Article

Inefficiency of C3H/HeN Mice to Control Chlamydial Lung Infection Correlates with Downregulation of Neutrophil Activation during the Late Stage of Infection

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We previously reported that massive infiltration of neutrophils in C3H/HeN (C3H) mice could not efficiently control *Chlamydia muridarum* (*Cm*) infection and might contribute to the high susceptibility of these mice to lung infection. To further define the nature of neutrophil responses in C3H mice during chlamydial infection, we examine the expression of adhesion molecules and CD11b related to neutrophils infiltration and activation, respectively, following intranasal *Cm* infection. The results showed that the expression of selectins (E-selectin, P-selectin and L-selectin), and intercellular cell adhesion molecule-1 (ICAM-1) in the lung of C3H mice increased more significantly than in C57BL/6 (B6) mice, the more resistant strain. These results correlated well with the massive neutrophils infiltration in C3H mice. In contrast, CD11b expression on peripheral blood and lung neutrophils in C3H mice exhibited a significant reduction compared with B6 mice during the late phage of infection (day 14). These findings suggest that the high-level expression of adhesion molecules in C3H mice may enhance neutrophils recruitment to the lung, but the decline of CD11b expression on neutrophils may attenuate neutrophil function. Therefore, CD11b down-regulation on neutrophils may contribute to the failure of C3H mice to control chlamydial lung infection. *Cellular & Molecular Immunology*. 2009;6(4):253-260.

Key Words: *Chlamydia trachomatis,* neutrophils, selectin, ICAM-1, CD11b

Introduction

C. trachomatis is an obligate intracellular bacterium that causes a variety of human and animal diseases, including trachoma, pelvic inflammatory disease, infertility and pneumonia. Histopathological studies showed that these infections were associated with a mass of polymorphonuclear leukocyte (PMN) infiltration at the site (1, 2). Neutrophils are professional phagocytes which contribute to host defense against numerous infections caused by extracellular and intracellular pathogens (3, 4), but excessive infiltration of them can also lead to tissue injury through release of toxic products. Although there was no direct evidence demonstrating the role of neutrophils in controlling *Chlamydia* infection, several reports have showed that neutrophils indeed play a role in chlamydial infection, through depletion of neutrophils with a monoclonal antibody (MoAb) (5, 6). In a pregnant Swiss OF1 mouse model following intraperitoneal infection with *C. psittaci* serotype 1, mice depleted of neutrophils aborted earlier and had a much higher mortality rate than nondepleted mice (6).

Recruitment and activation of PMN reflects an innate immunological response to invading pathogens and has also emerged as a hallmark of vascular inflammation. Neutrophil recruitment into infection site proceeds in a cascade-like fashion. Initial tethering to endothelium is mediated by the

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Abbreviations: *Cm, Chlamydia trachomatis* mouse pneumonitis; ICAM-1, intercellular cell adhesion molecule-1; IFU, inclusion-forming unit; PMN, polymorphonuclear leukocyte; MPO, myeloperoxidase; MoAb, monoclonal antibody.

selectin family of adhesion molecules, whereas firm adhesion and transmigration is dependent on CD11a/CD18 and CD11b/CD18 (Mac-1, CR3) molecules, both of which interact with up-regulated ICAM-1 and possibly other molecules on the endothelial cells. This eventually triggers diapedesis and transendothelial migration of neutrophils (8-10). Mac-1 can be up-regulated several folds from intracellular granules by inflammatory mediators (9). More importantly, the function of oxidative burst and phagocytosis of neutrophils is also mainly dependent on CD11b/CD18 (10-12). Neutrophil dysfunction caused by downregulation of CD11b/CD18 expression may lead to recurrent bacterial infections in human and animals (10). Therefore, CD11b/CD18 expression can be considered as a functional marker of neutrophils.

