (5). The adjuvant enhances Th1-mediated MØ activation and Th2-mediated antibody formation. Pathogenic roles of Th1/Th2 responses appear to be varied among autoimmune disease models, which may be related to the range of HK-mycobacteria concentrations (0.05 to 0.5 mg) used in these animal models (10). We conclude that, under chronic inflammatory conditions, splenic PGE_2-MØ develop *in vivo* from radiosensitive bone marrow precursors and promote a Th1-to-Th2 shift of immune responses.

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Heat-killed BCG induces biphasic cyclooxygenase 2^+ splenic macrophage formation --- role of IL-10 and bone marrow precursors

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Short Title: BCG-induced biphasic COX-2^+ splenic macrophage formation

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ABSTRACT

Previous studies have shown that PGE_2 release by splenic $F4/80^+$ cyclooxygenase (COX)-2^+ macrophages (M∅) isolated from mice treated with mycobacterial components plays a major role in the regulation of immune responses. However, splenic M∅, isolated from untreated mice and treated *in vitro* with LPS and IFNγ, express COX-1 and COX-2 within 1 day, but release only minimal amounts of PGE_2 following elicitation with calcium ionophore A23187. To further characterize *in vivo* requirements for development of PGE_2-releasing M∅ (PGE_2-M∅), C57Bl/6 (WT) and IL-10 deficient ($IL\text{-}10^{-/-}$) mice were treated ip with heat-killed *Mycobacterium bovis* BCG (HK-BCG). One day following injection, COX-2 was induced in splenic M∅ in an IL-10-independent manner. However, there was no increase in PGE_2 biosynthesis by these M∅. Thus, expression of COX-2 is not sufficient to induce PGE_2 production either *in vivo* or *in vitro*. In sharp contrast, 14 days after HK-BCG treatment, PGE_2 release by COX-2^+ splenic M∅ increased as much as 7-fold, and was partially suppressed by endogenous IL-10. To further determine whether the 14-day splenic PGE_2-M∅ could be derived from bone marrow precursors, we established a chimera in which bone marrow cells were transfused from green fluorescent protein (GFP)-transgenic donors to WT mice. Both donors and recipients were treated with HK-BCG simultaneously and marrow transfusion was performed on days 1 and 2. On day 14 after BCG treatment, a significant number of spleen cells co-expressed COX-2 and GFP indicating that bone marrow-derived, COX-2^+ M∅ may be responsible for the increased PGE_2 production.

INTRODUCTION

Prostaglandin E_2 (PGE_2) released by mononuclear phagocytes (M∅) regulates immune responses in autocrine and paracrine fashions. For example, PGE_2 inhibits IL-12 production by M∅ [1], reactive oxygen and nitrogen intermediates [2, 3]. PGE_2 also promotes IL-10 production by M∅ [4, 5], a Th1-to-Th2 shift of acquired immune responses [4, 5], dendritic cell antigen presentation [6], and regulatory T cell differentiation and function [7]. The effective *in vivo* expression of these responses, furthermore, may depend on the presence of an adequate number of MØ with appropriate functions in specific locations [5, 8, 9]. In the spleen, PGE_2-MØ interact closely with lymphocytes and induce a Th1-to-Th2 shift of immune responses in chronic inflammatory diseases including mycobacterial infections, *Leishmania* infection, syphilitic infection, HIV infection progressing to AIDS, and animal models of autoimmune diseases that are established with Freund's complete adjuvant (heat-killed [HK]-*Mycobacterium tuberculosis* in mineral oil) [5, 10-14].

In the production of PGE_2, M∅ metabolize endogenous arachidonic acid (AA) through cyclooxygenases (PGHS, EC 1.4.99.1), rate-limiting enzymes for PGH_2 synthesis. PGHS-1 (COX-1) is a constitutive isoform whereas PGHS-2 (COX-2) is induced in response to various inflammatory mediators [15]. PGH_2 is converted to PGE_2 by cytosolic PGE synthase (cPGES) and microsomal PGES-1 (mPGES-1) [16, 17]. PGE_2-releasing M∅ (PGE_2-M∅) are ontogenically heterogeneous populations and PGE_2 biosynthesis appears to be associated with differential expression of isoforms of the key enzymes [9, 18]. Although splenic COX-2^+ MØ develop in response to various inflammatory conditions, the exact mechanisms for splenic PGE_2-

MØ formation appear to be complex and controversial [19, 20]. Our *in vitro* studies suggested that unlike other tissue M∅, splenic M∅ in inflammation may not release PGE_2 in proportion to increased COX-2 expression [21]. Furthermore, endogenous IL-10, an immunosuppressive cytokine reported to inhibit COX-2 expression in bone marrow M∅, peritoneal M∅, neutrophils and cancer cells [22, 23], did not significantly modify COX-2 expression by these splenic MØ [21].

Previous murine studies [5, 9, 18, 24] indicate that intraperitoneal administration of HK-*M. bovis* BCG (HK-BCG) or *Corynebacterium parvum (Propionibacterium acnes)* results in formation of splenic COX-2^+ M∅ and 5 - 10-fold increases in PGE_2 release within 5 – 21 days. In previous whole animal studies, we found that the formation of splenic PGE_2-M∅ is dependent on radiosensitive bone marrow precursors but is independent of circulating monocytes [9, 18]. The PGE_2-M∅ formation appears to be down-regulated by endogenous IL-10 [24]. Preliminary studies showed that 1 day after HK-BCG treatment WT and IL-$10^{-/-}$ splenic MØ expressed COX-2 at relatively high levels in an IL-10-independent manner with little increase in the release of PGE_2. This was consistent with our previous report that WT splenic MØ treated *in vitro* with LPS and IFNγ expressed COX-2, but did not produce substantially increased amounts of PGE_2 [18]. In the present study, we have further characterized differences in day 1 COX-2^+ M∅ and day 14 PGE_2-MØ induced *in vivo* by HK-BCG treatment of IL-$10^{-/-}$ and WT mice.

An alternative explanation for our finding that radiosensitive bone marrow supplies precursors of PGE_2 MØ following treatment with HK-BCG is that an inflammatory cytokine “milieu” might convert resident splenic M∅ to PGE_2-M∅ [19, 20]. However, the *in vitro* studies showed that

PGE_2-M∅ do not develop from WT splenic MØ treated with the inflammatory mediators LPS and IFNγ [21]. Therefore, we hypothesize that the PGE_2-MØ present in the spleen on day 14 are derived from bone marrow precursor cells and do not result from maturation of resident splenic cells. To determine more directly whether bone marrow precursor cells could populate the spleen following treatment with BCG, we have performed adoptive transfer of bone marrow from green fluorescent protein (GFP)-expressing mice. Adoptive transfer of donor bone marrow cells is widely used in research on the function and metabolism of lymphoid and myeloid cells. The transfer of cells from GFP transgenic mice has demonstrated that donor cells can be engrafted and visualized in recipient mouse tissue for over 22 weeks after transfusion [25, 26].

MATERIALS AND METHODS

Animals. Non-pregnant female C57Bl/6 wild-type (WT) mice, 8-14 weeks old, were obtained from Harlan Laboratory (Indianapolis, IN). Healthy 8- to 12-wk-old IL-$10^{-/-}$ female mice on C57Bl/6 background were obtained from Jackson Lab (Bar Harbor, ME). Transgenic mice expressing GFP (C57Bl/6-TgN [ACTbEGFP] 1Osb) were provided by Dr. Kathryn Verbanac, East Carolina University, who obtained breeding pairs from Jackson Laboratory (Bar Harbor, ME). Mice were maintained in barrier-filtered cages and fed Purina laboratory chow and tap water *ad libitum*. Experimental protocols employed in this study were approved by the IACUC of the Brody School of Medicine at East Carolina University and Florida Atlantic University.

Treatment of mice with HK-BCG. As described previously [24], the cultured bacteria of *M. bovis* BCG Tokyo 172 strain were washed, autoclaved, and lyophilized. This HK-BCG powder was suspended in pyrogen-free saline and dispersed by brief (10 s) sonication immediately before use. These HK-BCG preparations contained undetectable levels of endotoxin (<0.03 EU/ml), as determined by the *Limulus* amebocyte lysate assay (Sigma, St. Louis, MO) [24]. Groups of mice (4 - 5/group) received 1 mg HK-BCG ip on day 0. Controls received 0.2 ml saline. Spleens were harvested on days 0, 1, 2, 3, 7 and 14.

Bone marrow transfusion. Groups of donor GFP mice (5/group) received 1 mg HK-BCG ip on day 0. Controls received 0.2 ml saline. To obtain bone marrow cells for transfusion, marrow cavities of femurs were flushed with ice-cold medium RPMI 1640; the expelled cell plugs were gently refluxed with a 20-gauge needle to form a single cell suspension. For preparation of bone

marrow transfusion recipients, 5 WT mice, 10 weeks of age, were treated with 1mg HK-BCG ip. On days 1 and 2, the WT recipients received 2 x 10^7 bone marrow cells isolated from BCG-treated GFP-donors [18]. In all experiments, bone marrow cells for transfusion were prepared from a pool of 5 donors.

Splenic MØ preparation. Spleens from each group of mice were isolated and pooled. Excised spleens were minced with scissors and digested with 50 U/ml collagenase D (C-2139, Sigma) in RPMI 1640 plus 10% FBS at 37°C for 60 min followed by filtration through 100-μm mesh. Cells were washed with RPMI 1640 in the presence of 100 μg/ml DNase (DN-25, Sigma), and resuspended in RPMI 1640 plus 10% FBS at 4 x 10^6 cells/ml. To enrich the MØ fraction [9, 18], spleen cell suspensions were layered over a discontinuous Percoll gradient (35/60%, Sigma). Following centrifugation (800 *g*, 30 min, 22°C), cells at the interface between 35% and 60% Percoll were collected, washed and suspended in RPMI 1640 plus 10% FBS. These cells were plated at 3 – 5 x 10^6 cells per 60 mm culture dish (Falcon, Oxnard, CA) and incubated at 37°C in 5% CO_2 in air. After 2 hr incubation, cells were washed with Ca^{2+} and Mg^{2+}-free 0.15 M phosphate-buffered saline (PBS) for removal of non-adherent cells. Culture dishes were placed on ice for 30 min before harvesting the adherent cells by scraping with a cell scraper (Corning, Corning, NY) and washing twice with serum-free RPMI 1640. Viability was >90% by trypan blue exclusion. Adherent spleen cells were >70% MØ, estimated by phagocytosis of IgG-opsonized sheep red cells and/or cytometrically following staining with anti-F4/80 [9, 18, 27].

For assay of PGE_2-release, plastic adherent splenic MØ (2 x 10^6 /ml) were incubated in serum-free RPMI 1640 with either 10^{-6} M calcium ionophore A23187 (Sigma) or 1 μg/ml arachidonic

acid (AA, Cayman) for 2 hrs. PGE_2 levels in the culture supernatants were measured by competitive ELISA (Cayman).

For determination of IL-10 production, splenic M∅ (2×10^6 /ml) were cultured in RPMI 1640 plus 5% FBS with 100 μg/ml HK-BCG or 1 μg/ml bacterial endotoxin (LPS) for 24 hrs. IL-10 levels in the culture supernatants were measured by sandwich ELISA (PharMingen). We always confirmed that IL-$10^{-/-}$ splenic M∅ used for our experiments lacked the production of IL-10 (Figure 5).

Magnetic separation of F4/80- or RB6-8C5- positive cells. Red cell-free spleen cells (10^8 cells) were stained with 5 μg/ml monoclonal antibody to F4/80 recognizing splenic M∅ (Accurate Chem, Westbury, NY), followed by addition of 200 μl magnetic microbead-conjugated goat anti-rat IgG (130-048-501, Miltenyi Biotec, Auburn, CA). F4/80- positive and negative cells were isolated according to the company's instructions. The $F4/80^+$ cells in positive and negative cell preparations were 87% and 0.8%, respectively (data not shown), determined cytometrically [5]. Similarly, RB6-8C5 (Gr-1)-positive and negative populations were also isolated using a specific antibody (Research Diagnostics, Flanders, NJ). The RB6-8C5 cells in positive and negative cell preparations were 91% and 0.9%, respectively (data not shown).

Western blotting. Splenic M∅ prepared as described above, were washed 3 times with cold saline. Washed cells were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1:500 protease inhibitor cocktail [P8340, Sigma], 1% Nonidet P-40, and 1% sodium deoxycholate) [5]. Debris was eliminated by centrifugation (5 min, 1000 g). Protein

concentration in the lysate was measured with a bicinchoninic acid assay (Pierce, Rockford, IL) and bovine serum albumin as standard. Equal amounts of protein were loaded onto SDS-polyacrylamide minigels and separated by electrophoresis (200 V for 45 min). Proteins were then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The membrane was blocked with 5% nonfat dry milk, and incubated with antibody (anti-COX-1, 1:1000; anti-COX-2, 1:4000; anti-cPGES, 1:1000; anti-mPGES-1, 1:1000, all from Cayman; anti-GAPDH, 1:4,000, from Novus Biologicals [Littleton, CO], and anti-actin, 1:4,000, from Sigma for the detection of GAPDH and actin, respectively, as constitutively expressed protein controls) in 5% nonfat dry milk, overnight at 4°C. Following incubation with peroxidase conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Glove, PA), proteins were detected by chemiluminescence (ECL, Amersham, Piscataway, NJ) following the manufacturer's instructions [5]. Intensity of the specific bands was quantified digitally with graphic imaging software (NIH Image 1.5).

Immunohistochemistry. Spleens were fixed with 4% paraformaldehyde overnight at 4°C, cryoprotected in 30% sucrose at 4°C for 24 hrs, embedded in TBS freezing medium (Triangle Biomedical Sciences, Durham, NC) and stored at -70°C until use. Frozen spleens were cryosectioned at -20°C, thaw-mounted onto Plus-treated glass slides and heat-set at 30°C for 3 min before air-drying overnight. For immunohistochemical analysis, 8-μm sections were stained with anti-COX-2 antibody at 1:250 (Cayman) followed by tetramethyl rhodamine-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch) at 1:100. Specific staining was detected by fluorescence microscopy (Zeiss Photomicroscope III, Carl Zeiss, Inc. New York, NY).

