Toll-Like Receptors 2 and 4 Regulate the Frequency of IFNγ-Producing CD4⁺ T-Cells during Pulmonary Infection with *Chlamydia pneumoniae*

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Abstract

TLR2 and TLR4 are crucial for recognition of *Chlamydia pneumoniae* in vivo, since infected TLR2/4 double-deficient mice are unable to control the infection as evidenced by severe loss of body weight and progressive lethal pneumonia. Unexpectedly, these mice display higher pulmonary levels of the protective cytokine IFNγ than wild type mice. We show here, that antigen-specific CD4⁺ T-cells are responsible for the observed IFNγ-secretion in vivo and their frequency is higher in TLR2/4 double-deficient than in wild type mice. The capacity of TLR2/4 double-deficient dendritic cells to re-stimulate CD4⁺ T-cells did not differ from wild type dendritic cells. However, the frequency of CD4⁺CD25⁺Foxp3⁺ T-cells was considerably higher in wild type compared to TLR2/4 double-deficient mice and was inversely related to the number of IFNγ-secreting CD4⁺ effector T-cells. Despite increased IFNγ-levels, at least one IFNγ-mediated response, protective NO-secretion, could not be induced in the absence of TLR2 and 4. In summary, CD4⁺CD25⁺Foxp3⁺ regulatory T-cells fail to expand in the absence of TLR2 and TLR4 during pulmonary infection with *C. pneumoniae*, which in turn enhances the frequency of CD4⁺IFNγ⁺ effector T-cells. Failure of IFNγ to induce NO in TLR2/4 double-deficient cells represents one possible mechanism why TLR2/4 double-deficient mice are unable to control pneumonia caused by *C. pneumoniae* and succumb to the infection.

Introduction

The obligate intracellular bacterium *Chlamydia pneumoniae* infects the respiratory tract and replicates in bronchial epithelial cells. The cells are infected by elementary bodies of *C. pneumoniae* which develop within hours post infectionem into reticulate bodies. The latter form divides several times and within 48 to 72 h a microscopically visible intracellular inclusion is generated. In vitro, the replication of *C. pneumoniae* is impaired by IFNγ. This cytokine exerts its effect indirectly via the induction of two enzymes: the inducible isoenzyme of the nitric oxide synthase (iNOS) and indolamine 2,3 dioxygenase (IDO). The former enzyme generates nitric oxide (NO), which is toxic for bacteria and impairs replication of *C. pneumoniae*. The latter degrades the aminoacid tryptophan, which is required by *C. pneumoniae* to generate nitric oxide (NO), which is toxic for bacteria and impairs the replication of the bacterium. In contrast, IFNγ-secreting CD4⁺ or CD8⁺ T-cells were protective since they impaired replication of *C. pneumoniae*. Thus, IFNγ-secreting cells of the adaptive immune system contribute to host defense against the bacterium.

Innate immune cells like bone marrow derived dendritic (BMDC) cells recognize *C. pneumoniae* via TLR2 and 4. In contrast to wild type animals, mice double-deficient for TLR2 and 4 were unable to control the replication of the bacteria and succumbed to progressive pneumonia. Moreover, although many immune responses in vivo, such as the secretion of pro-inflammatory cytokines and chemokines, depended almost exclusively on TLR2, the survival of infected mice required the presence of TLR2 but also of TLR4. Unexpectedly, TLR2/4 double-deficient but not TLR2-deficient mice displayed upon infection with the microorganism significantly higher pulmonary levels of IFNγ than wild type mice.

TLRs also influence the adaptive immune response. Thus, mice lacking MyD88, the most important adapter molecule of the TLR-signaling cascade, failed to mount a TH1 response upon immunization with the model antigen ovalbumin in complete Freund’s adjuvant while antigen-specific TH2 responses were not impaired. In particular, antigen-specific T-cells from MyD88-deficient mice were unable to produce IFNγ, but secreted TH2 cytokines like IL-13 and IL-4 at least as efficiently as T-cells from
wild type mice. Likewise, it was shown that endotoxin-stimulated wild type dendritic cells induced allogeneic CD4\(^+\) T-cells to secrete IFN\(\gamma\) while MyD88-deficient allogenic dendritic cells only stimulated a TH2 response [12]. However, upon vaccination of MyD88-deficient mice with Mycobacterium bovis BCG a TH1 response was observed as in wild type mice [13]. Interestingly, the adaptive immune response induced by the vaccination was only partially effective to prevent the lethal outcome of a challenging Mycobacterium tuberculosis infection in MyD88-deficient mice. In summary, the influence of MyD88 on adaptive immune responses appears to depend substantially from the model system used.

