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CX_3CR1^+ cell-mediated *Salmonella*-exclusion protects the intestinal mucosa during the initial stage of infection

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Abstract

During *Salmonella* Typhimurium infection intestinal CX_3CR1^+ cells can either extend transepithelial cellular processes to sample luminal bacteria or, very early after infection migrate into the intestinal lumen to capture bacteria. However, up to date, the biological relevance of the intraluminal migration of CX_3CR1^+ cells remained to be determined. We addressed this by using a combination of mouse strains differing in their ability to carry out CX_3CR1-mediated sampling and intraluminal migration. We observed that, the number of *S.* Typhimurium traversing the epithelium did not differ between sampling-competent/migration-competent C57BL/6 and sampling-deficient/migration-competent Balb/c mice. By contrast, in sampling-deficient/migration-deficient $CX_3CR1^{-/-}$ mice the numbers of *S.* Typhimurium penetrating the epithelium were significantly higher. However, in these mice the number of invading *S.* Typhimurium was significantly reduced after the adoptive transfer of CX_3CR1^+ cells directly into the intestinal lumen, consistent with intraluminal CX_3CR1^+ cells preventing *S.* Typhimurium from infecting the host. This interpretation was also supported by a higher bacterial faecal load in $CX_3CR1^{+/gfp}$ compared to $CX_3CR1^{gfp/gfp}$ mice following oral infection. Furthermore, by using real time *in vivo* imaging we observed that $CX3CR1^+$ cells migrated into the lumen moving through paracellular channels within the epithelium. Also, we reported that the absence of CX_3CR1-mediated sampling did not affect antibody responses to a non-invasive *S.* Typhimurium strain that specifically targeted the CX_3CR1-mediated entry route. These data showed that the rapidly deployed CX_3CR1^+ cell-based mechanism of immune-exclusion is a defence mechanism against pathogens that complements the mucous and secretory (s)IgA antibody-mediated system in the protection of intestinal mucosal surface.

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Introduction

One of the main tasks of the epithelium overlying mucosal surfaces of the intestinal tract is to provide an effective barrier to microorganisms present in the intestinal lumen. Firstly, this is achieved by the presence of tight junctions that allow the passage of water and ions but provide an effective mechanical barrier to macromolecules and microbes (1). Secondly, a combination of thick flowing mucus and secretory (s)IgA bathing mucosal surfaces provide an efficient gel that sequestrates harmful microorganisms and prevent them from crossing the epithelial barrier in a process known as immune-exclusion (2, 3). Furthermore, it has been recently shown that a few hours after infection the epithelium-intrinsic NAIP/NLRC4 inflammasone drove the expulsion of infected epithelial cells to restrict *S.* Typhimurium replication in the mucosa (4). Ultimately, the aim of these protective mechanisms is to prevent pathogens from traversing/colonizing the intestinal mucosa. We have previously reported that intestinal challenge with *S.* Typhimurium induced, very shortly after infection the migration into the intestinal lumen of *S.* Typhimurium-capturing cells expressing the high affinity receptor CX_3CR1 for the chemokine fractalkine (CX_3CL1) into the intestinal lumen (5), a chemokine that although expressed by a variety of cells is produced at its highest level by the intestinal epithelial cells (IECs) of the ileum (6). The migration of CX_3CR1^+ cells following challenge with *S.* Typhimurium was restricted to the small intestine in a flagellin/MyD88-dependent manner and did not affect the integrity of the epithelial barrier (5). These observations prompted us to test the hypothesis that *Salmonella*-capturing CX_3CR1^+ cells migrate rapidly into the intestinal lumen to limit the number of pathogens crossing the epithelial barrier. Interestingly, in the occurrence of infection with *S.* Typhimurium, CX_3CR1^+ cells displayed a dual behaviour. Indeed, these cells can also directly sample bacteria by using cellular extensions that protrude between epithelial cells and shuttle them across the epithelium to initiate immune responses (7, 8). Importantly, the presence of the fractalkine receptor CX_3CR1 appeared to be essential for both events (6, 9). However, while CX_3CR1-mediated sampling plays a role in the generation of immune responses (7) the biological relevance of the intraluminal migration of the CX_3CR1^+ cells during the early stages of infection remained to be determined. We sought to address this issue by using a combination of mouse strains that differed in their ability to undergo CX_3CR1-mediated direct sampling and intraluminal migration during *S.* Typhimurium infection. Indeed, while wild-type (wt) C57BL/6 mice responded to *S.* Typhimurium with CX_3CR1-mediated sampling (8) and migration (5), wt Balb/c mice lacked the ability to sample luminal antigen via this route (sampling-deficient) (10) but were migration-competent (5). Furthermore these two mouse strains were complemented with CX_3CR1-deficient mice that were both sampling- and migration-deficient (6, 9). We observed that the rapid *Salmonella*-induced intraluminal migration of CX_3CR1^+ cells reduced significantly the bacterial load in the intestinal tissue thus contributing effectively to the immune-exclusion provided by the mucous barrier and sIgA-based system.

Materials and methods

Mice

6-8 week old female $CX_3CR1^{gfp/gfp}$, Balb/c and C57BL/6 background (11) were used as CX_3CR1-deficient mice and bred with wt Balb/c or C57BL/6 mice to obtain heterozygotes $CX_3CR1^{+/gfp}$. $CX_3CR1^{-/-}$ (C57BL/6 background) were purchase from Taconic and 6-8 week old wt Balb/c and C57BL/6 were purchase from Charles River. Villin-Cre MyD88 ($MyD88^{\Delta IEC}$) (C57BL/6 background) were from the Welcome Trust Sanger Institute, Hinxton, UK (12). $CX_3CR1^{+/-}$ and $CX_3CR1^{-/-}$ on $RAG^{-/-}$ background (B6.129S7-Rag1tm1Mom/J, Jackson Laboratory) were obtained by an intercross between the knock-out mice. Mice on $RAG^{-/-}$ background were kept in SPF high barrier environment. Overall mice were kept under standardized conditions in groups of 3–5/cage. Food and water were provided *ad libitum*. Experiments were conducted under the guidelines of the Scientific Procedure Animal Act (1986) of the United Kingdom or at the University of Siena under the "Guiding Principles for Research Involving Animals and Human Beings". Intestinal surgery was performed under terminal anaesthesia induced and maintained throughout the procedure by inhalation of isoflurane.

Bacteria

The *S.* Typhimurium SL1344 *ΔinvA*::kan mutant was constructed using the Lambda Red recombination system as described previously (13). Briefly, using primers invARedF (TGAAAAGCTGTCTTAATTTAATATTAACAGGATACCTATA) and invARedR (ATATCCAAATGTTGCATAGATCTTTTCCTTAATTAAGCCC) the entire coding sequence of *invA* was replaced by a flippase recognition target (FRT)-flanked Km cassette from template plasmid pKD4. Recombinants were selected for kanamycin resistance and verified by PCR. The mutation was subsequently transduced by P22 into a clean SL1344 parent background and into SL3261 (Aro^-) to construct the double mutation.

Bacterial challenge

Isolated loops were injected with $1x10^7$ non-invasive/non-replicating $InvA^-Aro^-$ for intravital imaging experiment or invasive/non-replicating $InvA^+AroA^-$ *S.* Typhimurium for collecting intraluminal $CX_3CR1^{+/gfp}$ cells for phenotypic and quantitative analysis. Oral challenges were performed by gavages that were delivered five to ten minutes after administration of a solution of $NaHCO_3$ (10% wt/vol/200μl). In order to monitor intraluminal migration of CX_3CR1^+ cell or bacterial load in the gut tissue within 5h after infection mice received a single oral dose of $1x10^7$ of either $InvA^+AroA^-$, $InvA^-AroA^-$ or $InvA^-AroA^+$ *S.* Typhimurium; to determine long-term (5 days post-infection) bacterial load mice received a single dose of $1x10^7$ of $InvA^-Aro^+$ strain. To determine strain-specific susceptibility to *S.* Typhimurium infection mice received a single dose of $1x10^8$ wt $InvA^+AroA^+$ *S.* Typhimurium; finally to investigate antibody responses to non-invasive *S.* Typhimurium mice received three doses of $1x10^8$ $InvA^-Aro^-$ at three day interval. In order to monitor intraluminal migration of CX_3CR1^+ cells and faecal bacterial load mice received a single oral dose of $1x10^7$ of $InvA^+Aro^-$ *S.* Typhimurium. To determine translocation of non-invasive $InvA^-$ *S.* Typhimurium two approaches were undertaken. For short term experiments mice (n=8-10mice/group) were orally administrated with a single dose of

InvA^{-}*Aro*$^{-}$ *Salmonella* and sacrificed at 30, 60, 180 and 270 minutes post-infection. For long term experiments mice received the same dose of *InvA*$^{-}$*Aro*$^{+}$ and were sacrificed 5 days post-infection. Tissues (small intestine and PPs for short term experiments; PP, MLN and spleen for long term experiments) were harvested weighted and treated with gentamicin (1h at 37C°). After repeated washings in PBS tissues were homogenized. Serial dilutions of the homogenates were plated on LB agar and incubated overnight at 37C°. To determine antibody responses to non-invasive *Salmonella* strain mice received 3 doses of $1x10^{8}$ *InvA*$^{-}$*AroA*$^{-}$ *S.* Typhimurium at 3 day interval.

