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Integrin $\alpha 4\beta 1$ is necessary for CD4⁺ T cell-mediated protection against genital *C. trachomatis* infection¹

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Abstract

Chlamydia trachomatis infection is the most common sexually transmitted bacterial infection in the United States and a significant health burden worldwide. Protection from *Chlamydia* infection in the genital mucosa is dependent on interferon-gamma (IFN- γ) derived from CD4⁺ Th1 cells. These CD4⁺ T cells must home successfully to the genital tract to exert their effector function and decrease *C. trachomatis* burden. Although adhesion receptors expressed by CD4⁺ T cells in the genital tract have been characterized, the integrin receptor required for *Chlamydia*-specific CD4⁺ T cell-mediated protection has not been explored. Here we demonstrate that *C. trachomatis* infection of the upper genital tract results in recruitment of *Chlamydia*-specific CD4⁺ T cells robustly expressing the integrin $\alpha 4\beta 1$. Interfering with $\alpha 4\beta 1$, but not $\alpha 4\beta 7$, function resulted in defective CD4⁺ T cell trafficking to the uterus and high bacterial load. We conclude that integrin $\alpha 4\beta 1$ is necessary for CD4⁺ T cell-mediated protection against *C. trachomatis* infection in the genital mucosa. By identifying homing molecules required for successful CD4⁺ T cell trafficking to *C. trachomatis* infected tissues, we will be better equipped to design vaccines that elicit sterilizing, long lasting immunity without inducing immune pathologies in the upper genital tract.

INTRODUCTION

Chlamydia trachomatis is the most common cause of bacterial sexually transmitted infection in the United States and the leading cause of preventable blindness worldwide (1). *C. trachomatis* is an obligate intracellular pathogen that infects conjunctival and genital tract epithelial cells. In the upper genital tract, complications from *C. trachomatis* infection include pelvic inflammatory disease, ectopic pregnancy and infertility (2, 3). The high frequency of infection, low incidence of acquired immunity and lack of an effective vaccine make *C. trachomatis* a continuing public health concern.

Protection of the genital mucosa from *C. trachomatis* is dependent on the production of IFN- γ (4). IFN- γ protects through the upregulation of IDO, NOS and IRGs that interfere with various aspects of the pathogen's developmental cycle and reduce growth (5–8). Mice that

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are deficient in IFN- γ production have delayed resolution of infection in the genital mucosa (9). CD4⁺ T cells must produce IFN- γ in order to mediate protection, as transfer of *Chlamydia*-specific CD4⁺ T cells only protect naïve mice against challenge when IFN- γ is produced by those T cells. It is also critical that antigen-specific, IFN- γ -secreting CD4⁺ T cells efficiently traffic to the genital mucosa in response to *Chlamydia* infection in order to drive protective immunity (10, 11).

Homing receptors mediate the migration of immune cells towards specific signals in order to exit the circulation and enter target tissues (12). Integrins are a family of adhesion receptors consisting of α and β heteroduplexes that direct signaling from both outside and inside of the cell membrane (13). The role of certain integrin members on leukocytes has been studied extensively. For example, LFA-1 has been shown to play a crucial function in the arrest of leukocytes in the blood vessels at the site of inflammation (14, 15). Other integrin heterodimers, namely $\alpha 4\beta 1$ and $\alpha 4\beta 7$, provide tissue-specificity to T cells when homing to different areas of the body. Descriptions of how lymphocytes traffic to the gastrointestinal tract and central nervous system (CNS) have been reported. Lymphocyte recruitment to the gastrointestinal tract is largely mediated by the chemokine receptor CCR9 and the integrin receptor $\alpha 4\beta 7$ (16). On the other hand, integrin $\alpha 4\beta 1$ regulates trafficking to the CNS. In these models, interfering with $\alpha 4\beta 1$ and $\alpha 4\beta 7$ profoundly impairs immune cell recruitment to the respective tissues (17, 18). In fact, integrin-specific antibodies are used clinically to block immune cell infiltration and provide relief from autoimmune diseases such as ulcerative colitis and multiple sclerosis (19, 20). Unfortunately, our understanding of how CD4⁺ T cells traffic to the genital mucosa has been limited, including what combination of adhesion receptors is required for successful migration.

In this study, we interrogated the importance of $\alpha 4\beta 1$ and $\alpha 4\beta 7$ integrin heterodimers in promoting *Chlamydia*-specific CD4⁺ T cell recruitment to the genital mucosa and protecting mice from *C. trachomatis* infection. We show that integrin $\alpha 4\beta 1$ is dramatically increased on the surface of both polyclonal and *Chlamydia*-specific CD4⁺ T cells in the uterus following infection. We find that blocking or deleting integrin $\alpha 4\beta 1$, but not $\alpha 4\beta 7$, on pathogen-specific CD4⁺ T cells results in the impairment of trafficking to the uterus and a decrease in the protective capacity of CD4⁺ T cells. We conclude that integrin $\alpha 4\beta 1$ is necessary for CD4⁺ T cell-mediated protection against *C. trachomatis*. Identifying the receptors required for CD4⁺ T cell trafficking to the genital tract in response to *C. trachomatis* is important in designing a vaccine that elicits sterilizing, long lasting immunity against the pathogen while limiting the extent of tissue pathology.

MATERIALS AND METHODS

Mice

C57BL/6, B6.PL-Thy1a (CD90.1 congenic), C57BL/6, B6.SJL-Ptprca Pep3/BoyJ (CD45.1 congenic), B6.Cg-Tg548Jxm/J (*Lck-CRE*), C57BL/6-Itgb7^{tm1Cgn}/J (*Itgb7*^{-/-}), and B6;129-Itgb1^{tm1Efu}/J (*Itgb1*^{flox/flox}) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and NR1 mice were previously described (21). B6;129-Itgb1^{tm1Efu}/J (*Itgb1*^{flox/flox}) mice were crossed with C57BL/6 for greater than 10 generations. *C. trachomatis*-specific integrin-deficient mice were generated by breeding NR1 mice to either *Lck-CRE* and

Itgb1^{flox/flox} or *Itgb7*^{-/-} mice. All mice were maintained in facilities managed by the Harvard Medical School Center for Animal Resources and Comparative Medicine. To normalize for the murine estrous cycle, mice were treated subcutaneously with 2.5 mg of medroxyprogesterone 7 days prior to infection. Harvard's institutional animal care and use committee approved all the experiments described. A minimum of 4 mice per group was used in each experiment.

Growth, isolation and detection of *C. trachomatis*

C. trachomatis serovar L2 (434/Bu) was propagated using McCoy cell monolayers grown in Eagle's MEM (Invitrogen, Grand Island, NY) plus 10% FCS, 1.5 g/l sodium bicarbonate, 0.1 M nonessential amino acids, and 1 mM sodium pyruvate. Infected McCoy cells were detached from plates using sterile glass beads and then sonicated to disrupt *C. trachomatis* inclusions. Density gradient centrifugation was used to purify elementary bodies(22). Aliquots were stored at -80°C in a medium containing 250 mM sucrose, 10 mM sodium phosphate, and 5 mM L-glutamic acid.

Transfer of NR1 cells, infection of mice, and tissue preparation

C. trachomatis-specific CD4⁺ T cells were isolated from the lymph nodes and spleens of naïve donor NR1 mice. Recipient mice received 10⁶ NR1 cells and were infected the following day with 10⁶ *C. trachomatis* inclusion forming units (IFU) in 10 µl of sucrose-phosphate-glutamate media. We used the NSET device (ParaTechs) to bypass the cervix and directly infect the uterine horns (10). The uterus was harvested and disaggregated by digestion with 1 mg/ml of type XI collagenase (Sigma, St. Louis, MO) and 50 Kunitz/ml of DNase (Sigma) for 30 min at 37°C. Single cell suspensions from tissues were obtained by mechanical disaggregation prior to staining. Suspensions of splenocytes were treated with a hypotonic buffer to lyse red blood cells prior to use.

Flow cytometry

Single cell suspensions were stained immediately for activation markers or stimulated for 5 hours with 100 ng/ml PMA (Alexis Biochemical) and 1 µg/ml ionomycin (Calbiochem) in brefeldin A (BD Biosciences) for intracellular cytokine staining. Cells were treated with anti-FcγR (BioXCell) before staining with combinations of the following antibodies: anti-β1 Pacific Blue, anti-β7 FITC, anti-TCRα2 allophycocyanin, anti-CD90.1 peridinin chlorophyll protein, anti-CD45.2 phycoerythrin (PE), anti-CD90.2 FITC, anti-IFN-γ PE, anti-TNF-α PE-cy7, anti-CD25 PE, anti-CD44 PE or Pacific Blue, anti-CD62L FITC (Biolegend), anti-CD3e allophycocyanin, anti-α4 PE (BD Biosciences), anti-CD4 Qdot605 and a LIVE/DEAD dead cell stain kit (Invitrogen). The efficacy of all antibodies used in this study was confirmed extensively *in vitro* and compared to isotype control antibodies. For cytokine staining, cells were permeabilized using a Cytotfix/Cytoperm Plus Kit following manufacturer's instructions (BD Biosciences). Cell number was determined with AccuCheck Counting Beads (Invitrogen). Flow cytometry data were collected on a modified FACSCalibur (Cytex Development) or an LSR II (BD Biosciences) and analyzed using FlowJo (Tree Star).