Here, we explored adaptive immune responses in mice lacking TLR2 and 4 upon pulmonary infection with C. pneumoniae. The results demonstrate that an increased IFN\(\gamma\)-release by the adaptive immune system in the absence of both TLRs was associated with a lower frequency of regulatory T-cells. However, the cytokine was unable to trigger the release of NO by TLR2/4 double-deficient bone marrow-derived macrophages (BMDM). The latter finding presumably contributes to the increased lethality observed in TLR2/4 double-deficient mice.

**Results**

**Pulmonary recruitment of T-cells was not impaired in TLR2/4 double-deficient mice**

To explore whether adaptive immune responses were induced in vivo in the absence of TLR2 and 4 known to be of key importance for the recognition of C. pneumoniae, we infected wild type and TLR2/4 double-deficient mice pulmonary with C. pneumoniae. As shown in Fig. 1 these TLR-deficient mice lost a considerable part of their body weight between day 9 and day 12 post infection. In contrast, this was not observed in wild type mice which displayed a weaker weight loss between day 0 and 9. Similar data were demonstrated previously and that study also showed that the TLR2/4 double-deficient mice succumbed to the infection [10]. While recruitment of polymorphonuclear neutrophils into the infected lungs was considerably impaired in TLR2/4 double-deficient mice [10], CD4\(^+\) and CD8\(^+\) T-cells were present in wild type and TLR2/4 double-deficient mice in similar numbers (Fig. 2A, B).

**Presence of CD4\(^+\)IFN\(\gamma\)\(^+\) T-cells in the lung of TLR2/4 double-deficient mice**

To study whether C. pneumoniae-specific T-cells were detectable in the infected lung of TLR2/4 double-deficient mice, we re-stimulated T-cells isolated from the lung of infected animals with wild type BMDC in vitro, which were either infected or not infected with C. pneumoniae. In summary, the influence of MyD88 on adaptive immune responses appears to depend substantially from the model system used.

Figure 1. TLR2 and 4 are crucial to control pneumonia-induced loss of body weight of C. pneumoniae-infected mice. Wild type (n = 24 d0, n = 18 d6, n = 12 d9, n = 6 d12) and TLR2/4 double-deficient mice (n = 24 d0, n = 18 d6, n = 12 d9, n = 6 d12) were infected with C. pneumoniae. At the time point indicated in the graph the body weight of the mice was determined. Bars represent mean and SD of individual mice from two independent experiments. *p < 0.001, ANOVA posthoc Holm-Sidak. doi:10.1371/journal.pone.0026101.g001

Figure 2. Lack of TLR2/4 does not impair pulmonary recruitment of CD4\(^+\) and CD8\(^+\) T-cells upon infection with C. pneumoniae. Wild type (n = 3/time point) and TLR2/4 double-deficient mice (n = 3/time point) were infected with C. pneumoniae. At the time points indicated in the graph mice were sacrificed, lungs removed, single cell suspensions were prepared and the number of cells determined. Cells were stained with mAbs specific for CD4, CD8 and CD3 as described in Materials and Methods, analyzed by flow cytometry and the number of each subpopulation was calculated. CD4\(^+\)CD3\(^+\) T-cells are depicted in (A), CD8\(^+\)CD3\(^+\) T-cells in (B). Error bars represent SD of three individual mice. doi:10.1371/journal.pone.0026101.g002
TLR2/4 Regulate IFNγ-Secretion by CD4+ T-Cells

A

Mock

Infected

WT

TLR2/4−/−

B

CD4+ CD8+ IFNγ+ T-cells (%)

0 6 9 12

time (days)

WT, BMDC inf.

TLR2/4−/−, BMDC inf.