Cell-free PGE_2 biosynthesis assay. COX and PGES activities in cell lysates were measured as conversion of exogenous AA and exogenous PGH_2, respectively, to PGE_2 [16, 28]. For these assays, $F4/80^+$ splenic M∅ in 400 μl 10 mM Tris-HCl, pH 8.0, were disrupted by sonication using a Branson Sonifer (10 sec x 3 at 1-min intervals). After centrifugation of the sonicates at 1,700 *g* for 10 min, 4°C, protein concentrations in the supernatants were determined by bicinchoninic acid assay as described above, and adjusted to 1 mg/ml. For COX assay, 10 μl of each lysate (10 μg protein) was incubated for 15 min at 37°C with 3 μM AA in 0.1 M Tris-HCl, pH 8.0, containing 1 mM reduced L-glutathione (Sigma). For PGES assay, the lysate in the same buffer containing reduced L-glutathione was incubated with 0.5 μg of PGH_2 (Cayman) and 5 μg indomethacin for 30 s at 24°C. Reactions were terminated by addition of 100 mM $FeCl_2$ and PGE_2 in the supernatants was quantified by competitive ELISA (Cayman).

Cytometric detection. The expression of surface antigens on spleen cell preparations was determined by indirect immunofluorescence in the presence of 5% heat-inactivated newborn calf serum (Sigma), pH 7.2. Rat monoclonal antibodies used for the analyses were Mac-1 (β2 integrin), F4/80 (red pulp M∅), ER-TR9 (marginal zone M∅) and RB6-8C5 (Gr-1, neutrophils) (all from Research Diagnostics). Phycoerythrin (PE)-conjugated donkey anti-rat IgG (Jackson ImmunoResearch) was used as secondary antibody for indirect immunofluorescence.

To determine expression of cytosolic antigens, spleen cells prepared above were fixed with 4% paraformaldehyde, permeabilized with 1% saponin, and stained with rabbit antibodies specific for COX-1 or COX-2 at 2 μg/ml (Cayman) [29]. Presence of the primary antibody was determined by addition of PE-conjugated donkey anti-rabbit IgG at 1:1,000 (Jackson

ImmunoResearch). Fluorescence of 10^4 stained cells, unless stated, was quantitated with a FACScan flow cytometer using the CellQuest program (Becton Dickinson, Mountain View, CA). All cells as defined by forward and sideward scatter pattern were gated; only debris was excluded from analysis. Cells stained with the second antibody alone were used as negative controls in all experiments.

Statistics. Data for PGE_2 release were analyzed by one way analysis of variance. For cell culture studies, tissues isolated from at least 4 mice were pooled unless indicated; these cells were cultured in at least triplicate in each group. $P < 0.05$ is considered statistically significant.

RESULTS

IL-10-dependent splenic PGE_2-MØ formation 7 and 14 days after HK-BCG treatment. Results comparable to those in Figure 1 have been reported earlier and are included to validate interpretation of the present findings. Treatment of mice with 1 mg HK-BCG was chosen to achieve an inflammatory response like that associated with mycobacterial infection, and is also comparable to Freund's complete adjuvant used in models of autoimmune disease. Previous studies [5, 21] showed that splenic MØ isolated from untreated WT and IL-$10^{-/-}$ mice release minimal levels of PGE_2. In the present studies, plastic adherent splenic MØ obtained 7 and 14 days after treatment of mice with 1 mg HK-BCG and stimulated *in vitro* with A23187 released significantly more PGE_2 than did untreated controls (Figure 1). In addition, splenic MØ from IL-$10^{-/-}$ mice showed significantly higher PGE_2 release than WT cells. Neither IL-$10^{-/-}$ nor WT splenic MØ showed an increase in PGE_2 releasing activity on days 1 - 3 following HK-BCG treatment (Figure 1). Exogenous free AA elicited a similar pattern of PGE_2 release from splenic MØ (Figure 1). In previous studies with Balb/c mice, we have shown that PGE_2 biosynthesis by day 14 splenic MØ is inhibited by NS-398, nimesulide or indomethacin, indicating dependence on COX-2 [5].

Detection of COX-2 expressed by splenic MØ days 1, 7 and 14 after HK-BCG treatment. As shown in Figure 2, splenic MØ isolated from untreated WT and IL-$10^{-/-}$ mice expressed COX-1, mPGES-1 and cPGES but not COX-2. Following treatment with 1 mg HK-BCG, COX-2 was detected on days 1, 7 and 14. The levels of COX-1, mPGES-1 and cPGES were similar to those in untreated splenic MØ. Thus, on days 7 and 14, COX-2 levels, but not mPGES-1 levels,

were associated with the increase in PGE_2 release by splenic PGE_2-MØ. In sharp contrast, on day 1 after HK-BCG treatment, COX-2 expression by WT or IL-$10^{-/-}$ splenic MØ did not contribute to increased PGE_2 (Figures 1 and 2) or TxB_2 (data not shown) biosynthesis. On days 2 and 3 after HK-BCG treatment, splenic MØ from WT and IL-$10^{-/-}$ mice expressed little or no COX-2 (Figure 2).

MØ COX-2 expression on day 1 after HK-BCG is independent of IL-10, but is increased in IL-$10^{-/-}$ cells on day 14. To further confirm that splenic MØ express COX-2, cells expressing F4/80, a red pulp MØ antigen, were isolated by positive selection with magnetic beads from spleen cells obtained on days 1 and 14 following HK-BCG treatment of mice. In spleen cells from WT and IL-$10^{-/-}$ mice, COX-2 protein was concentrated in F4/80^+ cells on days 1 and 14 (Figure 3). The results further showed that COX-2 expression in F4/80^+ cells on day 1 was no distinction between WT and IL-$10^{-/-}$ mice (Figure 3), but on day 14 the increased expression by IL-$10^{-/-}$ cells was suppressed in WT cells (Figure 3). In contrast, COX-2 was not differentially expressed on positive and negative cells expressing Gr-1 (RB6-8C5) or Mac-1 (β2 integrin) (data not shown). The results indicate that on day 14 some Gr-1^+ cells, probably neutrophils, may also release PGE_2. A minor expression of COX-2 by Gr-1^+ or Mac-1^+ cells was also found on day 1 (data not shown).

PGE_2 biosynthesis, COX and PGES activities, and IL-10 production. As shown in Figure 4A, on day 1 after treatment with HK-BCG, despite the increase in COX-2, there was no increase in PGE_2 release elicited by 1 μg/ml exogenous AA (about 3 μM) from either WT F4/80^+ or IL-$10^{-/-}$ F4/80^+cells compared to untreated controls. In contrast, on day 14 after treatment,

there was a significant increase in PGE_2 release by both WT and IL-$10^{-/-}$ MØ (Figure 4A). For each day, the PGE_2 produced by IL-$10^{-/-}$ cells was significantly greater than that for WT MØ and on day 14 correlated with the increased COX-2 levels. Exogenous AA may serve directly as a COX substrate, independent of PLA_2 activity. However, exogenous AA is also reported to increase the level of intracellular Ca^{2+} [30], resulting in activation of PLA_2. Therefore, to assess further the COX and PGES enzymatic activities in splenic M∅, cellular lysates were mixed with exogenous AA or PGH_2, as substrates for COX or PGES, respectively. Day 1 cellular lysates did not convert exogenous AA to PGE_2 in either strain of mice (Figure 4B). PGES activity was present in untreated cells and was not significantly altered by treatment with HK-BCG (Figure 4C) on either day 1 or 14, strongly suggesting that COX activity is rate–limiting for PGE_2 biosynthesis. Thus, the COX catalytic activity in day 1 MØ is disproportionate to the COX protein level and would apparently account for the observation that PGE_2 production is not increased on day 1.

To examine the production of IL-10 by splenic M∅ isolated from HK-BCG-treated WT mice, splenic M∅ on days 0, 1 and 14 were stimulated *in vitro* with exogenous LPS or HK-BCG. Figure 5 shows that all M∅ produced relatively large amounts of IL-10: day 14 splenic M∅ produced more IL-10 than days 0 and day 1 M∅ (Figure 5).

Bone marrow-derived splenic PGE_2-M∅ developed in GFP-bone marrow chimera.

Previously our studies [9, 18] have shown that formation of PGE_2-M∅ is dependent on radiosensitive bone marrow cells. With the availability of GFP-transgenic mice as a source of bone marrow, we sought to assess, without the use of high dose radiation, whether splenic PGE_2-

M∅ are derived from bone marrow cells. We established a bone marrow chimera using GFP-transgenic mice as bone marrow donors and WT (C57Bl/6) mice as recipients. Both recipients and donors were treated with 1 mg HK-BCG ip on day 0. Based on previous results [18], 2 x 10^7 donor bone marrow cells were isolated and transfused immediately on both days 1 and 2.

Bone marrow cells, spleen cells and peritoneal cells were prepared from HK-BCG-treated chimeras and GFP expression was determined cytometrically. Figure 6 shows that small, but significant numbers of GFP^+ cells were detected in the tissues isolated from the chimera (Table 1). The chimera formed in animals treated with HK-BCG resulted in higher engraftment of donor cells than in the untreated chimera (Figure 6B, D). Our results suggest that treatment of mice with HK-BCG was essential to induce tissue localization of donor cells. This was most evident in the peritoneal cells (Figure 6). Selected splenic M∅ populations characterized by monoclonal antibodies against Mac-1, F4/80 and ER-TR9 and derived from GFP^+ cells were identified in the chimera (Table 1). Since numbers of donor bone marrow cells transfused are only a fraction of bone marrow cells in the recipients, GFP^+ cell numbers localized in the chimeric tissues appear to be relatively small even with HK-BCG treatment. For the HK-BCG-treated GFP chimeric mice, GFP^+ donor cells localized in the spleen were 1.27% of the total cells. Two-color cytometric analyses showed that 0.29% of the total spleen cells were both $COX\text{-}2^+$ and GFP^+ whereas 10.67% were $COX\text{-}2^+$ and GFP^-, suggesting that the $COX\text{-}2^+$ cells originated mainly from recipient cells (Table 1, Figure 7). Figure 8 illustrates immunohistologically the co-localization of COX-2 and GFP in spleen cells of BCG-treated mice following bone marrow transfusion.

DISCUSSION

The distinctive features of the biphasic COX-2^+ splenic M∅ formation described in this paper may be summarized as follows: (i) 1 day after ip injection of WT or IL-$10^{-/-}$ mice with HK-BCG, transient expression of MØ COX-2 protein was detected, but the enzymatic activity and resultant PGE_2 release are not increased proportionally (Figures 1-4); (ii) in contrast, 7 and 14 days after treatment, HK-BCG induces COX-2^+ PGE_2-M∅ with a 7-10 fold increase in PGE_2 release in an IL-10-dependent manner (Figures 1-4); and, (iii) the chimeras with GFP indicate that bone marrow-derived cells may serve as direct precursors of the COX-2^+ splenic M∅ that produce PGE_2 14 days after HK-BCG treatment (Figures 7 and 8).

An explanation for the dissociation between expression of COX-2 protein by day 1 splenic M∅ and the COX-2 activity indicated by the unchanged PGE_2 biosynthesis is not apparent. Analogous to the *in vivo* treatment with HK-BCG, our *in vitro* studies with WT splenic MØ treated with LPS and IFNγ resulted in increased COX-2, apparently independent of IL-10, but the release of PGE_2 was only minimally increased [21]. Certain tumor cells also express high levels of COX-2 with little PGE_2 biosynthesis due to catalytically inactive COX-2 [31]. Because PGES synthase activity is relatively constant in our cells, our results (Figure 4) also suggest that COX-2 induced on day 1 following HK-BCG treatment may lack catalytic activity in a post-translational regulation. It is also known that COX activation requires a small amount of peroxide, which may be supplied by peroxynitrite formed from superoxide anion and NO [32]. Thus, it is possible that the necessary peroxide is not present in the day 1 cells. Alternatively, PGE_2 may not be synthesized because of inadequate coupling of COX-2 with either a PLA_2

providing substrate or a terminal PGES. However, there do not appear to be reports clearly demonstrating this type of regulation of COX activity [33]. Regardless of the mechanism, the lack of PGE_2 biosynthesis by day 1 COX-2^+ splenic M∅ would imply that, unlike COX-2^+ splenic MØ on days 7 – 14, day 1 COX-2^+ M∅ do not regulate immune responses in a PGE_2-dependent manner.

Regulation of COX-2 expression by IL-10 appears to occur at the level of COX-2 mRNA. Berg et al. found that LPS stimulation of spleen cells from IL-$10^{-/-}$ mice resulted in a level of COX-2 mRNA that was 5.5-fold greater than in WT cells and correlated with increased COX-2 protein and PGE_2 production. In the IL-$10^{-/-}$ cells, the effect of LPS on COX-2 induction was reversed in the presence of neutralizing anti-cytokine antibodies to IFNγ, TNFα or IL-12, which suggests that the increase in COX-2 resulted from increased levels of these proinflammatory cytokines [19, 20]. In U937 cells, COX-2 mRNA was stabilized following treatment with LPS or IL-1β [34]. In addition, previous studies [35-37] suggest that LPS-induced COX-2 expression and PGE_2 synthesis are associated with p38 MAPK activation in human monocytes and neutrophils. The activity of p38 was necessary for stabilization of COX-2 mRNA [35-37]. IL-10 appears to down-regulate p38 MAPK activation [38-40]. p38 MAPK is an upstream kinase regulating NF-κB activation in neutrophils [41], suggesting that p38 MAPK might play a role in both transcriptional and post-transcriptional regulation of the COX-2 gene. Further studies will be required to dissect IL-10-dependent mechanisms regulating the expression of COX-2.

Different populations or compartments of mononuclear phagocytes (M∅) show considerable diversity in PGE_2 biosynthesis. Employing mice depleted of bone marrow cells and circulating

monocytes by the bone seeking isotope ^{89}Sr, we previously demonstrated that the development of splenic PGE_2-M∅ is dependent on radiosensitive bone marrow [9, 18], but that PGE_2-MØ development in the peritoneal cavity is independent of the bone marrow. Our results (Figure 6) clearly show that transfusion of WT recipients with GFP^+ bone marrow cells establishes a GFP-chimera in bone marrow, spleen and peritoneal cells following HK-BCG treatment. Thus, splenic PGE_2-M∅ may be derived directly from the bone marrow cells transfused on days 1 and 2 after HK-BCG treatment and that bone marrow does not solely supply an appropriate cytokine milieu. Taken together, it is likely that, at the early stages (1 – 2 days) after HK-BCG treatment, precursors of PGE_2-M∅ in the bone marrow are induced to migrate and localize in the spleen where mature forms of PGE_2-M∅ are established [9, 18]. Thus, COX-2^+ MØ present on day 1 and day 14 may be derived from distinct populations of cells, which may account, at least in part, for the deficient PGE_2 synthesis observed on day 1. Days 2 and 3, when COX-2 is not expressed, would be the transition from the day COX-2 expression by local M∅ to the day 7 COX-2 expression by M∅ newly derived from bone marrow.

