Protective antiviral antibody responses in a mouse model of influenza virus infection require TACI

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Antiviral Abs, for example those produced in response to influenza virus infection, are critical for virus neutralization and defense against secondary infection. While the half-life of Abs is short, Ab titers can last a lifetime due to a subset of the Ab-secreting cells (ASCs) that is long lived. However, the mechanisms governing ASC longevity are poorly understood. Here, we have identified a critical role for extrinsic cytokine signals in the survival of respiratory tract ASCs in a mouse model of influenza infection. Irradiation of mice at various time points after influenza virus infection markedly diminished numbers of lung ASCs, suggesting that they are short-lived and require extrinsic factors in order to persist. Neutralization of the TNF superfamily cytokines B lymphocyte stimulator (BLyS; also known as BAFF) and a proliferation-inducing ligand (APRIL) reduced numbers of antiviral ASCs in the lungs and bone marrow, whereas ASCs in the spleen and lung-draining lymph node were surprisingly unaffected. Mice deficient in transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI), a receptor for BLyS and APRIL, mounted an initial antiviral B cell response similar to that generated in WT mice but failed to sustain protective Ab titers in the airways and serum, leading to increased susceptibility to secondary viral challenge. These studies highlight the importance of TACI signaling for the maintenance of ASCs and protection against influenza virus infection.

Introduction

Antiviral Abs are critical for host protection and are the basis of successful vaccines. Abs produced in response to influenza infection are crucial for viral neutralization (1) and defense in a secondary viral infection. While the half-life of serum Igs is on the order of weeks (2), specific serum Ab titers can last a lifetime (3). Long-lived Ab-secreting cells (ASCs) and memory B cells are responsible for Ab maintenance and can persist for years (reviewed in ref. 4).

Influenza infection in mice induces virus-specific ASCs that are present for several months in various organs, including BM, spleen, lung-draining mediastinal lymph node (medLN), and lungs (5–9). ASC differentiation is governed by changes in gene expression mediated by key transcription factors including B lymphocyte–induced maturation protein 1 (BLIMP-1) (reviewed in ref. 10). ASCs have been subdivided into two subsets based on their lifespan, cell cycle activity, and expression levels of BLIMP-1: short-lived ASCs, representing early rapidly dividing cells with a life expectancy of 3–5 days and lower BLIMP-1 expression; and long-lived ASCs, persisting for the lifetime of the mouse, with decreased cell cycle activity and higher levels of BLIMP-1 (2, 11–13). Furthermore, long-lived ASCs survive when exposed to irradiation or treatment with cyclophosphamide, while short-lived ASCs do not (2, 14). Both ASC subsets can exist in the spleen of mice; however, long-lived ASCs preferentially localize to the BM (2, 13, 15).

The factors responsible for ASC longevity are not well understood. It has been postulated that ASC maintenance is independent of antigen (16), but critically dependent on access to a number of factors that constitute a survival niche (reviewed in ref. 17). These include physical association with BM stromal cells, signals through the Fc receptor CD32, and soluble factors such as the chemokine CXCL12 and the inflammatory cytokines IL-6 and TNF-α (18–20). Under conditions of inflammation, survival factors can be upregulated in tissues, leading to ASC recruitment and retention (reviewed in ref. 21). Whether the lung following a respiratory viral infection provides a survival niche for ASCs has yet to be determined. Indeed, reports in the literature are inconclusive regarding ASC persistence in the respiratory tract (RT) following influenza virus infection (5, 9).

Recent studies have emphasized the significance of the TNF superfamily members B lymphocyte stimulator (BLyS, also termed BAFF) and a proliferation-inducing ligand (APRIL) as key regulators of ASC survival (22–25). BLyS and APRIL can be produced by activated cells from the myeloid lineage, BM stromal cells, airway epithelial cells, and activated T cells (26–31). In both mice and humans, BLyS and APRIL expression by DCs and macrophages can induce Ig class switching and ASC differentiation (28, 32–35).

BLyS and APRIL bind two receptors, transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI) and B cell maturation antigen (BCMA), while BLyS also binds a third receptor, BLyS receptor 3 (BR3). BCMA is expressed on ASCs in the BM, which are severely reduced in *BCMA*$^{-/-}$ mice (25, 36). TACI is expressed on follicular B cells and at much higher levels on CD138^{+} ASCs and marginal zone (MZ) and B1 B cells (36–38).

Conflict of interest: Richard J. Bram is a co-inventor on patents regarding the *TACI* gene.

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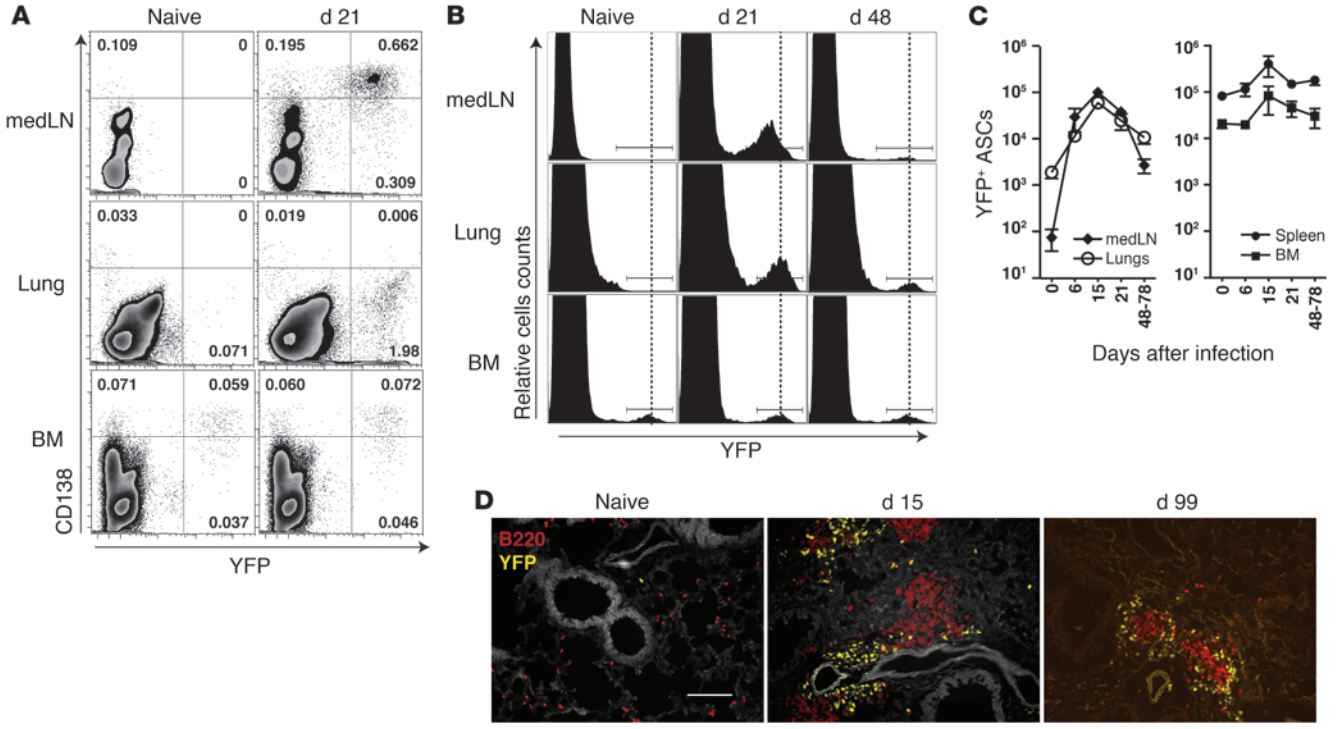