Our previous studies found that the massive neutrophil infiltration in C3H mice following *C. trachomatis* infection was paralleled with high-level expression of proinflammatory cytokines (interleukin-1β, IL-6 and tumor necrosis factor-α), CXC chemokines [macrophage inflammatory protein 2 (MIP-2), cytokine-induced neutrophil attractant (KC) and lipopolysaccaride-induced chemokine (LIX) and their receptor CXCR-2, indicating that the expression of these cytokines may contribute to the infiltration of neutrophils in chlamydial infection. Although much greater and persistent infiltration of neutrophils was observed in C3H mice than B6 mice, the former mice had more severe disease and higher *in vivo* chlamydial growth than the latter, revealing that infiltrated neutrophils during the late stage of infection is not efficient in controlling the infection (7). This raises a posibitigy that the state of neutrophil activation or function is different in different mouse strains. In the present study, in order to define whether the inefficiency of neutrophils in controlling chlamydial infection in C3H mice is related to functional deficiency of neutrophils, we compared the CD11b expression on peripheral blood and lung PMN between C3H and B6 mice during the different stages of infection with *Cm*. In addition, we also examined the expression of cell adhesion molecules correlated with the activation of CD11b such as selectins and ICAM-1 in the lung.

Materials and Methods

Mice and organism

Female C57BL/6(H-2^b) mice and C3H/HeN (6-8 weeks old) were kept in a specific pathogen-free facility for animals at the central-care facility in Tianjin Medical University with filtered air flow and autoclaved cage, food and water. The mouse penumonitis biovar of *C. trachomatis* (*Chlamydia muridarum*, *Cm*) was a gift from Dr. Weiming Zhao (School of Medicine, Shandong University) and grown in Hela 229 cells and purified by discontinuous density gradient centrifugation according to a well-established protocol (13).

Infection of mice

Mice were inoculated intranasally with 3×10^3 inclusion-forming units (IFUs) of *Cm* in a volume of 40 μl. The mice were monitored daily for body weight changes and viability following infection. To determine the growth of the organisms *in vivo*, the lungs from each mouse were aseptically isolated and homogenized in 4 ml sucrose phosphate glutamic acid (SPG) buffer. Tissue homogenates were spun down at 1,900 g for 30 min at 4°C, and the supernatants were divided into aliquots (1 ml/vial) and kept at -80°C until tested. Chlamydial infectivity in the lung homogenates was titrated by infection of Hela 229 cell monolayers for 48 h followed by enumeration of inclusions that were stained by a genus-specific monoclonal antibody and secondary antibody conjugated with horseradish peroxidase as described previously (13).

Lung inflammatory cell preparation

Lung tissues (50 mg/mouse) were harvested from *Cm*-infected mice on days 7 and 14 postinfection. Inflammatory cells in the lung tissues were isolated as described previously (14). Briefly, lung tissue was minced with scissors to affine slurry in 5 ml lung digestion buffer [RPMI-1640 with 10% fetal calf serum, 1 mg/ml collagenase A (Gibco), 30 μg/ml DNAse (Gibco)]. After incubation in the above medium for 30-45 min, undigested fragments were further dispersed by drawing the solution up and down through the bore of a 10-ml syringe. The total lung cell suspensions were centrifuged at 400 g for 40 min and washed once with 10 ml of cold PBS. Cell differentials were determined by Wright-Giemsa staining. The amount of each cell type was determined by multiplying the percentage of each type by the total number of cells.

Lung myeloperoxidase (MPO) assay

Lung MPO, as an indirect measurement of neutrophil infiltration, was quantified as described previously (15). Briefly, fresh lung samples were weighed (50 mg) and homogenized in 1 ml buffer A (3.4 mM KH_2PO_4, 16 mM Na_2HPO_4, PH 7.4). After centrifugation for 30 min at 10,000 g, the pellet was resuspended in 1 ml buffer B (43.2 mM KH_2PO_4, 6.5 mM K_2HPO_4, 10 mM ethylenediaminetetraacetic acid, 0-5% hexadecyltrimethylammonium, pH6.0) and sonicated for 10 sec. Following heating for 2 h at 60°C and spinning down at 10,000 g for 10 min, the supernatant was reacted with 3,3',5,5'-tetramethylbenzidine (Sigma) and read at 650 nm using human MPO as a standard. Results are expressed as per unit weight of lung.

