AIM2 contributes to the maintenance of intestinal integrity via Akt and protects against *Salmonella* mucosal infection

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The mechanism regulating the gastrointestinal epithelial barrier remains poorly understood. We herein demonstrate that Absent in melanoma-2 (AIM2) contributes to the maintenance of intestinal barrier integrity and defense against bacterial infection. AIM2-deficient mice displayed an increased susceptibility to mucosal but not systemic infection by *Salmonella typhimurium*, indicating a protective role for AIM2 in the gastrointestinal tract. In a *Salmonella* colitis model, compared with wild-type mice, AIM2^{-/-} mice exhibited more severe body weight loss, intestinal damage, intestinal inflammation, and disruption of basal and activated epithelial cell turnover. *In vivo* and *in vitro* data showed that AIM2 restricted the early epithelial paracellular invasion of *Salmonella* and decreased epithelial permeability. The decreased epithelial barrier in AIM2^{-/-} mice might be attributed to the altered expression of tight junction proteins that contribute to epithelial integrity. AIM2 promoted the expression of tight junction proteins through Akt activation. Together, these results suggest that AIM2 is required for maintaining the integrity of the epithelial barrier.

INTRODUCTION

The gastrointestinal (GI) tract is covered with a single-layered columnar epithelium that functions as a physical and biological barrier protecting the host against resident and foreign pathogens present in the gut.¹ To gain access to the host, pathogens must first breach the intestinal epithelial barrier.² Thus, understanding the mechanisms regulating this barrier is of fundamental importance to prevent microbial infection and disease progression.

The *Salmonella enterica* serovar *typhimurium* (*Salmonella*) is a prevalent foodborne pathogen that causes enteric disease in diverse animals and humans. After oral ingestion, *Salmonella* initially breaches the epithelial lining, triggers mucosal inflammation, disseminates into the mesenteric lymph nodes (MLNs), and even reaches and colonizes systemic sites such as the spleen and liver. Additionally, there are many non-typhoidal *Salmonella* strains typically cause self-limiting subacute/acute gastroenteritis in immune-competent individuals; the infection is in most cases confined to the

GI tract, and dissemination to the liver and spleen is atypical. *Salmonella* thus serves as a good model for studying the intestinal epithelial barrier against bacterial pathogens.^{3,4}

Absent in melanoma-2 (AIM2), a member of the HIN200 protein family, was originally identified as a tumor suppressor.⁵ The AIM2 gene has a microsatellite instability site that results in its inactivation in many tumors.⁶ Since its function as cytosolic DNA sensor was disclosed, research on AIM2 has predominantly focused on its role in innate immunity when assembled as an inflammasome.⁷ The AIM2 inflammasome is important in sensing both bacterial and viral pathogens and triggering anti-microbial innate immunity.⁸ Evidence has shown that the AIM2 inflammasome can be activated by *Francisella tularensis*,^{9–11} *Listeria monocytogenes*,¹¹ *Aspergillus fumigatus*,¹² *Streptococcus pneumoniae*,¹³ *Porphyromonas gingivalis*,¹⁴ vaccinia virus,¹¹ human papillomavirus,¹⁵ and mouse cytomegalovirus.¹¹ With regard to *Salmonella*, although previous work has reported that AIM2-deficient murine bone marrow-derived macrophages respond with interleukin

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Received 17 June 2015; accepted 16 November 2015; published online 3 February 2016. doi:10.1038/mi.2015.142

Figure 1 Absent in melanoma-2 (AIM2) deficiency increases the susceptibility to oral but not to systemic *Salmonella* infection. Wild-type (WT) and AIM2^{-/-} mice were treated with streptomycin before oral administration of 1×10^8 colony-forming units (CFU) of *Salmonella* ($n = 15$ each group), (a) survival and (b) bacterial dissemination in WT and AIM2^{-/-} mice was determined at day 5 post infection ($n = 5$ each group). WT and AIM2^{-/-} mice intraperitoneally infected with 5×10^4 CFU *Salmonella* ($n = 13$ each group), (c) survival and (d) bacterial dissemination in WT and AIM2^{-/-} mice was determined at day 3 post infection ($n = 5$ each group). $P < 0.05$, *** $P < 0.001$.

(IL)-1 β secretion that is similar to that of wild-type (WT) macrophages,¹¹ the biological implications of AIM2 during *Salmonella* infection have not been characterized *in vivo*.

