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Potential Role for Alternatively Activated Macrophages in the Secondary Bacterial Infection During Recovery from Influenza

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Abstract

Purpose—Secondary bacterial infections are a common complication of influenza. Innate immune host defenses appear to be impaired following influenza, leading to susceptibility to subsequent bacterial infections. Alternatively activated macrophages (AAM) in the lungs may play a critical role in eliciting the hypersusceptibility to secondary bacterial pneumonia.

Methods—C57BL/6 mice were challenged with sublethal doses of the mouse-adapted A/PR/8/34 (PR8) influenza virus or saline and allowed to recover. At complete recovery (day 14), mice were re-challenged with sublethal doses of *Streptococcus pneumoniae* serotype 3 (Sp3).

Results—PR8-recovered mice developed a rapidly fatal pulmonary infection to a 100-fold sublethal pneumococcal challenge, whereas PR8-naïve mice demonstrated no mortality or illness. The cytokines which induce AAM (IL-4 and IL-13) and the expression of genes associated with AAM (Arginase-1, FIZZ1, and YM1) were elevated after PR8 infection. Flow cytometry suggests that alveolar macrophages demonstrate the AAM-phenotype, as indicated by MGL-1 and MHCII expression, in response to PR8 infection. Recovery from PR8 was associated with blunted cytokine responses to TLR ligands.

Conclusions—The mechanisms of immune regulation during recovery from influenza are being elucidated. We provide evidence that pulmonary AAM are induced during influenza infection and may contribute to the elicitation of hypersusceptibility to a secondary bacterial infection.

Keywords

influenza; secondary pneumonia; alveolar macrophage; alternative activation

1. Introduction

A severe bacterial infection is commonly associated with influenza and is a significant contributor to the excess morbidity and mortality of influenza. In fact, the majority of deaths during the 1918 H1N1 [1], 1957 H2N2 [2,3], and 1968 H3N2 [4] pandemics are believed to

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be due to a secondary bacterial pneumonia. During interpandemic influenza, secondary bacterial infections may account for ~25% of influenza-associated hospitalizations [5,6]. Since more effective treatment strategies for influenza should address this frequent complication, studies directed at elucidating the mechanisms involved in inducing this hypersusceptibility to secondary infection are needed.

Although a secondary bacterial pneumonia often occurs during the primary viral infection, the risk continues to exist after resolution of influenza [2]. The late bacterial pneumonia has been hypothesized to be due, in part, to impaired host defenses [7,8]. Indeed, there is a sustained desensitization of alveolar macrophage Toll-like receptors (TLR) for at least 6 weeks after an influenza infection [9], indicating a window of susceptibility to secondary bacterial pathogens. Post-influenza resident pulmonary macrophages are impaired in their ability to engulf and kill bacteria [10,11]. Both the down-regulation and expression of the class A scavenger receptor MARCO on alveolar macrophages [12] and excessive IL-10 production [13] contribute to increased susceptibility to secondary bacterial pneumonia. Therefore, airway macrophage biology appears to be modulated after encountering an influenza infection.

The recognition of “alternatively activated macrophages” (AAM) has called attention to macrophage “plasticity” and the potential of environmental signals to alter macrophage differentiation. The pro-inflammatory “classical” macrophage (CM) is known for effective early host defenses and antimicrobial activity. CM differentiate in response to interferon- γ (IFN- γ) and produce inducible nitric oxide synthase (iNOS) to generate nitric oxide (NO) and other reactive oxygen species, for bacterial killing. In contrast, AAM demonstrate poor bactericidal activity. AAM are associated with the maintenance of tissue homeostasis or are involved with tissue remodeling, wound healing and the dampening of inflammatory responses [14,15]. Furthermore, AAM differentiate in response to Th2 cytokines, IL-4 and IL-13, and produce arginase-1 (Arg-1) which competes with iNOS for arginine to produce L-ornithine and urea, rather than NO.

Due to the importance of an intact early innate immune response on the success of defending against a secondary infection, we explored the potential role of AAM. The hypothesis is that AAM may be critically involved in limiting the potential damage of the inciting pro-inflammatory primary influenza infection and promote tissue repair and healing, as a result of the injury from this initial event. In so doing, AAM may also contribute to a hypersusceptibility to a secondary bacterial infection because of the induction of poor antibacterial activity.