WT, BMDC mock

TLR2/4−/−, BMDC mock

C

CD4+ IFNγ+ T-cells (%)

0 6 9 12

time (days)

WT, CD3+

TLR2/4−/−, CD3+

WT, CD3−

TLR2/4−/−, CD3−
strains of mice (Fig. 3B). However, on day 9 these cells were also enhanced in frequency in the infected TLR2/4 double-deficient mice. Furthermore, IFNγ-secretion by CD4+ but CD3− non T-cells was barely detectable (Fig. 3C). These results suggested that antigen-specific TH1 T-cell responses were efficiently generated in the absence of TLR2 and 4.

Enhanced *Chlamydia*-specific IFNγ-responses in the absence of TLR2/4 is not confined to the lung

Next, we assessed the local and systemic IFNγ-responses from lung and spleen cells of infected wild type or TLR2/4 double-deficient mice upon re-stimulation in vitro with *C. pneumoniae*-pulsed wild type BMDC. The results revealed that antigen-specific responses from lung and spleen cells isolated from infected TLR2/4 double-deficient mice secreted considerably more IFNγ than wild type cells after 3 days in culture (Fig. 4). These findings also suggested that increased responses by TLR2/4 double-deficient cells were not confined to the organ infected primarily.

TLR2/4 double-deficient BMDC are capable of stimulating antigen-specific IFNγ-responses

We then explored the capacity of TLR2/4 double-deficient BMDC to trigger an IFNγ response by *C. pneumoniae*-specific lung cells. We expected a weaker ability since activation of TLR2-deficient BMDC upon infection with the bacterium in vitro was impaired as analyzed by IL-12p40 and TNF secretion and induction of NF-κB [9]. *In vivo*, however, the infection induced the expression of the co-stimulatory molecules CD80 and CD86 as well as MHC class II by CD11c+ cells in wild type and TLR2/4 double-deficient mice with comparable efficiency (Fig. 5A, B). Based on the data presented in Fig. 3 we conclude that TLR2/4 double-deficient antigen-presenting cells triggered primary CD4+ T cells at least as efficiently as wild type cells. In addition, *C. pneumoniae*-infected TLR2/4 double-deficient BMDC re-stimulated lung cells, which were isolated from *C. pneumoniae*-infected wild type mice, as efficiently as wild type BMDC to secrete IFNγ (Fig. 5C). We therefore conclude that a deficiency of TLR2 and TLR4 by BMDCs did not explain the stronger IFNγ-response of antigen-specific T-cells.

Lungs of TLR2/4 double-deficient mice contain fewer CD4+CD25+Foxp3+ regulatory T-cells upon infection with *C. pneumoniae*

Several reports indicated that TLR2 influences the expansion and function of regulatory T-cells and express TLR2 on their cell surface upon activation with anti CD3 [14]. Additionally, co-stimulation with the TLR2-ligand Pam3Cys has been shown to induce their proliferation which is accompanied by a transient loss of their suppressive activity [14,15]. *In vivo*, TLR2 was also shown to be crucial for the expansion of regulatory T-cells in mice infected with *Schistosoma mansoni* [16]. Therefore, we analyzed the frequency of CD4+CD25+Foxp3+ T-cells in lungs of *C. pneumoniae*-infected wild type and TLR2/4 double-deficient mice. In mock-treated mice the percentage of CD4+CD25+Foxp3+ T-cells was not different in both strains (Fig. 6A, left panels). However, upon infection the frequency of these cells was almost 3-fold higher in wild type compared to TLR2/4 double-deficient mice (Fig. 6A right panels, 6C). Conversely, the percentage of IFNγ-producing CD4+ T-cells analyzed *ex vivo* (i.e. the cells were not re-stimulated)
TLR2/4 Regulate IFN-γ Secretion by CD4+ T-Cells

A

WT

100
80
60
40
20
0 10^6 10^7 10^8

CD86

25.8
14.3

100
80
60
40
20
0 10^6 10^7 10^8

CD80

37.5
22.7

100
80
60
40
20
0 10^6 10^7 10^8

MHC class II

B

% positive CD11c+ cells

WT mock
WT infected
TLR2/4+ mock
TLR2/4+ infected

CD86 CD80 MHC II

C

IFN-γ (ng/ml)

WT mock
WT infected
TLR2/4+ mock
TLR2/4+ infected

DC/well
was considerably increased in the latter mice (Fig. 6B). Thus, there is an inverse relation between the frequencies of CD4<sup>+</sup> CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T-cells and CD4<sup>+</sup>IFN<sub>γ</sub>-effector T-cells (Fig. 6C).