Intravital Two-Photon microscopy

Intestinal loops were performed as described (5); mice were then placed on a mouse holder; the temperature of the animals was maintained by an enclosed microscope temperature control system (Life Imaging Services, Basel, Switzerland). Two-photon excitation was done with a Chameleon Ultra II Ti: Sapphire laser and Chameleon Compact OPO (Coherent Inc., USA), and the fluorescence emission was measured with four PMTs with filters for 420/50, 525/50, 595/40 and 655/40 nm. The microscope system and data acquisition were controlled by the Imspector Pro 4.0 software. Image analysis was done with the Fiji/ImageJ package.

Immunofluorescence and transmission electron microscopy

Immunohistochemistry was carried out on 10 μm sections as described in detail elsewhere (14, 15). Briefly, non-specific binding sites were quenched with 5% bovine serum albumin; sections were then incubated with rabbit anti-entactin antibody (Abcam, Cambridge, UK) followed by Cy5-conjugated anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA) for 45 minutes. Sections were counterstained with TRITC-conjugated phalloidin (Sigma-Aldrich) and analysed with a Zeiss LSM 510 confocal microscope. For TEM analysis, samples were processed according to standard procedure (16) and examined with a Philips 201 electron microscope.

Antibody responses

S. Typhimurium-specific IgG and IgA were detected in serum and faeces. Briefly, serum was obtained after 1h incubation at 37C° and collected after centrifugation. Faecal samples were weighted and resuspended in PBS in presence of proteases inhibitors; debris-free supernatants were then collected after centrifugation. ELISA plates (Costar) were coated with lysate from wt *S.* Typhimurium obtained as described by others (17). Plates were blocked and then incubated with dilutions of both serum and faecal solution. After washing plate were incubated with anti IgA- and anti IgG-biotinylated antibody (Abcam, Cambridge, UK); this was followed by incubation with Streptavidin Peroxidase (Abcam). Also, numbers of single IgA-antibody forming cells (AFC) were detected using a modified ELISPOT assay developed in our lab (18) in 96-well membrane ELISPOT plates (Whatman) coated with lysate from wt *S.* Typhimurium as above.

Flow cytometry and isolation of CX3CR1^{+} cells

Following bacterial challenge, luminal contents were carefully recovered by gently flushing the intestine with PBS. Intraluminal $CX_3CR1^{+/gfp}$ cells were isolated and characterized by

flow cytometry as described in details elsewhere (5). Samples were analysed by BD FACSAria II (BD Biosciences). The following antibodies were used: CD11c (HL3) (BD Biosciences), CD103 (M290) (BD Biosciences), CD103 (2E7) (eBioscience), F4/80 (BM8) (eBioscience), MHC II (M5/114.15.2) (eBioscience), SiglecF (E50-2440) (BD Biosciences). For the isolation of CX_3CR1^{+} cells intestinal tissue from $CX_3CR1^{+/gfp}$ mice were collected and tissues repeatedly treated with HBSS containing EDTA (2mM). After each treatment tissues were shaken and supernatant discarded. After each wash an aliquot from the supernatant was analysed by microscopy to detect the presence of IECs; EDTA treatment was stopped (usually after 3-4 treatments) when epithelial cells were not present in the supernatant. Tissues were then treated for 50 minutes in RPMI 1640 with 10% FCS, 0.24mg/ml collagenase VIII (Sigma) and 40 U/ml DNase I (Roche) as described by others (19) ; after shaking cells suspensions were filtered and then purified by gradient separation as described before (5). Cells were sorted (>95% purity), suspended in PBS and injected into the intestinal lumen for pathogen exclusion assay.

Salmonella Typhimurium-exclusion assay

Experiments of adoptive transfer were performed to assess the ability of CX_3CR1 cells to prevent *S.* Typhimurium from traversing the epithelial barrier. $CX_3CR1^{+/gfp}$ and $CX_3CR1^{gfp/gfp}$ mice were used as donors and gfp-labelled CX_3CR1 cells were isolated as described above. $CX_3CR1^{-/-}$ mice (6 mice/group) were used as recipients and they received a single oral dose of $1x10^7$ invasive/non-replicating *InvA*$^{+}$*AroA*$^{-}$ *S.* Typhimurium approximately 5-10 minutes after the delivery of a solution of $NaHCO_3$ and then anesthetized. Initially four groups of mice were used. Group I was injected in the intestinal lumen with $0.5x10^3$ $CX_3CR1^{+/gfp}$ cells approximately 10 minutes after *S.* Typhimurium infection and sacrificed 30 minutes after infection; tissues were then removed, washed and homogenates plated on LB agar. Group II received the same number of $CX_3CR1^{+/gfp}$ cells after 10 minutes; subsequently the number of intraluminal $CX_3CR1^{+/gfp}$ cells was increased to $1x10^4$ with a second injection 30 minutes after infection. Mice were sacrificed 90 minutes after the initial *S.* Typhimurium infection and tissues treated as for group I. In group III mice received a total of $4.5x10^4$ *S.* Typhimurium in three injections administrated 10, 30 and 90 minutes after infection. This group of mice was sacrificed and tissue removed 120 minutes after infection. Group IV was treated as group I with the difference that the mice received $1x10^4$ $CX_3CR1^{+/gfp}$ cells after 10 minutes; mice were sacrificed 30 minutes post-infection. At each injection cells were equally divided into two injection sites approximately 1 cm and 3 cm from the pylorus. The same protocol was repeated in additional four groups of $CX_3CR1^{-/-}$ mice (V-VIII) that received adoptive transfer of $CX_3CR1^{gfp/gfp}$ cells

Permeability assay

Intestinal permeability to soluble (dextran) and particulate antigen (polystyrene micro particles) was measured in 6-8 week old $CX_3CR1^{gfp/gfp}$, $CX_3CR1^{+/gfp}$ and syngeneic wt mice (4 mice/group). FITC-labelled dextran (FD4; Sigma-Aldrich) was dissolved in PBS at a concentration of 100 mg/ml and administered to each mouse (44 mg/100 g body weight) by oral gavage. Blood samples were collected after 6 hours and the plasma analysed for FD4 concentration using fluorescence spectrometer at an excitation wavelength of 490 nm and

emission wavelength of 520 nm. Intestinal transport of polystyrene particles (Fluoresbrite YG Carboxylate Microspheres, 0.50μm) was assessed as described in detail elsewhere (20)

Statistics

Data are expressed as mean ± SD and statistical comparison made by the Student's unpaired t-test; p values were considered significant at $P < 0.05$.

Results

Bacteria translocation is increased in mice lacking intraluminal migration of CX_3CR1^+ cell but not antigen-sampling via the indirect route