2. Materials and methods

2.1. Influenza

The A/PuertoRico/08/1934 (PR8) virus was grown in allantoic fluid of 10-day-old specific pathogen-free embryonated chicken eggs, aliquoted, and stored in -80°C . The determination of viral titers in the viral stocks, bronchoalveolar lavage (BAL) fluid, or tissue homogenates was performed with a 50% tissue culture infective dose (TCID₅₀) assay. Titers are expressed as the reciprocal of the dilution that corresponds to 50% virus growth in Madine Darby canine kidney (MDCK) cells.

2.2 Bacteria

The *Streptococcus pneumoniae* serotype 3 (Sp3) isolate (ATCC 6303; Manassas, VA, USA) was grown in Brain Heart Infusion Broth at 37°C plus 5% CO₂ overnight, aliquoted into equal volumes of glycerol, and stored in -80°C . The counts of the bacterial stocks,

challenge inoculum, BAL fluid, or tissue homogenates was calculated from the colonies that grew on 5% sheep blood agar plates.

2.3 Animal Infections

All experiments involved 6–8 week old female, C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME, USA) that were handled in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine. Within one hour prior to challenge, frozen stocks of PR8 or Sp3 were diluted to the desired challenge concentration, in sterile, endotoxin-free phosphate buffered saline (PBS; Biosource International, Rockville, MD, USA). Mice were anesthetized with isoflurane (Baxter; Deerfield, IL, USA) prior to the deposition of 10–20 µl of challenge inoculum to a single nare. Each mouse was followed daily for weights and clinical scoring. Prospectively assigned mice were followed for survival or euthanized for harvesting of tissue, using sterile techniques.

2.4 Splenocytes

Individual spleens were obtained and homogenized as previously described [16]. Splenocytes, at a concentration of 1×10^6 cells/ml in 24-well plates, were stimulated overnight with the following agonists: *E. coli* O111:B4 LPS (List Biological Laboratories, Campbell, CA, USA), CpG ODN 10103 (Coley Pharmaceutical Group, Ontario, Canada), and Pam₃Cys (EMC Microcollections, Tübingen, Germany). Supernatants were collected for cytokine analysis.

2.5 Flow Cytometry

Lungs were perfused with ice cold PBS and incubated in Collagenase D (Roche, Indianapolis, IN, USA) plus DNase I (USB, Santa Clara, CA, USA) solution for 30 minutes at 37°C. After passing the cells through a cell strainer, RBCs were lysed and the cells were washed. Lung cells ($1-2 \times 10^6$) were re-suspended in FACS buffer (PBS, 3% FBS, 1% Sodium Azide, 1 mM EDTA), labeled and analyzed, as previously described [17]. Cells were stained for viability (Vivid, Live/Dead Fixable Dead Cell Stain kit; Invitrogen Corporation, Carlsbad, CA, USA) and the following fluorochemicals: CD19-eFluor450, CD11c-PE-Cy7, MHCII-Alexa700, NK1.1 PacBlue and F4/80-APC-Cy7 (eBioscience, San Diego, CA, USA); Gr1-PerCP-Cy5.5, and CD11b-APC (BD Bioscience, San Jose, CA, USA); CD3-PacBlue (Caltag, Burlingame, CA, USA); and MGL-1-PE (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as well as their isotype controls. Cells were fixed with 1% formalin and stored at 4°C until analyzed with a BD LSR II flow cytometer and FlowJo software (Tree Star Inc, Ashland, OR, USA).

2.6. Cytokine Expression and Production

Total cellular RNA in lungs was extracted and purified using TRIzol (Invitrogen). Real-time RT-PCR was performed, with an ABI 7900HT (Applied Biosystems, Carlsbad, CA, USA) sequence detection system and software, using primer sequences as previously described [18]. Relative mRNA level for specific genes are reported as relative gene expression normalized to untreated control samples. The cytokine production was quantified as previously described [16] using commercially available ELISA detection kits. Concentrations were calculated using the supplied standards and SoftPro (Molecular Dynamics, Sunnyvale, CA, USA).

2.8. Statistical analysis

Individual means were compared using a non-paired Student's *t* test, Mann-Whitney Rank Sum test, or McNemar's paired and ranked non-parametric test. Survival differences were

determined using Kaplan-Meier survival plots and analyzed by a Log-rank test. Results were considered significant with two-sided *p-values* < 0.05. GraphPad Prism v5.0 (San Diego, CA, USA) was used to perform these statistical calculations.