Th1 polarization and protection against *C. trachomatis*

CD4⁺ T cells were harvested from the lymph nodes and spleens of naïve NR1 mice and enriched with a mouse CD4 negative isolation kit (Invitrogen) following the manufacturer's protocol. CD4⁺ T cells were cultured in media consisting of RPMI 1640 (Invitrogen), 10% FCS, L-glutamine, HEPES, 50 µM 2-ME, 50 U/ml penicillin, and 50 mg/ml streptomycin. NR1 cells were activated by co-culture with irradiated or mitomycin-treated splenocytes pulsed with 5 µM of Cta1₁₃₃₋₁₅₂ peptide at a stimulator:T cell ratio of 4:1. Th1 polarization was achieved by supplying cultures with 10 ng/ml of IL-12 (Peprotech, Rocky Hill, NJ) and 10 µg/ml of anti-IL-4 antibody (Biolegend). After 5 days of stimulation, NR1 Th1 cells were transferred i.v. into naïve recipient mice. In integrin antibody blocking experiments, NR1 cells were treated with 100 µg of anti-α4, anti-α4β7 or isotype antibody (BioXCell) for 1 hour at room temperature prior to transfer into recipients. The next day, mice were challenged with 5×10⁶ *C. trachomatis* IFUs and the upper genital tract was analyzed for burden 5 days after infection. Mice were treated i.p. with 200 µg of the respective antibody at 1 and 3 days post-infection for integrin blocking experiments.

Competitive homing

For this assay an equivalent number of integrin wildtype NR1 cells (CD45.2/CD90.1) and congenic wildtype, *Lck-CRE/Itgb1*^{flox/flox}, or *Itgb7*^{-/-} NR1 cells (CD45.2/CD90.2) were combined and transferred into congenically mismatched CD45.1 hosts. The following day, mice were transcervically infected with 5×10⁶ *C. trachomatis* IFUs. Tissues were harvested and analyzed 7 days after infection.

Quantitative PCR

Bacterial burden was evaluated by quantifying *C. trachomatis* 16S DNA relative to mouse GADPH DNA(21). The uterus was homogenized and DNA was extracted using the DNeasy blood and tissue kit (Qiagen). DNA was analyzed using *C. trachomatis*- and mouse-specific primer pairs and dual-labeled probes. Threshold values were detected by an ABI Prism 7000 sequence system. The ratio of *C. trachomatis* to host DNA was obtained using a standard curve.

Statistical analysis

Statistical significance between groups was determined using an unpaired two-tailed t test and depicted within figures as * p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.0001.

RESULTS

C. trachomatis infection leads to robust integrin α4β1 surface expression on bulk CD4⁺ T cells in the uterus

Previous reports differ regarding the levels of α4β1 and α4β7 expression on T cells in the uterus during *Chlamydia* infection (23–25). As a first step to resolve these discrepancies, we examined the surface expression of α4, β1, and β7 on CD4⁺ T cells responding to *C. trachomatis* infection in the genital mucosa. To test differences in integrin expression, mice were infected transcervically with *C. trachomatis* (10). Seven days later, the uterus and

draining (iliac) lymph nodes were isolated and examined for the surface expression of integrins on endogenous CD4⁺ T cells by flow cytometry. We found that $\alpha 4$ and $\beta 1$ were dramatically upregulated on the surface of CD4⁺ T cells in the uterus relative to those present in the draining lymph nodes of infected mice (Fig 1A). In contrast, the surface expression of $\beta 7$ was only modestly increased on CD4⁺ T cells in the genital mucosa compared to $\alpha 4$ and $\beta 1$. We next quantified the absolute number of $\alpha 4^+ \beta 1^+$ - and $\alpha 4^+ \beta 7^+$ - expressing CD4⁺ T cells in the genital tract (Fig 1B). Very few CD4⁺ T cells were found in the uterus during steady state in naïve mice. These extremely low numbers precluded conclusive interpretations about integrin staining differences in naïve mice. Following infection, the absolute number of $\alpha 4^+ \beta 1^+$ CD4⁺ T cells in the upper genital tract significantly increased whereas the number of $\alpha 4^+ \beta 7^+$ cells did not. We next compared the number of activated $\alpha 4^+ \beta 1^+$ or $\alpha 4^+ \beta 7^+$ CD4⁺ T cells responding to the genital mucosa by gating for populations expressing high levels of CD44. Interestingly, both the number of activated $\alpha 4^+ \beta 1^+$ and $\alpha 4^+ \beta 7^+$ CD4⁺ T cells were significantly increased in infected animals relative to naïve controls (Fig 1C). Nonetheless, there was a more robust recruitment of activated $\alpha 4^+ \beta 1^+$ CD4⁺ T cells to infected uteri compared to $\alpha 4^+ \beta 7^+$ CD4⁺ T cells. Our results show that while both $\alpha 4^+ \beta 1^+$ and $\alpha 4^+ \beta 7^+$ CD4⁺ T cells are found in the infected genital mucosa, $\beta 1$ is more highly expressed. These observations on endogenous T cells suggest that $\alpha 4 \beta 1$ is the primary integrin driving CD4⁺ T cell recruitment to the genital mucosa in response to *C. trachomatis* infection.

Infection leads to increased $\alpha 4 \beta 1$ surface expression on *C. trachomatis*-specific CD4⁺ T cells in the uterus

During *Chlamydia* infection, antigen-specific T cells are primed and recruited specifically to the genital tract. However, inflammatory cytokines can also activate bystander T cells at the site of infection independently of antigen specificity (26). Previous work characterizing integrin receptors required for T cell recruitment to the genital mucosa focused exclusively on memory CD4⁺ T cell lines (23), polyclonal CD4⁺ T cells (24), or bulk CD4⁺ T cells (25) but never examined naïve *C. trachomatis*-specific CD4⁺ T cells. However, only by using naïve antigen-specific T cells is it possible to properly model primary infection. To directly interrogate the integrin profile of *Chlamydia*-specific CD4⁺ T cells, we took advantage of *C. trachomatis*-specific TCR transgenic CD4⁺ T cells that are locked into specificity for the *Chlamydia* antigen Cta1 (referred to subsequently as NR1 cells, 21). Based on the results described in the section above, we hypothesized that NR1 cells would display significantly increased surface $\beta 1$, rather than $\beta 7$, upon trafficking to infected uteri. To test this prediction, we transferred 10⁶ NR1 cells intravenously into congenic naïve animals that were then infected transcervically the following day with *C. trachomatis*. The integrin profile of naïve NR1 cells did not have major differences prior to transfer (data not shown). In agreement with our observations with polyclonal CD4⁺ T cells (Fig 1), 8 days after infection there was over a 10-fold increase in the surface expression of integrin $\beta 1$ on NR1 cells in the uterus compared to NR1 cells in the draining lymph nodes (Fig 2A). To better understand the dynamics of integrin expression we monitored the surface expression of integrins on NR1 cells in the genital tract and draining lymph nodes at three time points following *C. trachomatis* infection. Three, 8, and 13 days after infection were chosen to coincide with T cell activation, peak of infiltration to the site of infection, and contraction,

respectively (Fig 2B and C). There was a surge of both $\alpha 4^+ \beta 1^+$ and $\alpha 4^+ \beta 7^+$ NR1 cells in the genital tract 8 days after infection compared to what was observed with these populations 3 days after infection (Fig 2B). Even though both NR1 populations increased during infection, the magnitude of infiltration of the $\alpha 4^+ \beta 1^+$ NR1 cells to the uterus was far more robust ($***p < 0.001$) than the $\alpha 4^+ \beta 7^+$ population ($*p < 0.05$) (Fig 2B, left). Surface integrin profiles of NR1 cells in the draining lymph nodes were statistically indistinguishable between these two time points (Fig 2B, right). Thirteen days after infection the decline of NR1 cells (Fig 2C) corresponded to the resolution of *C. trachomatis* infection in the genital mucosa (10). Even at this late time point, $\alpha 4^+ \beta 1^+$ NR1 cells were found significantly more frequently than $\alpha 4^+ \beta 7^+$ NR1 cells in the genital tract. These observations suggest that $\alpha 4^+ \beta 1^+$ NR1 cells are either recruited or retained in the uterus for a longer period of time than other T cell populations. We confirmed that the limited $\beta 7$ expression observed in Figures 1 and 2 was not due to poor antibody staining. Robust $\beta 7$ staining was detected on NR1 cells activated *in vitro* in the presence of retinoic acid, a metabolite previously shown to induce $\alpha 4 \beta 7$ expression on T cells (data not shown). Taken together, these findings demonstrate that both polyclonal and *Chlamydia*-specific CD4⁺ T cells, preferentially express $\alpha 4$ and $\beta 1$ on their surface in the murine genital mucosa in response to *C. trachomatis* infection.