In this study, we reveal a role for AIM2 in maintaining intestinal barrier function during *Salmonella* infection. We found that AIM2 protected mice against *Salmonella* colitis and acted through Akt activation to promote the expression of tight junction proteins that are essential for maintaining intestinal barrier integrity.

RESULTS

AIM2 protects mice from mucosal but not systemic *Salmonella* infection

To explore the possible involvement of AIM2 in the host response to *Salmonella* infection, we initially examined the mortality rate of WT and AIM2-deficient (AIM2^{-/-}) mice following the oral administration of 10^8 colony-forming units (CFU) of *Salmonella* strain SL1344. WT and AIM2-deficient mice were treated with streptomycin before infection. Compared with WT mice, AIM2^{-/-} mice displayed significantly higher mortality (Figure 1a). On day 10 post infection (p.i.), all of the AIM2^{-/-} mice had died, whereas 69% of the WT control mice remained alive. To further characterize the role of AIM2 in host defense against *Salmonella*, the effect of AIM2 on bacterial burden was assessed in WT and AIM2^{-/-} mice. On day 5 p.i., AIM2^{-/-} mice showed increased bacterial loads in the spleen and liver (Figure 1b), which indicated that AIM2^{-/-} mice were more susceptible to bacterial dissemination. After oral ingestion, *Salmonella* crosses the intestinal barrier, disseminates from the GI tract to MLNs, and

colonizes systemic sites.¹⁶ Therefore, this event might be a consequence of either increased penetration and translocation of the epithelial layer by invasive *Salmonella* or impaired clearance of infection at systemic sites. Hence, we performed intraperitoneal infection of WT and AIM2^{-/-} mice to determine whether there is an impaired clearance of infection in AIM2^{-/-} mice. Interestingly, we observed identical lethality in WT and AIM2^{-/-} mice (Figure 1c) and similar bacterial loads in spleen and liver (Figure 1d), which indicated that WT and AIM2^{-/-} mice have a similar capacity to eliminate *Salmonella* systemic infection. Together, these data suggested that AIM2 might have an important role in the GI tract and contribute to the limitation of lethal colitis and bacterial dissemination during *Salmonella* mucosal infection.

AIM2 is critical in limiting *Salmonella* colitis

To determine whether AIM2 deficiency affects *Salmonella* colitis, the experiment was repeated with the administration of 5×10^7 CFU of *Salmonella* to study the phenotype of AIM2^{-/-} mice under milder conditions. We found that AIM2^{-/-} mice suffered from a greater degree of body weight loss from day 2 onward (Figure 2a). To further assess the severity of colitis, spleen and cecum weights were measured in WT and AIM2^{-/-} mice on day 2 p.i. AIM2^{-/-} mice exhibited severe splenomegaly (Figure 2b) and cecal weight loss (Figure 2c). Simultaneously, these clinical assessments were validated by the histological examination of the cecum using hematoxylin and eosin (H&E) staining (Figure 2d). Histological evaluation of the extent of epithelial damage, goblet cell deletion, submucosal edema, and inflammatory cell infiltration revealed that AIM2^{-/-} mice displayed significantly severe intestinal damage and exacerbated intestinal inflammation. Subsequently, we examined the bacterial load at the mucosal site and systemic site. The AIM2^{-/-} mice had a higher bacterial load in the cecum, MLN, spleen, and liver, indicating a higher bacterial dissemination in AIM2^{-/-} mice (Figure 2e).

To gain additional evidence of the role for AIM2 in limiting intestinal colitis, we measured the pro-inflammatory mediator production in the ceca of *Salmonella*-infected WT and AIM2^{-/-} mice on day 2 p.i. The amounts of pro-inflammatory cytokines/chemokines, including TNF α , IL-6, IL-1 β , IFN- γ , KC, CCL5, and CXCL10, in the cecum were significantly higher in AIM2^{-/-} mice compared with WT mice (Figure 3a–g). However, AIM2^{-/-} mice and WT mice had comparable levels of CCL2 production in cecum (Figure 3h). Additionally, Cox-2 and iNOS, two enzymes associated with inflammation, were also elevated in AIM2^{-/-} mice (Figure 3i). At a later time point, both WT and AIM2^{-/-} mice developed severe disease, and AIM2^{-/-} mice had a higher histological score of the cecum on days 3, 4, and 5 p.i. compared with WT mice (Supplementary Figure 2S online). Collectively, the extensive epithelium damage with significant inflammation present in the cecum and higher bacterial dissemination of AIM2^{-/-} mice indicated that AIM2 is critical for protection against *Salmonella* colitis.