Translocation of *InvA*$^-$ *S.* Typhimurium was investigated in mouse strains either able (C57BL/6) (8) or unable ($CX_3CR1^{-/-}$ or Balb/c) to sample luminal bacteria via the indirect route (6, 10; Supplemental Fig 1A-B). Following oral delivery of *InvA*$^-$ *S.* Typhimurium antigen sampling-competent/migration-competent C57BL/6 and sampling-deficient/migration-competent Balb/c had similar numbers of *S.* Typhimurium penetrating both the conventional epithelium of the small intestine (SI) and the specialized follicle-associated (FAE) epithelia of Peyer's patches (PPs) (Fig. 1A-B) at any time point during the initial stages of the infection. By contrast, sampling-deficient/migration-deficient $CX_3CR1^{-/-}$ mice showed significantly higher numbers of bacteria after 30 and 60 minutes post infection within the PPs and the small intestinal lamina propria that remained significantly higher throughout the experiment. In addition, the number of replicating *InvA*$^-$*AroA*$^+$ *S.* Typhimurium recovered from PP, mesenteric LN and spleen 5 days after oral delivery was higher in $CX_3CR1^{-/-}$ mice compared to their wt counterparts (Fig 1C). Increased bacterial translocation across the gut epithelium in CX_3CR1-deficient mice (both $CX_3CR1^{-/-}$ and $CX_3CR1^{gfp/gfp}$) was not the result of increased permeability of the epithelial barrier as shown by using soluble tracer and microparticles (Fig. 2A-B). Indeed, serum levels of orally delivered fluorescent FITC-dextran (Fig 2A) and numbers of orally delivered FITC-labelled latex microparticles (Fig 2B) were similar to wt mice. Increased bacterial transport in the gut of $CX_3CR1^{gfp/gfp}$ mice was also seen following infection with *InvA*$^+$ *S.* Typhimurium. Confirming a previous report (6) $CX_3CR1^{gfp/gfp}$ mice succumbed 7 days after oral delivery of lethal dose of invasive *S.* Typhimurium at a significantly faster rate compared to their $CX_3CR1^{+/gfp}$ counterparts (Supplemental Fig. 2A) and showed higher bacterial load in their organs (Supplemental Fig. 2B). Also, to rule out that some effects of CX_3CR1 deficiency on bacterial translocation might be related to altered activation of adaptive immune responses we assessed bacterial translocation in $CX_3CR1^{+/-}$ and $CX_3CR1^{-/-}$ on $RAG^{-/-}$ background. We observed that the $RAG^{-/-}$ background did not affect bacterial translocation (Supplemental Fig 2C). These results taken together would suggest that antigen-sampling via the indirect route does not play a significant role in *S.* Typhimurium up-take, at least at the initial stage of infection; instead it appeared that the lack of *Salmonella*-induced CX_3CR1^+ cells intraluminal migration favoured bacterial translocation.

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Intraluminal cell migration is triggered by epithelium-derived signals and it is higher in response to invasive *S.* Typhimurium

The potential critical role of intraluminal CX_3CR1^+ cells in controlling pathogen up-take prompted us to investigate this event in detail. First, we determined whether *Salmonella*-induced CX_3CR1^+ cell migration was abolished or simply delayed in CX_3CR1-deficient mice. After the introduction of *InvA*$^-$ *S.* Typhimurium into isolated intestinal loops of $CX_3CR1^{+/gfp}$ mice the number of CX_3CR1^+ cells appearing in the gut lumen 30 minutes post infection (Fig. 3A) reached $0.71x10^4 \pm 1.2x10^3$ which was approximately >30 fold higher than levels seen in $CX_3CR1^{gfp/gfp}$ mice ($1.3x10^2 \pm 1x10^2$). The numbers of intraluminal CX_3CR1^+ cells increased steadily between 90 and 270 minutes reaching $8.9x10^4 \pm 5x10^3$. In contrast, no increase in the number of intraluminal CX_3CR1^+ cells was observed in $CX_3CR1^{gfp/gfp}$ mice at any time point after infection showing that, the migration is completely abolished in mice lacking a functional fractalkine receptor. Furthermore, we assessed the role of epithelium-derived signals in the migration of CX_3CR1^+ cells in response to *S.* Typhimurium. To this end, $MyD88^{\Delta IEC}$ mice that lacked the adaptor molecule MyD88 solely in the intestinal epithelial cells (IEC) were challenged with *S.* Typhimurium. The migration was completely suppressed in $MyD88^{\Delta IEC}$ mice compared to syngeneic wt counterparts ($1.5x10^2 \pm 1x10^2$ and $6.5x10^4 \pm 1.1x10^4$ respectively at 5h post infection) (Fig. 3B) thus showing that signals from IEC-associated TLRs are the triggering event. We then evaluated the migration of CX_3CR1^+ cells in response to oral delivery of *S.* Typhimurium strains that differed in their capacity to invade the host. Intraluminal migration in $CX_3CR1^{+/gfp}$ mice (Fig. 3C), was significantly higher after infection with the invasive *S.* Typhimurium strain ($4.3.x10^2 \pm 1.2x10^2$) compared to non-invasive strain ($2.1.x10^2 \pm 1x10^2$) already within 30 minutes post-infection. The number of intraluminal cells steadily increased with time and, after 5h it reached $4.4x10^4 \pm 9x10^3$ and $1.7x10^4 \pm 1.2x10^3$ cells for invasive and non-invasive *S.* Typhimurium, respectively. Migration was significantly reduced in response to both *S.* Typhimurium variants at 12h post-infection consistent with intraluminal migration of CX_3CR1^+ cells being restricted to the initial stage of infection. We previously reported that CX_3CR1^+ cells were the only intraluminal cell population harbouring intracellular *S.* Typhimurium shortly after infection (5) suggesting a possible role in *Salmonella*-exclusion. In agreement with this interpretation we observed that at 5h after infection migration-competent $CX_3CR1^{+/gfp}$ mice had a significantly higher faecal (excluded) bacteria load compared to migration-deficient $CX_3CR1^{gfp/gfp}$ mice (Fig. 3D).

Salmonella-induced migration of CX_3CR1^+ cells occurred through paracellular channels in the epithelium

Then intravital two-photon microscopy was used to study in detail the transepithelial migration of CX_3CR1^+ cells in $CX_3CR1^{+/gfp}$ mice shortly (3h) after the introduction of *InvA*$^-$ *S.* Typhimurium into isolated ileal loops. First, the still image (orthogonal cross-sections through the 3D stacks in successive time-frames) (Fig. 4A-E) of the *in vivo* real time video (Supplemental Movie 1) showed a fluorescent $CX_3CR1^{+/gfp}$ cell protruded into the intestinal lumen from the surface of the intestinal epithelium (Fig. 4A) and progressed further into the lumen (Fig. 4B-C) before moving away from the entry site (Fig. 4D-E). It also appeared that the imaged cell was immediately followed by another $CX_3CR1^{+/gfp}$ cell migrating via the same opening in the epithelium (Fig. 4D-E, Supplemental Movie 1). This

migratory pattern was also investigated by both immunohistochemistry (Fig. 4F) and TEM (Fig. 4G-H). Figure 4G also showed three cells migrating (mC1-3) in single file through paracellular channel into the lumen, one of which (mC2) was in close contact with *S.* Typhimurium (Fig 4H). The migration of CX_3CR1^{+} cell is unidirectional; after collecting intraluminal $CX_3CR1^{+/gfp}$ cells and reintroducing them into freshly isolated intestinal ileal loop we never observed $CX_3CR1^{+/gfp}$ cells traversing the epithelial barrier to migrate back into the intestinal tissue. The majority of intraluminal $CX_3CR1^{+/gfp}$ cells (Fig. 5) at 5h post-infection displayed the phenotype of gut resident macrophages $MHCII^{+}$ $F4/80^{+}$ $CD11c^{+}$ $CD103^{-}$ $SiglecF^{-}$ that in steady-state situations do not migrate to mesenteric lymph-node, display poor T cell stimulatory capability and possess high phagocytic activity both *in vitro* and *in vivo* (19, 21).

Lack of CX_3CR1-mediated sampling did not affect antibody responses to *S.* Typhimurium

It has been suggested that antigen-sampling mediated by CX_3CR1^{+} cells in the lamina propria, also called the indirect route (22) plays a significant role in the generation of mucosal and systemic immune responses. However, so far a direct evidence of this was still lacking. Thus, we assessed mucosal and systemic antibody responses to *InvA*$^{-}$*AroA*$^{-}$ *S.* Typhimurium that specifically targets CX_3CR1-mediated entry route (22) in $CX_3CR1^{gfp/gfp}$ mice that lack a functional CX_3CR1 receptor. We observed that the levels of serum IgG was consistently higher in $CX_3CR1^{gfp/gfp}$ mice compared to $CX_3CR1^{+/gfp}$ mice starting at week 2 post-infection (Fig. 6A) although it did not reach statistical significance. Instead, sIgA production in $CX_3CR1^{gfp/gfp}$ mice, albeit significantly lower than the one induced by invasive/non-replicating *InvA*$^{+}$*AroA*$^{-}$ *S.* Typhimurium (data not shown) was significantly higher compared to wt mice (Fig. 6B) starting from week 3 post-infection. The higher *S.* Typhimurium-specific IgA response in $CX_3CR1^{gfp/gfp}$ mice was confirmed by assessing the numbers of antibody forming cells (AFC) in the PPs (Fig. 6C).