3. Results

3.1. Hypersusceptibility to secondary pneumococcal pneumonia post-influenza infection

When C57BL/6 mice are challenged intranasally with 2×10^4 TCID₅₀ of PR8, there is ~65% mortality by day 9. In contrast, mice that were challenged with a 10-fold sublethal dose of PR8 (2×10^3 TCID₅₀) developed illness without mortality. Clinical illness and weight loss peaked at day 7 post-sublethal PR8 (2×10^3 TCID₅₀), with an average loss of ~15% of original weight, and by day 10 mice were in recovery, as reflected by increased activity and weight gain [Figure 1A]. In sublethal infection, viable virus peaked between days 3 and 7 but was completely cleared by day 10, coinciding with clinical recovery [Figure 1B]. On day 14, the sublethally infected mice fully recovered their weights. There was no live virus present and histological examination of the lungs demonstrated the absence of inflammatory infiltrates and the return of ciliated epithelium [Figure 1C]. Therefore, mice infected with sublethal PR8 (2×10^3 TCID₅₀) appear to have fully recovered from the primary influenza infection by day 14.

It has been previously shown that mice are highly susceptible to a secondary bacterial infection when given a pneumococcal co-infection on day 7 of challenge with PR8, when the virus is still present [19,20,21]. However, when we administered a 100-fold sublethal dose of Sp3 (5×10^3 CFU) to PR8-recovered mice (day 14 from 2×10^3 TCID₅₀ PR8), we observed that mice remained highly-susceptible, rapidly succumbing to an otherwise sublethal pneumococcal pneumonia with deaths occurring within 3–4 days of the secondary infection [Figure 2A]. On the other hand, saline control mice (*i.e.* PR8-naïve) that received the 100-fold sublethal dose of Sp3 demonstrated no clinical illness, weight loss, or mortality. The mortality of the PR8-recovered mice (2×10^3 TCID₅₀) was associated with a marked inflammatory pulmonary infiltrate, by histological examination, as early as 48 hours from the onset of secondary infection [Figure 2B]. But, saline control mice (PR8-naïve) that received the same sublethal Sp3 challenge demonstrated little, if any, lung pathology. The clinical illness and pulmonary infiltrates of the PR8-recovered (2×10^3 TCID₅₀) mice were associated with overwhelming pneumococcal infection which rapidly and widely disseminated beyond the lungs, as demonstrated by bacterial colony counts from the lungs, blood, and spleens at 48 and 72 hours post-secondary infection [Figure 2C]. Meanwhile, the saline control mice (PR8-naïve) cleared the pneumococci from the lungs in <48 hours, and had no evidence of peripheral dissemination. Therefore, influenza-primed mice were hypersusceptible to a lethal overwhelming secondary bacterial infection, even during recovery from influenza and viral clearance.

3.2 The potential role of AAM

The pro-inflammatory response from an influenza infection must be balanced by regulatory and inhibitory effector mechanisms, thereby protecting bystander tissue damage from the effects of over-inflammation and promoting host tissue repair after viral clearance, preserving oxygenation [22,23]. Because AAM are associated with the regulation of inflammatory responses [15,24], we hypothesized that AAM would be present in the lungs of mice in recovery from influenza. Consistent with our hypothesis, there was an increase in gene expression (by RT-PCR) of the direct inducers of AAM, IL-4 and IL-13, on days 7 and 14 of influenza infection, when compared to uninfected controls [Figure 3A]. Naïve mice challenged with sublethal Sp3 alone demonstrated little to no detectable pulmonary cytokine response (data not shown). Furthermore, the prototypic markers of AAM: Arg-1, Ym1, and

“found in inflammatory zone 1” (FIZZ1) were also significantly elevated through influenza infection and recovery (day 14) [Figure 3B]. In addition, anti-inflammatory (IL-10 and TGF- β) cytokines, which are intimately associated with AAM, were elevated from day 3 (acute infection) through day 14 (recovery) of infection [Figure 3C]. As expected, and in agreement with previous reports [25], pro-inflammatory cytokines (TNF- α , IL-6, and IFN- β) were also detected [Figure 3D]. Importantly, the TNF- α and IL-6 response peaked early (day 3) and before the IL-10 and TGF- β responses (day 7), which suggests that immune-regulation is a dynamic process during active viral replication. These data imply the induction of pulmonary AAM, which is preceded by the pro-inflammatory response of the influenza infection.