Figure 1
Kinetics of ASCs during influenza virus infection. (**A**) Naive and influenza virus PR8–infected BLIMP-1–YFP mice (day 21 after infection) were analyzed for YFP$^+$CD138$^+$ ASCs in lung-draining medLN, lungs, and BM. Cells expressing CD4, CD8, and CD11b were excluded. Dot plots with percentages shown are representative of n = 3–5 mice from at least 2 independent experiments. (**B**) Level of YFP expression in ASCs from medLN, lungs and BM isolated from naive and influenza-infected BLIMP-1–YFP mice. Histograms are based on CD4$^-$CD8$^-$CD11b$^-$ cells and representative of n = 5–7 mice. (**C**) Kinetics of YFP$^+$ ASCs during influenza virus infection. Data with mean ± SEM are representative of n = 3–5 mice per time point. (**D**) Immunofluorescence of lungs from naive and infected BLIMP-1–YFP mice stained for B220 (red). Original magnification, ×20, Scale bar: 100 μm.

TACI has also been detected intracellularly in human DCs (39) and on activated T cells to varying degrees (reviewed in ref. 40). The role of TACI in isotype switching is controversial: this receptor augments class switching in some cases, as revealed by absence of functional TACI, such as in patients with common variable immunodeficiency disease (CVID) (41–43), but has an inhibitory function in others (44). In vivo studies in *TACI*$^{-/-}$ mice have found no reduction in Abs produced in T cell–dependent responses to NP-CGG (4-hydroxy-3-nitrophenylacetate coupled to chicken γ-globulin), while T cell–independent B cell responses to NP-Ficoll and NP-LPS are impaired (45–48). Others have found that use of TACI-Fc or BCMA-Fc fusion proteins for neutralization of both BLyS and APRIL results in diminished ASCs and Abs to the T cell–dependent antigens NP-CGG and keyhole limpet hemocyanin (KLH), and T cell–independent antigen Pneumovax (49–52).

In this study we address whether ASCs in the RT in comparison to lymphoid organs of influenza-infected mice are long-lived and what signals promote their survival. We demonstrate the importance of extrinsic signals such as the TACI ligands BLyS and APRIL for the longevity of humoral immunity and protection to influenza virus infection.

Results

Kinetics of ASCs during influenza virus infection. To study ASCs in vivo, we used transgenic BLIMP-1–YFP reporter mice, which express the yellow fluorescent protein (YFP) on a bacterial artificial chromosome under the control of regulatory sequences from the *Prdm1* gene locus (53). Analysis of the BM and spleen of naive BLIMP-1–YFP mice revealed a small population of cells expressing high levels of YFP, the majority of which coexpressed CD138, consistent with the surface phenotype of ASCs (Figure 1A and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI57362DS1). YFP$^+$ ASCs were CD4$^-$CD8$^-$CD11b$^-$ and expressed varying levels of CD19 depending on the tissue (Supplemental Figure 1A and data not shown). These mice facilitate the study of RT ASCs, since the collagenase digestion required to liberate cells from the lungs reduced cell surface levels of CD138 (Supplemental Figure 1B).

In naive mice, YFP$^+$ cells were rare in the lung-draining medLN and in the lungs (Figure 1, A, B, and D). However, after intranasal infection with influenza virus, we observed a significant increase in YFP$^+$ ASCs in these organs (10- to 100-fold), while the number of ASCs in spleen and BM changed little during the course of infection (Figure 1C). Noticeably, lung ASCs exhibited higher YFP expression compared with ASCs in medLN (Figure 1, A and B). Based on previous reports using *Blimp-1*^{gfp} reporter mice, which correlated higher GFP levels with increasing levels of BLIMP-1 expression and advanced stages of ASC differentiation (11), these results suggest that ASCs in the lungs were further differentiated than ASCs in the medLN.

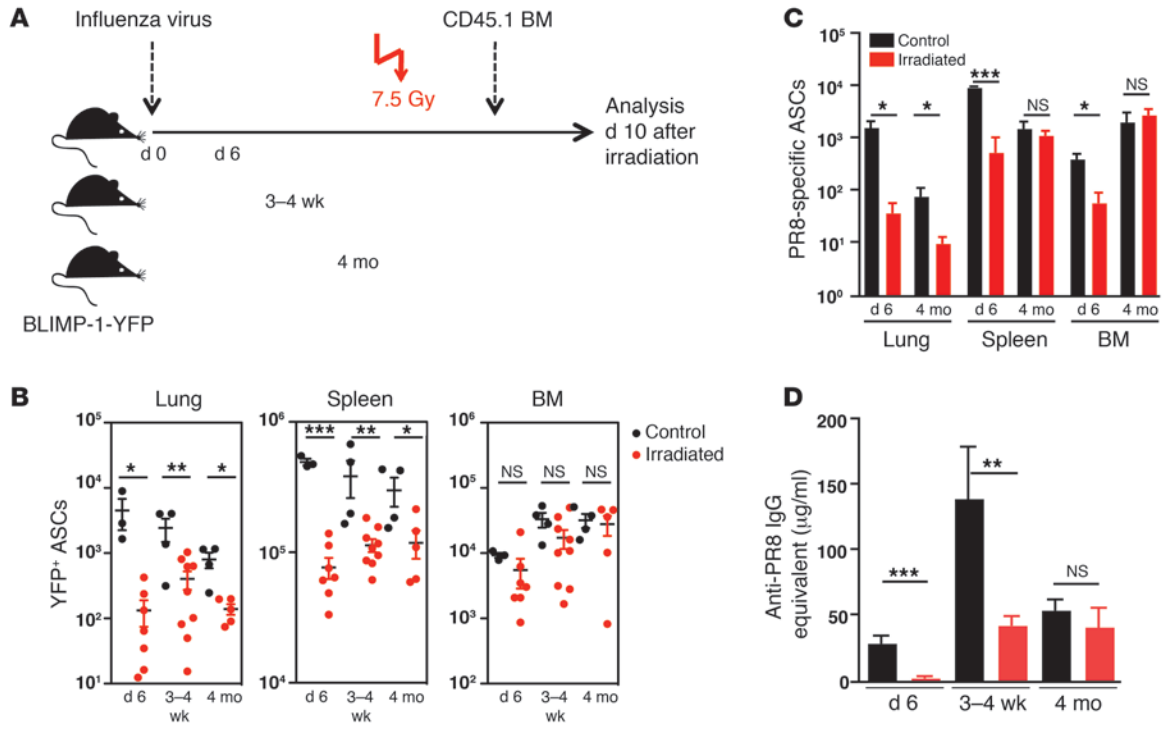

Figure 2

ASCs in lungs of influenza virus–infected BLIMP-1–YFP mice are sensitive to irradiation. (**A**) Influenza virus PR8–infected BLIMP-1–YFP mice (n = 3–9 mice/group) were subjected to whole-body irradiation (7.5 Gy) at 6 days, 3–4 weeks, or 4 months p.i. and analyzed 10 days later together with non-irradiated controls. (**B**) Total YFP$^+$ ASCs in lungs, spleen, and BM were determined by flow cytometry. (**C**) PR8-specific ASCs were determined by ELISPOT. (**D**) PR8-specific IgG Ab titers in sera from control and irradiated mice were measured by ELISA. All data with mean ± SEM represent at least 2 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