The presence of CX_3CR1^{+} cells in the intestinal lumen significantly reduced the number of *S.* Typhimurium penetrating the epithelial barrier

Finally, we tested the hypothesis that intraluminal CX_3CR1^{+} cells contributed to immune-exclusion of *S.* Typhimurium. At various intervals, shortly following oral delivery of $1x10^7$ *InvA*$^{+}$*AroA*$^{-}$ *S.* Typhimurium, CX_3CR1^{+} cells isolated from $CX_3CR1^{+/gfp}$ donors were injected into the intestinal lumen of migration-deficient $CX_3CR1^{-/-}$ mice (details of protocol in Supplemental Fig. 3). Four groups of mice were used and the number of adoptively transferred cells at any given time point was based on time course experiment carried out previously (Fig. 3C). The adoptive transfer of intraluminal CX_3CR1^{+} cells in $CX_3CR1^{-/-}$ mice significantly reduced the number of *InvA*$^{+}$ *S.* Typhimurium traversing the epithelial barrier (Fig. 7A). In the presence of $0.5x10^3$ CX_3CR1^{+} cells the number of tissue CFU of *InvA*$^{+}$ *S.* Typhimurium declined (from $1.2x10^3 \pm 3.3x10^2$ to $2.1x10^2 \pm 1.4x10^2$) at 30 minutes post-infection (group I). Similarly, significant reductions in bacterial load in the intestinal tissues were observed in group II and III. Finally, group IV was passively transferred, fifteen minutes after *S.* Typhimurium administration with a number of CX_3CR1^{+} cells ($1x10^4$) that far exceeded the number of CX_3CR1^{+} cells found in the small intestine at the beginning of the infection. In this case, the presence of intraluminal CX_3CR1^{+} cells almost completely abolished *Salmonella* invasion of intestinal tissue (CFU tissue <40). Importantly, when

$CX_3CR1^{gfp/gfp}$ were used in experiments of adoptive transfer (Figure 7B) we observed that these cells afforded a similar level of protection against *S.* Typhimurium invasion compared to $CX_3CR1^{+/gfp}$ cells. This demonstrated that transepithelial migration and no other intrinsic defects of $CX_3CR1^{-/-}$ cells is the critical event in immune-exclusion against *S.* Typhimurium. Taken together these results demonstrated that CX_3CR1^{+} cells that migrate rapidly into the lumen represented a rapidly deployed protective response that prevents harmful microbes such as *S.* Typhimurium from infecting the host.

Discussion

The presence of epithelial tight junctions, a thick layer of mucus and secretion of sIgA ensures that harmful microorganisms do not breach the intestinal epithelium. Here we demonstrated that when this barrier is breached by invading pathogenic bacteria the host rapidly responds by sending into the intestinal lumen pathogen (*S.* Typhimurium)-capturing CX_3CR1^{+} cells to limit the number of bacteria penetrating the epithelial barrier. The rapid migration is triggered by the initial interaction of pathogens with IEC since this migration is absent in mice lacking the adaptor molecules MyD88 solely within the epithelium. Interaction of *S.* Typhimurium and the IEC-associated TLR may lead to two different events. CX_3CR1^{+} cells can directly sample bacteria and shuttle them across the epithelium (6); alternatively these cells could also move through the epithelium and migrate into the lumen where they capture *S.* Typhimurium (5). However, while it was suggested that CX_3CR1-sampling was relevant to mounting antigen-specific responses the role and biological relevance of the rapid migration of *S.* Typhimurium-capturing CX_3CR1^{+} cells in the lumen remained to be determined. CX_3CR1-mediated sampling and migration showed significant differences. The most notable being that, while CX_3CR1-migration in response to *S.* Typhimurium takes place irrespective of the mouse strain, such as C57BL/6 and Balb/c (5) the ability to sample luminal bacteria via the indirect route may or may not be present according to the genetic make-up of the host (7, 8, 10), an observation that led to the conclusion that CX_3CR1-mediated sampling is not a universal phenomenon (23). We exploited this feature to investigate the biological relevance of these two CX_3CR1-mediated events during the initial stage of *S.* Typhimurium infection. We observed that, in spite of lacking the ability to sample bacteria via the indirect route, Balb/c mice showed very similar intestinal uptake of non-invasive *S.* Typhimurium, that specifically targets the indirect route compared to sampling-competent wt C567BL/6. This observation is of particular relevance when examined in the context of *S.* Typhimurium uptake in CX_3CR1-deficient mice that lacked both CX_3CR1-sampling (6) and -intraluminal migration (9). We observed that CX_3CR1-deficient mice showed a significantly higher bacterial load both at mucosal level and systemically compared to both C57BL/6 and Balb/c. Increased pathogen uptake in CX_3CR1-deficient mice did not depend on intrinsic alteration of the integrity of the epithelial barrier in both $CX_3CR1^{-/-}$ and $CX_3CR1^{gfp/gfp}$ mice since permeability to either soluble or particulate tracers was similar to what observed in their wt counterparts. Overall these results would suggest that sampling via the indirect route does not play a significant role in *S.* Typhimurium up-take through the intestinal epithelium, at least during the early stage of infection. Instead, they point to the lack of rapid intraluminal migration of CX_3CR1^{+} cells as the critical factor favouring bacterial translocation. This hypothesis is

further strengthened by the observation that intraluminal migration of CX_3CR1^+ cells in $CX_3CR1^{+/gfp}$ mice was associated with increased faecal bacterial counts compared to migration-deficient $CX_3CR1^{gfp/gfp}$ mice. Importantly, others have shown that $CX_3CR1^{-/-}$ displayed increased translocation of commensal bacteria to MLN (24), thus suggesting that intraluminal migration of CX_3CR1^+ cell might also play a role in controlling the access of commensal microbes to the intestinal tissue. Furthermore, CX_3CR1-deficient mice are more susceptible to *S.* Typhimurium infection, as seen by us and others (6); however, the reason for this remained to be determined. One possibility was that the lack of CX_3CR1-mediated sampling led to an impaired immunity to *S.* Typhimurium. We observed that CX_3CR1-deficient mice developed a systemic IgG response comparable to wt control and surprisingly, a significantly higher intestinal antibody response (IgA) to a non-invasive *S.* Typhimurium that targets the indirect route and is characterized by poor mucosal antigenic properties (22). It is likely that the latter observation could be attributed to the higher number of *S.* Typhimurium reaching the PPs, the inductive sites of IgA-mediated immunity, in CX_3CR1-deficient mice. Taken together these results strongly suggested that increased susceptibility to *S.* Typhimurium infection in CX_3CR1-deficient mice was due, at least partly to the lack of protection afforded by CX_3CR1-mediated pathogen exclusion in the early stage of infection. As a parallel observation, these data also confirmed previous reports suggesting that *S.* Typhimurium type III secretion system (*InvA*) facilitates up-take by FAE-microfold (M) cells but its absence does not totally compromise the ability of *S.* Typhimurium to target this entry route (25, 26). Intraluminal migration of CX_3CR1^+ cells is unidirectional and once into the lumen they do not traverse back the intestinal epithelium. Determining the fate of intraluminal *S.* Typhimurium-capturing CX_3CR1^+ cells was prompted by the important finding that in mice *T. gondii*-infected neutrophils that had migrated into the intestinal lumen during the infection can move back into the intestinal tissue at a later stage (one week post-infection) (27). The observation that $CX_3CR1^{+/gfp}$ in contrast to infected neutrophils undertake a one way journey suggested that, neutrophils may be intrinsically different from CX_3CR1^+ cells in their ability to traverse back the epithelium. Alternatively, it is possible that in contrast to *S.* Typhimurium, *T.* gondii might trigger the secretion/expression of cytokine/surface molecules in infected cells that favour this event. Finally, we demonstrated that the migration of CX_3CR1^+ cells into the lumen very early during *S.* Typhimurium infection contributed substantially to pathogen-exclusion. Direct introduction of these cells into the lumen of migration-deficient $CX_3CR1^{-/-}$ mice shortly after oral delivery of invasive *S.* Typhimurium significantly reduced the number of pathogens traversing the epithelial barrier. Furthermore, although at this time we cannot rule out the possibility that other cell types may participate to this protective response at a later stage in infection, the previous observation that CX_3CR1^+ cells was the only intraluminal cell population harbouring intracellular *S.* Typhimurium (5) implied that these cells are the main player in the exclusion of *S.* Typhimurium in the very early stage of infection. Previous study showed that intraluminal CX_3CR1^+ cells internalized *S.* Typhimurium (5); thus, intracellular killing is the most likely mechanism underlying *S.* Typhimurium-exclusion in the gut although at present other mechanisms such as production of anti-microbial products cannot be rule out. Here, we would like to propose that the CX_3CR1-mediated pathogen-exclusion is part of a defensive strategy that includes multiple effector mechanisms acting synergistically. First, the mucous and IgA that are constantly produced in large amount in the gut provide a

preventive barrier that in a steady state situation controls and limits the access of microbes to the intestinal epithelium. When this defensive barrier is breached, IEC-derived signals readily trigger the intraluminal migration of CX_3CR1^+ cells that are present in large number in the subepithelial area where they form an intricate cell network (19, 21). This strategy, implemented in the initial stage of the infection blocks further pathogen penetration and, in so doing may prevent the onset of infection by limiting the number of the offending pathogens that can trespass the epithelial barrier. At later stage this strategy is followed, if necessary by an additional defensive mechanism represented by the NAIP/NLRC4 inflammasone-mediated expulsion of infected enterocytes (4) to restrict/control *S.* Typhimurium replication/colonization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations

IEC	intestinal epithelial cell
PP	Peyers patch
MLN	mesenteric lymph node
lp	lamina propria

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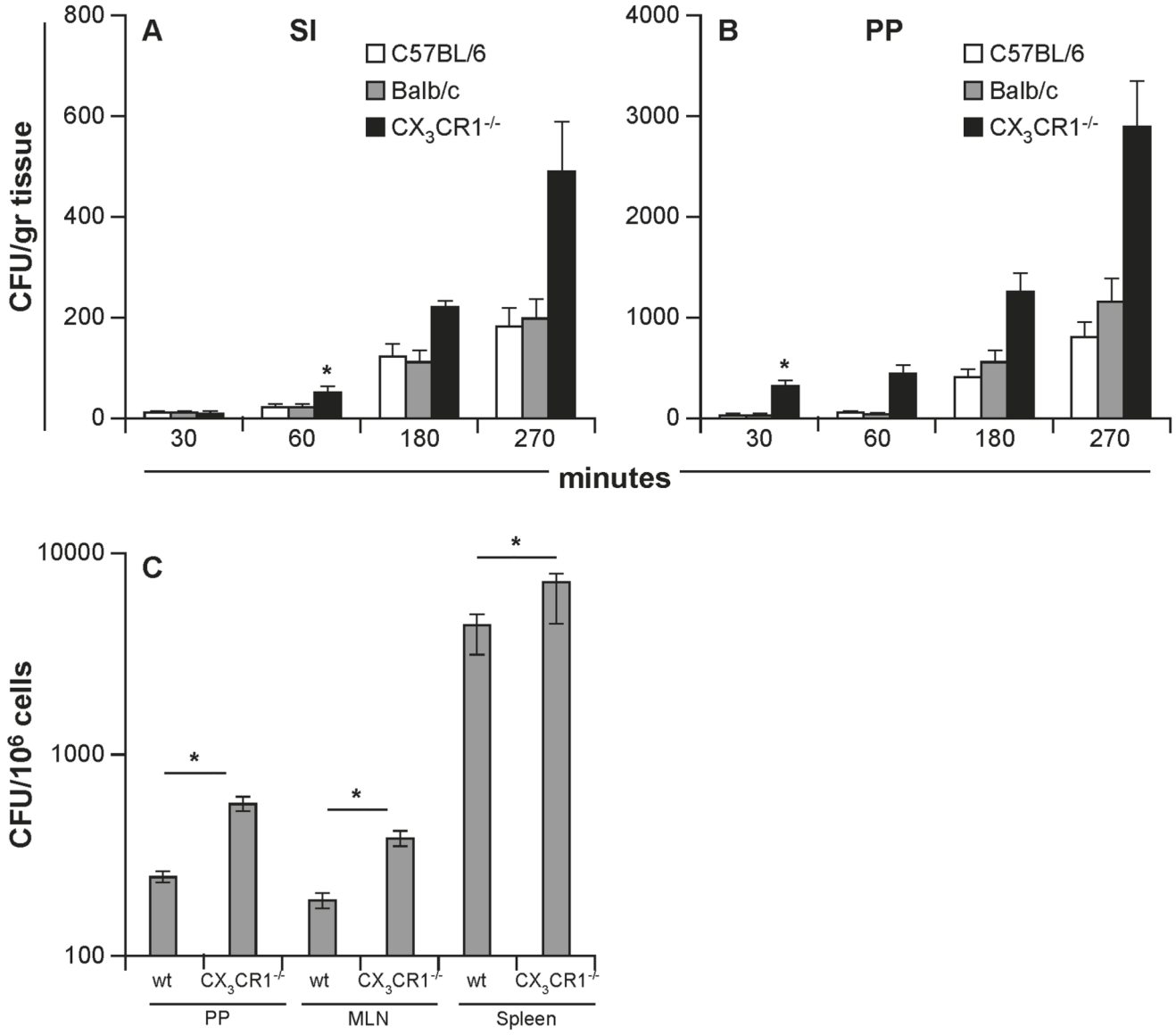

Fig. 1. Role of CX_3CR1-mediated sampling and migration in the uptake of non-invasive InvA$^-$ *S.* Typhimurium.

Typhimurium. Numbers of *S.* Typhimurium traversing the conventional (A) (small intestine, SI) and specialized (B) (Peyer's patches, PP) epithelia did not differ in mouse strains that have been shown to be either sampling-competent/migration-competent (C57BL/6) or sampling-deficient/migration-competent (Balb/c). In contrast, *S.* Typhimurium uptake was significantly higher in $CX_3CR1^{-/-}$ mice that were both sampling-deficient and migration-deficient (8 mice/group). Similarly higher numbers of *S.* Typhimurium were found to be higher in the GALT (PP and MLN) and spleen of $CX_3CR1^{-/-}$ mice compared to wt mice (10 mice/group), 6 days after a single oral delivery of non-invasive-replicating *InvA$^-$Aro$^+$ S.* Typhimurium. Asterisk (*) indicates significant statistical difference.

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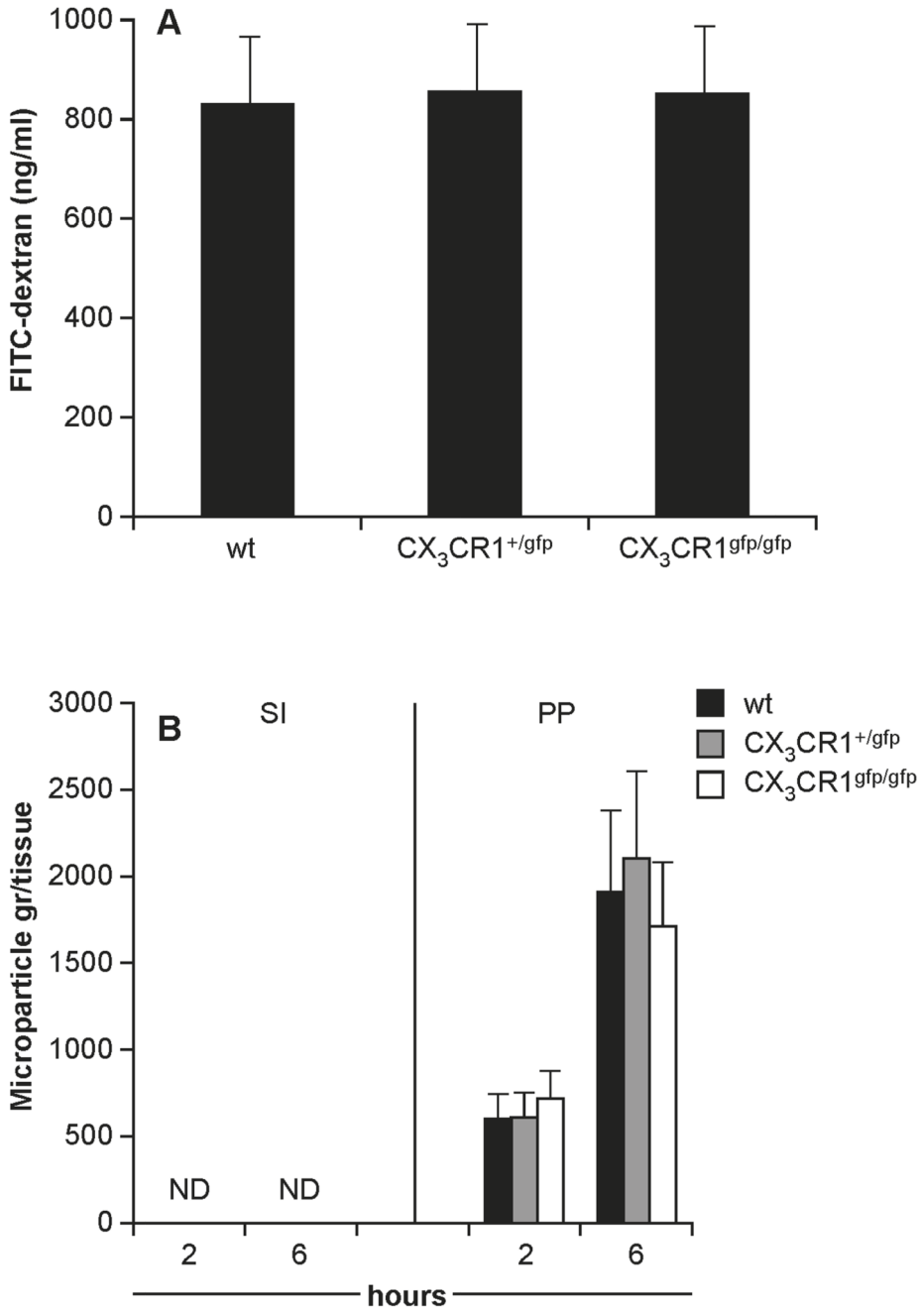

Fig. 2. Intestinal permeability in CX_3CR1-deficient mice.