Blocking integrin $\alpha 4$ but not $\alpha 4 \beta 7$ increases *C. trachomatis* burden

Since we identified a strong upregulation of $\alpha 4 \beta 1$ on the surface of CD4⁺ T cells responding to *C. trachomatis* infection, we next assessed the functional role of $\beta 1$ and $\beta 7$ in promoting protective immunity. Our group has previously shown that transfer of *in vitro*-activated Th1-skewed NR1 cells into naïve mice confers significant protection from *C. trachomatis* infection compared to mice that receive no T cells (10). We hypothesized that if specific integrins are required for recruitment to the genital mucosa, blocking these integrins on activated NR1 cells may alter their protective capacity. To test this and determine the relative contributions of $\beta 1$ and $\beta 7$ to CD4⁺ T cell-mediated protection, we selectively blocked integrin receptors using antibodies (Fig 3). NR1 cells from naïve mice were harvested and polarized *in vitro* to a Th1 phenotype. IFN- γ production by the NR1 cells was assayed to confirm their Th1 phenotype by flow cytometry prior to transfer (data not shown). One million NR1 Th1 cells were pre-treated with antibody that blocked $\alpha 4$ (blocks both $\alpha 4 \beta 1$ and $\alpha 4 \beta 7$), $\alpha 4 \beta 7$, or an isotype control and then transferred intravenously into host mice that were then transcervically infected with *C. trachomatis* the following day. To ensure robust blockade of integrins, each group of mice was also treated with antibody 1 and 3 days following infection. Five days after infection, *C. trachomatis* burden in the uterus was measured using quantitative PCR (qPCR). Mice that were treated with blocking antibody against $\alpha 4$ had a higher *C. trachomatis* burden in the genital tract than isotype control treated animals, similar to bacterial levels found in mice that received no NR1 cells at all. On the other hand, mice treated with $\alpha 4 \beta 7$ blocking antibody had significantly lower *C. trachomatis* levels than the no transfer group and similar bacterial burden to the isotype antibody treated mice. Despite the lack of an antibody that specifically blocks $\alpha 4 \beta 1$, we can infer the importance of $\alpha 4 \beta 1$ indirectly. Because blocking $\alpha 4$ with antibody prevents both $\alpha 4 \beta 1$ and $\alpha 4 \beta 7$ signaling, the differences observed in protection between $\alpha 4$ and $\alpha 4 \beta 7$ antibody treatment groups point towards a function of $\alpha 4 \beta 1$. Since the integrin $\alpha 4$ chain dimerizes with either $\beta 1$ or $\beta 7$ (27), the results shown in Figure 3 indirectly confirm that only

$\alpha 4\beta 1$ is required for CD4⁺ T cell-mediated protection against *C. trachomatis* in the genital mucosa.

A reduction in CD4⁺ T cells is responsible for the higher *C. trachomatis* burden in anti- $\alpha 4$ antibody-treated mice

Although blocking $\alpha 4$ was sufficient to prevent CD4⁺ T cell-mediated protection in the genital mucosa, we had not determined the mechanism responsible for higher burden. We predicted that the loss of protection seen in mice treated with $\alpha 4$ -blocking antibody was due to diminished recruitment of NR1 cells to the infected genital mucosa. To test this possibility, we monitored the trafficking of NR1 cells to the infected genital tract after integrin blockade. NR1 cells were skewed to the Th1 phenotype *in vitro*, and then transferred into congenically mismatched host mice. The next day mice were infected transcervically with *C. trachomatis*. Prior to transfer, NR1 cells were pretreated with individual integrin blocking antibodies or an isotype control. Mice were also treated with the same blocking antibody or isotype control 1 and 3 days after infection. Five days after infection, we examined the number of NR1 cells present in the genital tract and draining lymph nodes by flow cytometry. We noted that the number of NR1 cells in the genital mucosa was significantly diminished following treatment with the $\alpha 4$ blocking antibody relative to the isotype treated control mice (Fig 4A and B). In contrast, $\alpha 4\beta 7$ antibody treatment did not impact NR1 cell recruitment to the uterus, as absolute numbers were similar between isotype- and $\alpha 4\beta 7$ -treated groups. The absolute numbers of NR1 cells present in the draining lymph nodes were not significantly different between the groups suggesting no general defect in trafficking of NR1 cells following antibody treatment. We also calculated a migration index (the ratio of live NR1 cells in the uteri to draining lymph nodes within the same animal) to normalize for mouse-to-mouse variability (Fig 4C). We found that the migration index was profoundly decreased in mice treated with $\alpha 4$ blocking antibody compared to isotype-treated mice. These results show that blocking $\alpha 4$ prevents efficient CD4⁺ T cell trafficking from the draining lymph nodes to the uteri following *C. trachomatis* infection. The migration index of mice treated with $\alpha 4\beta 7$ antibody was not statistically different from isotype control treated mice demonstrating that $\alpha 4\beta 7$ plays a limited role in NR1 cell trafficking to the genital mucosa in response to *C. trachomatis* infection.

We next examined how antibody treatment directly alters the recruitment of effector CD4⁺ T cell populations in the genital mucosa. NR1 cells were stained for CD44 and CD62L in order to evaluate the presence of effector CD4⁺ T cells at the site of the infection. The absolute number of CD44⁺CD62L^{low} NR1 cells in the uterus was significantly decreased only in the $\alpha 4$ antibody-treated group compared to isotype-treated mice (Fig 4D). Moreover, anti- $\alpha 4\beta 7$ antibody treatment did not significantly decrease the absolute number of effector NR1 cells in the genital tract relative to the isotype-treated group. We confirmed the efficiency of $\alpha 4\beta 7$ antibody blockade *in vitro* (data not shown). These results show that disrupting the integrin $\alpha 4\beta 1$ but not $\alpha 4\beta 7$ on NR1 cells is sufficient to eliminate CD4⁺ T cell-mediated protection provided following *C. trachomatis* infection in the genital tract.

Integrin $\beta 1$ -deficient *C. trachomatis*-specific CD4⁺ T cells are unable to protect the uterus

To complement the antibody-blocking experiments showing that $\alpha 4\beta 1$ is required for *Chlamydia*-specific CD4⁺ T cells to home to and protect the genital mucosa, we generated TCR transgenic mice in which the T cells are deficient in either integrin $\beta 1$ or $\beta 7$. Because loss of $\beta 1$ results in embryonic lethality (28), we used a CRE-Flox system to generate NR1 cells conditionally deficient in $\beta 1$. NR1 transgenic mice were crossed to *Lck-CRE* and *Itgb1^{flox/flox}* animals such that only the lymphocytes were deficient in $\beta 1$. We also crossed NR1 transgenic mice with *Itgb7^{-/-}* mice to generate *Chlamydia*-specific CD4⁺ T cells deficient in $\beta 7$. We first confirmed that integrin surface expression was significantly altered for each knockout T cell genotype (Supp. Fig 1A and B). Interestingly, loss of $\beta 1$ led to a concomitant increase of surface $\beta 7$ on NR1 cells similar to a previous report showing that $\alpha 4\beta 7$ heterodimers form more readily in the absence of the $\beta 1$ chain (29). The loss of $\beta 7$ also led to an increase of the percentage of $\beta 1^+$ NR1 cells after *in vitro* activation. LFA-1 was robustly upregulated on NR1 cells from the three different genotypes. However, no compensatory LFA-1 expression was observed between integrin deficient T cells. In addition, NR1 cells do not express CD103 upon *in vitro* activation (data not shown). We next confirmed that NR1 cells deficient in individual integrins proliferated normally. Integrin-deficient NR1 cells were harvested from the knockout mice and polarized *in vitro* for 5 days to a Th1 phenotype. We found no significant difference between groups in the total number of recovered NR1 cells 5 days following activation, demonstrating that all the genotypes are viable (Fig 5A). We next examined the activation and cytokine production for each group. For all genotypes examined, NR1 cells were robustly activated as determined by staining for the activation markers CD25 and CD44 (Fig 5B). When we assayed cytokine profiles using intracellular cytokine staining, we found similar levels of IFN- γ and TNF- α in all groups, demonstrating that loss of integrin β chains does not negatively impact Th1 differentiation (Fig 5C). Therefore, the absence of either $\beta 1$ or $\beta 7$ does not interfere with expansion, activation, and Th1 cytokine production of NR1 cells *in vitro*.