RT-PCR

For analysis of mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR), lungs were harvested from mice, frozen immediately in liquid nitrogen, and stored at -80°. Total cellular RNA from frozen lungs was isolated using TRIzol Reagent (Gibco) according to the manufacturer's protocol. cDNA was synthesized by reverse transcription at 37°C for 1 h from 1.2 μg total cellular RNA with random hexamers to prime the RT. The cDNA was then amplified using specific primers for E-selectin, P-selectin, L-selectin, and ICAM-1, with a housekeeping gene (β-actin) as a control. Ten microlitres of each RT-PCR reaction was electrophoresed in a 1% agarose gel containing ethidium bromide (0.003%). The bands were visualized and photographed using

ultraviolet transillumination and were analysed for density on scion image software. The expression of adhesion molecules mRNA is presented as a percentage of β-actin. The primers used in the PCR analysis are as follows: E-selectin sense 5'-TGG CGA TTC AGA ACA AGG AA-3', antisense 5'-AGT CAG GGT GTT CCT GTG GT-3' to give a 420-bp product; P-selectin sense 5'-AGG AGG CAC TTC ACA GAC TT-3'; antisense 5'-GAC TTT TCC ACA CTC CTT GA-3' to give a 429-bp product; L-selectin sense 5'-AAC GAG ACT CTG GGA AGT-3', antisense-CAA AGG CTC ACA TTG GAT-3' to give a 380bp product; ICAM-1 sense 5'-TGC GTT TTG GAG CTA GCG GAC CA-3', antisense 5'-CGA GGA CCA TAC AGC ACG TGC AG-3' to give a 326-bp product; β-actin sense 5'-ATG GAT GAC GAT ATC GCT-3', antisense 5'-ATG AGG TAG TCT GTC AGG T-3' to give a 582-bp product.

FACS analysis

PMN were isolated from mouse peripheral blood as previously described using Ficoll-Paque leukocyte isolation medium (GE healthcare) (16). Isolated cells were double-labeled with fluorescein isothiocyanate (FITC)-labeled anti-mouse CD45 monoclonal antibody (BioLegend) and phycoerythrin (PE)-labeled GR1 MoAb (Caltag) to identify PMN purity, and the percentage of CD45 and GR1 double positive cell is >95%. So FITC-labeled anti-mouse CD11b MoAb (BioLegend) was directly used to assess CD11b expression on isolated PMN. Isotype-matched rat IgM (Caltag) labeled with FITC was used as isotype control. Saturating concentrations of antibodies were added to 100-μl aliquots of cell susupension and incubated at room temperature for 30 min in the dark. Unbound antibody was removed by washing twice in PBS (pH 7.4 containing 1% bovine serum albumin and 0.02% sodium azide) and resuspended in 1% paraformaldehyde in PBS. Lung inflammatory cell suspension preparation has been described above. Immediately after isolation, 100 μl lung cell suspensions (10^6 cells) were incubated with FITC-labeled CD11b MoAb and PE-labeled GR1 MoAb which assessed the identity of neutrophils in the dark at room temperature for 30 min. Cells were then analyzed on a FACScan flow cytometer (Becton Dickinson). Data acquisition and analysis were performed using the Cell Quest software programs (BD).

Statistical analysis

Statistical analysis was performed using SPSS 12.0 version software. The data were expressed as the mean ± SD. Statistical significance was assessed by one way ANOVA and followed by Bonferroni or Tambane's post Hos multiple comparisons.

Results

C3H mice show more severe infiltration of neutrophils in lung following intranasal Cm infection

Our previous studies have shown that C3H mice show more severe lung histopathological changes and massive neutrophil infiltration than B6 mice following *Cm* infection. Similarly, in the present study, infiltration of neutrophils was observed in both strains of mice following *Cm* infection, but the percentage of neutrophils ($p < 0.01$, $p < 0.05$) and MPO levels ($p < 0.01$) in the lung were significantly greater in C3H mice than B6 mice on day 7 and day 14 postinfection representing the early and late stages of infection (13) (Figure 1). The results confirmed previous reports that acute chlamydial lung infection can induce significant neutrophil infiltration and genetically different strains of mice mount different degrees of neutrophils recruitment following infection.