Oral delivery of a single dose of either FITC-dextran (A) or yellow-green fluorescent polystyrene microparticles (B) showed that intestinal permeability to both soluble and particulates tracers was not affect by the lack of functional fraktalkine receptor (4 mice/group). This demonstrates that the higher bacterial load in the intestine as shown in Fig 1 could not be attributed to an intrinsic "leaky" gut in $CX_3CR1^{-/-}$ mice. Asterisk (*) indicates significant statistical difference

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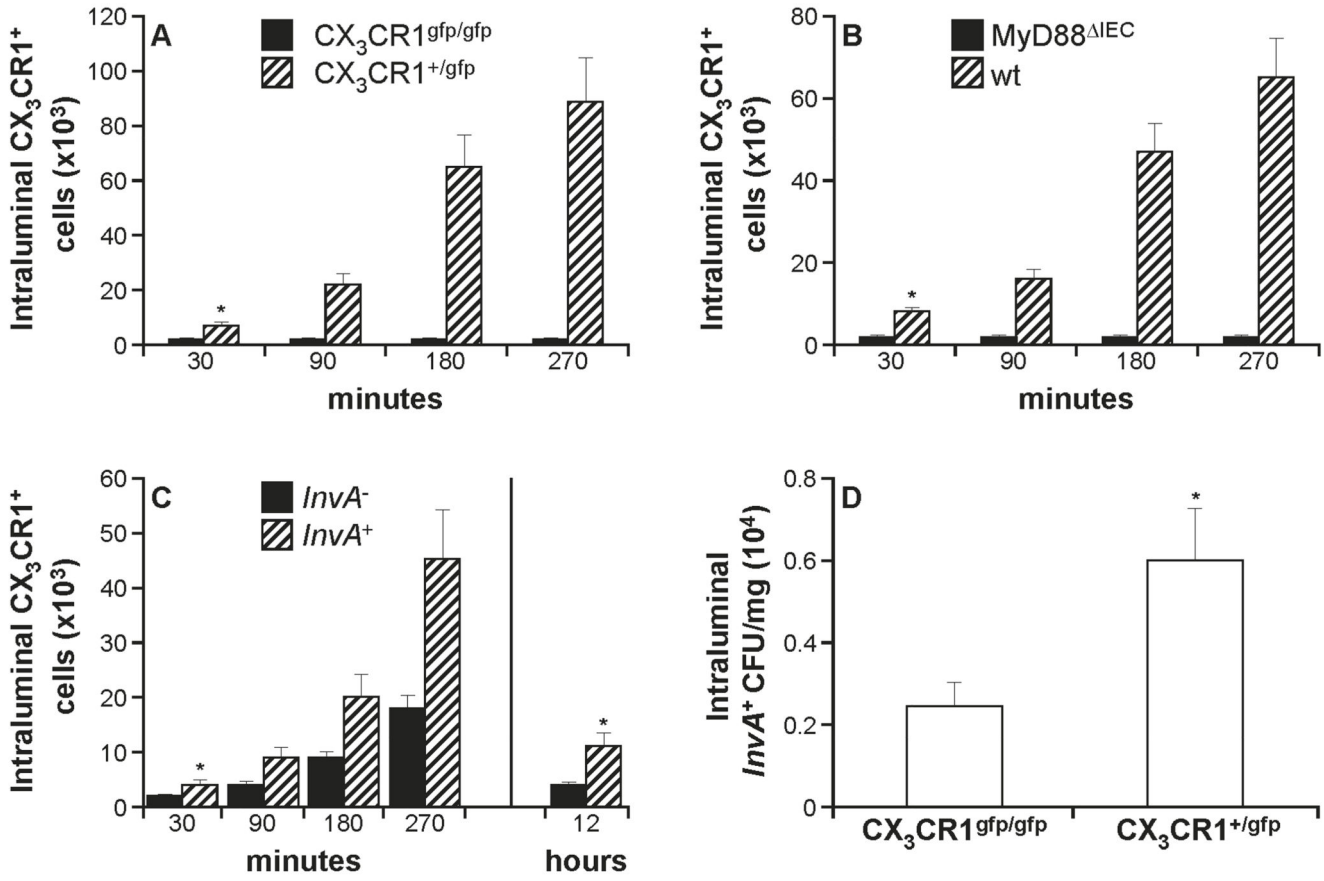

Fig. 3. Regulation of *S.* Typhimurium-induced migration of CX_3CR1^+ cells.

The role of the fraktalkine CX_3CR1 receptor in *S.* Typhimurium-induced migration was assessed in mice with a functional ($CX_3CR1^{+/gfp}$) or non-functional ($CX_3CR1^{gfp/gfp}$) receptor. Intraluminal migration of CX_3CR1^+ cells was absent in CX_3CR1-deficient mice (A) (7-8 mice/group) that had been challenged with $1x10^7$ *InvA*$^-$ *S.* Typhimurium The lack of the fraktalkine receptor completely abolished, and not simply delayed the pathogen-induced migration. IEC-derived signals are required for CX_3CR1^+ cells recruitment and migration; *S.* Typhimurium-dependent intraluminal recruitment of CX_3CR1^+ cells was also absent in mice with a target deletion of MyD88 in the IEC ($MyD88^{\Delta IEC}$ mice) (B) (5-6 mice group). In (C) it is shown that intraluminal migration is significantly more pronounced in response to oral challenge ($1x10^7$) with invasive (*InvA*$^+$) *Salmonella* variant. Migration appeared to be restricted at the initial stage of infection and it declined significantly 12h after infection for both invasive and non-invasive strains. The presence of intraluminal CX_3CR1^+ cells led to a significant increase in faecal bacterial load (D) compared to $CX_3CR1^{gfp/gfp}$ mice 5 hours after oral delivery of invasive *S.* Typhimurium as in (C). Asterisk (*) indicates significant statistical difference.