The localization of ASCs in tissues was examined using immunofluorescence. In the spleen, YFP$^+$ ASCs were detected mainly in extrafollicular foci between B220$^+$ follicles and CD4$^+$ T cell areas in the red pulp (Supplemental Figure 1, C and D). YFP$^+$ ASCs in the lungs of virally infected mice were often associated with B220$^+$ aggregates (Figure 1D), known as inducible bronchus-associated lymphoid tissue (iBALT) (54), and were present for several months after infection.

ASCs in the lungs of influenza-infected mice diminish after irradiation. Long-lived ASCs are resistant to irradiation (2). Thus, we used this measure to determine whether ASCs in the lungs, as well as other sites, of influenza-infected mice were short- or long-lived. BLIMP-1–YFP mice were subjected to whole-body irradiation (7.5 Gy) at various times after infection (6 days, 3–4 weeks, or 4 months), subsequently reconstituted with BM from CD45.1 allotypic mice, and analyzed 10 days later (Figure 2A). Splenic YFP$^+$ ASCs were sensitive to irradiation (Figure 2B), in contrast to YFP$^+$ ASCs in the BM, indicating that the majority of ASCs are short-lived in the spleen and long-lived in the BM. YFP$^+$ ASCs in the lungs were markedly decreased after irradiation, suggesting that they are short-lived.

Virus-specific ASCs as determined by ELISPOT were significantly reduced in all organs after irradiation at day 6 post-infection (p.i.) (Figure 2C). This was consistent with a complete ablation of virus-specific IgG in the serum (Figure 2D). Although irradiation at 3–4 weeks after infection reduced virus-specific serum IgG, the levels were comparable to those in mice at 4 months after infection. Irradiation 4 months after infection had no further effect, indicating that antiviral serum IgG at this time is derived from a pool of irradiation-resistant, long-lived ASCs. Virus-specific ASC numbers in spleen and BM were not diminished by irradiation (Figure 2C), whereas virus-specific ASCs in lungs were significantly reduced.

In anticipation that short-lived ASCs would correspond to proliferating plasmablasts, we determined the cell cycle activity of RT ASCs by BrdU incorporation. Continuous supply of BrdU in the drinking water for 8 days, initiated at either day 8 or day 50 after infection of BLIMP-1–YFP mice, revealed that the turnover of YFP$^+$ ASCs in the lungs, unlike their counterparts in medLN and spleen, was low and resembled the frequency of BrdU$^+$ ASCs in the BM 2 months after infection (Supplemental Figure 2, A and B). These results suggest that the maintenance of ASCs in the RT, including virus-specific ASCs, is likely not due to replenishment through proliferation but is instead regulated by survival factors produced by radiation-sensitive sources.

Treatment with TACI-Fc in vivo reduces lung and BM ASCs but does not affect ASCs in medLN and spleen. APRIL and/or BLyS support survival of long-lived BM ASCs (24, 52). To address whether these cytokines mediate survival of RT ASCs following infection, we infected BLIMP-1–YFP mice with influenza virus and 35 days later treated them for 2 weeks with TACI-Fc (Figure 3A), which neutralizes both BLyS and APRIL (50, 55). TACI-Fc treatment reduced the percentage and number of total YFP$^+$ ASCs, including virus-specific ASCs, in lungs and BM. The P value approached significance in spleen (P = 0.0523), but strikingly ASCs in medLN were not affected (Figure 3, B and C), suggesting an organ-specific role

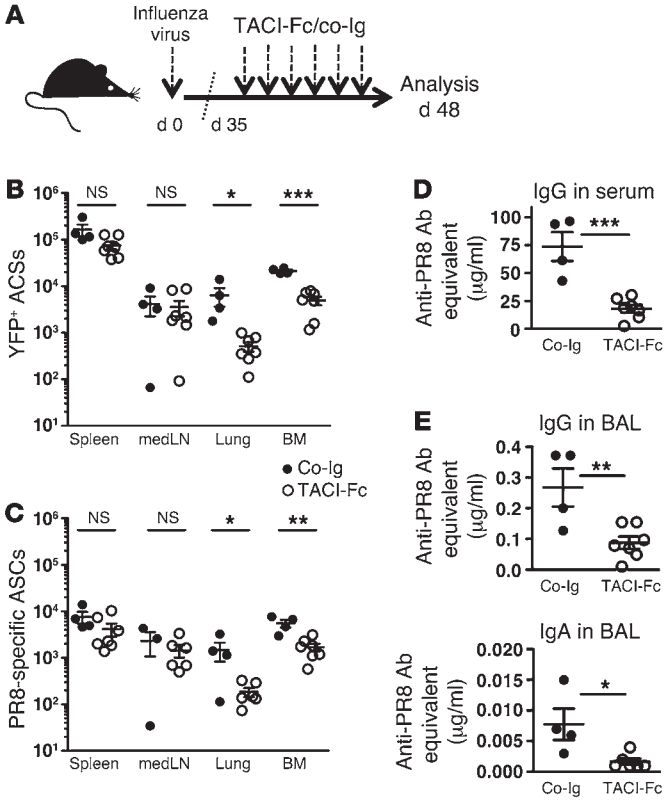

Figure 3
TACI-Fc treatment of BLIMP-1–YFP mice 1 month after influenza virus infection dramatically reduces virus-specific ASCs. (**A**) Thirty-five days after infection with influenza virus PR8, BLIMP-1–YFP mice were treated with 100 μg TACI-Fc ($n = 7$ mice) or human control IgG Ab (co-Ig) ($n = 4$ mice) every 2–3 days until analysis at day 48 p.i. (**B**) YFP$^+$ ASCs were enumerated in spleen, medLN, lungs, and BM. (**C**) PR8-specific ASCs were determined by ELISPOT. (**D**) PR8-specific IgG in serum and (**E**) PR8-specific IgG and IgA Ab titers in BAL from mice treated with TACI-Fc and co-Ig were determined by ELISA. All data with mean ± SEM represent 2 independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

for BLyS and/or APRIL in ASC survival. The decrease in ASCs in BM and lungs after TACI-Fc treatment was associated with a significant reduction in antiviral serum IgG (Figure 3D), as well as virus-specific IgG and IgA in the BAL (Figure 3E).