IFN<sub>γ</sub> fails to induce iNOS in C. pneumoniae-infected TLR2/4 double-deficient BMDMs

Normally, CD4<sup>+</sup>IFN<sub>γ</sub>-effector T-cells play a protective role during C. pneumoniae infection [8]. As demonstrated in Fig. 1, TLR2/4 double-deficient mice lost considerably more weight at day 12 post infection than wild type mice despite increased IFN<sub>γ</sub>-levels. We also demonstrated earlier that these mice displayed a higher lethality [10]. Therefore, we were interested in the effects of IFN<sub>γ</sub> on anti-chlamydial defense and investigated the influence of IFN<sub>γ</sub> on iNOS production in TLR2/4 double-deficient cells. This enzyme participates in the control of an infection with C. pneumoniae in vivo as revealed by the analysis of iNOS-deficient mice [7]. We also examined previously the role of MyD88, the adapter molecule used by TLR2 and TLR4, in the induction of iNOS and showed that the level of this enzyme was reduced in infected MyD88-deficient mice [17]. MyD88-deficient BMDM failed to release nitric oxide (NO) upon stimulation with C. pneumoniae and IFN<sub>γ</sub> since two important transcription factors, NF-kB and AP-1 which participate in the transcriptional regulation of the nos2 gene, were not induced, while induction of IRF-1 and phosphorylation of STAT-1 were normal [17]. As we show here, upon stimulation with IFN<sub>γ</sub>-IRF-1 induction is not affected in TLR2/4 double-deficient macrophages (Fig. 7A). Moreover, prior infection of the macrophages with C. pneumoniae did not alter the ability of IFN<sub>γ</sub> to induce IRF-1 in BMDM of both genotypes (Fig. 7A). As expected, infected BMDM with C. pneumoniae degraded IκB in wild type but not in TLR2/4 double-deficient cells (Fig. 7B). Since NF-κB is crucial for the induction of iNOS [17], it was not unexpected that the ability of IFN<sub>γ</sub> to induce iNOS-expression was severely impaired in C. pneumoniae-infected TLR2/4 double-deficient BMDM (Fig. 7C). Moreover, NO-secretion was completely abolished (Fig. 7D). Taken together, the failure of IFN<sub>γ</sub> to induce iNOS presumably contributes to lethality observed in TLR2/4 double-deficient mice.

Discussion

Using TLR2/4 double-deficient mice we show that TLR2 and TLR4 regulate IFN<sub>γ</sub>-secretion in vivo during pneumonia caused by C. pneumoniae. Our findings demonstrate that CD4<sup>+</sup> T-cells were responsible for the enhanced IFN<sub>γ</sub>-production, since CD4<sup>+</sup>CD25<sup>+</sup>IFN<sub>γ</sub>-cells were more frequent in lungs of infected TLR2/4 double-deficient mice. IFN<sub>γ</sub>-producing CD4<sup>+</sup> T-cells were antigen-specific, since release of IFN<sub>γ</sub> in vitro required re-stimulation of the cells with C. pneumoniae-infected BMDM. Enhanced IFN<sub>γ</sub>-secretion was not confined to CD4<sup>+</sup> T cells isolated from the infected lungs but was also observed in lymphocytes prepared from the spleen. Antigen-presenting cells appeared not to be involved in enhanced IFN<sub>γ</sub>-secretion by CD4<sup>+</sup> T-cells since BMDM from TLR2/4 double-deficient mice re-stimulated wild type cells as efficiently as wild type BMDM. However, upon infection TLR2/4 double-deficient mice displayed a lower frequency of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T-cells in vivo and the frequency of regulatory T-cells was inversely related to the frequency of CD4<sup>+</sup>IFN<sub>γ</sub>- T-cells. Despite the fact that IFN<sub>γ</sub>-content of the culture supernatant was analyzed by ELISA after three days of culture. Error bars represent SD of three replicate cultures. The experiment was repeated twice with similar results.