Increases in immature and mature M∅ and neutrophil numbers within 5 – 21 days are observed in animals treated with BCG or *P. acnes* [9, 42]. Our unpublished studies have also shown that depletion of RB6-$8C5^+$ cells by a specific antibody on day 14 in HK-BCG mice results in unchanged F4/80^+ red pulp M∅ numbers as well as PGE_2-release (data not shown). In addition, day 14 COX-2 was not differentially expressed on positive and negative cells expressing RB6-8C5 or Mac-1 (data not shown). However, ER-$TR9^+$ marginal zone M∅ and MOMA-1^+ metalophilic marginal zone M∅, both distinct from F4/80^+ red pulp M∅, potentially could be PGE_2-M∅. The expression of COX-2 by dendritic cells remains to be examined. Our results do

not rule out the possibility that HK-BCG induces cell types other than $F4/80^+$ MØ that contribute to the release of PGE_2.

In conclusion, our previous finding [18] that development of splenic PGE_2-M∅ is dependent on radiosensitive bone marrow and that these cells are probably derived directly from bone marrow precursors is further confirmed. Splenic PGE_2-M∅ populations persist for long periods, therefore, prolonging the effect of PGE_2 on immune regulation including the Th1-to-Th2 shift of immune responses against mycobacterial antigens [5]. Although resident splenic MØ can be induced to express COX-2, these cells do not produce increased levels of PGE_2. We find further that endogenous IL-10 regulates COX-2 expression and the resultant PGE_2 production in cells obtained 7-14 days following treatment with BCG. Thus, our study indicates that the generally accepted concept that resident $COX\text{-}2^-$ M∅ are converted to $COX\text{-}2^+$ M∅ under inflammatory conditions with release of relatively large amounts of PGE_2 may need further investigation particularly with respect to splenic MØ.

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Abbreviations used in this paper: COX, cyclooxygenase (PGHS, prostaglandin G/H synthase); WT, wild-type; M∅, macrophages; BCG, Bacillus Calmette-Guérin; PGE_2, prostaglandin E_2; HK, heat killed; GFP, green fluorescence protein; MZ, marginal zone

Table 1. Percent of spleen cells expressing selected cellular antigens in the HK-BCG-treated GFP-chimera.

	WT recipient control	GFP-transgenic donor control		GFP-chimera	
	GFP^-	GFP^-	GFP^+	GFP^-	GFP^+
Total cells	100%	27.96%	72.04 %	98.73%	1.27%
$COX\text{-}1^+$	39.48	0.56	13.14	37.53	0.50
$COX\text{-}2^+$	7.89	0.05	5.18	10.67	0.29
$Mac\text{-}1^+$ (β2 integrin)	32.83	10.03	8.11	18.74	0.54
$F4/80^+$ (red pulp M∅)	2.63	0.28	3.76	9.89	0.52
$ER\text{-}TR9^+$ (MZ M∅)	1.67	0.37	2.49	1.02	0.26
$RB6\text{-}8C5^+$ (neutrophil)	16.18	0.98	25.02	19.65	0.77

WT (C57Bl/6) recipients and GFP-transgenic donors were treated with HK-BCG. Donor bone marrow cells (2×10^7) were transfused into the recipients on days 1 and 2 after HK-BCG treatment. On day 14, recipients were sacrificed and spleen cells were isolated. Spleen cells were also isolated from separate groups of WT recipients and GFP-transgenic donors 14 days after HK-BCG treatment. Following staining with PE-labeled antibodies against selected cellular antigens, fluorescence was determined cytometrically as shown in Figure 7.

FIGURE LEGENDS

Figure 1. HK-BCG induces splenic PGE_2-M∅ formation in IL-$10^{-/-}$ and C57Bl/6 (WT) mice. Groups of IL-$10^{-/-}$and C57Bl/6 (WT) mice (4 /group) received 1 mg HK-BCG ip on day 0. At indicated intervals, spleens were harvested and plastic adherent splenic M∅ were isolated and pooled in each group. To determine PGE_2 biosynthesis, M∅ suspended in serum-free RPMI 1640 were incubated with saline (open bar), 1 μg/ml arachidonic acid (gray bar), or 1 μM A23187 (closed bar) for 2 hrs. PGE_2 in the supernatants was measured by ELISA. Mean ± SD, n=3. *, $p < 0.05$ compared to day 0 control group. Results shown are representative of results from three separate experiments.

Figure 2. PGHS-1 (COX-1), PGHS-2 (COX-2), mPGES-1 and cPGES levels in HK-BCG-treated mice. IL-$10^{-/-}$ and WT mice received 1 mg HK-BCG in saline, i.p., on day 0. Day 0 (untreated), day 1, day 2, day 3, day 7 and day 14, spleens were harvested. Plastic adherent splenic M∅ were isolated from each animal and analyzed by western blotting.

Figure 3. COX-2 levels in F4/80^+ cells. F4/80^+ and F4/80^- cells were isolated from day 1 and day 14 whole spleen nucleated cells obtained from WT and IL-$10^{-/-}$ mice as indicated in *Materials and Methods*. (A) COX-2 expression in each fraction was determined by Western blotting. (B) Intensity of COX-2 bands indicated at (A) was shown as density ratio to GAPDH that was quantified digitally using graphic imaging software (NIH Image 1.5). Mean ± SD, n=3. *, $p < 0.01$ compared to WT F4/80^+ group. Results are representative of two separate experiments.

Figure 4. PGE_2 biosynthesis, cell-free COX and PGES activities in $F4/80^+$ cells from HK-BCG-treated mice. $F4/80^+$ cells were isolated from days 0, 1 and 14 following BCG treatment, pooled in each group as indicated in the *Materials and Methods*. (A) To determine PGE_2 biosynthesis, cells suspended in serum-free RPMI 1640 were incubated with 1 μg/ml of AA for 2 hrs. PGE_2 was measured by ELISA. Mean ± SD, n=3. (B) COX and (C) PGES activities in cell lysates were measured as described in *Materials and Methods*. PGE_2 was measured by ELISA. Mean ± SD, n=3. *, $p<0.05$; **, $p<0.01$; #, $p<0.001$ for IL-$10^{-/-}$ compared to WT. In the absence of exogenous substrates (AA or PGH_2), endogenous PGE_2 levels were ≤1 ng/10 μg protein (data not shown).

Figure 5. Production of IL-10 by WT splenic M∅. Plastic adherent splenic M∅ were isolated from days 0, 1 and 14 following BCG treatments of WT and IL-$10^{-/-}$ mice as indicated in the *Materials and Methods*. M∅ (2 x 10^6 /ml) were stimulated with 100 μg/ml HK-BCG, 1 μg/ml LPS, or medium at 37 ˚C for 24 hr. The levels of IL-10 in culture supernatants were measured by ELISA. Mean ± SD, n=3; *, $p < 0.001$, compared to cells treated with saline at the same time point. The data shown are representative of two independent experiments.

Figure 6. Chimeras for bone marrow, spleen and peritoneal cells in C57Bl/6 recipients that received bone marrow cells from GFP-transgenic donors. WT recipients and GFP-transgenic donors were immunized with HK-BCG. As control groups, recipients and donors were immunized with saline. Donor bone marrow cells (2 x 10^7) were transfused into the recipients on days 1 and 2 after immunization. On day 14, recipients were sacrificed and bone marrow,

spleen and peritoneal lavage cells were isolated. GFP levels were determined cytometrically. As controls, cells were prepared from WT recipients and GFP-transgenic donors 14 days after HK-BCG immunization. (A) HK-BCG-treated WT recipient and GFP donor controls; (B) HK-BCG-treated WT recipient control and GFP-chimera; (C) Untreated WT recipient and GFP donor controls; and (D) Untreated WT recipient control and GFP-chimera.

Figure 7. Cytometric detection of spleen cells expressing both GFP and COX-2 in the HK-BCG-treated GFP-chimera. GFP-chimera mice following treatment with HK-BCG were prepared as described in *Materials and Methods*. Spleen cells were isolated from (A) GFP-chimera, (B) WT recipients and (C) GFP-donor controls. Intracellular COX-1 and COX-2 were stained with anti-COX-1 and anti- COX-2 followed by PE-conjugated donkey anti-rabbit IgG. Normal rabbit IgG served as negative control for primary antibody binding. Fluorescence of 10^5 stained cells was quantitated cytometrically. PE-positive cells and GFP-positive cells were counted by two-color analysis.

Figure 8. Immunofluorescent detection of spleen cells expressing both GFP and COX-2 in the HK-BCG-treated bone marrow chimera. HK-BCG-treated GFP- chimera was established as described in *Materials and Methods*. On day 14 after HK-BCG treatment, spleens of the chimera were harvested, fixed, frozen and sectioned as described in the *Materials and Methods*. For detection of COX-2^+ cells, sections were stained with anti-COX-2 primary antibody followed by tetramethylrhodamine (red)-conjugated secondary antibody. In the spleen cell sections shown at 100x magnification, GFP (green) was co-localized with COX-2 (red). Without

HK-BCG treatment in the donors and recipients, there were no COX-2^{+} cells or GFP^{+} cells in the spleen (data not shown).

Fig. 1

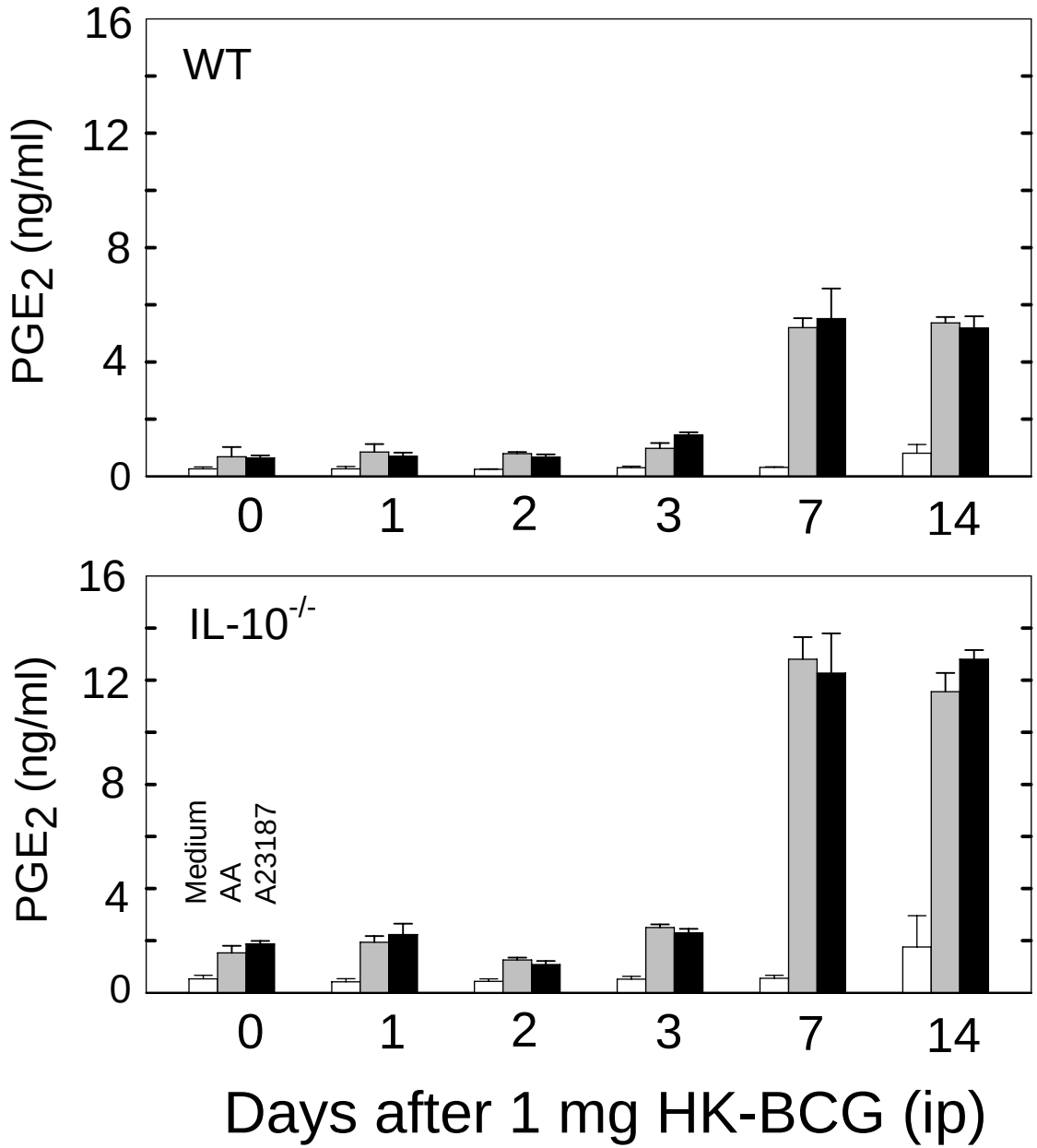

WT
IL-10-/-
PGE2 (ng/ml)
16
12
8
4
0
Medium
AA
A23187
0
1
2
3
7
14
Days after 1 mg HK-BCG (ip)