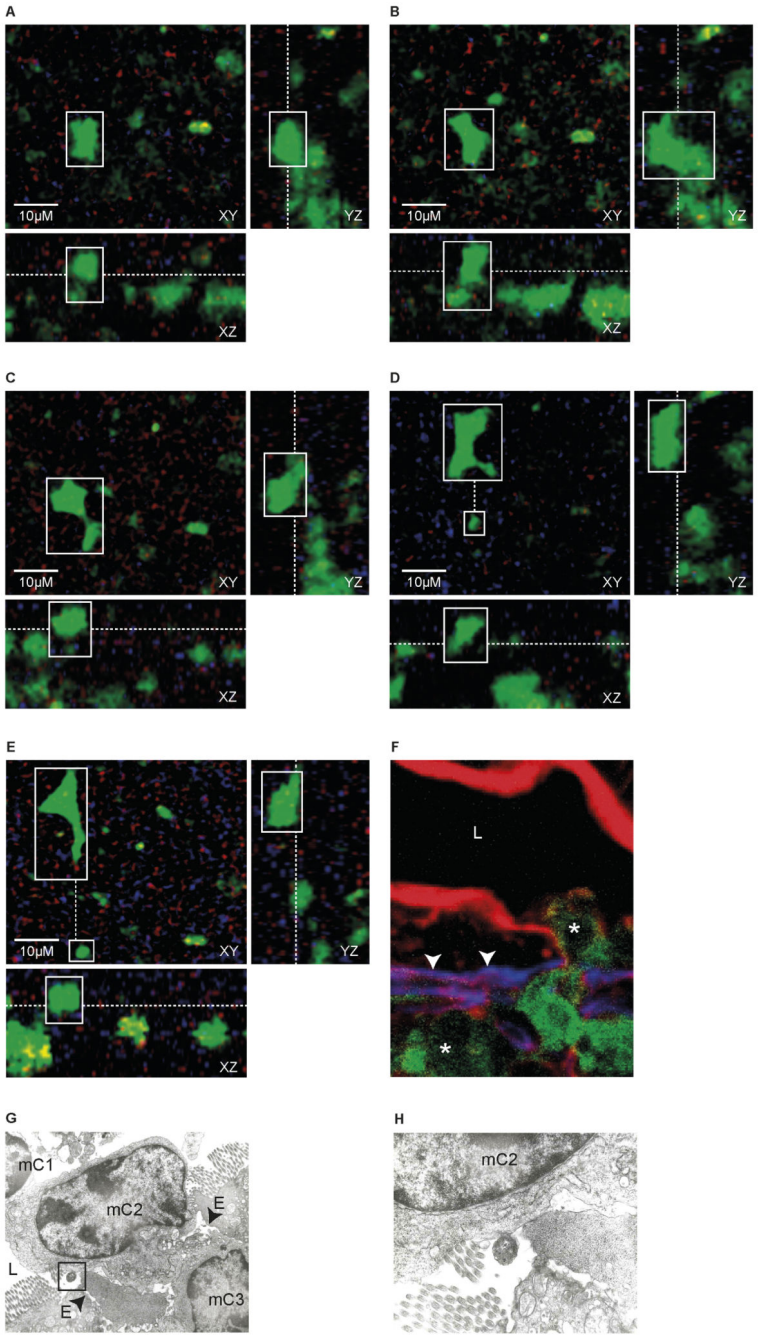

Fig. 4. CX_3CR1^+ cells passage into the intestinal lumen occurred through paracellular spaces.

Still images from *in vivo* real time video (Supplemental video 1): images of orthogonal cross-sections through the 3D stacks in successive time-frames. Detailed views show the movement of a fluorescent $CX_3CR1^{+/gfp}$ cell following challenge with *Salmonella*. In (A) the $CX_3CR1^{+/gfp}$ cell (white box) is protruding from the epithelial surface; the outward movement being more pronounced in (B). In C the migrating cell keeps protruding into the lumen until it completed its migration and moved away (dotted line) from the entry site (small white box) (D, E). The migrating cells is immediately followed by another

$CX_3CR1^{+/gfp}$ cells protruding into the lumen from the same opening (D,E, small white box). Images on YZ plan (A-E) allow seeing that, once into the lumen the cell progresses in a non-linear (side-to-side) pattern on the epithelial surface. Scale bar = 10 μm. Migration pattern was further investigated by immunofluorescence microscopy (F) and TEM (G, H). In (F), $CX_3CR1^{+/gfp}$ cells (asterisks) migrate into the intestinal lumen (L) across the basal membrane (arrow heads), identified by anti-entactin antibody (blue) and then the epithelium identified with phalloidin (red). In (G and H) a series of cells (mC1-3) moved into the lumen (L) via the paracellular space (arrow heads) between adjacent enterocytes (E) through the same paracellular channel. Also, in (G) one migrating cell (mC2) is in close contact with *Salmonella* (box) (detail in H).

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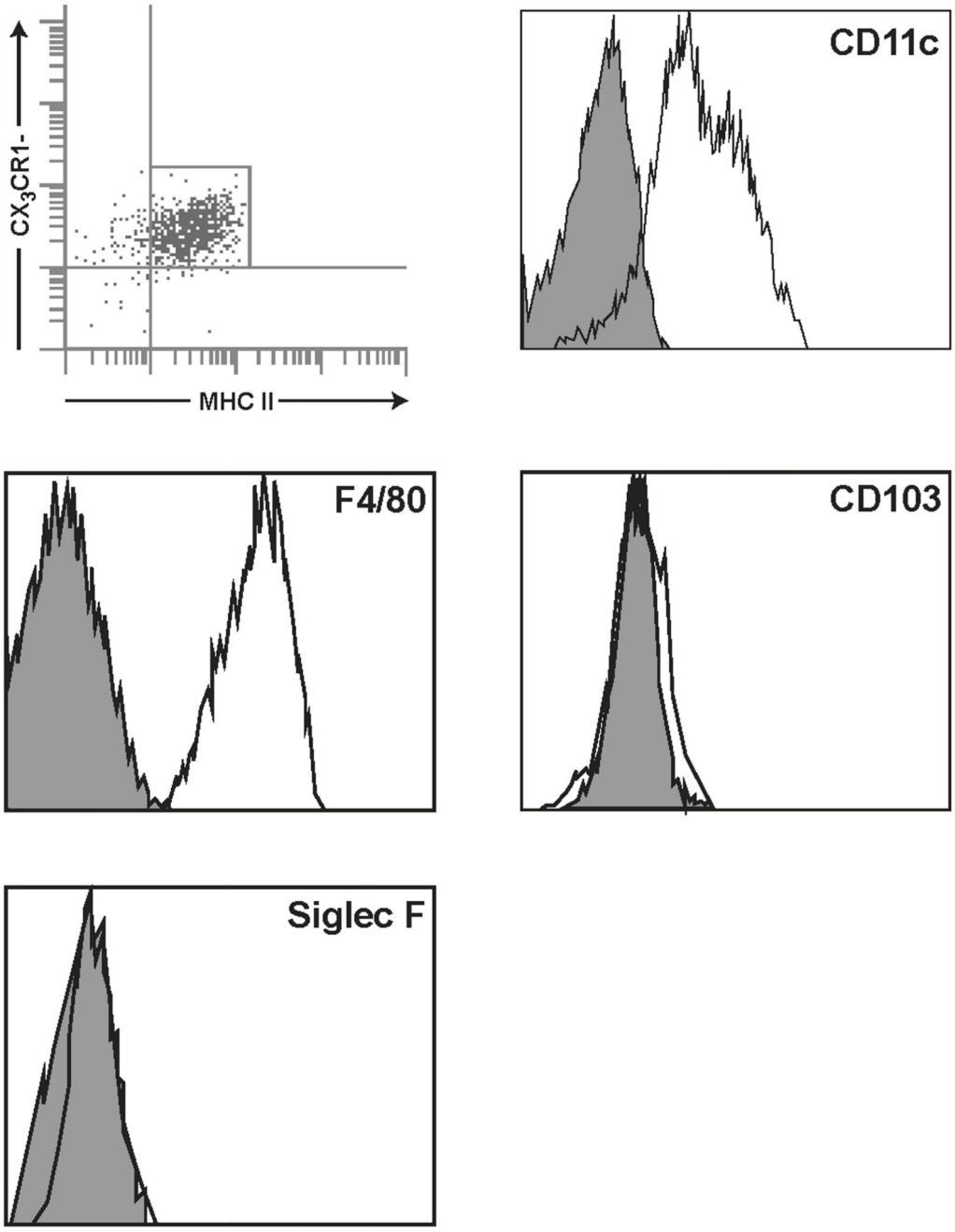

Fig. 5. Phenotypic analysis of intraluminal CX_3CR1^+ cell population.

Flow cytometry analysis of intraluminal cells in $CX_3CR1^{+/gfp}$ (Balb/c background) 5 hours following intestinal challenge with $1x10^7$ *InvA*$^-$ *Salmonella*. The vast majority of the cell population rapidly recruited into the intestinal lumen showed the phenotype of resident (stationary) macrophage with poor T cell stimulatory activity and high phagocytic activity. These cells were $CD11C^+F4/80^+MHCII^+$ but did not express the canonical marker for gut-derived dendritic cell (DCs), CD103; also, these cells lacked the neutrophil marker SiglecF