Flow cytometry was performed to further characterize the inflammatory infiltrate. The macrophage galactose-type C-type lectin (MGL-1; clone ER-MP23) has been described as a marker of AAM in the airways [26]. MGL-1 specifically identified AAM, when tested with *in vitro* generated AAM (primary peritoneal macrophages cultured with 40 ng/ml of IL-4, 48 hours); whereas CM (PBS treated primary peritoneal macrophages) did not stain for MGL-1 [Figure 4A]. We then used MGL-1 in combination with other markers of innate cells to assess the presence of AAM within the lungs of mice in our model of secondary bacterial infection. After eliminating lymphocyte lineage positive (removal of T and B lymphocytes as well as NK cells) and non-viable cells, innate immune cells were identified by the expression of Gr-1, CD11b, CD11c, MHCII, and F4/80. We found that alveolar macrophages (Gr-1⁻ CD11b⁻ CD11c^{high}, autofluorescent cells) stained positive for MGL-1. However neutrophils (Gr-1⁺) (not shown), conventional dendritic cells (Gr-1⁻ CD11b⁺ CD11c⁺, which were also MHCII⁺), and CM (Gr-1⁻ F4/80⁺ CD11b⁺ CD11c⁻) failed to stain for MGL-1 [Figure 4B].

Next, we measured these cellular populations during the course of influenza infection with or without a secondary pneumococcal infection, using the same sublethal doses of PR8 and Sp3 as in our previous experiments. In the resting state (naïve mice), alveolar macrophages expressed MGL-1, but had low constitutive expression of MHCII. We defined the activated state of AAM as the alveolar macrophage population which expressed both MGL-1 and upregulated MHCII expression [15]. During influenza infection, these pulmonary AAM were elevated in the lungs of mice at day 7 post-influenza infection and remained elevated through day 14 [Figure 5A]. We found that both the percentage and total numbers of AAM in the lungs remained elevated at day 14 [Figure 5B & 5C]. Although CM was also elevated during influenza infection [Figure 5D], the timing of the increase in AAM population correlated with the onset of gene expression data for AAM markers [Figure 4]. These data support the induction of an activated state of the AAM-like phenotype among alveolar macrophages during influenza infection and these cells, unlike CM, remain through recovery.

3.3. Host innate immunity is impaired during recovery from influenza

Since there is previous evidence that alveolar macrophages respond poorly to TLR ligands during recovery from influenza infection [9] and we imply that these may be AAM, we explored whether the cytokine responses of a distal, extra-pulmonary organ may also be affected (*i.e.* systemic versus local involvement). Splenocytes were harvested at 0 (control), 3 (active infection), and 14 days (recovery) after sublethal influenza infection and stimulated overnight with the following agonists: LPS 100 ng/ml, CpG 10 μ g/ml, Pam₃Cys 100 ng/ml, or media alone. These stimuli were selected to represent the TLRs most likely to be encountered during a bacterial infection. We found there was significant blunting of TNF- α , IL-6, IL-12, and IL-10 production following stimulation with any of the 3 ligands at both days 3 and 14 of influenza infection, compared to control splenocyte responses [Figure 6], which suggests systemic effects from an infection localized to the lungs. Similar trends for

decreased responses were also found for MIP-1 α and KC. (not shown) Therefore, we show that host innate immune hyporesponsiveness occurs as early as 3 days after infection and persists until at least 2 weeks post-infection. Since these responses were observed in splenocytes, we may conclude that the perturbation of the innate immune response is not compartmentalized only to the lungs.

4. Discussion

A serious secondary bacterial infection commonly complicates the recovery from a preceding influenza infection. Our mouse model faithfully reproduces the human clinical scenario, in that we show recovery from influenza infection is associated with susceptibility to a subsequent and normally sublethal dose of bacteria. The fact that the pulmonary system, open to the external environment, normally remains immunologically quiescent, despite encountering a constant cascade of inhaled foreign antigen, is a testament to the highly regulated nature of the lung milieu. When the host is infected with influenza, an effective pulmonary response should result in sterilizing immunity while avoiding excessive bystander tissue damage and preventing “cytokine storm” to maintain oxygenation. The balance and regulation of inflammation during infection and the promotion of resolution during recovery from influenza remain underappreciated. However, AAM are increasingly recognized as important contributors to wound healing and dampening of pro-inflammatory responses as well as eliciting pathologic Th2 (allergy) responses [14,15].