Fig. 2

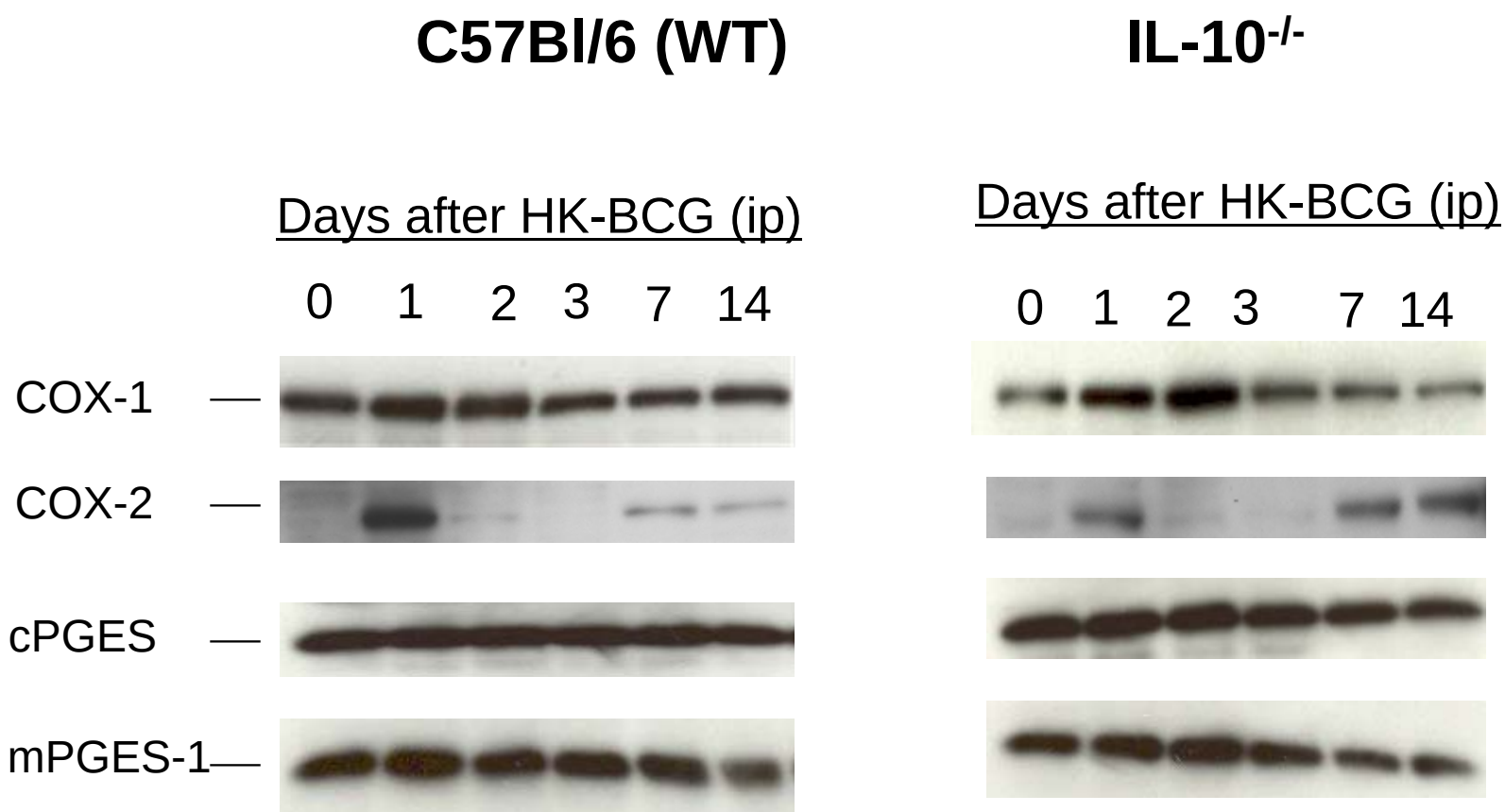
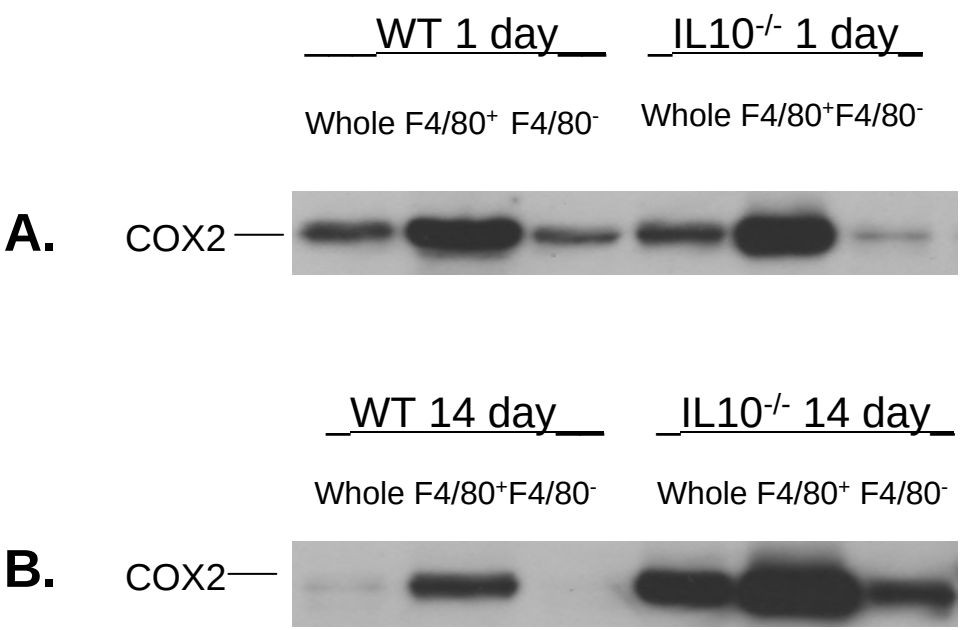
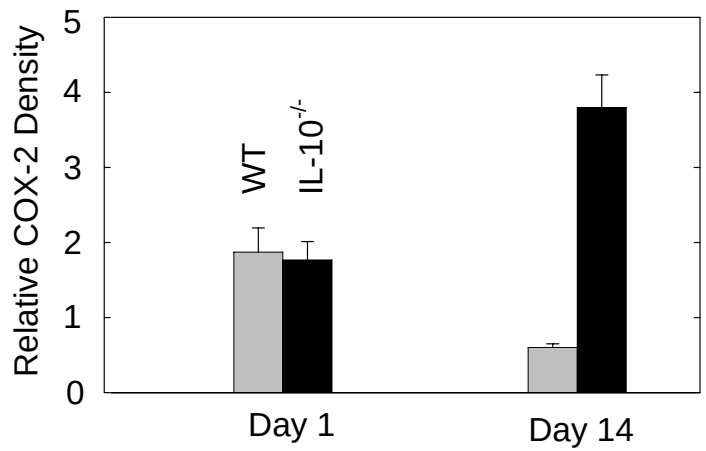

C57Bl/6 (WT)
IL-10-/-
Days after HK-BCG (ip)
0 1 2 3 7 14
Days after HK-BCG (ip)
0 1 2 3 7 14
COX-1
COX-2
cPGES
mPGES-1

Fig. 3


WT 1 day
IL10-/- 1 day
Whole F4/80+ F4/80-
Whole F4/80+F4/80-
A.
COX2
WT 14 day
IL10-/- 14 day
Whole F4/80+F4/80-
Whole F4/80+ F4/80-
B.
COX2


C. COX-2 density in F4/80+ cells
Relative COX-2 Density
0
1
2
3
4
5
WT
IL-10-/-
Day 1
Day 14

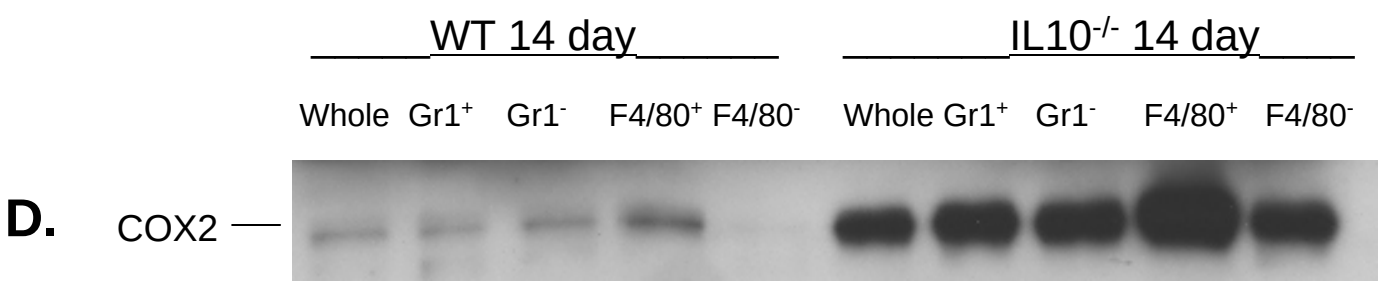

WT 14 day
IL10-/- 14 day
Whole Gr1+ Gr1- F4/80+ F4/80-
Whole Gr1+ Gr1- F4/80+ F4/80-
D.
COX2

Fig. 4

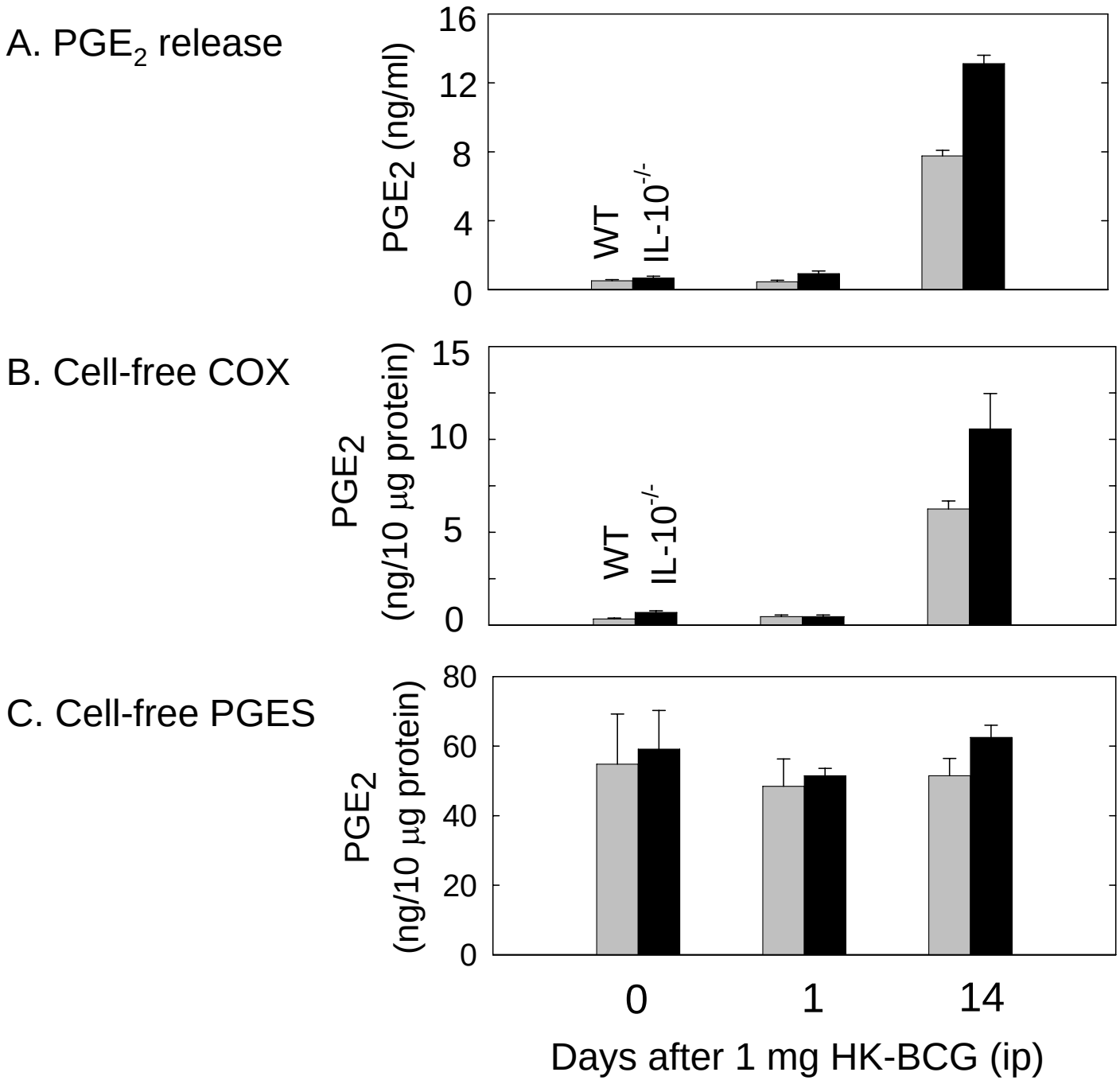

A. PGE2 release
PGE2 (ng/ml)
16
12
8
4
0
WT
IL-10-/-
B. Cell-free COX
PGE2
(ng/10 μg protein)
15
10
5
0
WT
IL-10-/-
C. Cell-free PGES
PGE2
(ng/10 μg protein)
80
60
40
20
0
0
1
14
Days after 1 mg HK-BCG (ip)