Figure 2 Absent in melanoma-2 (AIM2) deficiency leads to increased intestinal damage during *Salmonella* mucosal infection. Wild-type (WT) and AIM2^{-/-} mice were treated with streptomycin before the oral administration of 5×10^7 colony-forming units (CFU) of *Salmonella* for 2 days ($n=9$). (a) Body weight loss. (b) Spleen weight. (c) Gross image and weight of cecum. (d) Hematoxylin and eosin (H&E) staining of the cecum: upper panel, magnification $\times 40$, lower panel, magnification, $\times 100$. (e) Bacterial load in the cecum, mesenteric lymph node (MLN), spleen, and liver. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

AIM2 is required for maintaining intestinal tissue homeostasis

Maintaining tissue homeostasis is vital during infection and inflammation. Tissue homeostasis is the regulation of cell proliferation and cell death, also known as cell turnover. Loss of intestinal epithelial cell (IEC) homeostasis negatively affects intestinal barrier function.¹⁷ To determine whether there is a difference in cell proliferation and apoptosis that can explain the higher susceptibility of AIM2^{-/-} mice to *Salmonella* colitis, we first examined cell proliferation by immunofluorescence staining for PCNA, a nuclear antigen for proliferating cells. Our results revealed that the epithelial crypts presented

with more PCNA-positive cells in naive AIM2^{-/-} mice compared with WT mice; however, the level of epithelial cell proliferation significantly decreased following oral administration of *Salmonella* in AIM2^{-/-} mice (Figure 4a). Subsequently, TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) staining of histological sections of cecum showed that AIM2^{-/-} mice had greatly increased numbers of apoptotic cells in the epithelium and lamina propria (LP) on day 2 p.i, whereas WT mice showed only a mild increase in apoptotic cells in the epithelium (Figure 4b). Apoptosis was further confirmed by immunoblotting for cleaved caspase-3 in cecal lysates,

Figure 3 Absent in melanoma-2 (AIM2) deficiency results in elevated pro-inflammatory mediator production during *Salmonella* mucosal infection. On day 2 post infection, pro-inflammatory mediators in cecum were examined ($n = 9$ each group). (a) TNF- α ; (b) IL-6; (c) IL-1 β ; (d) IFN- γ ; (e) KC; (f) CCL5; (g) CXCL10; (h) CCL2 and (i) levels of iNOS and Cox-2 expression in the cecum were examined by western blotting. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

whereas AIM2^{-/-} mice had a higher level of cleaved caspase-3 production (Figure 4c). Collectively, these results revealed that AIM2^{-/-} mice have a defect in maintaining enterocyte proliferative/apoptotic homeostasis.

AIM2 restricts the early paracellular invasion of *Salmonella*

The significantly decreased proliferation and increased apoptosis in AIM2^{-/-} mice on day 2 p.i. maybe the result of exacerbated inflammation; however, the deregulation of basal proliferation in AIM2^{-/-} mice may contribute to bacterial dissemination, consequently exacerbating damage and inflammation in the GI tract. To explore this possibility, WT and AIM2^{-/-} mice were treated with streptomycin before the oral administration of 5×10^7 CFU of *Salmonella*. Bacterial burden was assessed in the cecal LP and lymph nodes at 3 h p.i. There were significantly more bacteria detected in both sites of AIM2^{-/-} mice compared with the counterparts of WT mice (Figure 5a). This result implied that the absence of AIM2 leads to a defect in the epithelial barrier and confers susceptibility to early bacterial invasion. Pathogens can engage different mechanisms for breaching the epithelial barrier. We hypothesized that the initial increase in bacterial invasion from the GI lumen into the LP in AIM2^{-/-} mice may be attributable to a functional defect in epithelial cells. To investigate this possibility, an AIM2-overexpressing Caco-2 cell monolayer was used to analyze the translocation of *Salmonella* (Figure 5b). As expected, the bacterial translocation was significantly decreased in AIM2-overexpressing Caco-2 cells (Figure 5c) but not in Akt inhibitor pretreated cells.

Bacteria can cross epithelial surfaces through paracellular and/or transcellular mechanisms. We thus assessed the intracellular bacterial loads of epithelial cells. Interestingly, there was no significant difference in bacterial load between AIM2-overexpressing Caco-2 cells and vector-transfected control cells (Figure 5d). Therefore, AIM2 might restrict bacterial paracellular penetration through the epithelial lining during early *Salmonella* infection.