This model of infection did not explore the potential for viral-induced (such as the role of PB1-F2 [27] or the neuraminidase [28,29]) or bacteria-specific mechanisms [30,31] which have been shown to play a role in the pathogenesis of the secondary bacterial pneumonia, during acute infection (concomitant with the pro-inflammatory response). Rather, the focus of our model was to allow recovery from the initial influenza infection, such that there may be no biasing of our observations with a dual (virus and bacteria) infection. The majority of secondary bacterial infection models interrogate influenza post-infection day 7 (references), when the virus is still present, and only a few have investigated the host after recovery (reference). Furthermore, by allowing recovery from influenza, we minimized the potential effect of denuded respiratory epithelium and exposed basement membrane as contributory factors that may allow the bacteria to evade host defenses.

Although these studies were intended to define the model, we show that pulmonary AAM are induced during influenza infection and remain present during recovery. The cytokines necessary to induce alternative activation, IL-4 and IL-13 [15,32], were detected and the markers unique for AAM (Arg-1, FIZZ1, and Ym1) [33] were also expressed. Finally, alveolar macrophages, with an AAM-like phenotype were identified. Surprisingly, we also found evidence for systemic hyporesponsiveness to non-replicating TLR agonists, as evidenced by the altered responses of *ex vivo* splenocytes. These data support the presence and a potential role for the AAM in post-influenza secondary bacterial infection.

In the present study we do not definitively show AAM directly contributed to the hypersusceptibility phenotype. Further mechanistic studies are ongoing. However, another potential explanation for the presence of AAM is that they represent a bystander population; a result of the inflammation by influenza but having little role in eliciting the hypersusceptibility phenotype. On the other hand, there is substantial circumstantial evidence that AAM do contribute to hypersusceptibility. A reduction in bacterial opsonophagocytosis following influenza has been well-established [11,34]; impaired opsonophagocytosis is also observed with AAM [35]. And, we do find that AAM are unable to generate NO in response to LPS (unpublished data). Alveolar macrophage engulfment of apoptotic inflammatory cells (efferocytosis), such as during influenza infection, triggers a

reduction in clearance of *S. pneumoniae* and the release of TGF- β and IL-10 [34]. Furthermore, alveolar macrophage responses to bacterial stimuli are blunted [9] and the negative homeostatic pressure, through the upregulation of the repressive activity of CD200R ligation, increases in response to influenza infection and through resolution (day 14) [36].

Our results add to the increasing evidence that host defenses, especially those of alveolar macrophages, are impaired following influenza infection. We show that AAM may play a role in eliciting the susceptibility to a secondary bacterial infection subsequent to an influenza infection, even after clearance and recovery from the acute viral infection. Future mechanistic studies should be performed to determine whether AAM are necessary and sufficient for eliciting the hypersusceptibility to a secondary bacterial infection.

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Abbreviations

AAM	alternatively activated macrophage
CM	classical macrophage
PR8	mouse-adapted A/PR/8/34 influenza virus
Sp3	<i>Streptococcus pneumoniae</i> serotype 3

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Highlights

- Mice that have fully recovered from a primary influenza infection are highly susceptible to a secondary bacterial infection.
- Cytokine responses to TLR stimulation are blunted, after influenza infection.
- The initial pro-inflammatory cytokine (IL-6 and TNF- α) response of an influenza infection is followed by anti-inflammatory cytokines (IL-10 and TGF- β).
- Gene expression of markers of AAM (Arg-1, FIZZ1, YM1) and the cytokines necessary to induce AAM (IL-4 and IL-13) are elevated after influenza infection.
- Alveolar macrophages which demonstrate an AAM phenotype are induced after influenza infection.