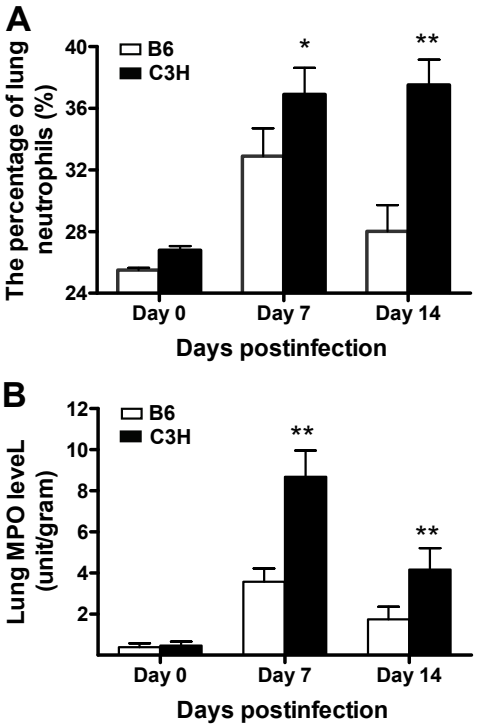

Figure 1. The level of neutrophil infiltration into lungs of B6 and C3H mice after intranasal infection with *Cm*. (A) The percentage of lung neutrophils in total inflammatory cells were determined by Wright-Giemsa staining. (B) Lung MPO levels in B6 and C3H mice were assessed at various time points after *Cm* infection. The results were presented as mean ± SD. *$p < 0.05$, **$p < 0.01$, comparison between C3H and B6 mice.

Cm-infected C3H mice show higher selectins and ICAM-1 mRNA levels in the lung than B6 mice

Up-regulated E-selectin and P-selectin on inflamed endothelium can interact with L-selectin expressed on leucocytes including neutrophils and promote the initial rolling of leukocytes along the postcapillary venules in the systemic circulation (8). Although the rolling mediated by selectins may not occur within the pulmonary capillary bed because their diameters are similar to or narrower than the diameters of neutrophils, selectins combined with β_2-integrin