To test whether blockade of BLyS alone results in the reduction in antiviral ASCs, we treated BLIMP-1–YFP mice with anti-BLyS (56). CD19$^+$ B cells were significantly reduced in spleen, medLN, and lungs (Supplemental Figure 3A). Although YFP$^+$ ASCs were diminished in the spleen, neither total ASCs (Supplemental Figure 3B) nor influenza virus–specific ASC numbers (Supplemental Figure 3C) in any other of the organs analyzed were affected by neutralization of BLyS, and antiviral Ab titers remained undisturbed in serum and the bronchoalveolar space (Supplemental Figure 3, D and E). Taken together these results show that blockade of BLyS and APRIL, but not BLyS alone, regulates ASC survival in response to influenza virus infection.

We demonstrated that this tissue-specific dependence on BLyS and APRIL was not due to cell-intrinsic differences in receptor expression (Supplemental Figure 4). YFP$^+$ ASCs and B220$^+$YFP$^-$ B cells were sorted from medLN, spleen, lung, and BM of influenza-infected BLIMP-1–YFP mice on day 28 p.i. to determine the level of *BR3*, *TACI*, and *BCMA* mRNA. *TACI* and *BR3* were expressed similarly in all ASCs, while *BCMA* was elevated in BM ASCs compared with ASCs from the other organs.

Maintenance of influenza-specific Ab titers requires TACI. To address the impact of TACI on antiviral Ab responses, we infected *TACI*$^{-/-}$ and C57BL/6 (WT) mice with influenza virus. The strains had similar viral titers in their lungs at days 3 and 6 p.i., indicating a comparable course of infection (Supplemental Figure 5A). Virus-specific serum IgM peaked at similar levels by day 8 p.i. in WT and *TACI*$^{-/-}$ mice (Figure 4A). Moreover, no difference in virus-specific IgG in the serum was observed until 30 days p.i., when the titers declined significantly in *TACI*$^{-/-}$ mice. Thus, we conclude that early production of influenza-specific IgM and IgG is unperturbed in *TACI*$^{-/-}$ mice, whereas TACI is required for the maintenance of antiviral IgG.

We next measured the Ig content in the BAL from WT and *TACI*$^{-/-}$ mice 8 days and 1 month p.i. As in the serum, we detected no difference in virus-specific IgM in the BAL from either mouse strain on day 8 p.i. However, by day 32 p.i., virus-specific IgG and IgA were 2- and 10-fold reduced in BAL of *TACI*$^{-/-}$ mice, respectively (Figure 4B). The concentration of total IgG in BAL was not different, while total IgA was reduced in *TACI*$^{-/-}$ mice (Figure 4C). This finding is consistent with reports that *TACI*$^{-/-}$ mice, as compared with WT mice, have similar levels of IgG but reduced IgA in serum (45, 46) and that APRIL deficiency, like TACI deficiency, has a more marked effect on IgA (57).

The early influenza-specific ASC and CD8$^+$ T cell response in the medLN is normal in TACI$^{-/-}$ *mice.* To investigate whether early ASC differentiation was affected in *TACI*$^{-/-}$ mice, we enumerated influenza-specific ASCs 6–8 days p.i. in medLN, where the early B cell activation and ASC differentiation are primarily initiated (58, 59). WT and *TACI*$^{-/-}$ mice generated comparable frequencies and numbers of antiviral IgM-, IgG-, and IgA-producing ASCs (Figure 5A and data not shown), suggesting that early initiation of virus-specific ASCs is TACI independent, consistent with the observed serum Ab levels (Figure 4A).

Others have reported impaired CD8$^+$ T cell activation in the absence of TACI (60). However, CD8$^+$ T cells specific for influenza virus nucleoprotein (NP) were comparable in WT and *TACI*$^{-/-}$ mice at day 8 p.i. (Supplemental Figure 5, B and C).

Survival of ASCs in the BM and lungs, but not in medLN and spleen, is TACI dependent. The reduced virus-specific Ab titers in the serum after 1 month p.i. in *TACI*$^{-/-}$ mice (Figure 4A) coincided with significantly lower numbers of anti-influenza IgG and IgA ASCs in the BM (Figure 5B). A significant reduction in virus-specific IgA ASCs was also observed in the lungs. IgG ASCs were reduced, but the values were not statistically significant at that time point. In contrast, WT and *TACI*$^{-/-}$ mice harbored similar numbers of virus-specific ASCs in the medLN and spleen, suggesting a TACI-independent pathway of ASC survival in these organs. We showed that these differences were not due to altered total B cell or germinal center B cell numbers (Supplemental Figure 6, A–C).

Recent work by Bessa et al. has suggested that TACI is required for optimal ASC response after mucosal but not systemic immunization with virus-like particles (34). To test whether TACI-mediated effects on ASC responses to influenza virus are independent of the route of administration, we intravenously injected purified,

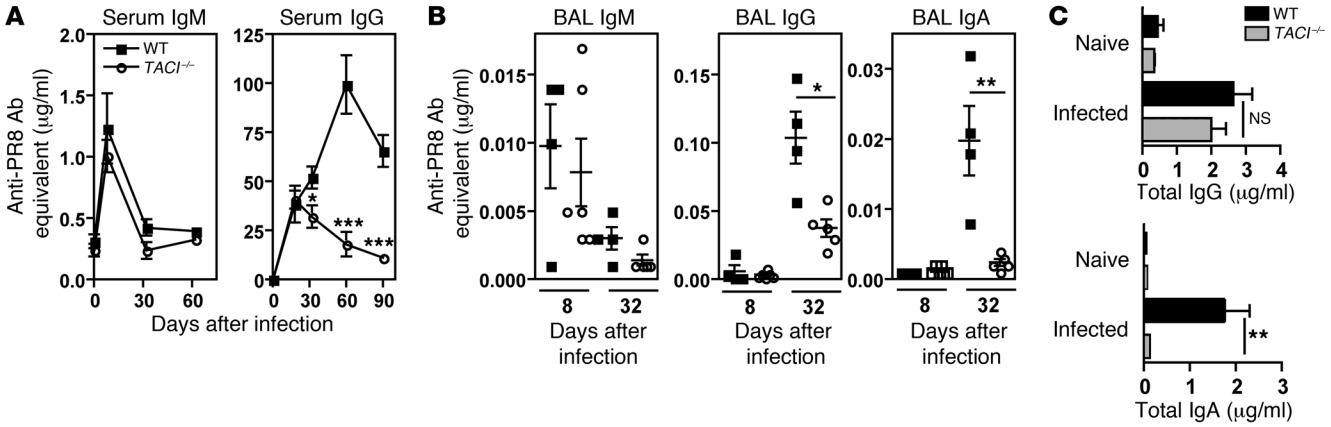

Figure 4
Maintenance of virus-specific Abs to influenza virus requires TACI. (**A**) Sera from WT and *TACI*$^{-/-}$ mice were assayed for PR8-specific IgM and IgG by ELISA. Blood samples were collected from naive mice (n = 3 mice) and on days 8, 18, 33, and 60 p.i. (n = 3–12 mice per time point). (**B**) PR8-specific IgM, IgG, and IgA Ab titers in BAL were measured at days 8 and 32–34 p.i. (**C**) Total IgG and IgA in BAL from WT and *TACI*$^{-/-}$ mice (n = 8–11 mice/group) were determined (days 32–34 p.i.). Data with mean ± SEM are representative of at least 2 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