AIM2 promotes tight junction protein expression and decreases epithelial permeability

The integrity of the GI epithelium is sustained through cell-cell junctions. The tight junction is the major type of cell-cell junction and is composed of scaffolding proteins, including Claudin, Occludin, and Zonula occludens (ZO)-1. The tight junction acts as a permeable barrier against microbes. Based on the data above, we set out to characterize the effects of AIM2 on tight junction and the paracellular permeability of the epithelial lining. Compared with WT mice, the basal levels of Claudin3 (Figure 6a–c) and Occludin (Figure 6d) in AIM2^{-/-} mice were lower whereas there was no difference in the basal level of ZO-1 (Figure 6e). Following the oral administration of *Salmonella*, there was a decrease in Claudin3 and Occludin in WT mice, although there was no additional decrease in Claudin3 or Occludin in AIM2^{-/-} mice which might due to the already lower level of tight junction protein expression. Moreover, before or after *Salmonella* challenge, AIM2-overexpressing Caco-2 cells had a higher level of Claudin3 expression and a lower epithelial permeability

Figure 4 Absent in melanoma-2 (AIM2) deficiency leads to perturbed epithelial cell proliferation and apoptosis following *Salmonella* oral challenge. (a) Immunofluorescence was performed on cecum sections derived from wild-type (WT) and AIM2^{-/-} mice on day 2 post *Salmonella* oral infection for the examination of proliferating cells (PCNA). (b) Apoptotic cells were identified in the cecum by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining. (c) Levels of cleaved caspase-3 expression in cecum lysates were determined by immunoblotting. Magnification, $\times 200$.

Figure 5 Absent in melanoma-2 (AIM2) restricts *Salmonella* dissemination and translocation. (a) Bacterial dissemination in the mesenteric lymph node (MLN) and lamina propria (LP) of wild-type (WT) and AIM2^{-/-} mice following *Salmonella* infection for 3 h ($n=8$ each group). (b) AIM2 expression. Cells were treated with Akt and caspase-1 inhibitor, respectively; and bacterial translocation (c) and invasion (d) in vector-transfected Caco-2 cells and AIM2-overexpressing Caco-2 cells were determined ($n=3$). ND stands for not detected. * $P<0.05$, *** $P<0.001$.

compared with vector-transfected cells (Figure 6f,g), this effect can be reduced by an Akt inhibitor but not by a caspase-1 inhibitor. Thus, these results indicated that AIM2 promotes the

expression of tight junction proteins, and the loss of AIM2 leads to the deregulation of tight junction, consequently making the epithelial barrier fragile enough to be disrupted by *Salmonella*. Permeability through the paracellular space can be measured by evaluating the trans-epithelial resistance (TER), a hallmark of epithelial integrity. There was a higher TER across monolayer in AIM2-overexpressing Caco-2 cells compared with control cells, which indicated that AIM2 overexpression resulted in decreased permeability and a tightened epithelial barrier (Figure 6h). Collectively, these data suggested that AIM2 protects against *Salmonella* infection via the enhancement of tight junction barrier function.

Akt mediates AIM2-induced enhancement of tight junction proteins

Previous reports have indicated that Akt alters the expression of tight junctions.^{18–20} AIM2-overexpressing cells had a higher level of Claudin3 expression (Figure 6f), reduced bacterial translocation (Figure 5c), and increased trans-epithelial electrical resistance (Figure 6g). However, this effect can be diminished by an Akt inhibitor but not by a Caspase-1 inhibitor. Thus, we evaluated whether Akt activity is affected by AIM2 and whether Akt mediates the AIM2-induced increase in expression of tight junction proteins. Our results showed that AIM2-overexpressed Caco-2 cells had a higher level of Akt phosphorylation compared with vector-transfected cells (Figure 7a,b). *Salmonella* challenge promoted Akt phosphorylation and Claudin3 expression at 120 min

Figure 6 Absent in melanoma-2 (AIM2) enhances the expression of tight junction proteins. Claudin3 expression was examined on cecum sections by (a) immunofluorescence and (b) immunoblotting. The mRNA levels of (c) Claudin3, (d) Occludin, and (e) Zonula occludens (ZO-1) in cecum were examined by real-time PCR. The data were normalized to GAPDH expression and are shown as the fold increase in mRNA. (f) Claudin3 expression levels in Caco-2 cells and AIM2-overexpressing Caco-2 cells were determined. (g) Levels of Claudin3 expression in AIM2-overexpressed Caco-2 cells and vector-transfected cells were determined by western blotting following *Salmonella* infection for 30 min. (h) Trans-epithelial resistance (TER) levels in Caco-2 cells and AIM2-overexpressing Caco-2 cells were determined. ** $P < 0.01$, *** $P < 0.001$.