Upon showing that the various genotypes of NR1 cells had normal effector phenotypes, we then tested whether deficiency in either the $\beta 1$ or $\beta 7$ chain would adversely affect the protective capacity of *Chlamydia*-specific CD4⁺ T cells *in vivo*. Based on our previous results from antibody blocking experiments, we hypothesized that $\beta 1$ -deficient NR1 cells would be unable to protect the genital mucosa from *C. trachomatis* infection. We transferred 10^5 wildtype, $\beta 1^{-/-}$ or $\beta 7^{-/-}$ Th1-skewed NR1 cells into naive mice 1 day prior to transcervical infection with *C. trachomatis* (Fig 5D). Five days after infection, we harvested the genital tract and quantified the levels of *C. trachomatis* using qPCR. As expected, mice that received wildtype NR1 cells showed a significant reduction in *C. trachomatis* burden compared to mice that received no transfer. Mice that received $\beta 1^{-/-}$ NR1 cells were not protected against *C. trachomatis* infection as indicated by bacterial burdens similar to mice receiving no transferred NR1 cells. In line with our previous findings using antibody blockade, we also found that mice receiving $\beta 7^{-/-}$ NR1 cells were significantly protected against *C. trachomatis* infection relative to mice that received no transfer, and trended toward being even more protective than wildtype NR1 cells. These findings definitively show that integrin $\beta 7$ on NR1 cells is dispensable for protecting the uterus from *C.*

trachomatis infection. In summary, integrin $\beta 1$, but not $\beta 7$, is necessary for *Chlamydia*-specific CD4⁺ T cells to protect against infection in the genital mucosa.

Integrin $\beta 1$ deficiency impairs *C. trachomatis*-specific CD4⁺ T cell homing to the uterus

We next sought to understand the mechanisms responsible for the loss of protective capacity in $\beta 1^{-/-}$ *Chlamydia*-specific CD4⁺ T cells. Our previous data using antibody blocking showed that T cell trafficking to the genital mucosa was inhibited and therefore provided limited protection. Here we used a competitive homing assay to test the trafficking potential of integrin-deficient *Chlamydia*-specific CD4⁺ T cells. We directly compared the migration of integrin-sufficient and deficient NR1 cells under identical conditions within the same infected host. We transferred an equal number of CD45.2/CD90.2 $\beta 1^{-/-}$, $\beta 7^{-/-}$ or wildtype NR1 cells and CD45.2/CD90.1 wildtype NR1 cells into congenically mismatched CD45.1 recipients. The next day, we infected mice transcervically with *C. trachomatis*. Seven days after infection, we isolated and processed tissues to quantify the numbers of both NR1 populations using flow cytometry. We found that $\beta 1^{-/-}$ NR1 cells were far less efficient than wildtype NR1 cells in trafficking to the genital mucosa, while $\beta 7^{-/-}$ NR1 cells outcompeted their wildtype counterparts (Fig 6A). None of the experimental groups showed defects in their migration to the draining lymph node. Interestingly, we noted a higher percentage of $\beta 1^{-/-}$ NR1 cells in the lymph nodes compared to the wildtype NR1 cells, inverse of what we observed in the genital mucosa. These results are likely due to a decreased ability of $\beta 1^{-/-}$ NR1 cells to leave the circulation to enter the infected genital mucosa. To normalize for mouse-to-mouse variation in absolute T cell numbers, we calculated a migration index for each mouse by comparing the ratio of the two transferred NR1 cell populations in the uterus (integrin deficient CD90.2% : integrin sufficient CD90.1%) to the ratio of the transferred populations in the draining lymph nodes or spleen within the same animal (Fig 6B). A smaller migration index indicated less efficient trafficking of integrin-deficient NR1 cells specifically to the uterus relative to the circulation. We found a dramatically lower migration index for $\beta 1^{-/-}$ NR1 cells demonstrating that trafficking to the uterus during infection was significantly impaired relative to wildtype NR1 cells. Intriguingly, the migration index for $\beta 7^{-/-}$ NR1 cells was significantly higher than wildtype, demonstrating enhanced homing of *Chlamydia*-specific CD4⁺ T cells to the uterus in the absence of integrin $\beta 7$. These results suggest that integrin $\beta 7$ is not only dispensable, but that deficiency of $\beta 7$ enhances antigen-specific CD4⁺ T cell migration to the genital tract during *C. trachomatis* infection. Our findings collectively reveal that integrin $\beta 1$ plays a crucial role in trafficking of CD4⁺ T cells to the genital mucosa and that absence of $\beta 1$ negatively affects the protective capacity of *C. trachomatis*-specific CD4⁺ T cells.

Discussion

The orchestration of events required for a successful T cell response determines whether an intracellular pathogen will be eliminated from the host. In the case of *C. trachomatis* infection, a robust CD4⁺ Th1 cell population that homes to the genital tract provides the vigorous IFN- γ response necessary for bacterial clearance (10). The integrin receptors important for *C. trachomatis*-specific CD4⁺ T cell-mediated protection have not been

previously described. Using *C. trachomatis*-specific CD4⁺ T cells, we demonstrated that perturbing integrin $\alpha 4\beta 1$ but not $\alpha 4\beta 7$, through antibody blockade or genetic deletion, results in impaired T cell trafficking to the genital tract and a loss of protective capacity following *C. trachomatis* infection. Together these observations demonstrate that integrin $\alpha 4\beta 1$ is necessary for CD4⁺ T cell-mediated protection against *C. trachomatis* infection in the murine genital tract.

We previously demonstrated that the chemokine receptors CXCR3 and CCR5 are required for *Chlamydia*-specific CD4⁺ T cells to home to and protect the genital mucosa following infection with *C. trachomatis* (22). Here we extended these studies to identify integrin receptors that are important for CD4⁺ T cell-mediated protection. When we examined the surface expression of $\beta 1$ and $\beta 7$ on both bulk and antigen-specific CD4⁺ T cells, we found that relative $\beta 1$ expression was dramatically increased on the majority of CD4⁺ T cells found in the uterus following *C. trachomatis* infection (Fig 1A and 2A). These findings are in line with Perry et al who demonstrated that surface $\beta 1$ is upregulated on bulk lymphocytes localized in the genital tract but not in the intestinal mucosa following infection with the mouse-adapted species *Chlamydia muridarum* (25). Although most CD4⁺ T cells were expressing high levels of surface integrin $\beta 1$, this report also noted a minor percentage of $\beta 7^+$ CD4⁺ T cells in the genital tract (25). Separate reports suggested that $\alpha 4\beta 7$ was the dominant integrin receptor expressed on CD4⁺ T cells in the genital tract. However, these studies had significant experimental constraints that limit their interpretation. For example, Kelly et al did not interrogate the integrin profile during the primary response or differentiate between antigen-specific and bystander CD4⁺ T cells in the genital tract (30). Another report examined the surface integrin receptors on a memory CD4⁺ T cell line in culture following stimulation but did not assess *in vivo* integrin dynamics (23). Memory CD4⁺ T cells can exert effector activities with greater ease than primary T cells (31), therefore examining memory cells is not indicative of the trafficking properties of T cells in a primary response. Because these studies did not examine naïve, antigen-specific CD4⁺ T cells they were unable to recapitulate the initial activation events during primary *C. trachomatis* infection. In addition, these reports did not examine how perturbation of distinct integrin complexes alters the protective capacity of CD4⁺ T cells responding to genital *C. trachomatis* infection.

The results presented here show that $\alpha 4\beta 1$ drives *Chlamydia*-specific CD4⁺ T cells to the infected genital mucosa (Fig 4 and 6) and is required to mediate protective immunity following *C. trachomatis* infection (Fig 3 and 5). While $\alpha 4\beta 1$ was the dominant integrin in the genital mucosa we did identify a second $\beta 7^+$ population that when perturbed did not alter immunity to *Chlamydia* infection. The existence of two NR1 cell populations with distinct integrin profiles and identical TCR specificity shows that integrin levels are not hardwired but rather imprinted during T cell activation as has been suggested previously (32). Our results also suggest that only CD4⁺ T cells with the correct integrin profile can extravasate into the infected genital mucosa. We speculate that while $\alpha 4\beta 1$ -expressing T cells can enter the infected tissue efficiently, the $\alpha 4\beta 7^+$ CD4⁺ T cells remain in the circulation and fail to provide protection in the genital mucosa. Because the entire uterus, including its associated vasculature, was harvested in all experiments it was not possible to distinguish between

those T cells present in blood vessels and those that had completed transendothelial migration. It is possible that the $\alpha 4\beta 7^{+}$ $CD4^{+}$ T cell population still contributes to immunity elsewhere in the mouse, but this remains to be tested. Interestingly, one recent report uncovered a mechanism that drives distinct integrin expressing T cell populations in the lungs of mice. Ruane et al found that a subset of residing dendritic cells (DCs) can imprint lung T cells to express $\alpha 4\beta 7$. The T cells expressing $\alpha 4\beta 7$ do not mediate protection in the lungs but rather provide gut mucosal immunity (33). Given that a subset of lung DCs can imprint a population of T cells to express $\alpha 4\beta 7$, we speculate that a similar process also occurs in the genital tract. While the majority of $CD4^{+}$ T cells are primed to express $\alpha 4\beta 1$, which mediates immunity in the genital mucosa during *C. trachomatis* infection, perhaps a subset of uterine DCs also imprints $\alpha 4\beta 7$ on a fraction of the $CD4^{+}$ T cell population that may provide systemic mucosal immunity. This hypothesis will need to be examined in future experiments.