non-infectious influenza virus into WT and *TACI*$^{-/-}$ mice. As with intranasal infection, PR8-specific ASCs were reduced in the BM of *TACI*$^{-/-}$ mice, while ASC numbers in the spleen were not different (Figure 5C). Virus-specific ASCs were not detectable in lungs with this route of immunization (data not shown). Together, these results indicate that maintenance of virus-specific ASCs in the BM, but not in medLN and spleen, require TACI after both mucosal and systemic administration of influenza virus.

BCMA has been shown to play an important role in survival of BM ASCs (25). To test whether BCMA plays a role in the humoral Ab response to influenza virus infection, we compared WT and *BCMA*$^{-/-}$ mice. Antiviral serum IgG titers as well as PR8-specific IgG and IgA ASCs were similar in WT and BCMA-deficient mice up to 2 months p.i. (Supplemental Figure 7, A–C), suggesting that BCMA is dispensable for the generation and maintenance of influenza-specific Abs.

T cell help is required for the induction but not maintenance of humoral immunity to influenza virus infection. Previous studies have found that *TACI*$^{-/-}$ mice have impaired responses to T cell–independent antigens, whereas no reduction was observed in Abs produced in T cell–dependent responses (45–48). To test the contribution of T cell help in the induction and maintenance of the anti-influenza Ab response, we performed blocking experiments with anti-CD40L Ab (MR1). WT and *TACI*$^{-/-}$ mice were treated with MR1 or hamster Ig control Ab starting at day 3 p.i. every 3 days until day 15 p.i., and serial bleeds were examined for antiviral IgG. Similar to studies in *CD40*$^{-/-}$ mice (61), blockade of CD40L resulted in significantly reduced virus-specific serum IgG level in WT mice. An analogous reduction in antiviral IgG was also observed in the serum of *TACI*$^{-/-}$ mice, suggesting that the antiviral Ab response in both, WT and *TACI*$^{-/-}$ mice is dependent in large part on T cell help (Figure 6A).

To address whether T cell help is required to sustain virus-specific ASCs and Ab titers, we infected BLIMP-1–YFP mice with influenza virus PR8 and after 38 days treated them with anti-CD40L. Treated mice showed a reduction in total YFP^{+} ASCs (Figure 6B) and in germinal center cell numbers (data not shown), but virus-specific ASCs were not affected in any of the organs examined

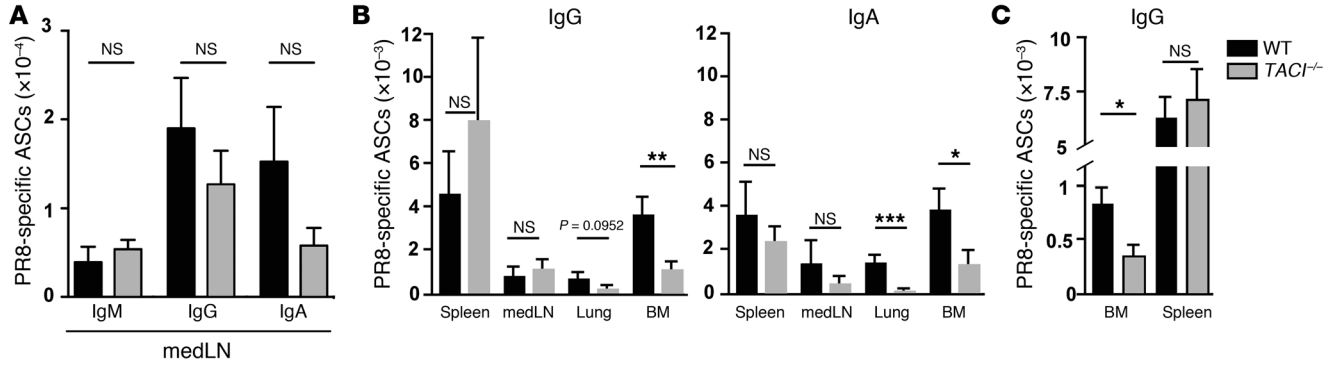

Figure 5
Maintenance but not early generation of virus-specific ASCs is impaired in *TACI*$^{-/-}$ mice. (**A**) MedLNs from WT and *TACI*$^{-/-}$ mice (n = 10–14 mice/group) were harvested 6–8 days p.i. and assayed for PR8-specific IgM, IgG, and IgA ASCs by ELISPOT. (**B**) PR8-specific IgG and IgA ASCs from indicated organs of WT and *TACI*$^{-/-}$ mice were determined by ELISPOT at days 32–34 p.i. (n = 5–8 mice/group). (**C**) WT and *TACI*$^{-/-}$ mice (n = 5 mice/group) received 1,000 HAU/100 μl purified PR8 virus intravenously, and PR8-specific IgG ASCs were enumerated 30 days later in BM and spleen. Data with mean ± SEM are representative of at least 2 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