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Figure 5. Antigen-presentation is not impaired in TLR2/4 double-deficient BMDC. In (A) and (B) lung cells were prepared three days post infection with C. pneumoniae. The cells were stained with antibodies specific for CD11c, CD86, CD80 or MHC class II. All events are gated on CD11c. Error bars represent SD of three individual mice. The experiment was repeated once with similar results. (C) Pulmonary cells were prepared from wild type mice infected nine days earlier with C. pneumoniae (MOI = 5). The IFN<sub>γ</sub>-content of the culture supernatant was analyzed by ELISA after three days of culture. Error bars represent SD of three replicate cultures. The experiment was repeated twice with similar results.
TLR2/4 Regulate IFN-γ-Secretion by CD4+ T-Cells

A

Mock

Infected

CD25

10^4

10^1

10^2

10^3

10^4

WT

1.4

2.7

4.4

8.7

TLR2/4−

2.2

2.1

2.5

3.6

Foxp3

B

Mock

Infected

CD4

10^4

10^1

10^2

10^3

10^4

WT

0.01

10.8

0.39

0.65

TLR2/4−

0.02

0.08

24.2

0.47

IFN-γ

C

CD4+ IFN-γ+ cells (%)

CD4+ CD25+ FoxP3+ cells (%)

WT mock

TLR2/4− mock

WT Inf−

TLR2/4− Inf.

IFN-γ

FoxP3

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be responsible for the expansion of CD4⁺CD25⁺Foxp3⁺ Tregs in vivo.

Our results also imply that the adaptive TH1 immune response is unable to prevent the lethal outcome of pulmonary infection caused by C. pneumoniae, if TLR2 and TLR4 are absent. In association, previous studies have shown the importance of IFNγ-producing CD4⁺ T-cells in increasing resistance of RAG-1/IFNγ-deficient mice against an infection with C. pneumoniae [8]. Similar observations were obtained in a model where MyD88-deficient mice were infected with M. tuberculosis. Vaccination of MyD88-deficient mice with M. bovis BCG induced IFNγ-producing CD4⁺ T-cells in vivo, but the TH1 cells only partially prevented lethal pneumonia upon challenge with M. tuberculosis [13]. Upon challenge with C. pneumoniae MyD88-deficient mice succumbed like TLR2/4-deficient mice to progressive pneumonia [10], although pulmonary IFNγ-levels were increased like in wild type mice six days post infection [2]. Furthermore, the ability of IFNγ to induce iNOS was impaired in MyD88-deficient macrophages [17]. Full induction of iNOS required the transcription factors AP-1, NF-kB, IRF-1 and STAT-1. The former two were not activated in MyD88-deficient bone marrow-derived macrophages (BMDM) upon infection with C. pneumoniae in vitro leading to a complete failure to induce iNOS. In vivo, iNOS-levels were considerably reduced in MyD88-deficient mice post infection with C. pneumoniae. Identical to these published findings we show here, that in C. pneumoniae-infected TLR2/4-deficient BMDM IFNγ only weakly up-regulated the expression of iNOS and failed to induce the secretion of NO, since the infection of these cells with C. pneumoniae did not induce NF-kB. iNOS is crucial to control the replication of C. pneumoniae in vivo, since the chlamydial burden is considerably increased in iNOS-deficient mice [7]. Thus, although C. pneumoniae-specific CD4⁺ T-cells produce enhanced amounts of IFNγ in TLR2/4-deficient mice, the cytokine cannot completely exert its protective effects in TLR2/4 double-deficient mice, since it is impaired to induce iNOS.

In summary, expansion of CD4⁺CD25⁺Foxp3⁺ T-cells is impaired in C. pneumoniae-infected BMDC and BMDM double-deficient mice. This is accompanied by an increased frequency of CD4⁺IFNγ⁺ effector T-cells which cannot prevent lethal pneumonia.

Materials and Methods

Ethic statement

All animal experiments were reviewed and approved by the local authorities (Regierung von Oberbayern, file number 211-2531-59/06).