(Figure 7b). Although the basal level of Akt phosphorylation in vector-transfected cells remained very low or undetectable up to 60 min after *Salmonella* challenge, inhibition of Akt phosphorylation accompanied by decreased Claudin3 expression was observed in AIM2-overexpressing cells. Therefore, a short exposure of cells to *Salmonella* inhibited Akt activity and Claudin3 expression, whereas prolonged *Salmonella* treatment promoted Akt activity and Claudin3 expression. Moreover, pretreatment with an Akt inhibitor prevented the induction of Akt activation and Claudin3 expression by *Salmonella* (Figure 7c). Collectively, these results indicated that AIM2-induced enhancement of tight junction proteins may be mediated through Akt.

DISCUSSION

Acting as a DNA sensor, AIM2 is assembled as an inflammasome and is involved in sensing various bacterial and viral pathogens. However, the biological implications of AIM2 during *Salmonella* infection remain unknown. We orally infected mice and found that AIM2^{-/-} mice were more

susceptible to *Salmonella* infection, as indicated by increased mortality and bacterial dissemination. This observation highlights the importance of AIM2 in protection against *Salmonella* infection.

After oral ingestion, *Salmonella* initially breaches the intestinal epithelium and then disseminates from the GI tract to colonize systemic sites. Therefore, increased susceptibility of AIM2^{-/-} mice to *Salmonella* mucosal infection might be a consequence of either an impaired epithelial barrier or an impaired systemic clearance. To further characterize the role of AIM2 in the host defense against *Salmonella*, we intraperitoneally infected mice. In this situation, AIM2 deficiency did not affect mortality or bacterial dissemination. The discrepancy between oral and intraperitoneal infection suggests that AIM2 may function in the GI tract to defend against *Salmonella* infection.

The GI tract provides physical and biological barriers against local and foreign bacteria entry into deeper tissue. It is an extremely complex organ and has a unique immune system. Deregulation of the intestinal immune response appears to have

Figure 7 Absent in melanoma-2 (AIM2)-induced enhancement of tight junction proteins is mediated through Akt. (a) Levels of phospho-Akt in both AIM2-overexpressing Caco-2 cells and control cells were measured by western blotting. (b) Levels of phospho-Akt and Claudin3 in both AIM2-overexpressed Caco-2 cells and control cells were determined following *Salmonella* infection for different time points by western blotting. (c) Pretreated Caco-2 cells with Akt inhibitor (MK-2206). Claudin3 expression was examined following *Salmonella* infection for 120 min by western blotting.

a principal function in the pathogenesis of inflammatory bowel disease.²¹ A healthy gut has evolved diverse regulatory mechanisms that tightly control the immune reaction and cooperate to maintain intestinal immune homeostasis.^{22,23} For example, the Toll-like receptor and Nod-like receptor family members are compartmentally expressed throughout the intestine and recognize microbial components.^{23,24} It has been shown that various members of Toll-like receptors and Nod-like receptors are essential for the maintenance of intestinal homeostasis.^{24–28} To explore whether AIM2 regulates the local immune response in the GI tract, a *Salmonella* colitis model was established by the oral administration of 5×10^7 CFU of *Salmonella*. Our results showed that AIM2 has the potential to be a pattern recognition receptor that limits gut inflammation. AIM2^{-/-} mice were more susceptible to *Salmonella* mucosal infection and suffered from heavier intestinal damage with higher pro-inflammatory mediator production. The uncontrolled inflammatory response and the exorbitant intestinal damage in AIM2^{-/-} mice indicate an essential contribution of AIM2 in limiting inflammatory response and preserving intestinal immune homeostasis. Thus, it is worthwhile to investigate how intestinal immune homeostasis is impaired in the absence of AIM2.