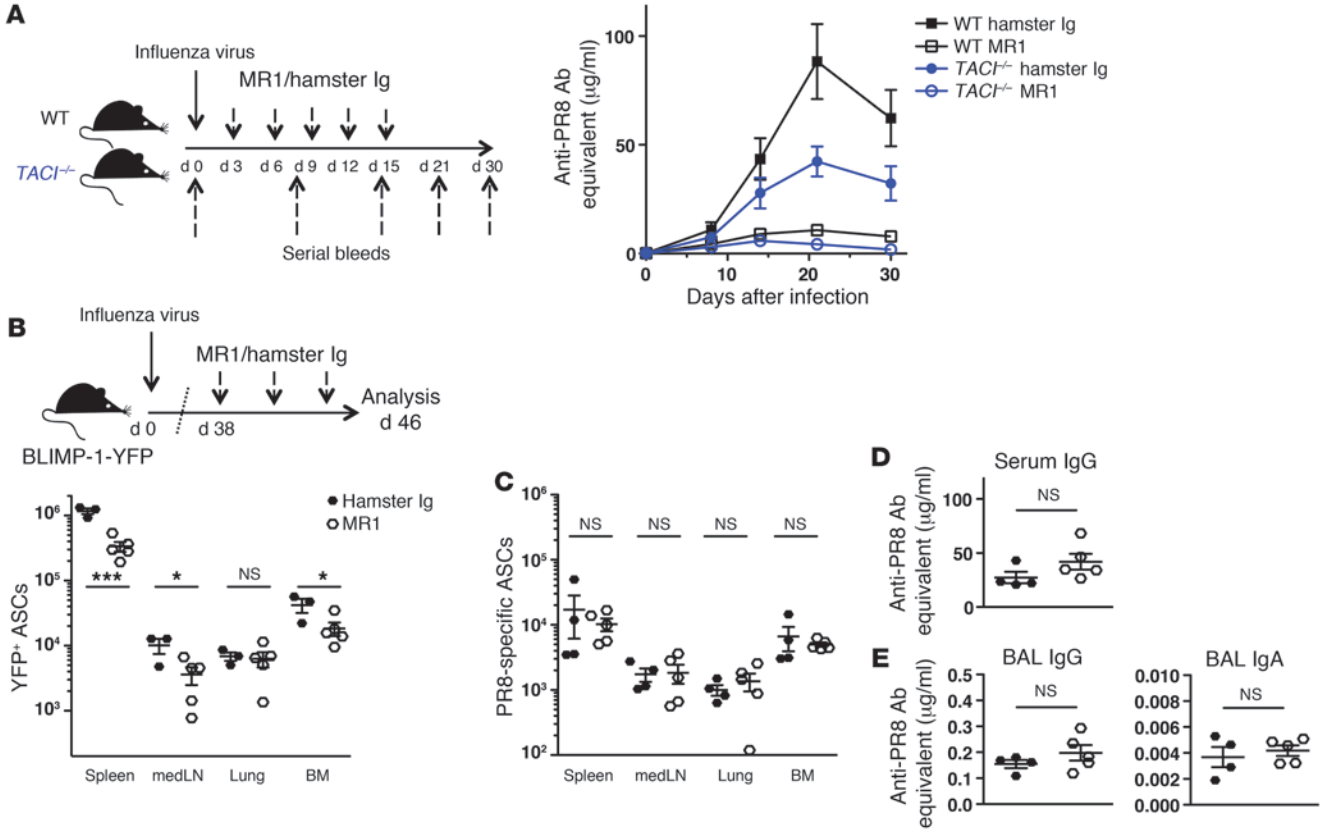

Figure 6

Induction but not maintenance of the antibody response to influenza is dependent on T cell help. (**A**) WT and *TACI*$^{-/-}$ mice (n = 7–8 mice/group) were infected with influenza virus PR8. Starting on day 3 p.i., WT and *TACI*$^{-/-}$ mice received 5 i.p. injections of MR1 or hamster Ig control Ab (150 μg/mouse) in 3-day intervals. Blood samples were collected prior to (day 0) and days 8, 14, 21, 30, and 45 after infection and PR8-specific IgG Ab titers were determined by ELISA. (**B**–**D**) BLIMP-1–YFP mice were infected with influenza virus PR8 and treated 3 times in 3-day intervals starting on day 38 p.i. with MR1 or hamster Ig Ab (150 μg/mouse). At day 46 p.i., spleen, medLN, lungs, and BM were harvested. Diagram in panel **B** applies to panels **B**–**E**. (**B**) YFP$^+$ ASCs were enumerated by flow cytometry and (**C**) PR8-specific ASCs determined by ELISPOT. (**D**) PR8-specific IgG Ab titers in serum and (**E**) IgG and IgA in BAL were measured by ELISA. All data with mean ± SEM combine the results of 2 independent experiments. *P < 0.05, ***P < 0.001.

(Figure 6C). Likewise, antiviral serum IgG (Figure 6D) as well as virus-specific IgG and IgA in BAL (Figure 6E) were similar in MR1-treated and control cohorts, suggesting that sustaining virus-specific ASCs and Ab production is independent of CD40L-mediated T cell help at this later time point.

TACI$^{-/-}$ *mice have reduced BLyS- and APRIL-expressing CD11b*$^+$ *cells in the RT.* Studies using human myeloid cells indicated a role for TACI and its ligand BLyS in the activation and maturation of these cells (39, 62). To determine whether CD11b$^+$ cells are altered in *TACI*$^{-/-}$ mice following influenza virus infection, cells from lungs, BAL fluid, spleen, and medLN were stained for CD11b and CD11c and enumerated. Strikingly, at day 34 p.i., CD11b$^+$CD11c$^-$ cells, which accounted for a high percentage of innate cells in the lungs of WT mice, were significantly reduced in *TACI*$^{-/-}$ mice as compared with WT mice (Figure 7, A and B). The percentage of CD11b$^+$CD11c$^-$ cells was also significantly reduced in spleen and lungs of naive *TACI*$^{-/-}$ mice and early after infection (day 6 p.i.) (Supplemental Figure 8A). In contrast, CD11b$^+$CD11c$^+$ cells were present at similar frequencies in both mouse strains.

CD11b$^+$CD11c$^-$ cells include monocytes, macrophages, eosinophils, and basophils (Supplemental Figure 8B). To investigate which of the myeloid cells contribute to the production of BLyS and APRIL, we sorted cells from lungs of naive and infected mice (3–4 weeks p.i.) into subsets based on Abs to CD11c, CD11b, and Gr-1, which recognizes Ly6G and Ly6C (Figure 7C). *BLyS* and *April* expression was assessed by real-time PCR. Gr-1$^+$ neutrophils expressed *BLyS* but not *April*. Gr-1^{lo} cells expressed both *BLyS* and *April* mRNA (Figure 7D). In contrast, CD11b$^+$CD11c$^+$ cells predominantly expressed *April*, but not *BLyS*, and CD11b$^-$CD11c$^-$ cells showed insignificant levels of both cytokines. Interestingly, although the frequency of CD11b$^+$CD11c$^-$ cells was significantly reduced in *TACI*$^{-/-}$ mice as compared with WT mice, there was no preferential reduction in individual Ly6C- and Ly6G-expressing cell subsets (Supplemental Figure 8, C and D).

IL-6 plays an important role in humoral immunity to influenza virus infection and can be produced by myeloid cells and DCs (35, 63). To test whether the reduction of myeloid cells in *TACI*$^{-/-}$ mice has an impact on IL-6 production, we measured *Il6* expression by real-time PCR on whole mouse lung homogenates from WT and *TACI*$^{-/-}$ mice at various times after infection. *Il6* was similarly induced on day 6 p.i.; however, by day 15 p.i. *TACI*$^{-/-}$ mice had reduced expression of *Il6* as compared with WT mice. *Il6* was

