T cell immunoglobulin and mucin protein-3 (Tim-3)/Galectin-9 interaction regulates influenza A virus-specific humoral and CD8 T-cell responses

Shalini Sharma a, Aarthi Sundararajan b, Amol Suryawanshi a, Naveen Kumar c, Tamara Veiga-Parga a, Vijay K. Kuchroo d, Paul G. Thomas a, Mark Y. Sangster b, and Barry T. Roues a,1

aDepartment of Pathobiology, College of Veterinary Medicine, University of Tennessee, Knoxville, TN 37996-0845; bDavid H. Smith Centre for Vaccine Biology and Immunology, Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, NY 14642; Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA 30322; cCenter for Neurologic Diseases, Brigham and Women’s Hospital and Harvard Medical School, Boston, MA 02115; and dDepartment of Immunology, St. Jude Children’s Research Hospital, Memphis, TN, 38105-3678

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Reactions to pathogens are usually tuned to effect immunity and limit tissue damage. Several host counterinflammatory mechanisms inhibit tissue damage but these may also act to constrain the effectiveness of immunity to acute infections, as we demonstrate in mice acutely infected with influenza A virus (IAV). We show that compared with wild type (WT), galectin-9 knockout (G9KO) mice mounted a more robust acute phase virus-specific CD8 T-cell response as well as higher and more rapid virus-specific serum IgM, IgG, and IgA responses and also cleared virus more rapidly than did WT mice. Blocking galectin-9 signals to Tim-3 via a Tim-3 fusion protein resulted in improved immune responses in WT mice. When IAV immune mice were challenged with a heterologous IAV, the secondary IAV-specific CD8 T cells were also present on day 10 postinfection (p.i.) in the spleen (Fig. 2J). Using tetramers and the intracellular cytokine staining (ICCS) assay to detect DNP66-74 (ASNENMETM)-specific CD8 T cells, up to 75% IAV nucleoprotein (NP) tetramer-specific cells were Tim-3+ (Fig. 1A and B) as well as CD44hi and CD62Llo (Fig. S1B, Lower). Furthermore, after NP peptide stimulation, the majority (around 75%) of IFNγ+ CD8 T cells were Tim-3+ (Fig. S1A). Endogenous levels of Gal-9 in the lung extracts of IAV-infected animals were also quantified by both Western blotting (Fig. S1C) and ELISA (Fig. S1D). Basal levels of Gal-9 were detectable in control lung extracts and these were moderately increased after IAV infection. A significant increase (2–2.5 fold) was observed at around 7 d p.i. We could also show at day 10 p.i. that around 20–22% of total CD4 T cells (Fig. 1C) and Tim-3+ CD8 T cells in the BAL samples of the WT animals. Moreover, using the ICCS assay to detect NP311–325 (QVYSLIRPNENPAHK) peptide-specific CD4 T cells, up to 65–70% (on average) of virus-specific IFNγ+ CD4 T cells were Tim-3+ (Fig. 1D). Tim-3 expression was not detected on CD3+ B220+ CD19+ B cells.

Gal-9 Induces Apoptosis of IAV NPtet+CD8 T Cells ex Vivo. Ex vivo experiments were performed with splenocytes from IAV-infected mice at day 10 p.i. to determine whether the NPtet+ population (the majority of which were Tim-3+) would undergo apoptosis upon exposure to recombinant Gal-9. As shown in Fig. S2 B and D at an optimal dose the majority of NPtet+ CD8 T cells became annexin V+, indicative of their undergoing apoptosis, an effect inhibited by adding an excess of α-lactose (Fig. S2 A and B, Lower), the sugar that binds to Gal-9 and reduces its binding to Tim-3 (14, 15). The major effect of Gal-9 was observed in Tim-3+ cells (Fig. S3D) and was dose dependent. However, there was a low level of apoptosis of Tim-3+ cells (Fig. S3E) consistent with recent reports that Gal-9 could regulate T-cell function independently of Tim-3 (16). Our ex vivo results indicate that Gal-9 binding to Tim-3+ effectors causes them to die by apoptosis in vitro, an effect that might similarly occur in vivo.