Maintaining intestinal immune homeostasis and generating a proper inflammatory response to a pathogen depend on the integrity of the intestinal barrier. Epithelial cell turnover is required in sustaining intestinal barrier function.²⁹ Cell turnover,

also known as tissue homeostasis, is the regulation of cell proliferation and cell death and has an important role in maintaining normal tissue function and architecture.³⁰ IECs at the villus tips constantly undergo cell death and cell shedding, which contributes to epithelial cell turnover and prevents persistent bacterial colonization.^{1,31} These cells are constantly renewed from cryptic stem cells. If the death and renewal homeostasis of the GI epithelium is broken, then there is an increased risk of microbial infection, tissue injury, or hyperplasia. Our results showed that naive AIM2^{-/-} mice have increased epithelial cell proliferation and a similar level of epithelial cell apoptosis. Following *Salmonella* challenge, AIM2^{-/-} mice were unable to respond normally compared with WT mice, as indicated by the significantly decreased proliferation and increased apoptosis on day 2 p.i. These results suggest that AIM2 is required to maintain the proliferation/apoptosis homeostasis of IEC.

Although we cannot exclude the possibility that abnormal epithelial proliferation/apoptosis in AIM2^{-/-} mice on day 2 p.i. is a consequence rather than the cause of exacerbated inflammation, the deregulated proliferation in naive AIM2^{-/-} mice may contribute to the higher bacterial dissemination and excessive inflammation response. To explore this possibility, bacterial burden was assessed in the cecal LP and lymph nodes as early as 3 h p.i. Higher bacterial loads in both sites of AIM2^{-/-} mice suggest that AIM2 deficiency results in an impaired epithelial barrier. The pathogen can engage IECs or LP phagocytes for transversing the epithelium to access the LP. Based on a previous report that AIM2-deficient macrophages respond to *Salmonella* with normal IL-1 β production¹¹ and our data regarding increased IEC proliferation in naive AIM2^{-/-} mice, we hypothesized that the initially increased bacterial invasion from the GI lumen into the LP in AIM2^{-/-} mice may be attributable to abnormal IEC function. This hypothesis was tested using a human intestinal epithelial cell line, Caco-2. As expected, AIM2 overexpression restricted *Salmonella* translocation across epithelial cells. Moreover, we found that the altered capacity of paracellular rather than transcellular penetration was responsible.

The integrity of the gut epithelium is also sustained through tight junction, which acts as a permeable barrier in the defense against foreign antigens and microbes. A loss of tight junction structure and function is frequently observed in inflammatory bowel disease, leaky diarrhea, and epithelium-derived cancers.^{32–34} Disruption of intestinal tight junction by microbes benefits their dissemination inside the host. We thus examined the effects of AIM2 on the expression of tight junction proteins. Our results showed that the basal level of Claudin3 and Occludin in naive AIM2^{-/-} mice was significantly lower compared with those in WT mice. This effect was confirmed *in vitro*. Moreover, AIM2 overexpression resulted in an increased TER. Collectively, these data suggest that AIM2 contributes to maintain the integrity of intestinal barrier through enhancing the expression of tight junction proteins. Due to the disrupted integrity of the intestinal barrier, AIM2^{-/-} mice are more readily invaded by pathogens and consequently exhibit a higher mortality rate, bacterial

dissemination, and overwhelming inflammation. Although barrier defects were found in AIM2^{-/-} mice, spontaneous inflammation was not observed. This result suggests that intestinal barrier dysfunction is insufficient to cause spontaneous intestinal inflammation. There might be a compensatory mechanism helping to maintain homeostasis in uninfected AIM2^{-/-} mice under uninfected condition. However, in a hostile environment, AIM2^{-/-} mice are more readily infected due to the fragile intestinal barrier.

Subsequently, we observed that AIM2-overexpressing Caco-2 cells had a higher level of Akt phosphorylation compared with that in control cells. *Salmonella* infection activated phospho-Akt at 120 min, and a corresponding increase in Claudin3 expression was observed. The chemical inhibition of Akt activation prevented the induction of Claudin3 by *Salmonella* challenge. Thus, we found that AIM2 regulates Akt signaling, thus mediating the enhancement of tight junction proteins. How AIM2 regulates Akt phosphorylation in epithelium upon *Salmonella* challenge remains elusive. Recently, a report has shown that AIM2 suppresses colon tumorigenesis by interfering with Akt activation in an inflammasome-independent manner.³⁵ In our study, IL-1 β production was higher and IL-18 transcript (Supplementary Figure S3) remained comparable in the cecum of AIM2^{-/-} mice compared with WT mice after *Salmonella* challenge. However, we still cannot make sure that AIM2 contributes to the maintenance of intestinal integrity in an inflammasome-independent manner. To address this possibility, caspase-1-deficient mice and mice deficient in both caspase-1 and AIM2 need to be analyzed with respect to inflammatory responses and tight junctions.