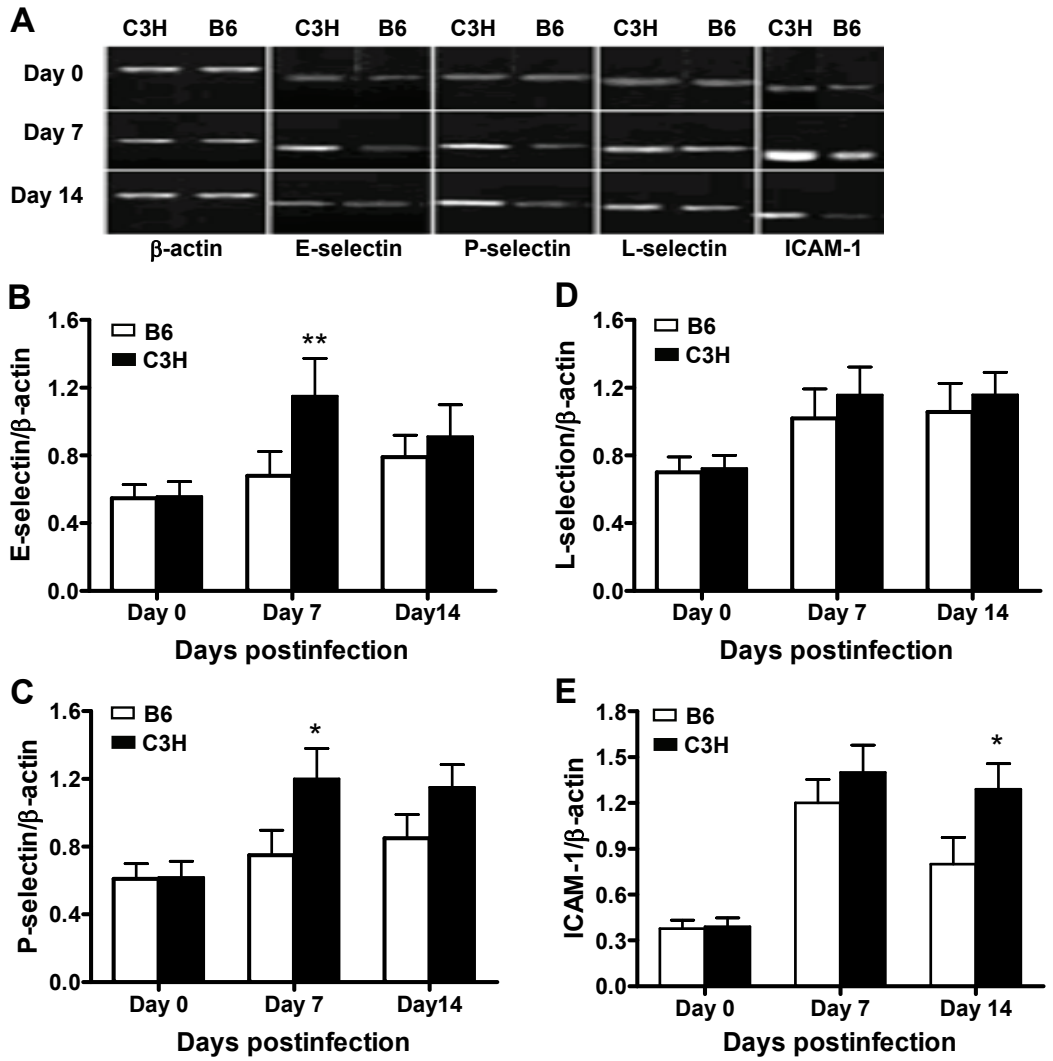

Figure 2. The expression of lung selectins and ICAM-1 mRNA in B6 and C3H mice after intranasal infection with *Cm*. (A) The mRNA levels of lung E-selectin, P-selectin, L-selectin and ICAM-1 in B6 and C3H mice at various time points after *Cm* infection were detected by RT-PCR. (B, C, D, E) Relative densities were obstained by comparing selectins and ICAM-1 with β-actin. The results were presented as mean ± SD. *$p < 0.05$, **$p < 0.01$, comparison between C3H and B6 mice.

(CD11b/CD18) are required at a later stage for prolonged PMN sequestration within the pulmonary capillaries (17). We therefore compared the mRNA level of them in lungs elicited by *Cm* infection in C3H and B6 mice. As shown in Figure 2, L-selectin, P-selectin and E-selectin mRNA levels in both strains of infected mice exhibited significant increases compared with control group (naive mice) on day 7 and day 14 following *Cm* infection. E-selectin and P-selectin mRNA levels in C3H mice are significantly higher than that in B6 mice on day 7 (Figures 2B, 2C). Therefore, the more severe infiltration of neutrophils in C3H may be partially due to their relative high expression of selectins induced by *Cm*.

ICAM-1 is expressed on the surface of most leukocytes, fibroblasts, and endothelial cells, but not on neutrophils, and is up-regulated in many tissues after stimulation with inflammatory mediators. The up-regulated ICAM-1 displayed on the inflamed endothelium can interact with activated integrins (Mac-1 and LFA-1) expressed on neutrophils, promoting PMN adhesion, migration, and respiratory burst, it is a pivotal event in PMN-mediated tissue injury (9, 10). The data showed that lung ICAM-1 mRNA level was significantly up-regulated in C3H and B6 mice following intranasal *Cm* infection (Figures 2A and 2E), indicating that endothelial cells of both strains of mice produced high-level ICAM-1 in chlamydial infection. The continuous high level of ICAM-1 expression in C3H mice paralleled with continuous infiltration of neutrophils.

CD11b expression on peripheral blood and lung PMN in C3H mice show a significant reduction during the late stage of Cm infection