Fig. 5

A. HK-BCG treatment of GFP-chimera

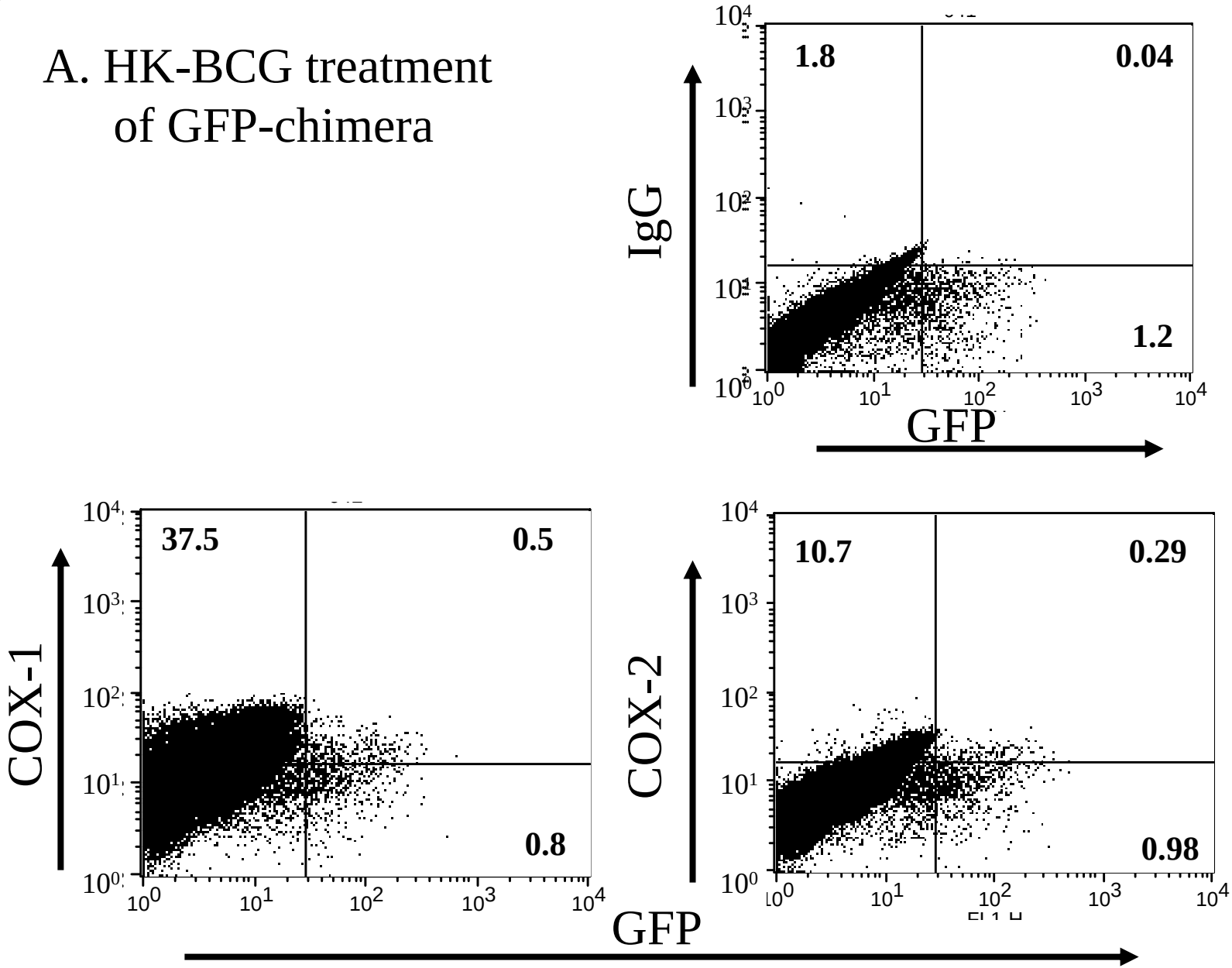


Fig. 5

B. HK-BCG Treatment of WT recipients

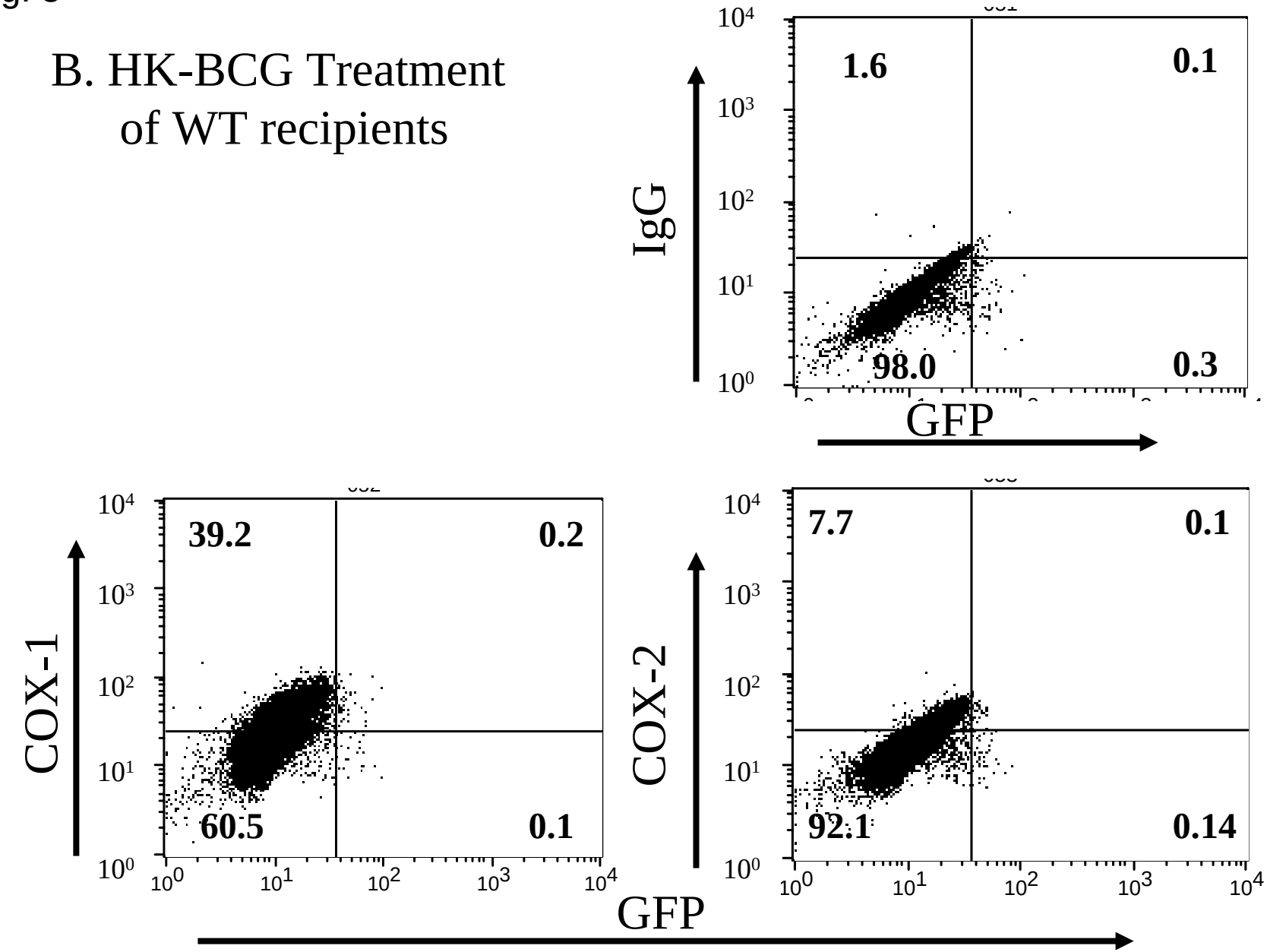


Fig. 5

C. HK-BCG treatment of GFP-donors

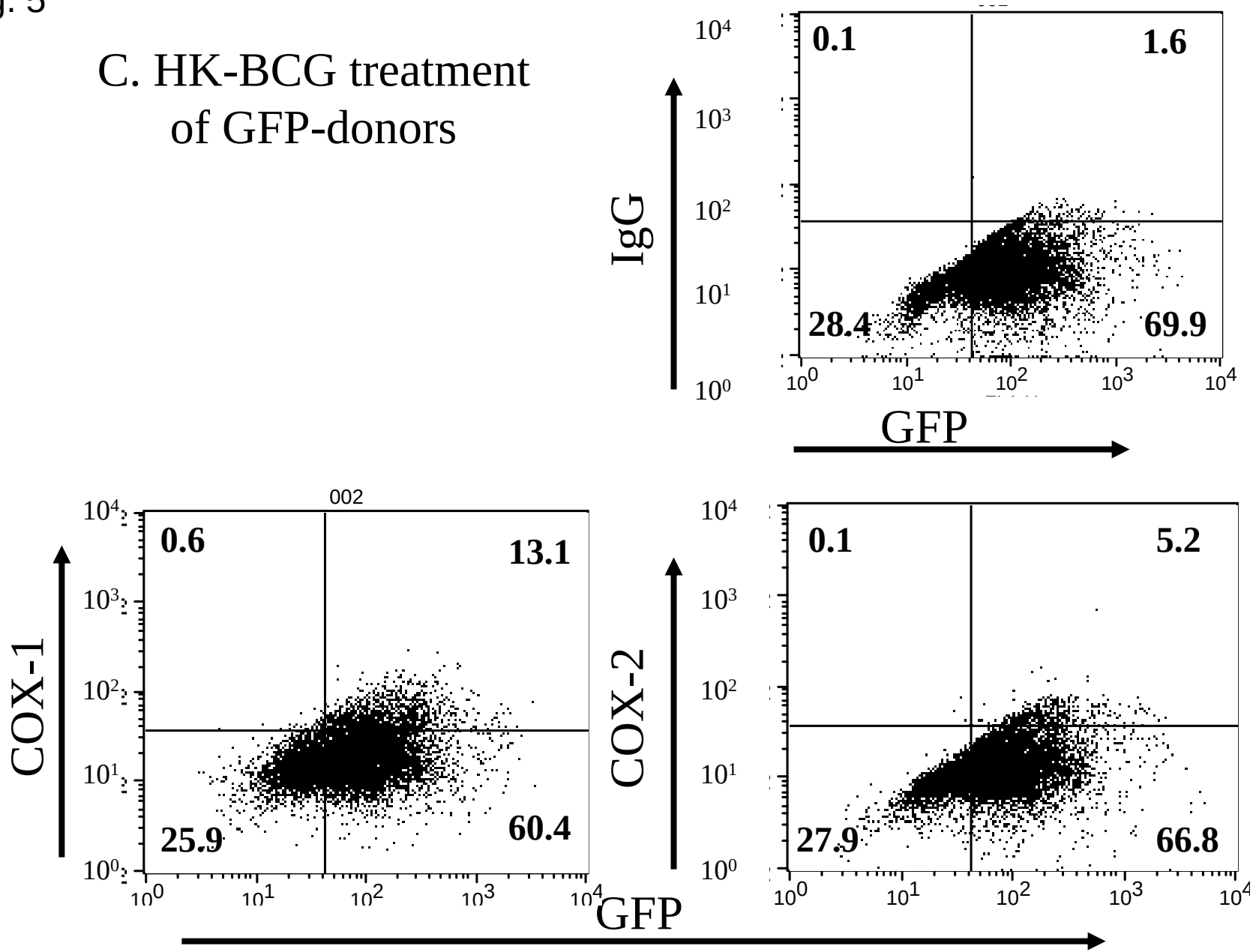


Fig. 6

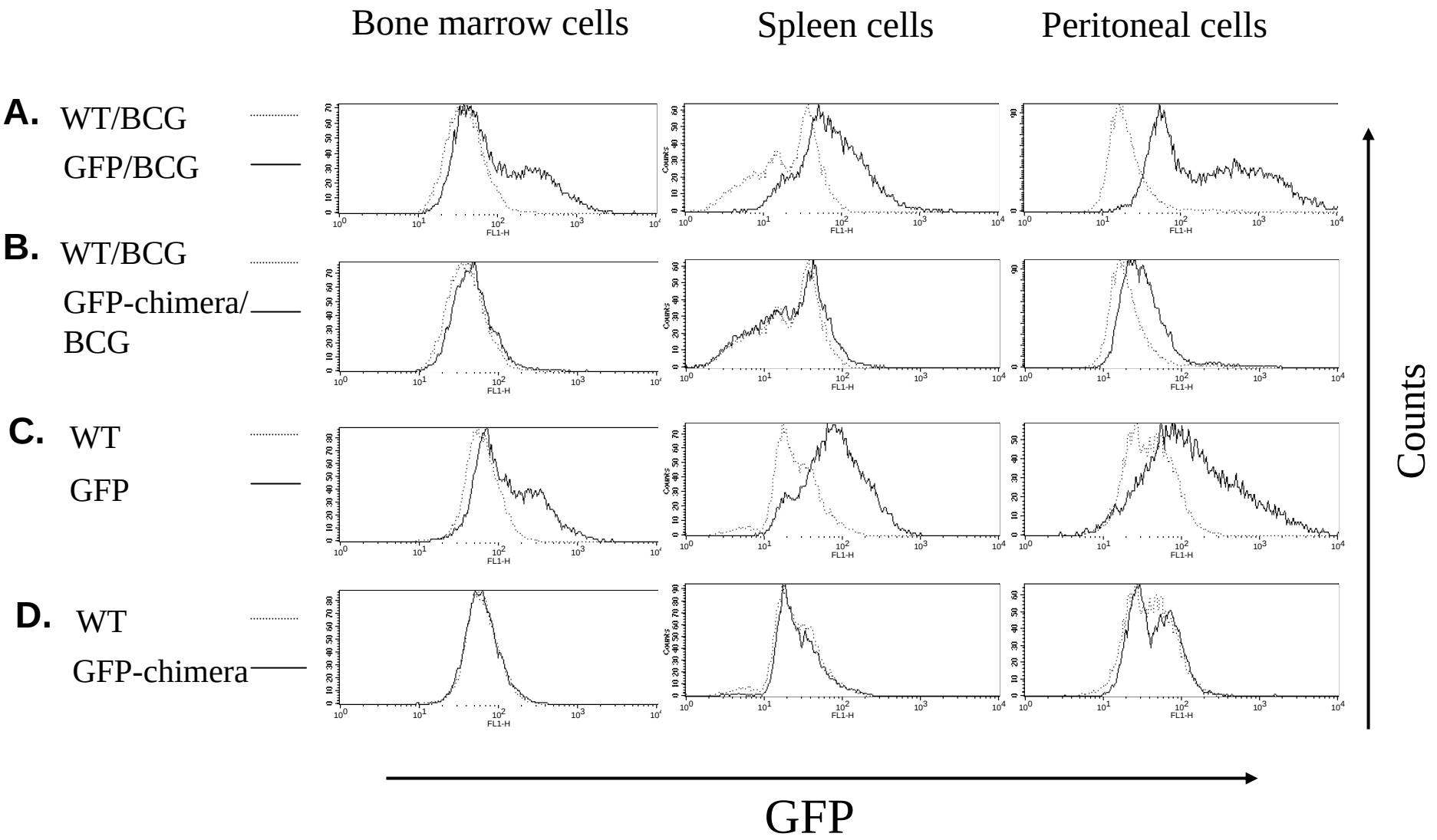
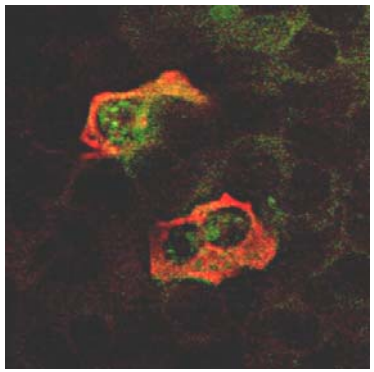
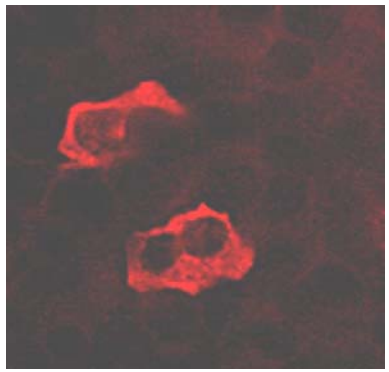

Bone marrow cells
Spleen cells
Peritoneal cells
A. WT/BCG
GFP/BCG
B. WT/BCG
GFP-chimera/ BCG
C. WT
GFP
D. WT
GFP-chimera
Counts
GFP
FL1-H

Fig. 7

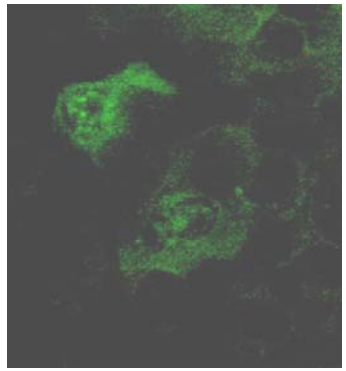
Overlay of COX-2$^+$ and GFP$^+$ Cells



COX-2$^+$ Cells



GFP$^+$ Cells



Phagocytosis of *N*-acetyl-D-glucosamine particles, a Th1 adjuvant, results in MAPK activation and TNF-*α*, but not IL-10, production

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ABSTRACT

A practical Th1 adjuvant should induce Th1 cytokines (IL-12, IL-18 and TNF-α) but not the Th2 cytokine IL-10, an inhibitor of Th1 responses. In this study, phagocytosis of *N*-acetyl-D-glucosamine polymer (chitin) particles by RAW264.7 macrophage-like cells resulted in phosphorylation of MAPK (p38, Erk1/2 and JNK) and production of relatively high levels of TNF-α and COX-2 with increased PGE_2 release. Similar results were observed in response to bacterial Th1 adjuvants (oligonucleotides with CpG motifs and mycobacterial components) and endotoxin. However, these bacterial components also induced a large amount of IL-10. Chitin particles, in contrast, induced only minimal levels of IL-10, although the production of high levels of PGE_2 and TNFα and the activation of MAPK's are potentially positive signals for IL-10 production. Thus, our results indicate that in macrophages chitin particles act as a unique Th1 adjuvant without inducing increased production of IL-10.