Salmonella have evolved various mechanisms to penetrate the epithelial monolayer, such as dysregulating tight junctions through effector proteins. It was previously reported that SopB, SopE, SopE2, and SipA can induce ruffling of cell membrane and change the localization and expression of ZO-1 and occludin, facilitating bacterial invasion.³⁶ *Salmonella* also secrete AvrA to stabilize tight junctions.³⁷ In this study, we found that Claudin3 expression gradually decreased until 60 min after *Salmonella* infection, and then increased at 120 min. This dynamic expression of tight junction protein might be due to the regulation of a panel of effectors, which facilitates pathogen invasion or intracellular survival. AIM2 enhances intestinal barrier function to restrict *Salmonella* invasion. There also might be some *Salmonella* effectors, which promote invasion by hampering AIM2 signaling.

In summary, our findings establish a role for AIM2 in maintaining intestinal barrier integrity, pave the way for future studies aiming to better define the role of AIM2 in regulating innate immunity, and provide clues regarding the control of intestinal diseases.

METHODS

Mice. AIM2^{-/-} mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and were subsequently backcrossed onto the C57BL/6J background for another eight generations. Heterozygous

breeding pairs were used to generate WT mice. WT and AIM2^{-/-} mice carry a mutant Nramp1 allele (Supplementary Figure S1). All animal studies were conducted according to the experimental practices and standards approved by the Animal Welfare and Research Ethics Committee at Jilin University.

In vivo infections. Six- to eight-week-old sex-matched mice were used in this study. The *Salmonella*-induced colitis model was established as previously described.³⁸ Briefly, following the administration of 20 mg of streptomycin per mouse, WT and AIM2-deficient mice were orally challenged with 5×10^7 CFU of *Salmonella* strain SL1344 for 3 h, 2 days, or 5 days p.i. Log CFU per organ values were determined. Cecum inflammation was analyzed 2 days p.i. For systemic infection, mice were intraperitoneally infected with 5×10^4 CFU of *Salmonella* and log CFU per organ values were determined at 3 days p.i. For the survival study, WT and AIM2^{-/-} mice were orally infected with 1×10^8 CFU or intraperitoneally infected with 5×10^4 CFU of *Salmonella*. Bacterial loads were measured as follows. Spleen, liver, and MLN were weighed and homogenized in cold phosphate-buffered saline (PBS) at a ratio of 4 mL per gram of tissue; for the cecum, tissue was washed free of feces and weighed and subsequently homogenized in cold PBS. For the LP, the bacterial load was examined as previously described.³⁹ Briefly, the cecum was washed free of feces with PBS three times and then cut into small pieces and incubated in Hank's Balanced Salt Solution containing 5 mM EDTA, 10 mM hydroxyethyl piperazine ethanesulfonic acid, and 5% fetal bovine serum at 37°C for 20 min, followed by vigorous vortexing to remove the epithelial cell layers. This process was repeated two times. The remaining tissue was homogenized in sterile PBS, and serial dilutions were plated on lysogeny broth agar.

Histology and immunofluorescence. At 2 days p.i., the cecum was harvested, washed free of feces with cold PBS, it was then fixed in formalin and embedded in paraffin; 5 μ m sections were used for H&E or immunofluorescence staining. Histological assessment was performed randomly using a scoring system as previously described.³⁸ Immunostaining was performed using anti-PCNA (1:50, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-Claudin3 (1:100, Abcam, Cambridge, MA) antibodies. Epithelial cell apoptosis was analyzed by TUNEL staining using a commercial kit (KeyGEN BioTECH, Nanjing, China).

Western blotting analysis. The ceca of mice were harvested and washed free of feces with PBS, and then homogenized in lysis buffer containing 1% Triton X-100, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1 mM Na₃VO₄, 0.1 mM NaF, 1 mM PMSF, 5 μ g mL⁻¹ Aprotinin, 5 μ g mL⁻¹ Leupeptin, and 5 μ g mL⁻¹ Pepstatin A. Homogenates were centrifuged at 13,000 r.p.m. per minute for 30 min and supernatants were collected and used for immunoblotting analysis. The lysates (30 μ g) were subjected to 12% sodium dodecylsulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA). After blocking with 5% milk, the membrane was blotted with antibodies against iNOS (Abcam), Cox-2 (Abcam), Claudin3 (Abcam), Phospho-Akt (Cell Signaling, Beverly, MA), Cleaved caspase-3 (Cell Signaling), AIM2 (Proteintech, Wuhan, China), and β -Tubulin (Sungene Biotech, Tianjin, China).