Our previous study has shown that the massive infiltration of

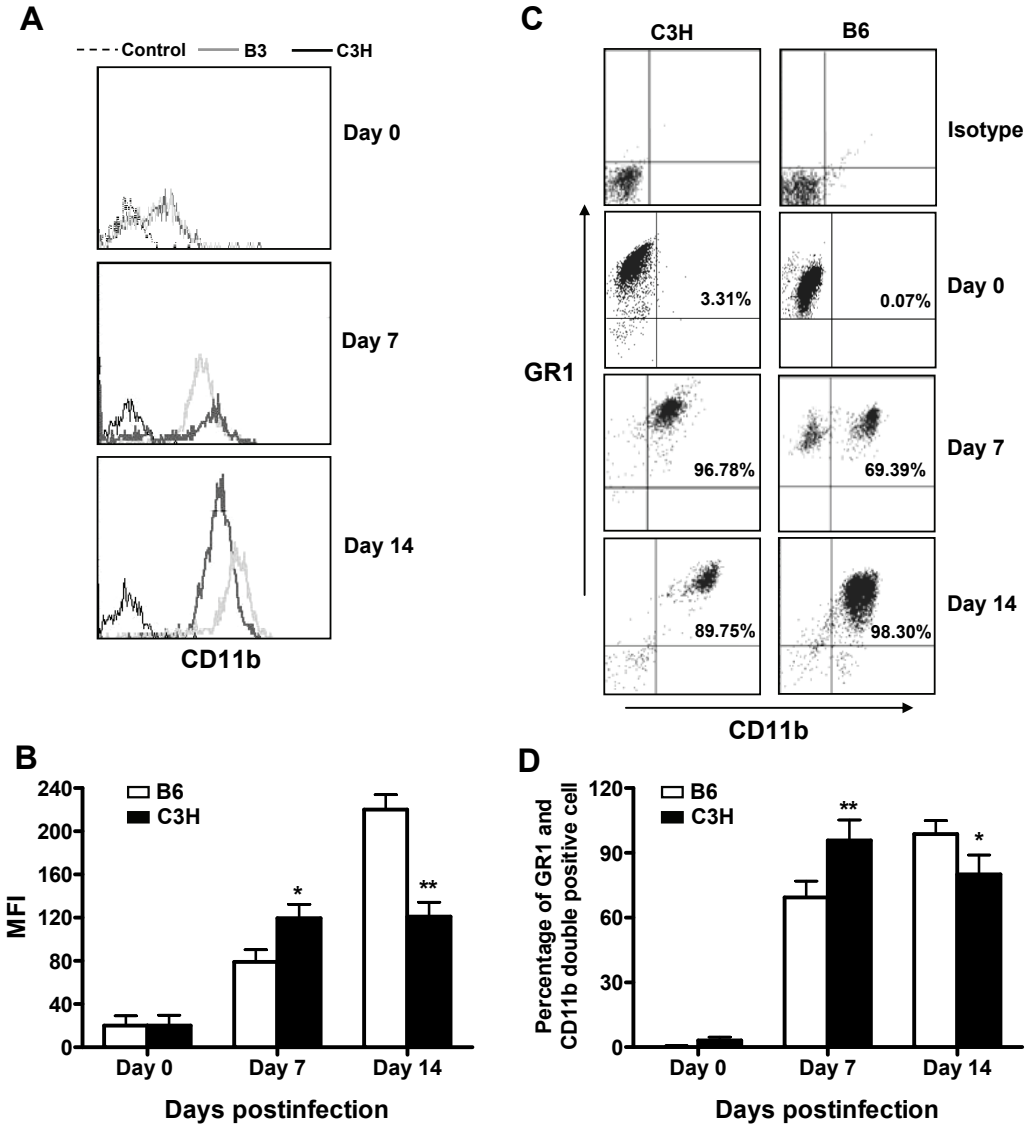

Figure 3. Flow cytometric analysis of CD11b expression on peripheral blood and lung PMN in B6 and C3H mice after intranasal infection with *Cm*. (A) The fluorescence intensity of CD11b expression on peripheral PMN of B6 and C3H mice at various time points after *Cm* infection were detected by flow cytometry. (B) The mean fluorescence intensity (MFI) analysis of CD11b expression on peripheral PMN. (C) CD11b expression on lung PMN of B6 and C3H mice at various time points after *Cm* infection were detected by flow cytometry. Percentage of double positive cell represents the expression of CD11b on neutrophils. (D) The analysis of CD11b expression based on the percentage of $GR1^+/CD11b^+$ cells in the total pulmonary cells. Values are expressed as mean ± SD (n = 5). *$p < 0.05$, **$p < 0.01$, comparison between C3H and B6 mice.

neutrophils in C3H mice can not efficiently control chlamydial infection. Therefore, we prospected that neutrophil activity in C3H mice may differ from that of B6 mice. CD11b is a molecule marking the activation of neutrophils, we therefore detected the CD11b expression on peripheral blood and lung PMN in the two strains of mice by flow cytometry. The peripheral blood PMN vitality showed by trypan blue dye staining was >95%, and PMN purity by flow cytometry was >95%. CD11b expression on peripheral blood PMN in infected C3H and B6 mice increased significantly on days 7 and 14 postinfection compared with uninfected mice (Figures 3A and 3B). However, CD11b expression in C3H mice was significantly higher than B6 mice on day 7 postinfection ($p < 0.05$) (Figure 3B), which was consistent with the magnitude of neutrophil infiltration in these two strains of mice. In contrast, the CD11b expression on peripheral blood PMN in B6 mice continuously increased on day 14, but not in C3H mice, leading to a significantly lower CD11b expression in C3H than B6 mice on day 14 postinfection ($p < 0.01$) (Figure 3B).