Key words: *N*-acetyl-D-glucosamine polymer particles, macrophages, phagocytosis, mitogen-activated protein kinases, IL-10

1. Introduction

A general approach to increasing host defense against multi-drug resistant intracellular pathogens would be the use of a practical Th1 adjuvant to stimulate innate immunity and promote acquired cell-mediated immune responses (CMI) that eliminate infected cells. However, an appropriate Th1 adjuvant is not currently available for this application.

Because of their exceptional capacities for the induction of CMI and Th1 responses, mycobacteria and their components have been used as immunotherapeutic agents and Th1 adjuvants [1]. In addition, Freund's complete adjuvant (heat-killed [HK]-*Mycobacterium tuberculosis* in mineral oil) and HK-BCG in mineral oil have been widely used to establish animal models of autoimmune diseases, such as experimental autoimmune encephalomyelitis, neuritis, uveitis, thyroiditis, orchitis, and adjuvant arthritis [2,3]. These adjuvants enhance Th1-mediated macrophage (MØ) activation and/or Th2-mediated antibody formation in a dose-dependent manner [3-7]. Pathogenic roles of Th1/Th2 responses appear to be varied among autoimmune disease models [3]. Furthermore, Th2-mediated antibody formation against mycobacteria does not have a decisive protective role against infections [8]. Recently, bacterial oligonucleotides (ODN) with unmethylated CpG motifs (CpG-ODN) have been studied extensively as Th1 adjuvants [9-11]. Several CpG-ODN compounds with nuclease-resistant phosphorothioate backbones are under pre-clinical and clinical evaluation against infectious diseases, cancer and allergic asthma [12]. These bacterial Th1 adjuvants, however, also induce the production of IL-10 [13,14], which reduces protective innate and acquired immune responses against intracellular infections [15-17].

We originally found [18-20], and other groups [21,22], confirmed that phagocytosable chitin (*N*-acetyl-D-glucosamine [GlcNAc] polymer) particles, a Th1 adjuvant, induce the activation of MØ leading to an innate immune response characterized by production of Th1 cytokines (IL-12, IL-18 and TNF-α). The activation of MØ induced by chitin particles is initiated by phagocytosis through C-type lectin receptors [19]. Since soluble chitin or >50 μm chitin particles fail to activate MØ [5,15,18-20], post-receptor events of phagocytosis including internalization and phagosome maturation are required for cellular activation.

For MØ stimulated by mycobacteria, CpG-ODN or bacterial endotoxin (LPS), it is well established that the activation of mitogen-activated protein kinases (MAPK) regulates the production of Th1 cytokines as well as IL-10 [23-29]. Activated MAPK family members include phosphorylated p38 MAPK (p38), extracellular signal-regulated kinase (Erk) 1/2, and c-Jun N-terminal kinase (JNK). The endogenous mediators TNF-α and prostaglandin E_2 (PGE_2) produced in response to bacterial Th1 adjuvants or LPS are reported to induce IL-10 production [26,30-32] . The expression of both TNF-α and cyclooxygenase-2 (COX-2), a rate-limiting enzyme for PGE_2 synthesis, is also regulated by MAPK activation [33]. In our initial studies we found that, unlike bacterial Th1 adjuvants or LPS [13,14] and in contrast to our earlier report [15], chitin particles induce far less, if any, IL-10. Comparative studies among the IL-10-inducing Th1 adjuvants and chitin particles may identify distinctive pathways for production of Th1 cytokines and IL-10. The chitin particle-induced activation of MAPK associated with Th1 cytokine production, COX-2 expression, and PGE_2 biosynthesis has not been previously investigated.

2. Materials and methods

2.1. Reagents and antibodies

Chitin particles were purchased from Sigma (St. Louis, MO) and phagocytosable (1 – 10 μm) and non-phagocytosable (>50 μm) chitin particles were prepared as described previously [19]. Soluble chitin oligosaccharide was provided by Kyowa Technos (Chiba, Japan). Chitin particles, 1 – 10 μm, were de-acetylated with 8 M sodium hydroxide at 55 °C for 16 hr, washed three times with saline and used as 1 – 10 μm chitosan particles. Latex beads (1.1 μm, polystyrene) and LPS (*Escherichia coli* serotype 0111:B4, phenol extraction) were purchased from Sigma. Both endotoxin-free CpG-ODN (5' TCC ATG ACG TTC CTG ACG TT 3'; unmethylated) and GpC-ODN (5' TCC ATG AGC TTC CTG AGC TT 3'; unmethylated) with phosphorothioate backbones were purchased from TriLink (Sorrento Mesa, CA). Cultured *M. bovis* BCG Tokyo 172 strain were washed, autoclaved and lyophilized. The HK-BCG powder was suspended in endotoxin-free saline and dispersed by brief sonication (10 sec) immediately before use. The stimulating reagents were suspended or dissolved in endotoxin-free saline as 10 mg/ml stock solutions. Selective p38 inhibitor SB203580, actin polymerization inhibitor cytochalasin D, calcium ionophore A23187, and arachidonic acid (AA) were purchased from Sigma. PD98059, a selective inhibitor for MAPK/Erk kinase (MEK)-1, which is an upstream activator of Erk 1/2, and SP600125, a selective JNK inhibitor, were purchased from Calbiochem (La Jolla, CA). These reagents, except for AA, were dissolved in DMSO to prepare 10 mM stock solutions. AA was dissolved in 100% ethanol to prepare a 1 mg/ml stock solution. Stock solutions were stored at -20 ˚C until use. Rabbit polyclonal anti-p38, anti-phospho-p38 (anti-p-p38), anti-Erk 1/2, anti-

phospho-Erk 1/2 (anti-p-Erk 1/2), anti-JNK, and anti-phospho-JNK (anti-p-JNK) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal anti-COX-2 antibody (anti-COX-2) was purchased from Cayman Chemicals (Ann Arbor, MI).

2.2. Cell culture

Murine MØ-like RAW 264.7 cells (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 (Life Technologies, Gaithersburg, MD) containing 5% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B at 5% CO_2 and 37 ºC in a 100% humidified incubator. Cells were passaged every 2 – 3 days to maintain logarithmic growth until they were used.

2.3. Cytokine production

Cells (5×10^5 /ml) were stimulated with 100 μg/ml 1 – 10 μm chitin particles, 5 μg/ml CpG-ODN, 0.1 μg/ml LPS, or saline in 96-well plates at 37 ºC and 5% CO_2. After 3, 6 or 24 hr, culture supernatants were harvested and stored at -80 ºC until used for ELISA. To examine the effects of SB203580, PD98059, and SP600125 on cytokine production, cells were pretreated with each MAPK inhibitor or its vehicle (0.1% DMSO in saline) at 37 ºC for 30 min prior to stimulation.

2.4. PGE_2 production

Cells (3×10^5 /ml) were stimulated with 100 μg/ml 1 – 10 μm chitin particles, 5 μg/ml CpG-ODN, 0.1 μg/ml LPS, or saline in 96-well plates at 37 ºC and 5% CO_2. After 24 hr, cells were washed and media was replaced with serum free RPMI 1640 with 1 μM A23187, 1 μg/ml AA, or saline and incubated at 37 ºC for an additional 2 hr. Culture supernatants were harvested and stored at -80 ºC until used for ELISA.

2.5. ELISA

Levels of TNF-α and IL-10 in culture supernatants were measured by two-site ELISA specific for the respective cytokines (BD Pharmingen, San Diego, CA). PGE_2 levels in the culture supernatants were measured by competitive ELISA (Cayman Chemicals).

2.6. MAPK activation

Cells (10^6 /ml) were pre-incubated in serum-free RPMI 1640 for 2 hr and then stimulated with chitin particles, bacterial Th1 adjuvants and their controls as described above. Cells were incubated at 37 ºC for 0, 10, 20, 30, and 40 min. To further characterize MAPK phosphorylation, cells were pretreated with each inhibitor at 10 μM or its vehicle (0.1% DMSO in saline) at 37 ºC for 30 min prior to stimulation.

2.7. Protein extraction and western blotting

Cells were lysed with SDS-lysis buffer (50 mM Tris-HCl, pH 7.5, 10 mM NaF, 1 mM Na_3VO_4, 5 mM EDTA, and 1% SDS) by heating at 95 ˚C for 5 min and then chilled on ice. Debris in the

lysate was eliminated by centrifugation (15,000 *g*, 3 min). Protein concentration in the supernatants was measured by a bicinchoninic acid reagent (Pierce, Rockford, IL), using bovine serum albumin as standard. Equal amounts of cellular protein were loaded onto SDS-11% polyacrylamide gel and separated by electrophoresis. Separated proteins were electroblotted onto PVDF membrane. After blocking with non-fat dry milk, membranes were stained with antibodies against total MAPK (anti-p38, anti-Erk 1/2, or anti-JNK), dual phosphorylated MAPK (anti-p-p38, anti-p-Erk 1/2, or anti-p-JNK), or COX-2. Following incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch, West Glove, PA), specifically labeled proteins were detected by chemiluminescence (ECL Western Blotting Detection Reagents, Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions. Intensity of the specific bands was quantified digitally using graphic imaging software (NIH Image 1.5).

2.8. Endotoxin removal

Endotoxin was removed from soluble materials for culture by filtration and sterilization through a 0.22-μm Zetapore membrane (AMF-Cuno; Cuno, Meriden, CT) [34]. The final preparations were monitored for endotoxin by the *Limulus* amebocyte assay (Sigma) [15].

2.9. Statistics

Differences between mean values were analyzed by Student's *t* test. $P < 0.05$ is considered statistically significant.

3. Results

3.1. Chitin particle-induced production of TNF-α but not IL-10

Treatment of RAW 264.7 cells with 1 – 10 μm chitin particles resulted in production of relatively large amounts of TNF-α within 3 hr, which increased at 6 and 24 hr (Fig. 1). However, at 24 hr, chitin particles induced only a minimal amount of IL-10 (Fig. 1A). In contrast, bacterial Th1 adjuvants (CpG-ODN and HK-BCG) and LPS all induced large amounts of IL-10 and TNF-α in a time-dependent manner (Fig. 1).

As controls, soluble chitin oligosaccharide, non-phagocytosable chitin particles (>50 μm), 1 – 10 μm chitosan particles, 1.1 μm latex beads induced only minimal levels of TNF-α and undetectable levels (<15 pg/ml) of IL-10 in RAW 264.7 cells (Fig. 1). The results confirm that chitin-induced TNF-α production requires both recognition of GlcNAc and internalization associated with phagosome formation [18,19]. Although chitin particles induce TNF-α, a possible mediator of IL-10 production in LPS-stimulated MØ [30], with a time course similar to that for LPS, chitin particles do not induce significant IL-10 production (Fig. 1).

3.2. Chitin particle-induced COX-2 expression and PGE_2 release

CpG-ODN and LPS are known to induce COX-2 expression and PGE_2 release by MØ [33,35]. Because PGE_2 is reported to induce IL-10 production [31,32], we examined COX-2 expression and PGE_2 release in response to chitin particles. Following 2 hr treatment of RAW 264.7 cells

with 1 – 10 μm chitin particles, COX-2 protein was detected at levels only slightly lower than those induced by CpG-ODN and LPS (Fig. 2A). After incubation with the agonists for 24 hr, PGE_2 release from the cells treated with chitin was twice that seen with CpG-ODN-treated cells and comparable to that for cells treated with LPS (Fig. 2B). Interestingly, PGE_2 release from cells elicited with A23187 or AA was not significantly increased above that for the spontaneous (constitutive) release from cells treated with saline, suggesting that, under these conditions, treatment of the cells with chitin particles, CpG-ODN or LPS is sufficient to maximally stimulate prostaglandin production (Fig. 2). Soluble chitin oligosaccharide-, >50 μm chitin particle-, or 1 – 10 μm chitosan particle- treated cells showed only minimal levels of COX-2 expression (Fig. 2) and no increase in PGE_2 release (data not shown). Our results indicate that chitin particle phagocytosis, dependent on specific recognition of GlcNAc, significantly enhances PGE_2 biosynthesis. Despite reports that LPS-induced PGE_2 release contributes to IL-10 production [31,32], it appears to be insufficient to induce IL-10 production in chitin particle-stimulated MØ.

3.3. Chitin particles induce MAPK phosphorylation

One or more MAPK (p38, Erk1/2 and JNK) pathways regulate IL-10 and TNF-α production and COX-2 expression by MØ or monocytes [24,26,36]. To determine the role of MAPK pathways in regulation of IL-10 production in RAW 264.7 cells, we examined early MAPK activation in response to treatment with chitin particles. As shown in Fig. 3, 1 – 10 μm chitin particles induced p38, Erk 1/2 and JNK phosphorylation. The peak of phosphorylation was 30 min for p38, 30 and 40 min for Erk 1/2, and 40 min for JNK (Fig. 3). In contrast, soluble chitin, >50 μm

chitin particles, 1 – 10 μm chitosan particles, or 1.1 μm latex beads failed to induce phosphorylation of any MAPK by 40 min after stimulation (Fig. 3).