Animals Unable to Produce Gal-9 Mount Better Acute-Phase Virus-Specific CD8 T-Cell Responses. We reasoned that if the presence of endogenous Gal-9 acts to limit the magnitude of CD8 T-cell
responses in the WT animals, then mice unable to produce Gal-9 due to gene knockout (G9KO), should respond better than WT animals to IAV. To analyze this possibility, WT and G9KO animals were infected intranasally with 5,000 egg infective dose, 50% (EID₅₀) of IAV (x31) and the magnitude of CD8 T-cell responses were compared 6, 8, and 10 d later. As shown, the responses of virus-specific CD8 T cells in both BAL and spleen, as measured by tetramers (Fig. 2 A–E) and ICCS (Fig. 2 H–J) were significantly higher at multiple time points in the G9KO mice. In both WT and G9KO animals up to 70–73% IFNγ⁺ CD8 T cells were Tim-3⁺ (Fig. 2F). However, a higher proportion of Tim-3⁺ and NPtet⁺ T cells in G9KO were CD44⁺ (Fig. 2 K and L) and CD62L⁻ (Fig. 2 M and N), indicating that more virus-specific CD8 T cells in G9KO animals expressed the activation phenotype. We also evaluated and compared the mean fluorescence intensity (MFI) of IFNγ and coexpression of multiple cytokines, both indicative of high quality T cells (17). G9KO animals expressed significantly higher MFI of IFNγ compared with WT animals (Fig. 2F). Collectively our data indicate that G9KO animals have a 2.5- to 3.5-fold augmented IAV-specific CD8 T-cell responses that have a higher proportion of CD8 T cells with properties of high quality.

ICCS was also performed on the BAL fluid cells using NP₃₁₁–₃₂₅ peptide stimulation (to detect IAV-specific CD4 T cells) at day 10 p.i. G9KO mice had two- to threefold higher frequencies of IFNγ⁺ CD4 T (IAV-specific CD4 T) cells in the BAL compared with the WT (Fig. 1D). Additionally, around 39–40% of CD4 T cells were Tim-3⁺ in the BAL of G9KO (Fig. 1C) compared with only 18–20% in the WT mice. It is conceivable that better CD4 T-cell responses of G9KO mice play a role in maintaining higher CD8 T-cell responses and also provide help for antibody (Ab) responses (18). Additionally, compared with WT mice, G9KO animals had fewer Tregs both in the BAL (Fig. S4A–C) and spleen (Fig. S4D) particularly that were Tim-3⁺ and CD103⁺ (Fig. S4E and F).

Gal-9 Knockout Mice Generate a More Rapid IAV-Specific Humoral Response. WT and G9KO mice were also compared for their plasma virus-specific Ab responses. Plasma levels of virus-specific IgM and IgG were substantially higher in G9KO compared with WT mice on day 7 (Fig. 3A and B), indicating a markedly more rapid Ab response in the G9KO mice. IgG levels remained significantly higher in G9KO mice than in WT mice at day 8 p.i. in the BAL of G9KO (Fig. 1C) compared with only 18–20% in the WT mice. It is conceivable that better CD4 T-cell responses of G9KO mice play a role in maintaining higher CD8 T-cell responses and also provide help for antibody (Ab) responses (18). Additionally, compared with WT mice, G9KO animals had fewer Tregs both in the BAL (Fig. S4A–C) and spleen (Fig. S4D) particularly that were Tim-3⁺ and CD103⁺ (Fig. S4E and F).

Gal-9 Knockout Mice Develop More Robust Recall Responses to IAV Infection. To compare recall response to IAV, animals were primed with x31 then challenged intranasally (i/n) 41 d later with the heterologous IAV strain PR8. The NPtet⁺ CD8 T-cell responses were around four- to fivefold higher in the G9KO mice compared with WT animals in the BAL (Fig. 4A and D) and spleen (Fig. 4E, H, and J). Additionally G9KO mice had a fourfold greater proportion of NPtet⁺ cells that were Tim-3⁺ in the BAL (Fig. 4C) and spleen (Fig. 4G). More interestingly, when primary and secondary responses were compared, WT mice expressed two- to threefold higher frequencies of NPtet⁺ CD8 T cells compared with the primary response whereas in G9KO mice the NPtet⁺ T-cell frequencies were enhanced eightfold. Furthermore a significantly higher proportion of NPtet⁺ CD8 T cells were CD62L⁺ (fivefold higher) in the BAL (Fig. 4B) and (ninefold higher) in the
spleens (Fig. 4F) of G9KO animals. Using the ICCS assay IFNγ+TNFα+ CD8 T cells in BAL (Fig. 4F and K) and spleen (Fig. 4L) in G9KO mice were increased over WT, indicative of larger and higher quality responses. The significantly higher proportion of CD62Llo and Tim-3lo IAV NP tetramer-specific CD8 T cells in the G9KO mice that followed secondary infection suggests that a greater proportion of these cells were in an activated state in G9KO mice during recall responses. In an attempt to address the issue of whether G9KO CD8 T cells were intrinsically more responsive or being less inhibited in G9KO environment, adoptive transfer experiments were conducted (Fig. S8A). The accumulation of WT DNPtet+ donor Thy1.1+ CD8 T cells was significantly
greater in G9KO than WT recipient animals (Fig. S8 G and H), indicating that Gal-9/Tim-3 signaling is responsible for the observed T-cell phenotype. The reverse experiment, where WT or G9KO (Thyl.1) cells were transferred to WT (Thyl.1) animals resulted in no observed differences in expansion. Taken together, our results suggest that the Tim-3/Gal-9 interaction acts in normal animals to limit the magnitude and efficiency of recall CD8 T-cell responses.