We therefore analysed CD11b expression on lung PMN in C3H and B6 mice by flow cytometry (Figures 3C, 3D).

The data showed that lung PMN CD11b expression was in a similar pattern to peripheral blood PMN, which was significantly higher in C3H mice than B6 mice on day 7 postinfection, and lower on day 14 postinfection. From day 7 to day 14, the CD11b expression exhibited a significant increase in B6 mice but a slight reduction in C3H mice. These results together imply that the functional alterations may exist during the late phage of infection in C3H mice. Therefore, the downregulation of CD11b in C3H mice may contribute to the inefficiency of neutrophils in controlling the infection.

Discussion

Genetically different inbred mouse strains have been shown to exhibit differences in susceptibility to chlamydial disease. Our previous studies combined with other studies have shown that B6 mice consistently have less pathogen burden or disease compared with C3H mice in models of acute genital tract, respiratory, and systemic infections (18-20). These studies demonstrated that differences in the innate immunity mainly featured by neutrophil infiltration determine susceptibility to *Chlamydia* in inbred mice. In the present study, in order to further investigate the role played by neutrophils in susceptibility difference of inbred mice to *Chlamydia,* we compared the expression of adhesion molecules related to neutrophils infiltration and activity following *Cm* infection. Our data showed that the expression of selectins (E-selectin, P-selectin and L-selectin), ICAM-1 and CD11b in the lung increased more significantly in C3H than B6 mice on day 7 postinfection, which paralleled the magnitude of infiltrated neutrophils and MPO levels in the lung. More importantly, during the late phage of infection (day 14), the CD11b expression on neutrophils in C3H mice exhibited a significant reduction compared with B6 mice, which supported our hypothesis that the down-regulation of neutrophil activation during the late phage of infection contributed to the susceptibility of C3H mice to *C. trachomatis* infection.

The processes of neutrophil priming, adhesion, emigration and activation are considered important regulatory processes in PMN function. Induction and control of these events involve the expression and binding of adhesion molecules on the PMN to cellular ligands. Selectins which initiate the recruitment of leukocytes from the blood stream on inflamed endothelium (21) and combined with β_2-integrin (CD11b/CD18) are required at a later stage for prolonged PMN sequestration within the pulmonary capillaries (17). In addition, cross-linking of L-selectin and P-selectin glycoprotein ligang 1 (PSGL-1) expressed on neutrophils following binding to corresponding selectin ligands can cooperatively provide an important accessory signal to trigger the activation of integrin CD11b/CD18 (22, 23). Firm adhesion and transmigration is predominantly dependent on β_2-integrins on PMN interaction with their counterreceptors, believed to be predominantly ICAM-1 (8-10). Mice receiving anti-CD11b antibody and/or anti-ICAM-1 antibody demonstrated a dramatic reduction in pulmonary neutrophils recruitment compared with control mice (24). In the present study, we observed a significant increase in the expression of selectins, ICAM-1 and CD11b in C3H and B6 mice following infection with *Cm* on day 7 postinfection. And compared with B6 mice, the expression of these adhesion molecules in C3H mice increased more significantly, which paralleled the massive infiltration of neutrophils. Combined with other studies, we infer that the high-expression of these adhesion molecules during the early stage of infection coordinately promote more neutrophils migration into lungs participating in defense against the invasion of *Cm* in C3H mice. During the late stage of infection, although the percentage of infiltrated neutrophils in C3H mice was still more than B6 mice, the CD11b expression on peripheral blood and lung PMN in C3H mice was significantly lower than B6 mice, revealing that other alternative mechanisms such as CD11a/CD11b mediated adhesion may also be responsible for neutrophil infiltration.