It is well established that integrin $\alpha 4\beta 1$ adheres to the addressin VCAM-1 and the extracellular matrix protein fibronectin (34, 35). Surface VCAM-1 increases on endothelial cells lining microvessels following inflammation (27). For example, patients with CNS autoimmune disorders are often treated with natalizumab, an anti- $\alpha 4$ antibody that blocks $\alpha 4\beta 1$ and $\alpha 4\beta 7$ interactions with their ligands. It is thought that natalizumab treatment decreases undesirable inflammation in the CNS by interfering with $\alpha 4\beta 1$ -mediated immune cell recruitment to this sensitized area in the body (36). Previous studies have also shown that surface VCAM-1 becomes abundant on murine and human genital mucosa following *Chlamydia* infection. In contrast, the expression of the mucosal addressin cell adhesion molecule-1 (MAdCAM-1), the binding partner for $\alpha 4\beta 7$, has been reported to be expressed robustly in the gut but only modestly in the genital tract (25, 37). The noticeable increase of surface VCAM-1 in the genital mucosa following *Chlamydia* infection corresponds to the upregulation of surface $\alpha 4\beta 1$ on *Chlamydia*-specific $CD4^{+}$ T cells in the uterus observed in this study.

Several other signals stimulate the rapid increase of VCAM-1 on vaginal epithelial cells including IFN- γ treatment and herpes simplex virus infection (38). Given that VCAM-1 can be selectively upregulated on both endothelial and non-endothelial cells, responding $CD4^{+}$ T cells may require $\alpha 4\beta 1$ signaling for multiple steps in order to provide protection. Previous studies have characterized the importance of $\alpha 4\beta 1$ to slow/arrest lymphocytes in the blood vessel, but $\alpha 4\beta 1$ may also mediate the interactions between effector $CD4^{+}$ T cells and infected epithelial cells as has been suggested to occur during *Chlamydia* infection (39). It remains unknown whether *C. trachomatis*-specific $CD4^{+}$ T cells directly interact with infected epithelial cells in the genital mucosa or if their antimicrobial effects occur by altering the cytokine milieu at the site of infection. In this study we observed that integrin $\beta 1$ and $\beta 7$ were dispensable for proliferation and differentiation to a Th1 phenotype (Fig 5A, B and C). Consequently, we conclude that the function of $\beta 1$ in mediating protection (Fig 5D) is to allow successful *Chlamydia*-specific $CD4^{+}$ T cell trafficking to the uterus (Fig 6) rather than playing a role in activation or production of IFN- γ . Future studies must further explore the interaction between IFN- γ -producing $CD4^{+}$ T cells and the infected epithelium to determine whether $\beta 1$ is required for cellular interactions *in vivo* within the genital mucosa.

Additional studies will need to determine if T cell recruitment and effector activity, mediated by $\alpha 4\beta 1$, contributes to genital tract pathology following *Chlamydia* infection. Although integrin $\beta 1$ is found primarily in complex with $\alpha 4$ on T cells, our findings do not entirely eliminate the possibility that $\beta 1$ can form additional heterodimers on CD4⁺ T cell and that these heterodimers may play a role in the retention of T cells in the genital tract following infection.

The results obtained through this study further elucidate the essential homing receptors required for an effective CD4⁺ T cell defense in the genital mucosa. It remains to be determined whether CD4⁺ T cells responding to other pathogens in the genital tract also require the homing receptors we have identified (CXCR3, CCR5 and $\alpha 4\beta 1$) as required for successful trafficking during *C. trachomatis* infection. Generating a robust and long-lived protective T cell response is crucial to clear infection and avoid recurrent cycles of inflammation and associated pathology. Vaccine efforts against intracellular pathogens should examine whether the appropriate T cell population, with the necessary homing molecules, is being generated to ensure protection and minimize pathology. In addition to the current treatments for autoimmune diseases in the gut and the CNS, integrin and chemokine receptor targeted therapies could be used to selectively shape the recruitment of desired T cells to other mucosal tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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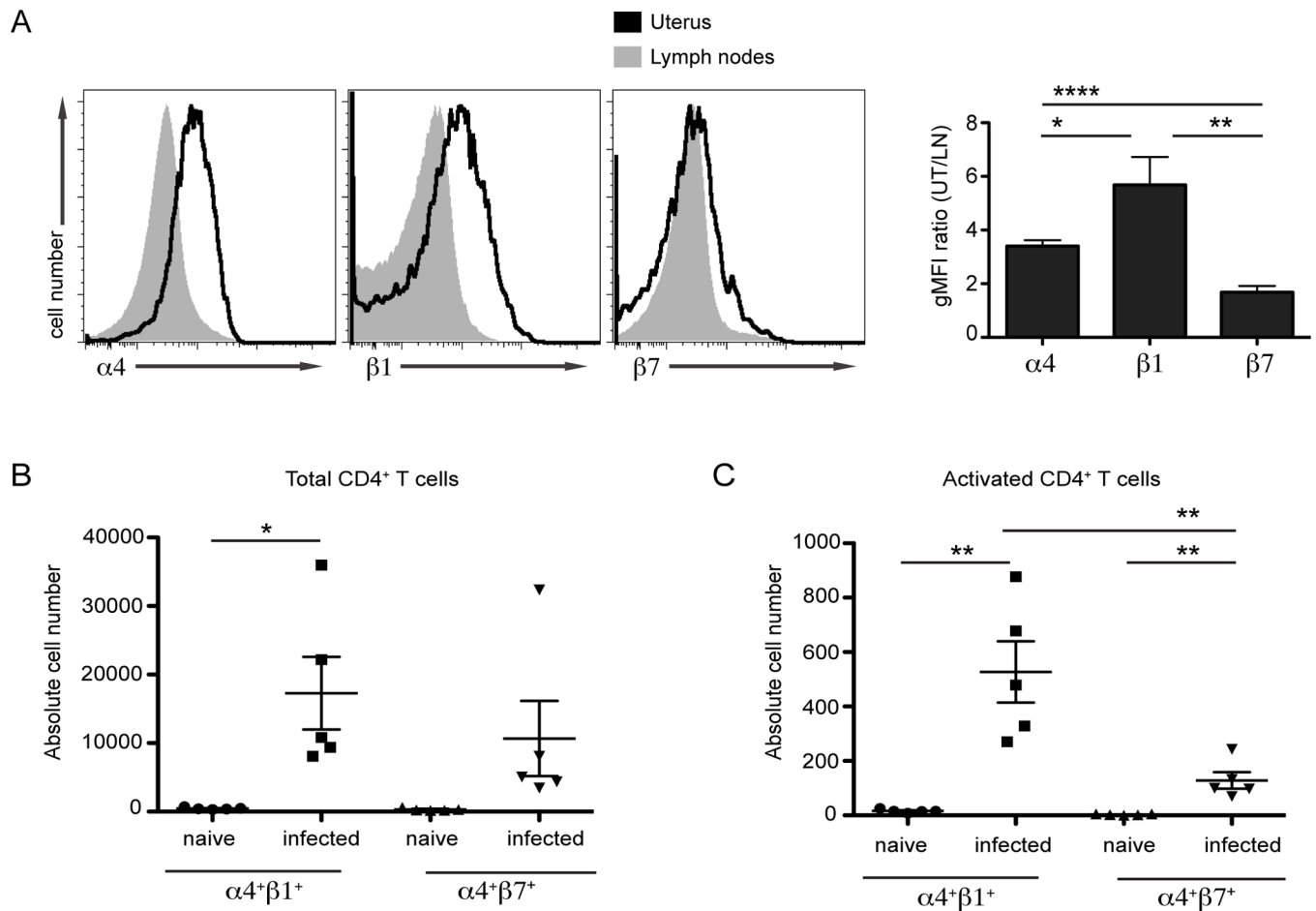


FIGURE 1.