In Vivo Blockade of Tim-3 Pathway Results in Augmented Primary IAV-Specific CD8 T-Cell Responses. Our results indicated that Gal-9 may be playing a role in vivo to limit the extent of antiviral CD8 T-cell responses. To further evaluate the regulatory effect of Tim-3/Gal-9 interaction, this pathway was inhibited in vivo with a Tim-3 fusion protein (Tim-3 Ig). Using tetramers and the ICCS assay to record virus-specific CD8 T-cell responses in the BAL and spleen, Tim-3 blockade resulted in ~1.6- to twofold higher frequencies and fourfold higher absolute numbers of NPtet$^+$ and IFNγ$^-$/TNFα$^+$ CD8 T cells in BAL (Fig. 5A and B) and spleen (Fig. 5E) at day 10 p.i. The blockade also resulted in better viral control (Fig. 5C). Additionally a higher proportion of virus-specific CD8 T cells were CD62L$^+$Tim-3$^+$ in the BAL (Fig. 5D) and spleen (Fig. 5F) of Tim-3 Ig-treated mice. A similar phenotype was observed when the Tim-3 fusion protein administration was begun at day 4 p.i. (Fig. S9).

Discussion

The host response to pathogens is usually tuned to effect immunity and to minimize any bystander tissue damage resulting from the immune reaction to the invader (1). Tissue damage is limited by several counterinflammatory events that act to functionally inhibit or destroy damaging cells as occurs when Gal-9 binds to one of its receptors, Tim-3 (14). Counterinflammatory events benefit the host in some chronic inflammatory processes (10, 19), autoimmunity, and some viral immunopathological lesions (11, 14) but, as we demonstrate in this report, Tim-3/Gal-9 interactions can also act to limit the effectiveness of immunity to acute infectious agents, such as influenza virus. Accordingly, we show that mice lacking the ability to produce Gal-9 because of gene knockout generate more robust antiviral T-cell responses, more rapid antibody responses, and control intranasal infection more effectively than WT animals. The more effective responses to IAV by G9KO animals was explained by the observation that virus-specific CD8 and CD4 T cells up-regulate Tim-3 early after infection, making them susceptible to apoptosis upon binding to Gal-9, as we demonstrated in ex vivo studies. G9KO animals also developed better recall responses to IAV and generated superior CD8 T-cell responses compared with WT upon heterologous IAV infection. Our results could mean that manipulating signals that are usually provided by Gal-9 could result in improved responses to influenza vaccines.

Our data indicate that decreased cell death responses observed in WT animals were likely the consequence of the elevated endogenous production of Gal-9 that occurred after infection, along with the fact that most of the responder T cells, both CD8 and CD4, up-regulated the Tim-3 receptor. This scenario would set the stage for T-cell apoptosis as demonstrated in our ex vivo studies. G9KO animals also had lower numbers of Foxp3$^+$ Tregs compared with WT. Such cells could be responsible for suppressing the magnitude of influenza-specific T-cell responses as some have reported (20), at least as a minor effect. In addition to the observation of two- to threefold higher responses in G9KO animals, on the basis of multipletokine production and the levels of cytokine produced by antigen-stimulated CD8 T cells (17), the responses in G9KO mice were also of higher quality than were those in WT mice. This may mean that the levels of Tim-3 differ between responding T cells, accounting for differential suscep-
response to Gal-9–mediated killing or inhibition. However, such effects were not formally investigated.