Strains of mice

C57/HeN mice were purchased from Harlan Winkelmann GmbH (Borchen, Germany). Breeding pairs of C57BL/6 TLR2⁻/⁻ mice came from Tularik (South San Francisco, CA). They were backcrossed six times to TLR4⁻/⁻ C3H/HeJ mice to generate TLR2/4 double-deficient mice. The genotype of the mice was verified phenotypically as described in [21]. Alternatively, TLR2⁻/⁻ mice were crossed with TLR4⁻/⁻ mice and the resulting TLR2/4 double-deficient mice were further backcrossed nine times to C57/HeN mice. The genotype of these mice was analyzed by PCR (Fig. S1). All mice were bred in our own animal facility under specific pathogen-free conditions.

Reagents

The peroxidase-conjugated AffiniPure F(ab')2 fragment Donkey anti-Rabbit IgG (H+L) and peroxidase-conjugated AffiniPure F(ab')2 fragment Goat anti-Mouse IgG (H+L) were purchased from Dianova (Germany), and the monoclonal antibody to β-actin was provided by Sigma-Aldrich (Germany). The monoclonal antibodies specific for IRF-1 (M-20; sc-640), iNOS and iκB (clone E130) were provided by Santa Cruz Biotechnology, Inc. (CA, USA), Upstate (Millipore, Germany) and Epitomics (USA), respectively. Murine IFNγ (315-05) was purchased from PeproTech Inc. (New Jersey, USA).

Infection protocol

Anaesthetized mice were infected intranasally with Chlamydia pneumoniae (CM-1, ATCC VR-1360, 2.5×10⁶ IFUs). Infected mice were inspected every day and body weight was determined on day 6 and 9 and 12 post infection.

Generation of BMDC and BMDM

BMDC were generated according to Inaba et al with slight modification [22]. Mice were sacrificed and tibiae and femora were removed, cleaned and flushed with cell culture medium. They were plated on bacterial petri-dishes overnight in culture medium (RPMI 1640, 10% heat-inactivated FCS, 100 μg/ml penicillin, 100 μg/ml streptomycin, 5×10⁻⁵ M 2-ME) to remove adherent cells. Non-adherent cells were plated at a density of 5×10⁶ cells/dish, and cultivated for another five days in complete medium in the presence of GM-CSF (10% v/v). The cells were counted, plated in medium devoid of FCS and antibiotics and exposed to C. pneumoniae at a multiplicity of infection (MOI) of 5. After 30 hours the BMDC were counted and used for re-stimulation assays.

BMDM were generated according to Rutschman et al. [23]. Briefly, femora and tibiae of mice were rinsed with cell culture medium applied through a 26-gauge syringe. Bone marrow cells were cultured in petri dishes at a density of 5×10⁶ cells/dish in the presence of L-cell-conditioned medium as a source of M-CSF-1 (10% v/v). The medium used was very low endotoxin DMEM (PAA Laboratories GmbH, Austria) supplemented with 10% FCS (Biochrom AG, Germany), 2-ME (50 μM; Life Technologies, Germany) and the antibiotics Vancomycin and Gentamicin, both provided by Sigma-Aldrich (Germany). Cells were washed vigorously and only adherent macrophages were used 6–7 days after plating. FACS analysis showed that these BMDM were F4/80⁺ and CD11b⁺ as described previously (data not shown) [10].