Bacterial Th1 adjuvants (CpG-ODN and HK-BCG) and LPS also activated MØ MAPKs as shown previously [24-26] with profiles generally comparable to those of MØ stimulated with chitin particles, except that maximal phosphorylation occurred at later time points with chitin (Fig. 3). As a control for CpG-ODN, phosphorothioate GpC-ODN which induces some other pro-inflammatory responses [37], did not activate MAPK (Fig. 3) or stimulate production of TNF-α or IL-10 (Fig. 1).

To further elucidate the mechanism of chitin-induced MAPK activation, cytochalasin D, an inhibitor of actin polymerization [38], was used to investigate the contribution of the actin cytoskeleton. As shown in Fig. 4, cytochalasin D reduced chitin particle-induced phosphorylation of p38, Erk 1/2, and JNK (60 – 70% reduction each), indicating that MAPK activation requires internalization of chitin particles. Our previous studies showed that the same dose of cytochalasin D significantly reduced chitin particle-induced TNF-α and IL-12 production by mouse splenic MØ [19]. Except for LPS-induced p38 phosphorylation (40% reduction; data not shown and [39]), cytochalasin D did not have a significant effect on CpG-ODN- or LPS- induced MAPK phosphorylation.

Our results indicate that chitin particle-induced MAPK activation and TNF-α production are dependent on GlcNAc recognition followed by particle internalization and phagosome formation. Among the three Th1 adjuvants, the effects of cytochalasin D on cytoskeletal reorganization and

MAPK activation were distinct for chitin consistent with participation of different cell-surface receptors in the responses to the adjuvants.

3.4. Effects of MAPK inhibitors on chitin particle-induced IL-10 production

To examine the roles of each MAPK in cytokine production, cells were treated with selective inhibitors of the three MAPK pathways. Table 1 shows that the inhibitors employed did not significantly enhance IL-10 production in chitin particle-stimulated cells. Similar results were obtained with murine splenic MØ stimulated with 1 – 10 μm chitin particles (data not shown) confirming the lack of IL-10 production in response to chitin and the effects of the inhibitors. With CpG-ODN as agonist, IL-10 production was slightly enhanced by PD98059, MEK-1 inhibitor, but reduced by SB203580, p38 inhibitor, and SP600125, JNK pathway inhibitor (Table 1). Each of the MAPK inhibitors reduced LPS-induced IL-10 production by approximately 90% (Table 1).

TNF-α production induced by chitin particles, CpG-ODN and LPS was significantly reduced by SB203580 and SP600125, but not by PD98059 (Table 1). The large reduction seen in CpG-ODN-induced TNF-α production in the presence of SP600125 suggests a major dependence on JNK activation for this agonist (Table 1). Thus, the three MAPK pathways differentially regulate the responses to the three agonists studied and also have distinct roles for the two individual cytokines.

The production of PGE_2, which inhibits TNF-α release and enhances IL-10 release from

macrophages [31,32] is dependent primarily on the expression of COX-2. In seeking to understand the effects of the three MAPK inhibitors on cytokine production, we have determined the effect of these inhibitors on COX-2 expression. As shown in Fig. 5, chitin particle-induced COX-2 expression was significantly reduced by each MAPK inhibitor. Therefore, the inhibition of chitin-induced TNF-α production by each of the three MAPK inhibitors would not appear to be related to increased PGE_2 biosynthesis. Even without inhibition of COX-2 expression, IL-10 in response to chitin was below the detection limits of the assay. Although PGE_2 is expected to enhance IL-10 production, it is apparently not sufficient for induction of this cytokine. Thus the MAPK inhibitors affected both cytokine and COX-2 expression, but there does not appear to be a direct relationship between the inhibition of COX-2 expression and production of TNF-α in this system. Rather, the expression of each appears to be independently mediated by MAPK pathways.

4. Discussion

Our previous studies indicate that phagocytosis of chitin particles results in the production of IL-12p70, IL-18 and TNF-α by murine splenic MØ [15]. Since the detection of MAPK phosphorylation is more stable and reproducible in RAW 264.7 cells compared to splenic MØ, these studies were performed employing murine MØ-like RAW 264.7 cells. When RAW 264.7 cells phagocytose 1 – 10 μm chitin particles, p38, Erk 1/2 and JNK are activated in a phagocytosis-dependent manner. MAPK activation is not induced by soluble chitin or non-phagocytosable (>50 μm) chitin particles. Confirming the chitin particle dependence of MAPK activation, phagocytosis of latex beads or chitosan (de-acetylated chitin) fails to induce either MAPK activation or cytokine production by RAW 264.7 cells. Although our RAW 264.7 cells do not produce IL-12p70, IL-12p40 or IL-18 in response to chitin particles, bacterial Th1 adjuvants (CpG-ODN, HK-BCG) or LPS, they produce TNF-α and IL-10 dependent on the agonist. Taken together, our present and previous studies [15,19] clearly indicate that specific GlcNAc post-receptor ligation events of phagocytosis, particularly internalization and probably phagosome maturation are required for MAPK activation and Th1 cytokine production.

Studies with selective MAPK inhibitors indicate that TNF-α production is partially dependent on MAPK (p38, Erk 1/2 and JNK) activation in chitin particle-stimulated RAW 264.7 cells. Further, activation of these kinases is not sufficient for IL-10 production. The role of these MAPK pathways in IL-10 production is agonist (for Th1 adjuvants) dependent. Interestingly, CpG-ODN-induced IL-10 production is up-regulated by p38 and JNK pathways but down-regulated by the Erk 1/2 pathway. However, our study and others indicate that MAPK-regulated

IL-10 production appears to be distinct for different IL-10-inducing agents and cell types. Foey *et al*., for example, reported that, for LPS-activated human monocyte IL-10 production, p38 pathway provides a positive signal, whereas Erk 1/2 pathway provides no signal [30]. Ma *et al.*, further demonstrated that activation of transcriptional factor Sp1 via p38 activation is required for IL-10 expression in LPS-stimulated THP-1 cells [36]. In contrast, for *Leishmania*-infected murine peritoneal MØ, Mathur *et al.* reported that CD40-induced IL-10 production is up-regulated by Erk 1/2 and down-regulated by p38 [40].

As for other bacterial Th1 adjuvants, phagocytosis of chitin particles by RAW 264.7 cells results in the induction of COX-2 and PGE_2 biosynthesis. COX-2 induction is at least partially dependent on MAPK activation, and is not induced by phagocytosis of latex beads or chitosan particles nor by soluble chitin or non-phagocytosable (>50 μm) chitin particles. It is well established that PGE_2 and TNF-α act as endogenous mediators to induce IL-10 production possibly mediated through MAPK pathways [41-43]. Our results clearly indicate that despite significant MAPK activation and production of relatively large amounts of PGE_2 and TNF-α, chitin particle-stimulated MØ produce only minimal levels of IL-10. This raises the question of whether chitin particles might specifically inhibit IL-10 production. However, IL-10 production by chitin-stimulated RAW 264.7 cells was not altered by the MAPK pathway inhibitors, which indicates that activation of these pathways does not suppress IL-10 production. Previously, we reported the chitin-stimulated production of IL-10 by murine splenic MØ [15]. However, since we have begun using a commercially available ELISA kit to measure IL-10, we have not observed IL-10 production by MØ in response to chitin. Results for induction of TNF-α in splenic MØ are comparable to those shown in Figure 1 (unpublished). Further studies

comparing features of Th1 adjuvant stimulation of RAW 264.7 cells may assist in identifying intermediate steps critical for limiting IL-10 production.

In conclusion, MØ from immunocompromised populations (the aged, neonates, tumor-bearers, atherosclerotics, and diabetics) frequently express high levels of IL-10 [44-47], potentially suppressing host defense capabilities. The expression of IL-10 reduces both innate immunity and CMI. *M. bovis* BCG components including oligonucleotides with unmethylated CpG motifs are known to induce IL-10 production and Th1 cytokine production. In sharp contrast, chitin as a Th1 adjuvant does not stimulate IL-10 production. Thus, chitin may be unique among presently available Th1 adjuvants and an attractive candidate for stimulating host defense in immunocompromised populations.

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TABLE 1. The effects of inhibitors for MAPK pathways on chitin particle-, CpG-ODN- and LPS- induced IL-10 and TNF-α production by RAW 264.7 cells.

Agonist	Inhibitor [a]	% Production [b] (% Suppression) IL-10	TNF-α
Chitin particles (1 – 10 μm)	DMSO	ND [c]	100 (0)
	SB	ND [c]	66# (34)
	PD	ND [c]	79 (21)
	SP	ND [c]	47# (53)
CpG-ODN	DMSO	100 (0)	100 (0)
	SB	63# (37)	60* (40)
	PD	123* (NS)	77 (23)
	SP	25# (75)	9# (91)
LPS	DMSO	100 (0)	100 (0)
	SB	5# (95)	72* (28)
	PD	8# (92)	82 (18)
	SP	5# (85)	39# (61)

[a] Cells were pre-treated with 10 μM SB203580 (SB), 10 μM PD98059 (PD), 10 μM SP600125 (SP), or its vehicle (0.1% DMSO in saline) at 37 ºC for 30 min prior to stimulation with 100 μg/ml chitin particles, 0.1 μg/ml LPS or 5 μg/ml CpG-ODN for 3 hr (for TNF-α) or 24 hr (for IL-10). Cytokine levels were measured by specific ELISA.

[b] The amounts of cytokines produced by agonist-stimulated cells in the absence of inhibitors (DMSO) were considered as 100%.

[c] ND, Not detected (<15 pg/ml IL-10). ( ), % Suppression; NS, no suppression. Mean, n=3; *, $p < 0.05$; #, $p < 0.01$ compared to agonist-stimulated cells (DMSO), respectively. The data shown are representative of two independent experiments.

LEGENDS FOR FIGURES

Fig. 1. Production of IL-10 and TNF-α by RAW 264.7 cells. Cells at 5×10^5 /ml were stimulated with 100 μg/ml 1 – 10 μm chitin particles, 5 μg/ml CpG-ODN, 5 μg/ml GpC-ODN, 100 μg/ml HK-BCG, 0.1 μg/ml LPS, 100 μg/ml soluble chitin oligosaccharide, 100 μg/ml >50 μm chitin particles, 100 μg/ml 1 – 10 μm chitosan particles, 100 μg/ml 1.1 μm latex beads, or saline at 37 ˚C for 3 hr (black bars), 6 hr (white bars), and 24 hr (gray bars). The levels of IL-10 (A) and TNF-α (B) in culture supernatants were measured by ELISA. Mean ± SD, n=4; *, $p < 0.05$; #, $p < 0.01$, compared to cells treated with saline at the same time point. The data shown are representative of two independent experiments.

Fig. 2. Production of COX-2 and PGE_2 by RAW 264.7 cells. *A*. Cells at 10^6 /ml were stimulated with 1 – 10 μm chitin particles, CpG-ODN, LPS and their controls at 37 ˚C for 2 hr. Their concentrations were identical to those shown in Fig. 1. For detection of COX-2, 2 μg protein was separated on SDS-10% polyacrylamide gel, and electroblotted to PVDF membrane. COX-2 was detected with specific antibodies. *B*. Cells were primed as described under MATERIALS AND METHODS and elicited with saline (black bars), calcium ionophore A23187 (white bars) and free AA (gray bars) for PGE_2 production. PGE_2 in culture supernatants was measured by ELISA. Mean ± SD, n=4; #, $p < 0.01$, compared to the corresponding cells stimulated with saline. The data shown are representative of two independent experiments.

Fig. 3. Chitin particle-induced phosphorylation of p38, Erk1/2, and JNK in RAW 264.7 cells. *A.* Cells at 10^6 /ml were stimulated with 1 – 10 μm chitin particles, bacterial Th1 adjuvants (CpG-ODN and HK-BCG), bacterial endotoxin (LPS) and their controls as indicated in Fig. 1. Cells

were incubated at 37 ˚C for 0, 10, 20, 30, and 40 min. One μg protein for detection of p38 and Erk 1/2, or 4 μg protein for detection of JNK were separated on SDS-11% polyacrylamide gel and electroblotted to PVDF membrane. Total MAPK and dual phosphorylated MAPK were detected with specific antibodies and chemiluminescence of horseradish peroxidase conjugated secondary antibody. The level of total JNK in HK-BCG-treated cells was not determined due to non-specific detection by the antibodies. *B – E*. Band intensities (Fig. 3A) were quantified digitally using graphic imaging software (NIH Image 1.5). Intensity of phosphorylated MAPK bands was normalized to that of total MAPK bands. Normalized intensity of phosphorylated MAPK band detected in cells stimulated with agonist for 0 min (background phosphorylation) was considered as 1-fold phosphorylation. Black bars, p38; White bars, Erk1/2; Gray bars, JNK. The data shown are representative of three independent experiments.

Fig. 4. Effect of cytochalasin D on chitin particle-induced phosphorylation of p38, Erk 1/2, and JNK. Cells were treated with 10 μM cytochalasin D or 0.1% DMSO (0 μM) at 37 ˚C for 30 min and then stimulated with 1 – 10 μm chitin particles at 37 ˚C for 30 min. Normalized intensity of phosphorylated MAPK bands detected in non-stimulated cells (background phosphorylation) was subtracted from that of phosphorylated MAPK bands detected in chitin particle-stimulated cells to determine the percentage phosphorylated MAPK. The data from one experiment representative of three are shown.

Fig. 5. The effects of inhibitors of MAPK pathways on chitin particle-induced COX-2 production by RAW 264.7 cells. *A*. Cells were pre-treated with 10 μM SB203580 (SB), 10 μM PD98059 (PD), 10 μM SP600125 (SP), or its vehicle (0.1% DMSO in saline) at 37 ºC for 30 min

prior to stimulation with 100 μg/ml 1 – 10 μm chitin particles for 2 hr. Two μg protein was separated on SDS-11% polyacrylamide gel, and electroblotted to PVDF membrane. COX-2 was detected as described under Fig. 3. *B*. Band intensities were quantified digitally using graphic imaging software (NIH Image 1.5). The intensity of the COX-2 band detected in non-stimulated cells (background production) was subtracted from that of COX-2 bands detected in chitin particle-stimulated cells. The data from one experiment representative of three are shown.