Cytokine analysis. The ceca of mice were harvested, washed free of feces with PBS, and subsequently, weighed and homogenized in cold PBS at a ratio of 4 mL per gram tissue. The homogenates were centrifuged at 13,000 r.p.m. per minute for 30 min, and supernatants were used for cytokine examination. Cytokines and chemokines in tissue homogenate were detected by an enzyme-linked immunosorbent assay following the R&D Systems (Minneapolis, MN) instruction.

RNA isolation, reverse transcription-PCR, and real-time PCR. Total RNA was extracted from tissue samples using TRI-Reagent (Sigma-Aldrich, Shanghai, China) according to the manufacturer's instructions. The reverse transcription-PCR was performed using a RT-PCR Kit (TaKaRa, Kyoto, Japan). Quantitative real-time PCR was

performed on an IQ5 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) using SYBR Green PCR Master Mix (Roche Diagnostics, South San Francisco, CA). The levels of cDNA were determined using a standard curve and normalized to GAPDH. Each example was run in triplicate. The relative changes in gene expression were analyzed by the $2^{-E_{ac}}$ method, and a melting-curve analysis was performed to ensure the specificity of the products. The following primers were used: Claudin3, 5'-CCTAGGAAGTGTCC AAGCCG-3' and 5'-CCCGTTTCATGG TTTGCCTG-3'; Occludin, 5'-CAGCCTTCTGCTTCATCG-3' and 5'-GTCGGGTCACTCCC AT TA-3'; ZO-1, 5'-GACCTTGAGCAGCCGTCATA-3' and 5'-CCGTAGG CGATGGTCATAGTT-3'; GAPDH, 5'-CACCCAGC AAGGACACT GAGCAAG-3' and 5'-GGGGGTCTGGGATGGAAATTGTGAG-3'.

In vitro infection and translocation assay. Caco-2 cells and AIM2-overexpressing Caco-2 cells were seeded at a high density of 5×10^5 cell per transwell filter (3 μ m pore size, Thermo Fisher, Odense, Denmark, DM) for bacterial translocation and invasion studies. The medium was refreshed daily until the transepithelial electrical resistance was $\geq 250 \Omega \text{ cm}^{-2}$, as measured using a Millicell Electrical Resistance System (Millipore). Before infection, the medium was changed to Dulbecco's Modified Eagle's Medium (DMEM)/F12. *Salmonella* was added at 1×10^7 CFU per well. For translocation, 1 h p.i., samples were taken from the basolateral chamber and rates of translocation were determined. To determine infection, 1 h p.i., the medium was replaced with fresh medium containing $100 \mu\text{g mL}^{-1}$ gentamycin for 30 min to kill the extracellular bacteria. The filter was washed apically and basolaterally three times with PBS and then lysed in 1 mL of 0.1% Triton X-100 for 10 min. Serial dilutions were plated on lysogeny broth agar plates.

AIM2-overexpressing Caco-2 cell construction. AIM2 overexpression was performed with Lipofection2000 (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. Caco-2 cells were stably transfected with plasmid pcDNA3.0 or pcDNA3.0 encoding human AIM2. Forty-eight hours post transfection, G418 was added at a final concentration of 500 ng mL^{-1} ; the culture medium was replenished every other day until single clones were formed.

Inhibition of Akt and caspase-1 activity in vitro. Cells were pretreated with $1 \mu\text{M}$ Akt inhibitor (MK2206) for 2 h and $5 \mu\text{M}$ caspase-1 inhibitor (VX-765) for 45 min.

Statistical analyses. All experiments were independently performed three times in triplicate. The results are showed as mean $\pm$ s.e. The differences between mean values were assessed with ANOVA Dunnett's multiple comparison test and Student's *t*-test, and a value of $P < 0.05$ was considered to be statistically significant. Statistics were calculated with the Prism software (GraphPad, La Jolla, CA).

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

ACKNOWLEDGMENTS

This work was supported by National Natural Science Foundation of China (No. 31372410), National Key Basic Research Program of China (No. 2011CB944404), Changjiang Scholars and Innovative Research Team in University (No. IRT1248).

DISCLOSURE

The authors declared no conflict of interest.

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