Isolation of lung and spleen cells

Mice were sacrificed with CO₂ on day 6, 9 or 12 post infection with C. pneumoniae. Lungs were flushed with 10 ml PBS and digested after manual mincing. The lungs were digested with collagenase VIII (400 U/100 μl 10 min at room temperature, 400 U/2 ml RPMI 0% FCS, 30 min at 37°C), washed and
Figure 7. IFN-γ is severely impaired to increase iNOS expression and fails to release NO in *C. pneumoniae*-infected TLR2/4 double-deficient BMDMs. (A) Wild type or TLR2/4 double-deficient BMDMs (7.5 x 10⁵ cells/well) were left untreated, or stimulated with IFN-γ (10 ng/ml) for 24 h, or were infected with *C. pneumoniae* (MOI = 10, for 48 h), or were infected with *C. pneumoniae* for 48 h and treated with IFN-γ 24 h post infection. IRF-1 was detected by Western blot, detection of β-actin was used as loading control. (B) Wild type or TLR2/4 double-deficient BMDMs (7.5 x 10⁵ cells/well) were or were not infected with *C. pneumoniae* (MOI = 10) for the time periods indicated in the graph. IκB was detected by Western blot, detection of β-actin was used as loading control. (C) Wild type or TLR2/4 double-deficient BMDMs were treated as described in (A). iNOS was determined by Western blot, detection of β-actin was used as loading control. (D) Wild type or TLR2/4 double-deficient BMDMs (7.5 x 10⁵ cells/well) were left untreated, or stimulated with IFN-γ (10 ng/ml) for 48 h, or were infected with *C. pneumoniae* (MOI = 10, for 72 h), or were infected with *C. pneumoniae* for 72 h and treated with IFN-γ 48 h post infection. Subsequently, NO-levels were determined in the culture supernatant. Error bars represent SD of four or in the case of untreated samples of two individual cultures. *not detectable. The experiment was repeated once with similar results.
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separated through a cell strainer. Spleens were removed and a single cell suspension was prepared. Erythrocytes were lysed with ammonium chloride (0.15 M, 5 min, RT) and washed subsequently with PBS containing 3% FCS. Co-cultures for intracellular staining were performed with 4×10^6 lung cells and 1×10^6 BMDC for 13 h. Brefeldin A (Golgi-Plug, BD Biosciences, San Jose, CA, USA) was added for the last 12 h of culture. For long-term cultures, 1×10^6 pulmonary or spleen cells and 1×10^6 BMDC were co-cultured in 96-well plates.

Determination of IFNγ levels
IFNγ levels were determined using a commercially available ELISA system (DuoKit BD Biosciences). The assay was performed according to manufacturer’s manual. Data were analyzed using SigmaPlot 10.0 (Systat Software, Inc.).

Flow cytometry
Cells were counted and concentrated to 2×10^6 cells per ml. Cells were washed with PBS (3% FCS) and pre-incubated with anti-human CD11c (2 µg/ml, BD Biosciences, San Jose, CA, USA) for 20 min on ice. After another wash step intracellular permeabilization was performed with FITC-labelled mAb directed against CD4 (2 µg/ml, Caltag Laboratories, Invitrogen, Carlsbad, CA, USA) to block Fc-receptors. Cells were stained with different combinations of APC- or PE-labelled mAb specific for CD3, APC-labelled mAb directed against CD8 (2 µg/ml, Caltag Laboratories) and PE-labelled mAb directed against CD4 (2 µg/ml, Caltag Laboratories) for 30 min on ice. The cells were washed again and then fixed and permeabilized with Perm/Wash (BD Biosciences, San Jose, CA, USA) for 20 min on ice. After another wash step intracellular staining was performed with FITC-labelled mAb directed against IFNγ (1 µg/ml, BD) for 30 min on ice. Cells were washed with Perm/Wash (BD) twice and fixed again with 2% paraformaldehyde.

To detect regulatory CD4^+ T-cells, cells were also stained with a mAb specific for CD25 (5 µg/ml, 30 min, 4°C, BD) and with a mAb specific for Foxp3 (clone FJK-16s, eBioscience, Frankfurt, Germany) using the Foxp3 Staining Buffer Set from eBioscience (00-5523-00) according to the manufacturer’s recommendations. The induction of co-stimulatory molecules on CD11c^+ pulmonary cells was analyzed by staining the cells with antibodies specific for CD11c (2 µg/ml, Caltag Laboratories), CD80 (5 µg/ml, BD), CD86 (2 µg/ml, BD) and MHC class II molecules I-A/I-E (5 µg/ml, BD). Flow cytometry was performed with a Calibur instrument (BD Biosciences, San Jose, CA, USA), the data were analyzed using the FlowJo software (Tree Star Inc, OR, USA).