Although the elimination of the intracellular parasites including *Chlamydia* depends on the activation of the antimicrobial effector mechanisms in the infected macrophages, many studies have demonstrated that neutrophils represent the first leukocyte population migrating to the sites of infection and play an essential role in defense against some intracellular pathogens (3, 4, 25). A basic mechanism of recognition of microorganism by neutrophils is CD11b/CD18 (CR3) mediated opsonophagocytosis. It has been reported that the uptake of some intracellular pathogens such as *Leishmania major*, *Mycobacterium kansasii* and *Bordetella pertussis* by neutrophils is all dependent on CR3 (11, 12, 26). Blocking of CR3 *in vitro* with anti-CD11b MoAb significantly reduced the phagocytosis of *L. major* promastigotes by PMN (26). Although CR3 can mediate nonopsonic phagocytosis to invading microorganisms, it has been demonstrated that only opsonin-dependent uptake of *leishmania* activates PMN. Activated PMN are able to activate antimicrobial effector mechanisms and to eliminate the intracellular pathogens (26). However, the pathogens including *Chlamydia* can survive in inactivated PMN (27). In the present study, CD11b expression on peripheral blood and lung PMN in C3H and B6 mice on day 7 after *Cm* infection increased significantly compared with control group, indicating that invasion of *Cm* can potently induce neutrophil activation which is more intense in C3H mice than B6 mice in the early immune response. These activated neutrophils may play a protective role in defense against *Cm* invasion through the direct killing of pathogen. However, the toxic product released by them may also lead to tissue injury, partially leading to susceptibility of C3H mice to *Chlamydia.* Indeed, blocking the interaction between CD11/CD18 complex and ICAM-1 or creating ICAM-1 deficiency can reduce tissue injury and organ dysfunction following endotoxin challenge in animal models (28).

A major finding in the present study was that the CD11b expression on neutrophils in C3H mice exhibited a significant reduction compared with B6 mice on day 14 postinfection. This may be related to the failure of C3H mice

to control *Cm* infection. In addition to promoting neutrophil recruitment into inflamed tissue and pathogen phagocytosis, more recent data suggest that CD11b/CD18 also modulate neutrophil apoptosis (29, 30). Mac-1-dependent phagocytosis of complement-opsonized pathogens triggers rapid neutrophil apoptosis. In mice selectively deficient in CD11b/CD18, thioglycollate-induced neutrophil accumulation in the peritoneal cavity was increased and was associated with a significant delay in apoptosis of extravasated cells. The extravasated cells had a near absence of neutrophil phagocytosis and a reduction in oxygen free radical generation (30). Therefore, more neutrophils infiltrated in the lungs of C3H mice than B6 mice during the late stage of infection may be due to delayed apoptosis of neutrophils caused by downregulation of CD11b expression on them, and these delayed apoptosis of neutrophils may be inefficient in controlling *Cm* infection due to loss of phagocytosis and reduction in oxygen free radical generation. At the same time, the persistent infiltration of these neutrophils might otherwise result in sustained inflammatory tissue damage.

Recently, it has been suggested that neutrophils can regulate specific immune responses and even function as antigen-presenting cells (APCs). In a pregnant mouse model with *C. psittaci* serotype I infection and an anti-neutrophil MoAb (RB6-8C5), the lack of neutrophils led to a substantial decrease in the number of macrophages and T lymphocytes in the liver of mice (6). Indeed, using confocal microscopy and immunoelectron microscopy Gavin P, et al. provided evidences that CD80, CD86 and MHC class II antigen are colocalized within neutrophil secretory vesicles, and the cross-linking of neutrophils CD11b was required for the translocation of these molecules from pre-existing cytoplasmic stores onto the cell surface (31). Therefore, during the late stage of infection, the down-regulation of CD11b expression on neutrophils in C3H mice might attenuate the antigen-presenting function of neutrophils, which may be another mechanism of the high susceptibility of C3H mice to *C. trachomatis* infection. However, the exact mechanism for the protective role of CD11b in chlamydial infection still needs to be studied further.

In summary, the present study enhanced our understanding on the molecular basis of neutrophils recruitment in chlamydial infection. More importantly, we found a significant decrease of CD11b expression on neutrophils in C3H mice during the late phage of infection. This may lead to the reduction of the phagocytosis, apoptosis and antigen-presenting of neutrophils, thus partially explained the severe disease of C3H mice.

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