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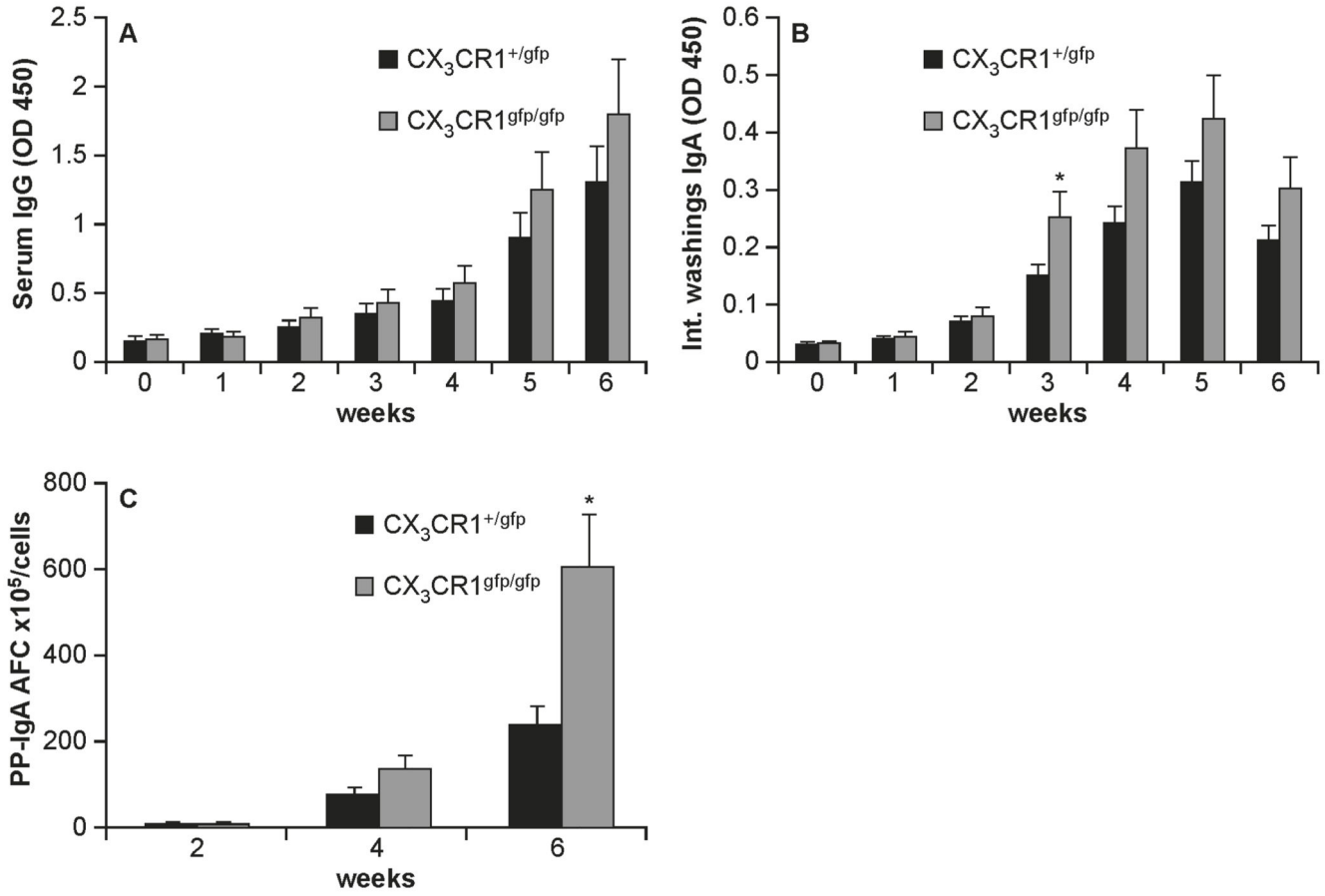

Fig. 6. Humoral immunity to non-invasive Salmonella in $CX_3CR1^{-/-}$ mice.

Mice (9-10 mice/group) received 3 consecutive doses of $1x10^7$ of non-invasive/non-replicating *InvA⁻AroA⁻ Salmonella* at 3 day interval. Levels of serum IgG (A) and intestinal IgA (B) *Salmonella*-specific antibodies were determined by ELISA. Both responses appeared to be higher in $CX_3CR1^{gfp/gfp}$ compared to $CX_3CR1^{+/gfp}$ mice although only intestinal levels of IgA were significantly different starting from week 3 after infection (*). Higher mucosal IgA immunity in $CX_3CR1^{gfp/gfp}$ mice was further confirmed by monitoring numbers of AFC in PPs at weeks 2, 4 and 6 post-infection (C). Asterisk (*) indicates significant statistical difference.

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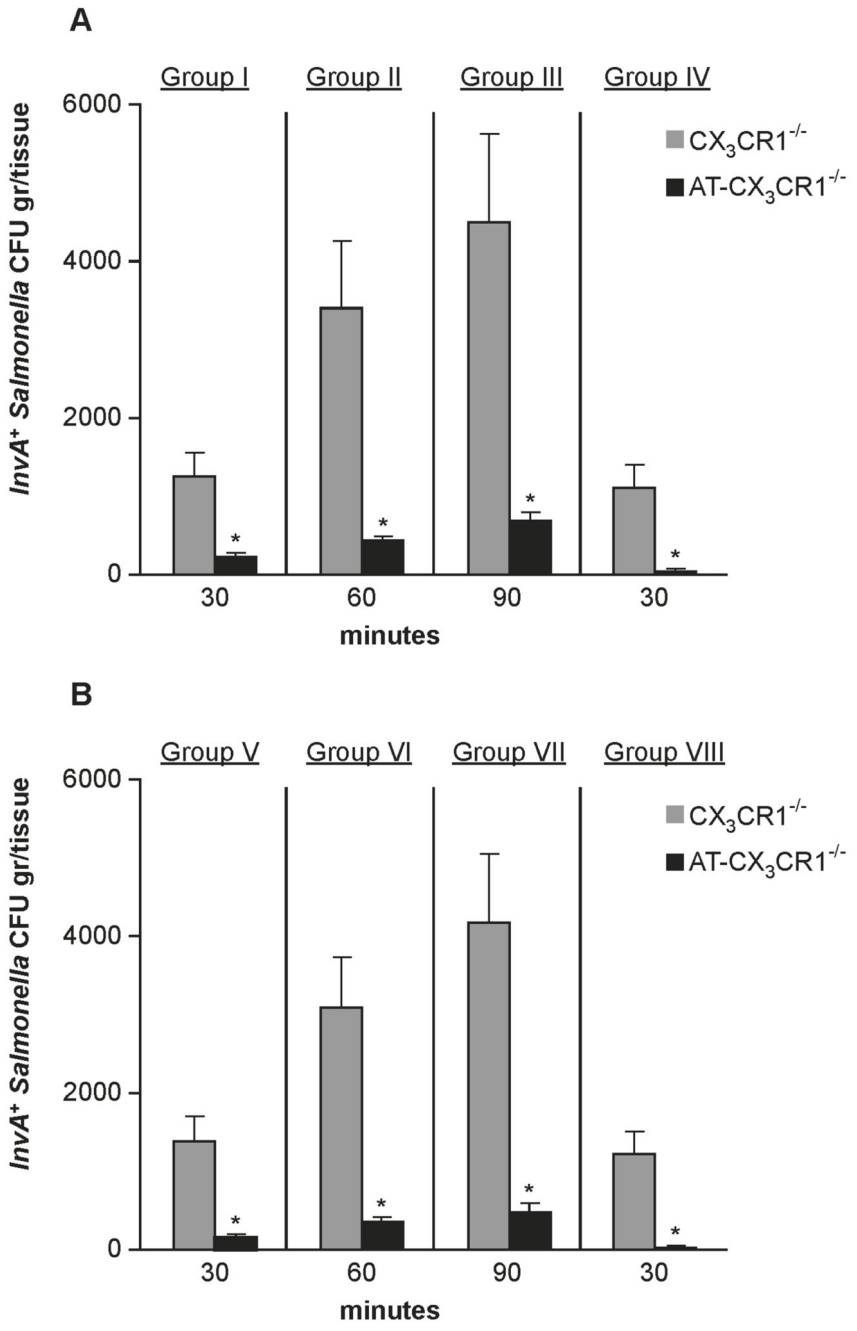

Fig. 7. CX_3CR1^+ cell-mediated pathogen-exclusion.

$CX_3CR1^{-/-}$ mice (6 mice/group) were infected with a single oral dose ($1x10^7$) of invasive/non-replicating *InvA*$^+$*Aro*$^-$*Salmonella*. *Salmonellae* infecting the intestinal tissue were determined in the absence ($CX_3CR1^{-/-}$, grey bars) or presence (AT-$CX_3CR1^{-/-}$, black bars) of $CX_3CR1^{+/gfp}$ (A) or $CX_3CR1^{gfp/gfp}$ (B) cells that were adoptively transferred (AT) directly into the intestinal lumen. A significant decline of *S.* Typhimurium CFU gr/tissue was observed in group I (A) 30 minutes after infection following adoptive transfer of 0.5x103 $CX_3CR1^{+/gfp}$ cells. Significant reduction in the number of pathogens invading the host was

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also seen in group II and III that received increasing numbers of CX_3CR1^+ that were determined according to the time course study shown in Fig 3C. In group IV the introduction in the lumen of a larger, non-physiologically high number of intraluminal CX_3CR1^+ cells ($1x10^4$) of higher (20 fold increase compared to the number of cells usually found in the gut 15 minutes after infection) nearly completely abolished *S.* Typhimurium infection. A similar pattern was observed in parallel experiments when $CX_3CR1^{gfp/gfp}$ cells were used for the adoptive transfer (Group V-VIII) (B) showing that the lack of transepithelial migration and no other intrinsic defects of $CX_3CR1^{-/-}$ cells is critical for immune exclusion of *S.* Typhimurium. Asterisk (*) indicates significant statistical difference.