Figure 1. Recovery from primary PR8 influenza strain infection

(Panel A) The changes in weight after challenge with saline (n=10), PR8 2×10^3 TCID₅₀ (n=20), and PR8 2×10^4 TCID₅₀ (n=17) were recorded and show recovery of weight by day 14 among mice receiving sublethal (PR8 2×10^3 TCID₅₀) infection. * indicates that at day 9, there is incomplete data due to 65% mortality. The viral counts from lungs and BAL fluid **(Panel B)** and H&E stains on lungs **(Panel C, 40X magnification)** were performed on the days indicated after challenge with sublethal (PR8 2×10^3 TCID₅₀) infection and show clearance of virus by day 10 and normalization of lung histology at day 14. ND = not detectable.

Figure 2. Lethality of secondary Sp3 infection during recovery (14 days) from a primary influenza infection

(Panel A) Kaplan-Meier curves were created for mice that recovered from primary saline (n=11) or PR8 2×10^3 TCID₅₀ (n=25) followed by challenge with sublethal 5×10^3 CFU of Sp3 and show significant hypersusceptibility during recovery from influenza; Logrank p-values are indicated. Mice challenged with sublethal Sp3 (5×10^3 CFU) show significant inflammatory cell infiltration **(Panel B)** on lung H&E staining (40X magnification) and pneumococcal dissemination on organ bacterial counts **(Panel C)** at the times indicated among mice in recovery (day 14) from sublethal PR8 (2×10^3 TCID₅₀) infection. *p=0.002 **p=0.0002

Figure 3. Gene expression associated with AAM

Gene expression was measured from the lungs of mice at the indicated times after sublethal PR8 ± Sp3 infection for the direct inducers (**Panel A**) and prototype signature markers (**Panel B**) of AAM and for anti-inflammatory (**Panel C**) and pro-inflammatory (**Panel D**) cytokines. Gene expression by real-time PCR is reported as relative gene expression compared with lungs of mice exposed to saline only. Experiments were performed in triplicate and data are presented as mean ± SEM. *p < 0.05 **p < 0.005

Figure 4. Flow cytometric identification of the AAM phenotype

(Panel A) Primary murine macrophages that were treated with IL-4 (AAM, solid line) stained positive for MGL-1, whereas un-treated macrophages (CM, dashed line) lacked the expression of this marker. **(Panel B)** Lung homogenates were stained with a multichromatic flow cytometry panel (Naïve mouse shown). The plot depicts CD11b and CD11c following exclusion of dead cells (ViviD) and granulocytes (Gr1⁺). Populations identified were interrogated for the expression of MGL-1, MHCII, F4/80, and autofluorescence. Only alveolar macrophages (AM, Gr1⁻ CD11b⁻ CD11c^{high}) stained positive for MGL-1; they also were MHCII^{dim} and highly autofluorescent. DC (dendritic cell) and CM (classical macrophage) do not express MGL-1.

Figure 5. Kinetics of lung AAM

AAM were defined as alveolar macrophages (autofluorescent Gr1⁻ CD11b⁻ CD11c^{high} F4/80^{dim}) which express both MGL-1 and MHCII. **Panel A** depicts the MGL-1 and MHCII positive alveolar macrophage population after sublethal PR8 ± Sp3 infection. **(Panel B)** The percentage of lung AAM (percentage relative to alveolar macrophages) after sublethal PR8 ± Sp3 are summarized in 5–95 percentile box-whisker plots (line indicates mean values). **(Panel C)** The numbers of AAM per gram of lung tissue after sublethal PR8 ± Sp3 are summarized in minimum-maximum box-whisker plots (line indicates mean values) with dots that indicate individual animal data. **Panel D** summarizes the number of CM (Gr1⁻ MGL-1⁻ CD11b⁺ CD11c⁻ F4/80⁺) per gram of lung tissue after sublethal PR8 ± Sp3, by minimum-maximum box-whisker plots (line indicates mean values) with dots that indicate individual animal data. The data are from 3 separate experiments. *p < 0.05.

Figure 6. Blunted splenocyte responses to TLR stimuli

Splenocytes from mice at the indicated times after sublethal PR8 infection were stimulated with: medium alone (non-stimulated), 100 ng/mL LPS, 10 µg/mL CpG, or 100 ng/mL Pam3Cys. After 18 h incubation, supernatants were harvested and cytokine production was measured by ELISA. Data are expressed as the mean ± SEM of cytokine production and are representative of three independent experiments for Day 0 and 14, two experiments for Day 3. *p < 0.05 **p < 0.005