C. trachomatis infection leads to robust integrin $\alpha 4 \beta 1$ surface expression on polyclonal CD4⁺ T cells in the genital tract. C57BL/6 mice were transcervically infected with 10^6 IFU of *C. trachomatis*. Seven days following infection the indicated tissues were harvested and prepared for flow cytometry. After gating on live cells that were CD3⁺CD4⁺ the surface expression of $\alpha 4$, $\beta 1$ and $\beta 7$ was quantified. (A) The integrin surface expression was analyzed by comparing the geometric mean fluorescence intensity (gMFI) ratio of CD4⁺ T cells localized in the genital mucosa to those in the draining lymph node. (B) The absolute number of $\alpha 4^+ \beta 1^+$ and $\alpha 4^+ \beta 7^+$ CD4⁺ T cells were quantified in the genital tract of naïve or mice infected with *C. trachomatis* for seven days. (C) The absolute number of activated $\alpha 4^+ \beta 1^+$ and $\alpha 4^+ \beta 7^+$ CD4⁺ T cells was quantified in the genital tract of naïve or infected mice; activation was determined by CD44⁺ staining. Shown are representative results from one of two independent experiments. * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$.

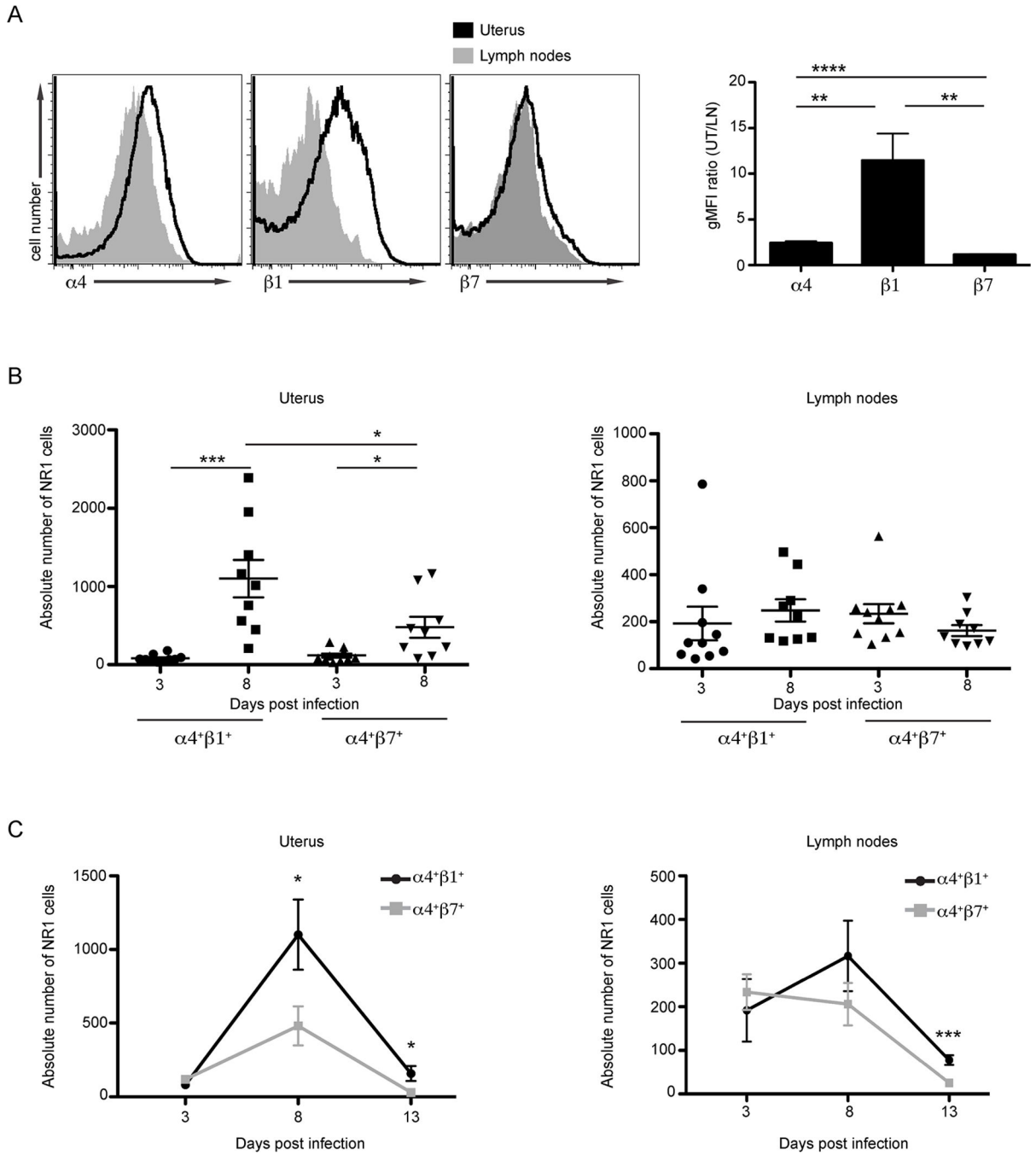


FIGURE 2.

C. trachomatis infection leads to robust α4β1 surface expression on *C. trachomatis*-specific CD4⁺ T cells responding to the genital tract. One million CD90.1⁺ NR1 cells were transferred intravenously into CD90.2⁺ recipient mice. The following day mice were infected transcervically with 10⁶ IFU of *C. trachomatis*. The uterus and draining lymph nodes were harvested at the indicated time points following infection and prepared for flow cytometry. For integrin surface staining analysis, cells were pre-gated as live Vα2⁺CD4⁺CD90.1⁺ cells and then examined for the surface expression of α4, β1, and β7. **(A)** Eight days after *C. trachomatis* infection, the integrin surface expression on NR1 cells was analyzed by comparing the gMFI ratio of NR1 cells in uterus to those localized in the draining lymph nodes. **(B)** The absolute numbers of α4⁺β1⁺ and α4⁺β7⁺ NR1 cells was

quantified 3 and 8 days after transcervical infection in the uterus (left) and the draining lymph nodes (right). (C) Quantification of the trafficking kinetics of $\alpha 4^+ \beta 1^+$ and $\alpha 4^+ \beta 7^+$ NR1 cells in the uterus (left) and draining lymph nodes (right) at the indicated time points. Shown are representative results from one of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

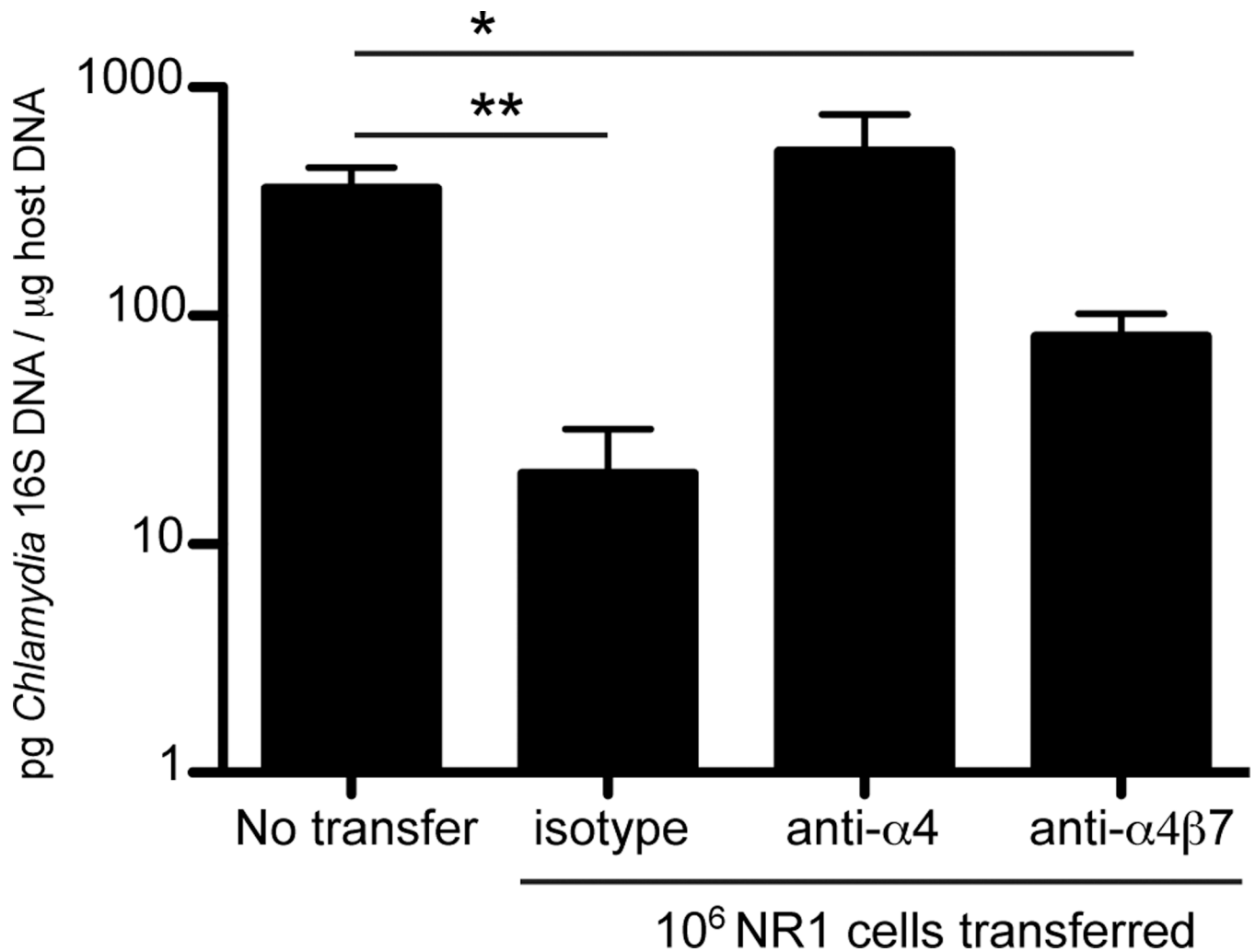


FIGURE 3.