Whereas a number of previous studies reported the modulating effects of Tim-3/Gal-9 on T-cell–mediated lesions and immunity, few if any have analyzed the influence on Ab responses. We show that the early production of virus-specific antibody responses to influenza infection was strikingly enhanced in the absence of Gal-9. Because we could not demonstrate Tim-3 expression on B cells, their destruction as a consequence by Gal-9 binding would seem an unlikely mechanism. A better explanation may be that the enhanced responses of G9KO animals reflected the absence of modulating effects of Gal-9 on Tim-3–expressing helper CD4 T cells. Antibody responses to influenza virus are largely T cell dependent (21). Thus, accelerated B-cell help due to an increase in the availability or effectiveness of CD4 T cells could explain the stronger early antibody responses observed in G9KO mice (22, 23). We did not evaluate CD4 T-cell activation in lymph nodes draining the respiratory tract where cognate help for early B-cell responses is delivered. However, Gal-9 deficiency resulted in higher frequencies of virus-specific CD4 T cells in the airways after influenza infection, consistent with a more vigorous CD4 T-cell response in the draining lymph nodes. The rapidity of the B-cell response in G9KO mice suggests an increase in antibody-secreting cell generation via the extrafollicular pathway of B-cell differentiation, an arm of the B-cell response that is enhanced by increased availability of T-cell help (24). The expression of Tim-3 by activated Th1 but not Th2 cells suggests that the Th1 response may be preferentially enhanced in G9KO mice. This could fit with the pattern of isotype expression in the antibody response of G9KO mice, because enhanced IgG2c production (driven by Th2-type cytokines). We also observed that the frequency of germinal center B cells was significantly lower in G9KO than in WT mice, likely reflecting differentiation of activated B cells via the extrafollicular pathway at the expense of germinal center formation in G9KO mice. At this time, we cannot exclude other mechanisms that may also contribute to the enhanced Ab response in G9KO mice. For instance Gal-9 deficiency may limit other mechanisms of suppression (25), resulting in increased levels of factors that act directly on B cells to promote their activation (26).

Antiviral antibody production in response to influenza infection contributes in large part to viral control (27). We analyzed virus-specific antibody levels in the airways as a measure of antibody-mediated antiviral activity at the site of viral replication. This strategy also permitted an evaluation of the effect of Gal-9 deficiency on the virus-specific IgA response, because IgA-secreting cells generated in lymphoid tissues rapidly home to the respiratory tract submucosa and secrete IgA that is transported to the airway lumen (21, 28). IgA that is recovered from the airways is thought to be primarily derived from circulating anti-

![Fig. 4](image-url)  
**Fig. 4.** Gal-9 knockout mice develop more robust recall responses to influenza virus upon heterologous challenge. Both WT and Gal-9 KO animals primed 41 d previously with x31 were challenged intranasally with 8,000 EID50 of heterologous IAV (PR8) and the response in the BAL and spleen measured at day 8 postchallenge by tetramers and ICCS assays. Representative FACS plots showing the frequencies of NPtetIgG9KO IFNγ D8 T cells are shown in BAL (A) and spleen (E and H), respectively. Absolute numbers of NPtetIgG9KO CD8 T cells are shown in BAL (D) and spleen (I). Coexpression of NPtet and CD62L are shown in BAL (B) and spleen (F). Tim-3 expression on NPtetCD8 T cells are isolated from BAL fluid (C) and spleen (G) of WT and G9KO animals. Representative FACS plots showing the frequencies (J) and absolute numbers (K) of IFNyTNFα CD8 T cells in the spleen of x31 immune WT and G9KO animals. Data are representative of three independent experiments. Error bars represent SEM.
body by transudation (29). The levels of virus-specific IgG and IgA recovered from the airways on day 10 after infection were generally higher in G9KO compared with WT mice, but differences were not statistically significant. However, the overall kinetic pattern suggested earlier production of both IgG and IgA in G9KO mice and a contribution of these antibodies to antiviral activity in the lung during the phase of viral clearance. A strong IgA response in G9KO mice, as for the IgG response, may reflect a more vigorous and sustained CD4 T-cell response (21). However, this mechanism must be weighed against evidence that strong Th1 responses and IFNγ production, as might be expected in G9KO mice, are antagonistic to IgA production (30–32). Further studies are required to clarify this situation.

Materials and Methods

Mice and Viral Infections. Female 6–8-wk-old C57BL/6 were purchased from Harlan Laboratories and housed in the animal facilities at the University of Tennessee, Knoxville. G9KO were kindly provided by Gal Pharma. Stocks of IAV strains HKx31 (H3N2)(x31) and A/Puerto Rico/8/34 (H1N1)(PR8) for mice infections were grown and titrated as described previously (33). Mice were infected intranasally with 5,000 EID50 of IAV HKx31 in 30-μl volume. To assess secondary CDT cell responses, mice were infected with x31 and challenged intranasally at least 4 wk later with 8,000 EID50 of PR8. The animal care and use committee of the University of Tennessee approved all animal procedures.

Tissue Sampling. Spleen, BAL fluid, and plasma and lung samples were recovered from mice at acute phases of the primary and secondary infections. BAL samples were obtained from individual mice as described previously (34). Cells in the BAL were collected by centrifugation and the supernatants were stored at –80 °C for ELISA. BAL-associated cells were pooled from three mice in each group and single-cell suspensions were prepared from individual spleens. IAV x31 titers in lungs were determined by plaque assay (35).