Cell lysis, SDS-PAGE and Western blotting
Cell extracts were prepared for Western blotting using RIPA buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% Sodium Deoxycholate, 1 mM EDTA, 0.1% SDS) which was supplemented with Sodium Orto-Vanadat (Sigma-Aldrich, Germany) and a protease inhibitor cocktail (Roche Diagnostics GmbH, Germany). Cell lysates were clarified by sonication and centrifugation before electrophoresis. Proteins were separated by SDS-PAGE (10% acrylamide) in TANK Buffer (25 mM Tris, 0.2 M glycine, 0.1% SDS), using Laemmli buffer (62.5 mM Tris, 50% glyceral, 2% SDS, 2 mM EDTA) for sample loading. After transfer to nitrocellulose membrane by semidy electroblotting for 1.5 h (2 mA/cm²) in Transfer Buffer (25 mM Tris, 230 mM glycine, 20% Methanol, 0.35% SDS), membranes were blocked in TBST (2.4 g/l Tris, 8 g/l NaCl, 0.1% Tween, pH 7.6, containing 5% milk powder, 2 h, room temperature). Thereafter, membranes were incubated with antibodies specific for iNOS (diluted 1:1000), IRF-1 (diluted 1:500), IkB (diluted 1:5000) and β-actin (diluted 1:20000). All primary antibodies were incubated overnight at 4°C. After three washing steps with TBST the secondary antibody was added (diluted 1:8000 in TBST containing 5% milk powder, 2 h, room temperature). The blot was washed again three times with TBST and visualized using the Western lightning™ Chemiluminescence Reagent (Perkin Elmer LAS Inc, MA, USA) as described by the manufacturer.

Nitrite measurement
Nitrite production was measured from wild type and TLR2/4 double-deficient BMDM supernatants. Briefly, the cells were cultured in 12-well plates in 1 ml of culture medium until confluence. The cells were stimulated with IFNγ or C. pneumoniae and the culture supernatants were collected. Nitrite was measured by adding 50 µl of Griess reagent (1% sulfanilamide and 0.1% naphthylenediamide in 5% phosphoric acid) to 50 µl samples of culture medium. The optical density at 550 nm (OD_{550}) was measured using a microplate reader and the nitrite concentration calculated by comparison with the OD_{550} produced using standard solutions of sodium nitrite in the culture medium.

Statistics
Comparison of two equally treated groups was analyzed by Mann-Whitney Rank sum test. More than two equally treated groups were tested for significant differences with one way ANOVA, post hoc test Holm-Sidak. Statistical analysis was performed with SigmaStat (SPSS Inc., IL, USA).

Supporting Information
Figure S1 Identification of TLR2/4 double-deficient mice. Genomic tail DNA was prepared from 19 mice bred from a cross of TLR2- or TLR4-deficient mice. Primer sequences used were as follows: TLR2 WT1 5'-CTTCTCGTAATTTGGTGCCAGTGACCCAG-TACAGG-3' TLR2 WT2 5'-TCGACCTCGAACCAGGAGAAGGG-3' TLR2 KO 5'-GCGCCCGCTATCTGTCCTCCG-ACTCT-3' TLR4 WT1 5'-GGTGGAGATCTTGTTGGC-TGTGGGAGAC-3' TLR4 WT2 5'-TATATGCGGCAGCT-TATCGTCG-TGTGGGTTCACGCC-3' TLR4 KO 5'-TGTGGTGCGTTTGTGGTGGACGTCG-3'. PCR amplification using primers TLR2 WT1 and TLR2 WT2, or TLR2 WT1 and TLR2 KO, or TLR4 WT1 and TLR4 WT2, or TLR4 WT1 and TLR4 KO detected the wild type TLR2 gene, TLR2-deficiency, the wild type TLR4 gene or TLR4-deficiency, respectively. The different PCRs were run with slight variations (details upon request): 94–95°C 60–180 s 72°C 30–60 s 59–67°C 30–60 s 70–74°C 60–180 s 72°C 120 s 4°C.

(TIF)

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Author Contributions
Conceived and designed the experiments: CC LEL HW TM. Performed the experiments: NW NR CC TE SD. Analyzed the data: NW NR CC TE SD TM. Contributed reagents/materials/analysis tools: CC. Wrote the paper: LEL TM.
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