Antibody blockade of $\alpha 4$ but not $\alpha 4\beta 7$ exacerbates *C. trachomatis* burden in the genital mucosa. NR1 cells were skewed *in vitro* to a Th1 phenotype for 5 days. NR1 cells were pretreated with the indicated antibodies and then transferred intravenously into naïve recipients. The following day, mice were infected transcervically with 5×10^6 IFU of *C. trachomatis*. Groups were injected with the respective integrin or isotype control antibody 1 and 3 days following infection. Five days after infection, the genital tract was isolated and genomic DNA was purified. The levels of *Chlamydia* 16S DNA relative to the levels of host GADPH were quantified using qPCR. Shown are representative results from one of two independent experiments. * $p < 0.05$, and ** $p < 0.01$.

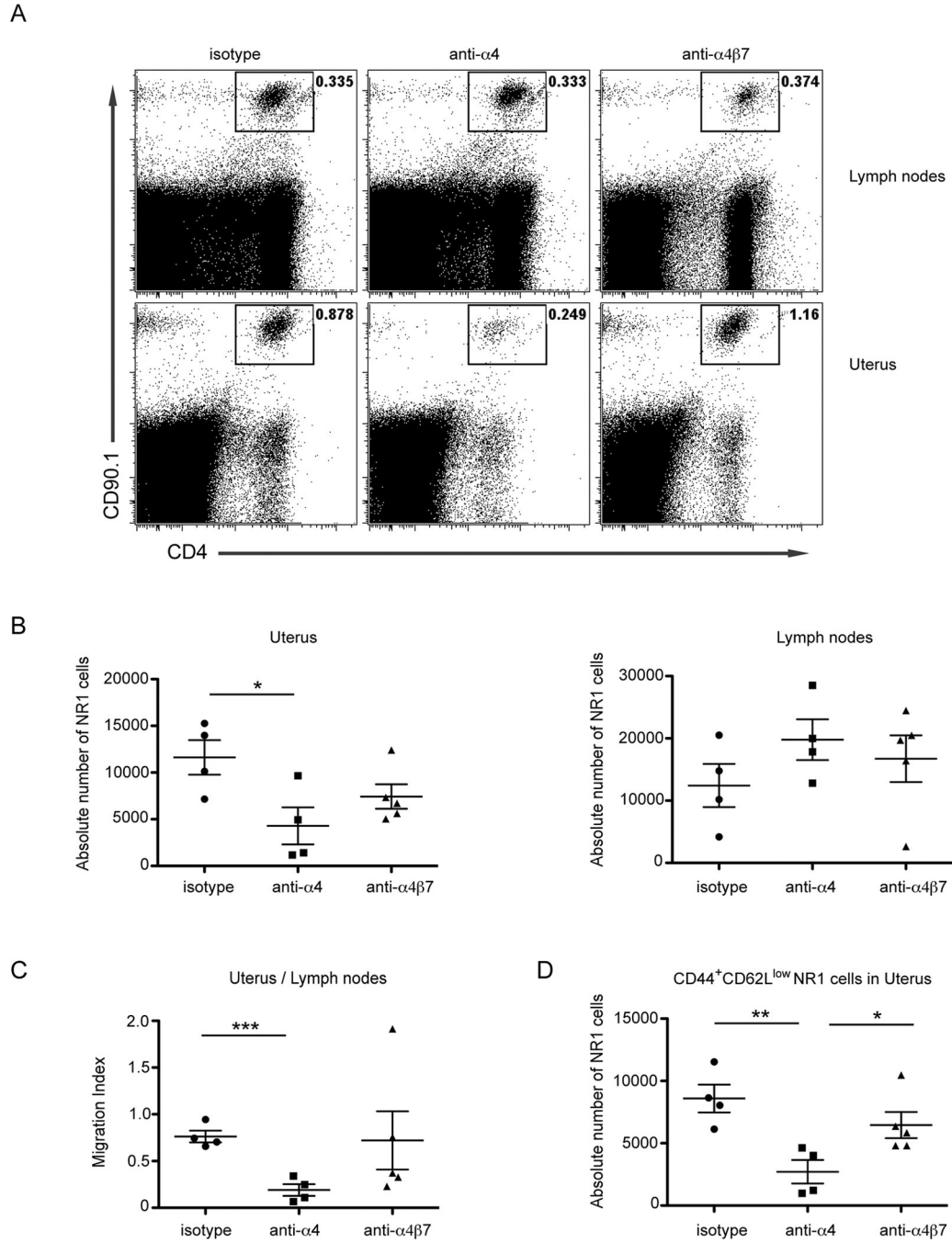


FIGURE 4.

Blockade of $\alpha 4$ but not $\alpha 4\beta 7$ impairs *C. trachomatis*-specific CD4⁺ T cell trafficking to the genital tract following infection. NR1 cells were skewed *in vitro* to a Th1 phenotype for 5 days. NR1 cells were pretreated with the indicated antibodies and then 10^6 CD90.1⁺ NR1 cells were transferred intravenously into CD90.2⁺ host mice. The following day mice were infected transcervically with 5×10^6 IFU of *C. trachomatis*. Groups were injected with the respective integrin or control antibody 1 and 3 days following infection. The uterus and draining lymph nodes were harvested 5 days following infection and prepared for flow cytometry. For quantification of NR1 cell trafficking, we gated on live V $\alpha 2^+$ CD4⁺CD90.1⁺ cells. (A) Representative flow cytometry plots indicate the percentage of NR1 cells following the indicated antibody treatment in the draining lymph nodes

(top) and uterus (bottom) **(B)** The absolute number of NR1 cells were quantified in the uterus (left) and draining lymph nodes (right). **(C)** A migration index for each antibody treatment was calculated by comparing the absolute number of live NR1 cells in the uterus directly to the number of NR1 cells in draining lymph nodes within each animal. A lower migration index ratio indicates decreased NR1 cell recruitment to the uterus. **(D)** The absolute number of effector NR1 cells was determined by examining the CD44⁺CD62L^{low} gated population in the genital mucosa. Shown are representative results from one of two independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