Fig. 1.

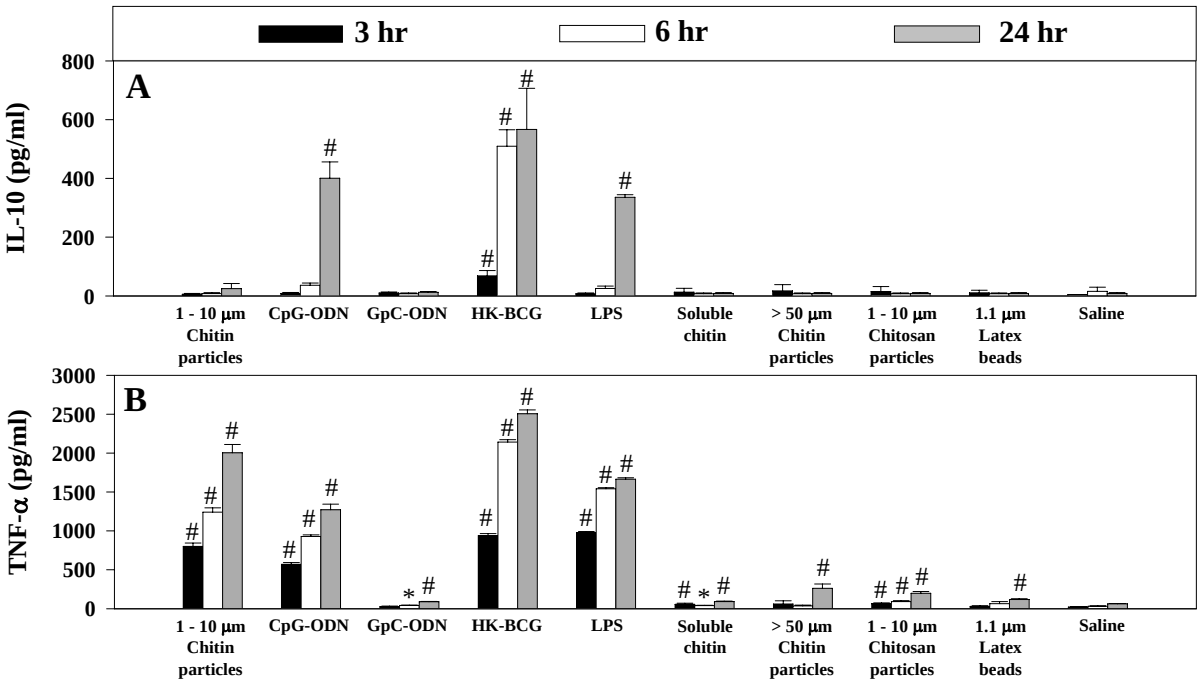
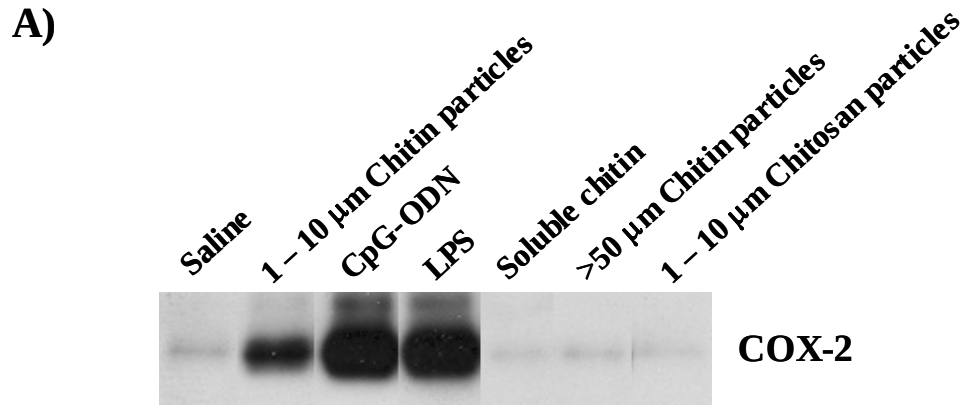

3 hr
6 hr
24 hr
A
IL-10 (pg/ml)
800
600
400
200
0
B
TNF-α (pg/ml)
3000
2500
2000
1500
1000
500
0
1 - 10 µm Chitin particles
CpG-ODN
GpC-ODN
HK-BCG
LPS
Soluble chitin
> 50 µm Chitin particles
1 - 10 µm Chitosan particles
1.1 µm Latex beads
Saline

Fig. 2.


A)
Saline
1 – 10 μm Chitin particles
CpG-ODN
LPS
Soluble chitin
>50 μm Chitin particles
1 – 10 μm Chitosan particles
COX-2

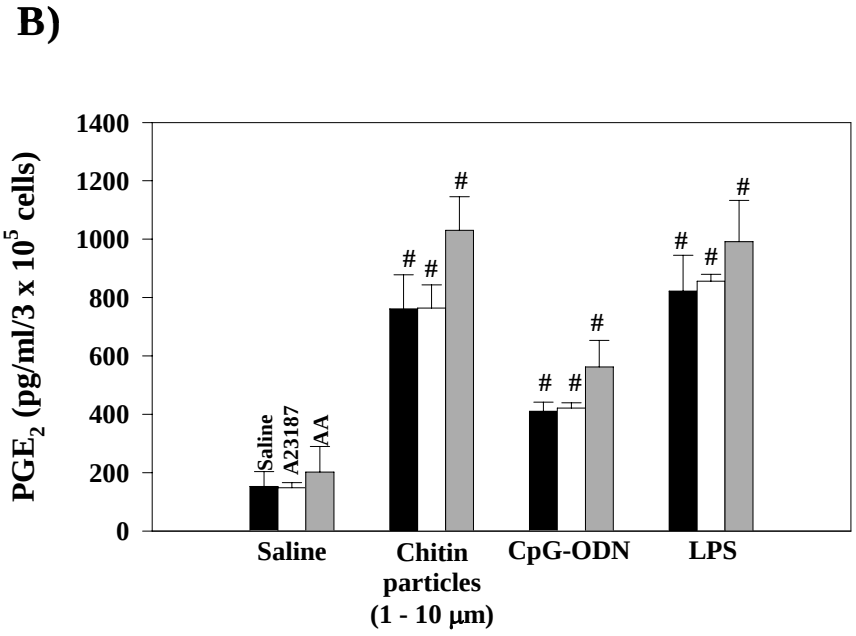

B)
PGE2 (pg/ml/3 x 10^5 cells)
1400
1200
1000
800
600
400
200
0
Saline
A23187
AA
#
Saline
Chitin particles (1 - 10 μm)
CpG-ODN
LPS

Fig. 3.

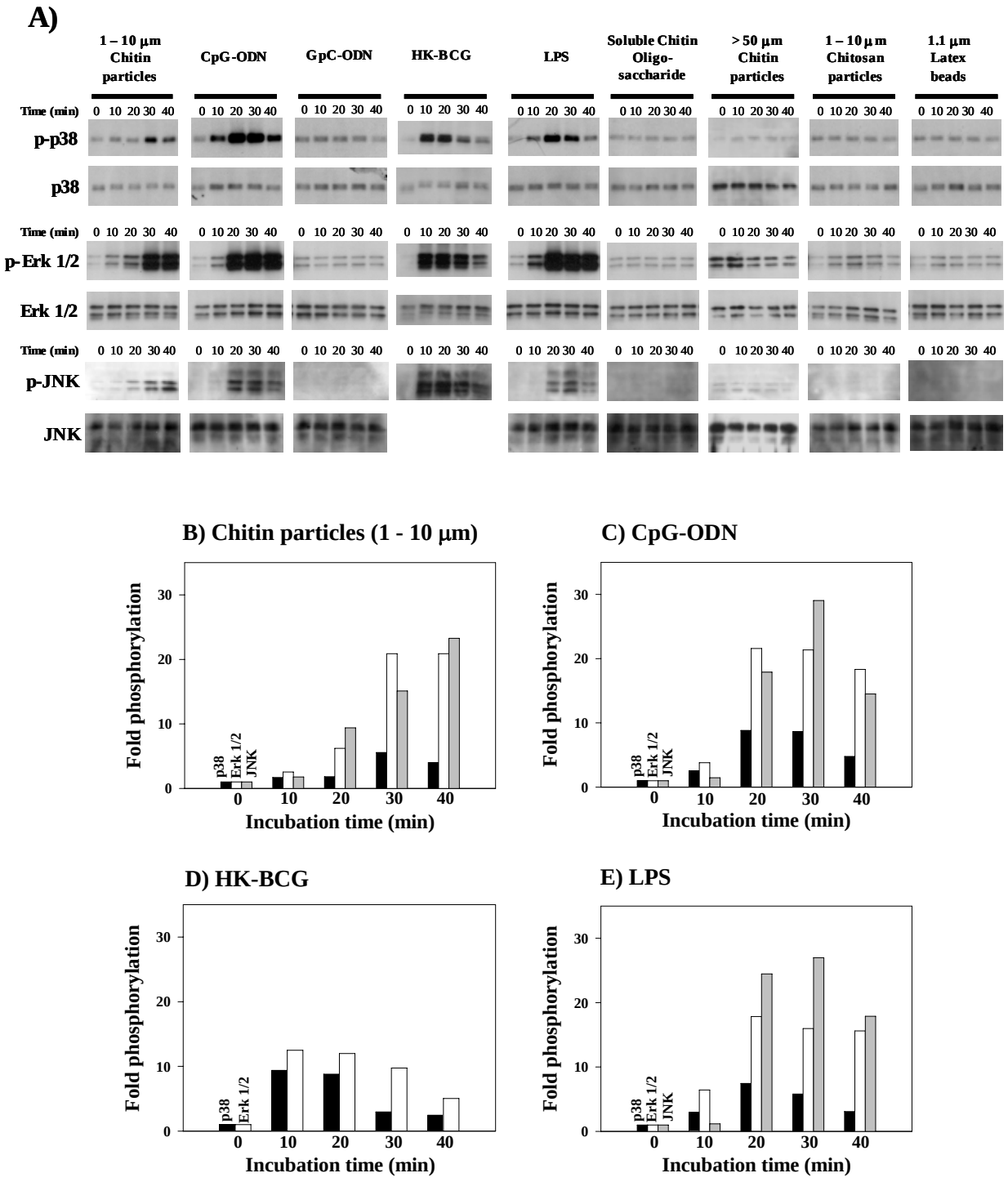

A)
1 – 10 µm Chitin particles
CpG-ODN
GpC-ODN
HK-BCG
LPS
Soluble Chitin Oligo-saccharide
> 50 µm Chitin particles
1 – 10 µm Chitosan particles
1.1 µm Latex beads
Time (min) 0 10 20 30 40
p-p38
p38
p-Erk 1/2
Erk 1/2
p-JNK
JNK
B) Chitin particles (1 - 10 µm)
C) CpG-ODN
D) HK-BCG
E) LPS
Fold phosphorylation
Incubation time (min)
p38
Erk 1/2
JNK

Fig. 4.

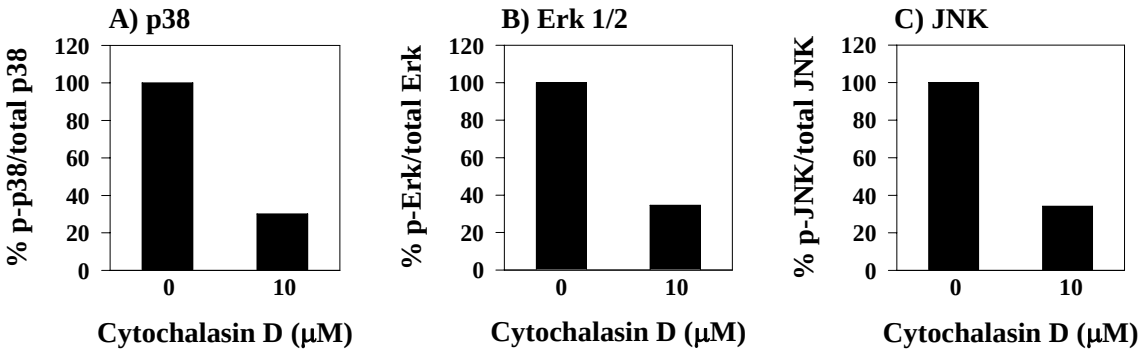
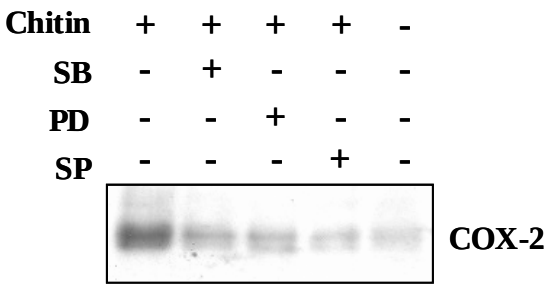
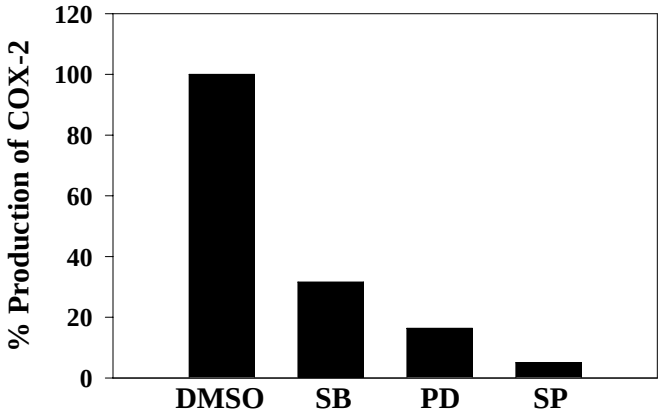

A) p38
% p-p38/total p38
120
100
80
60
40
20
0
0
10
Cytochalasin D (μM)
B) Erk 1/2
% p-Erk/total Erk
120
100
80
60
40
20
0
0
10
Cytochalasin D (μM)
C) JNK
% p-JNK/total JNK
120
100
80
60
40
20
0
0
10
Cytochalasin D (μM)

Fig. 5

A)


Chitin + + + + -
SB - + - - -
PD - - + - -
SP - - - + -
COX-2

B)


% Production of COX-2
120
100
80
60
40
20
0
DMSO
SB
PD
SP