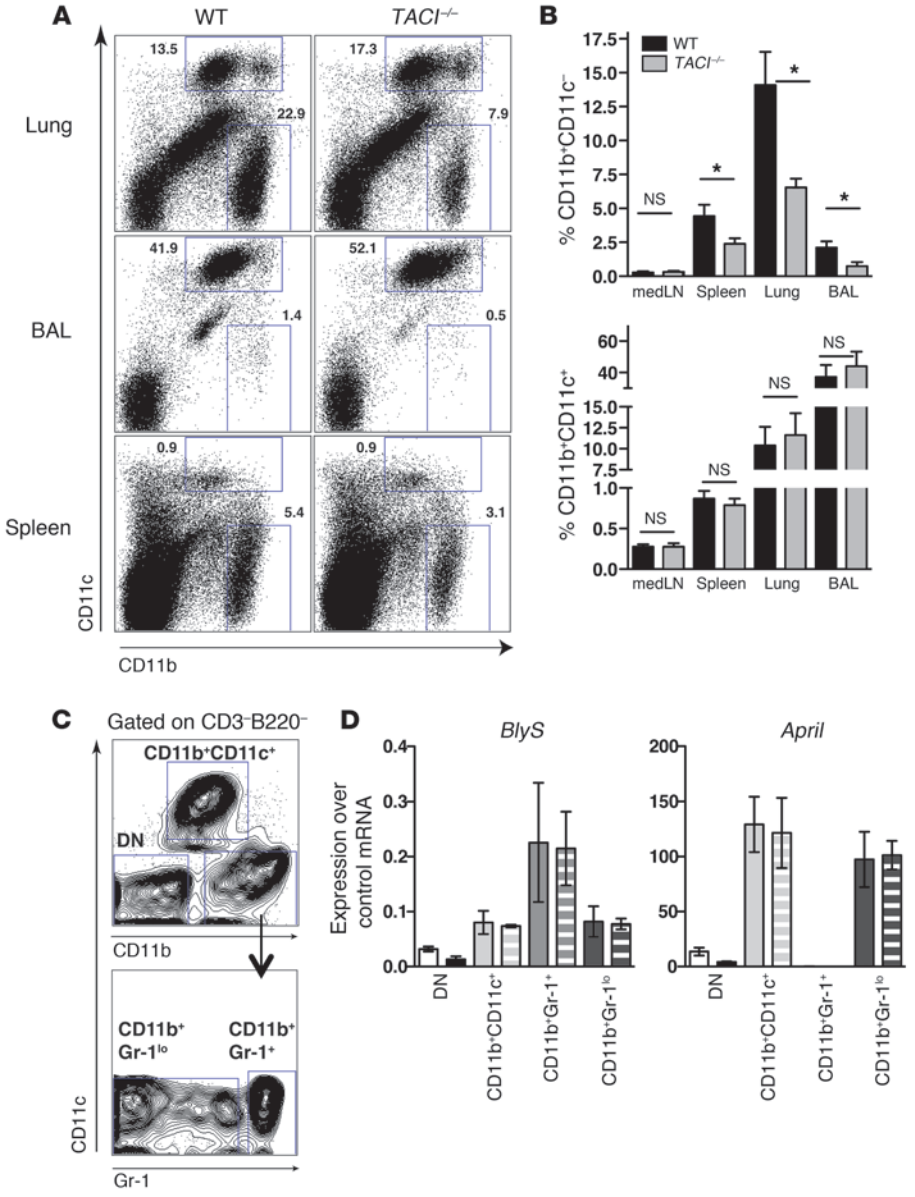

Figure 7
Reduction of BLyS- and APRIL-expressing CD11b$^+$CD11c$^-$ cells in lungs of *TACI*$^{-/-}$ mice after influenza virus infection. (**A**) Flow cytometry plots with percentages of CD11b$^+$CD11c$^+$ and CD11b$^+$CD11c$^-$ cells in lungs, BAL, and spleen isolated from WT or *TACI*$^{-/-}$ mice at day 34 p.i. (**B**) Frequency of CD11b$^+$CD11c$^-$ and CD11b$^+$CD11c$^+$ cells in various organs of WT and *TACI*$^{-/-}$ mice at day 34 p.i. Data with mean ± SEM combine the results of 3 independent experiments (n = 8 mice/group). (**C**) Identification of cell subsets in lungs of mice based on expression of CD11b, CD11c, and Gr-1 used for purification by FACS. DN, double negative (CD11b$^-$CD11c$^-$). (**D**) Real-time PCR analysis of *BLyS* and *April* mRNA expression in cell subsets sorted from lungs as shown in **C** of naive mice (solid bars) and mice 3–4 weeks p.i. Data with mean ± SEM are from 2 independent experiments. *P < 0.05.

undetectable in both mouse strains by day 20 p.i. and thereafter (Supplemental Figure 8E).

To investigate whether the reduction in CD11b$^+$CD11c$^-$ cells was due to a cell-intrinsic function of TACI, we generated mixed BM chimeras by reconstituting irradiated congenic CD45.1 hosts with a 1:1 mixture of BM cells from CD45.1 WT and CD45.2 *TACI*$^{-/-}$ mice (Supplemental Figure 9). When mice were bled 6 weeks after reconstitution to determine the ratio of CD45.1$^+$ to CD45.2$^+$ cells, WT BM donors represented 61.6% ± 2.64% of cells in the blood of recipient mice. For subsequent analysis, the ratio of WT to *TACI*$^{-/-}$ was set to equal 1. Next, BM chimeras were infected with influenza virus and analyzed 31 days later for the ratio of WT to *TACI*$^{-/-}$ donor cells among CD138$^+$ ASCs and CD11b$^+$CD11c$^-$ cells (Supplemental Figure 9, B and C). CD138$^+$ ASCs in all organs analyzed showed a significant overrepresentation of WT-derived donor cells, suggesting that ASCs from *TACI*$^{-/-}$ mice have an intrinsic disadvantage compared with ASCs that express TACI. In contrast, no bias for cells derived from WT or *TACI*$^{-/-}$ mice was observed within the CD11b$^+$CD11c$^-$ cell population. These data imply that the reduction in CD11b$^+$CD11c$^-$ cells in *TACI*$^{-/-}$ mice compared with WT mice (Figure 7, A and B, and Supplemental Figure 8A) is due to cell-extrinsic regulatory mechanisms.

TACI$^{-/-}$ *mice exhibit reduced Ab-mediated protection*. Because Abs are important for viral neutralization (1), we determined whether the reduced antiviral Ab titers in *TACI*$^{-/-}$ mice (Figure 4A) resulted in a lack of virus-neutralizing capacity and decreased protection against viral challenge. Serum was collected from WT and *TACI*$^{-/-}$ mice 2 months after infection and tested for the ability to prevent rbc hemagglutination by virus using an in vitro hemagglutination inhibition (HI) assay (64). The HI capacity of sera from *TACI*$^{-/-}$ mice was reduced (Figure 8A). To examine whether lower Ab titers in *TACI*$^{-/-}$ mice could alter the ability to neutralize antigenic variant influenza viruses, we extended our studies to a different H1N1 isolate, influenza A/SW/31 (SW) virus. Abs induced with PR8