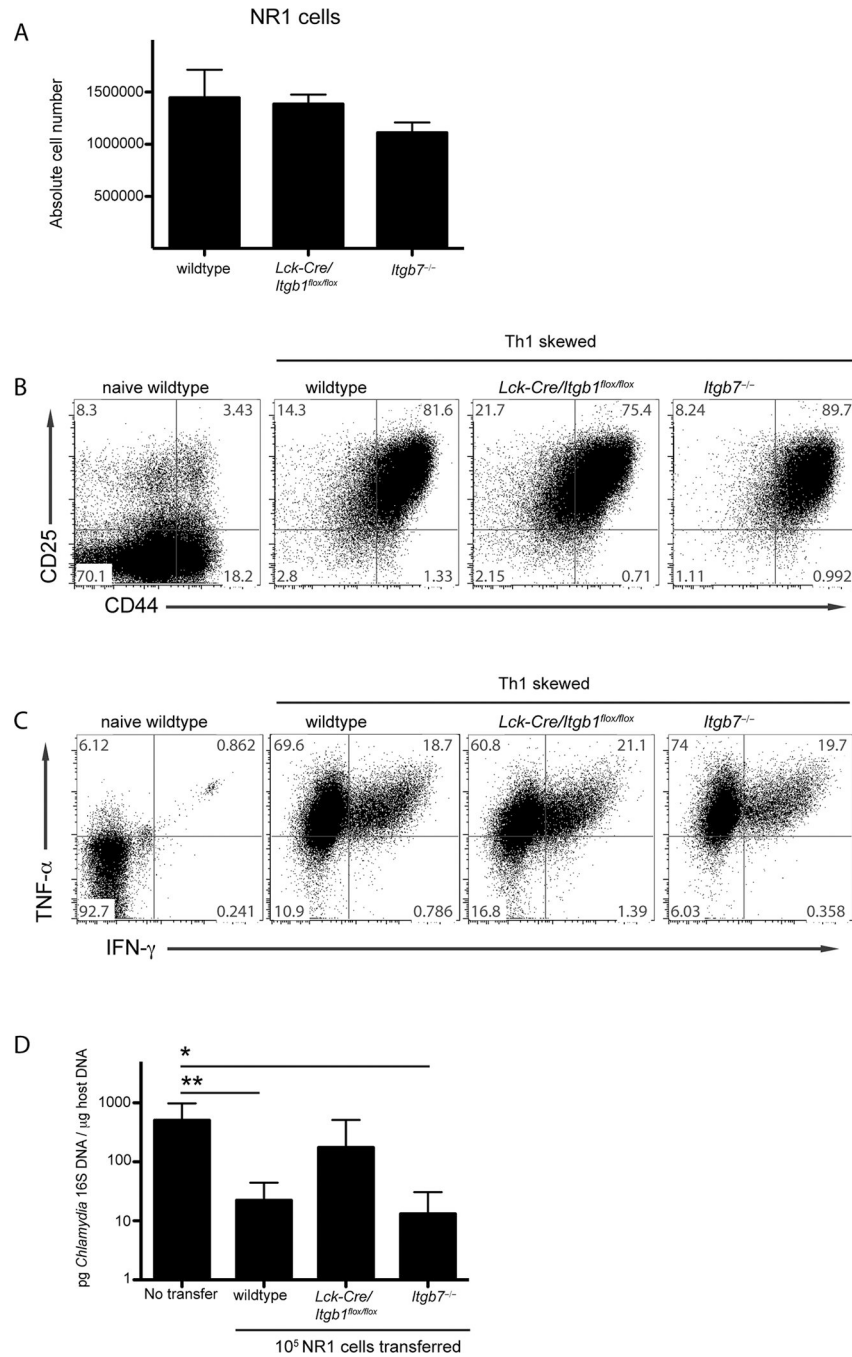
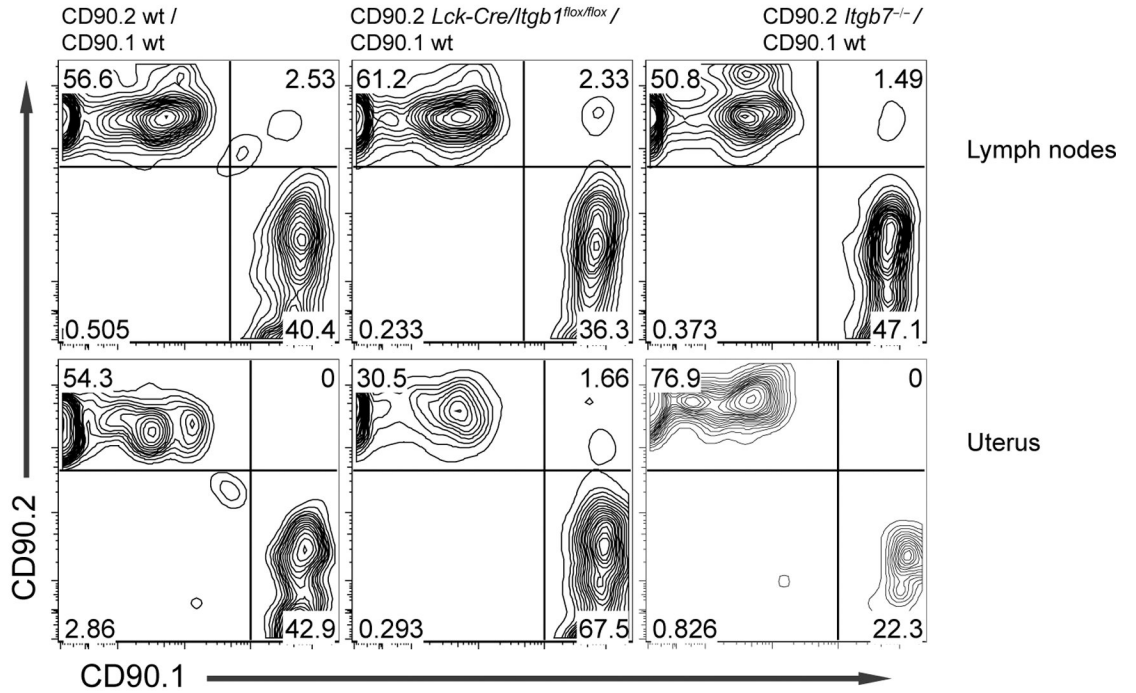


FIGURE 5.

Chlamydia-specific CD4⁺ T cells deficient in integrin β1 are unable to protect mice from *C. trachomatis* infection. The indicated NR1 genotypes were skewed *in vitro* to a Th1 phenotype for 5 days. **(A)** The expansion of NR1 cells was compared for the three genotypes. **(B)** The activation of live NR1 cells was assessed by gating for CD25⁺CD44⁺ cells. **(C)** TNF-α and IFN-γ production was examined using intracellular cytokine staining. **(D)** After 5 days of *in vitro* stimulation, 10⁵ NR1 cells were transferred intravenously into naïve hosts. The following day, mice were transcutaneously infected with 5x10⁶ IFU of *C. trachomatis*. Five days after infection, the genital tract was harvested and genomic DNA was purified. The levels of *Chlamydia*

16S DNA relative to the levels of host GAPDH were quantified using qPCR. Shown are representative results from one of three independent experiments. * $p < 0.05$ and ** $p < 0.01$.

A



B

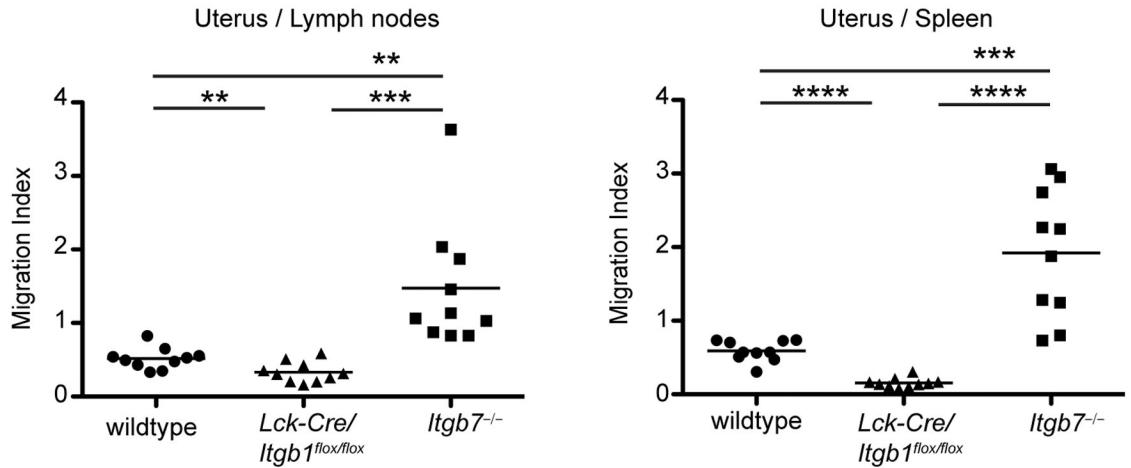


FIGURE 6.

$\beta 1$ deficient *Chlamydia*-specific CD4⁺ T cells are unable to traffic efficiently to the genital tract following infection. An equivalent number of CD45.2/CD90.1 integrin wildtype NR1 cells and CD45.2/CD90.2 wildtype, $\beta 1^{-/-}$, or $\beta 7^{-/-}$ NR1 cells were transferred intravenously into CD45.1/CD90.2 host mice. The next day, mice were infected transcervically with 10^6 IFU of *C. trachomatis*. The genital tract, draining lymph nodes and spleen were harvested 7 days after infection and prepared for flow cytometry. We examined the recruitment of NR1 cells by pre-gating on live Va2⁺CD4⁺CD45.2⁺ cells. We then differentiated the competing NR1 populations by examining the number of CD90.1⁺ or CD90.2⁺ cells. (A) Shown are representative plots of integrin sufficient and deficient NR1 cells in the draining lymph node (top) and genital mucosa (bottom). (B) The migration

index within each group was calculated by comparing the percentage of CD90.2⁺ to CD90.1⁺ NR1 cells in the uterus to the CD90.2⁺ to CD90.1⁺ NR1 cells in the draining lymph nodes (left) or spleen (right). A lower migration index indicates less efficient trafficking of integrin-deficient NR1 cells specifically to the uterus relative to the circulation. Shown are representative results from one of three independent experiments. ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.