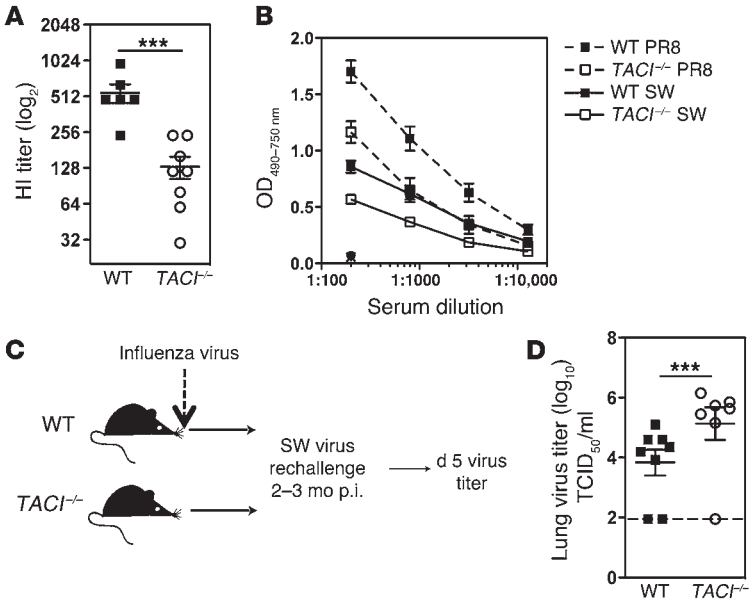

Figure 8

TACI$^{-/-}$ mice have reduced Ab-mediated protection. (**A**) HI assay with sera from WT and *TACI*$^{-/-}$ mice collected 3 months after infection with influenza virus PR8. (**B**) Sera from WT and *TACI*$^{-/-}$ mice (n = 8–9 mice/group) 2–3 months after infection were tested for binding to PR8 (dashed line) and SW (solid line) virus by ELISA. As a control, sera from naive mice are shown at a dilution of 1:200. (**C**) Two to 3 months after primary infection with influenza virus PR8, WT and *TACI*$^{-/-}$ mice were challenged with 2,000 $TCID_{50}$/30 μl of SW virus, and lungs were assayed for viral titers 5 days later. (**D**) Viral titers in lungs from PR8-infected WT and *TACI*$^{-/-}$ mice 5 days after challenge with SW virus. Dotted line represents limit of detection for this assay. Data with mean ± SEM summarize 2 independent experiments. ***$P < 0.001$.

infection exhibited poor cross-reactivity to SW virus (Figure 8B). Moreover, Abs in sera from PR8-infected *TACI*$^{-/-}$ mice bound SW virus less efficiently than Abs in sera from WT mice. To test protection in vivo, we challenged WT and *TACI*$^{-/-}$ mice with SW virus 2–3 months after primary infection with PR8 virus (Figure 8C). *TACI*$^{-/-}$ mice displayed 10-fold higher viral titers in the lungs as compared with WT mice 5 days after challenge (Figure 8D). Thus, protection against a secondary infection with an antigenic variant influenza virus is significantly impaired in the absence of TACI.

Discussion

Here we identify a critical role for TACI and its ligands BLyS and APRIL in the maintenance of protective antiviral ASCs. Following infection with influenza, ASCs increase at various anatomical sites, including the RT, where they are sustained at elevated numbers for several months (Figure 1C and Figure 2C). The majority of lung ASCs exhibit low cell cycle activity, suggesting that they are long-lived (Supplemental Figure 2C). However, unlike long-lived ASCs resident in the spleen and BM, ASCs in the lungs of influenza-infected mice are susceptible to the effects of whole-body irradiation (Figure 2, B and C), which points toward a cell-extrinsic mechanism for ASC survival.

We demonstrate that neutralization of BLyS and APRIL, but not BLyS alone, 5 weeks after infection results in substantially decreased virus-specific ASCs in BM and lungs, correlating with reduced Ab titers in serum and BAL (Figure 3 and Supplemental Figure 3). Interestingly, *April*$^{-/-}$ mice or *April*-transgenic mice showed normal early antiviral ASC and Ab responses (65); however, the persistence of ASCs was not investigated in these studies. Here we demonstrate that the early antiviral Ab and ASC response (day 8 p.i.) was similar in WT and *TACI*$^{-/-}$ mice (Figure 4, A and B, and Figure 5A), but by 3–4 weeks p.i. TACI-deficient mice exhibited significantly lower antiviral Ab titers in serum and BAL correlating with reduced numbers of virus-specific ASCs (Figure 4, A and B, and Figure 5B). In contrast to *TACI*$^{-/-}$ mice, there was no difference in the humoral response to influenza virus in *BCMA*$^{-/-}$ mice (Supplemental Figure 7). Although BCMA has been implicated in survival of BM ASCs (25), our data and those of others (66) suggest that BCMA does not play a major role in mediating survival of virally induced ASCs.

In vitro studies have indicated that signals through TACI can play an important role in the induction of class switch recombination (41–43, 48). Interestingly, our data suggest that the initial induction of isotype switching after influenza virus infection is largely TACI independent in vivo: The number of virus-specific IgG- and IgA-producing cells in medLN of WT and *TACI*$^{-/-}$ mice was similar at 6–8 days p.i. (Figure 5A). We cannot exclude the possibility that TACI functions in ASC class switching at later times, particularly in the lung, when costimulatory signals and help from effector T cells may have become sparse. However, we demonstrate that blockade of CD40L 5 weeks p.i. does not impact antiviral ASCs and Ab production locally in the lungs (Figure 6, B–D).

In humans, individuals carrying mutations in the *TNFRSF13B* gene encoding TACI are prone to developing CVID, characterized by low serum IgG and IgA concentrations and recurrent bacterial infections (67, 68). TACI mutations also result in severely decreased proportions of isotype-switched B cells, suggesting defects in the generation and/or maintenance of memory B cells in the absence of functional TACI (68, 69). TACI mutations can interfere with class switch recombination in humans, potentially through a failure to properly induce activation-induced deaminase (*AID*) expression (43, 67, 68). In addition, naive B cells from TACI-mutated individuals fail to secrete IgA Abs after stimulation in vitro with BLyS or APRIL, suggesting an important role for TACI in human mucosal immunity (67). In line with this observation, CVID patients often experience pneumonia, potentially leading to chronic lung disease (70). Moreover, defects in the expression of TACI, which we showed in the present study to be important for the survival of long-lived ASCs in the BM, may also account for the decreased serum IgG and IgA concentrations observed in patients with TACI mutations (67–69).

Strikingly, we show a site-specific requirement for TACI in the antiviral ASC response. Lung and BM ASCs, but not ASCs in medLN and spleen, were significantly reduced after treatment

with TACI-Fc or in *TACI*$^{-/-}$ mice 5 weeks p.i. (Figure 3, B and C, and Figure 5B). Our findings raise the question as to what could account for the difference in TACI-dependent survival of virus-specific ASCs at these sites. Organ-specific access to TLR ligands, for example at mucosal sites, could positively regulate BLyS and/or APRIL expression (26, 30, 71) and thus influence local isotype switching and/or ASC survival. Studies with human B cells have suggested that mucosal IgG and IgA responses to poly(I:C), a surrogate for viral dsRNA and a ligand for TLR3, can be potentiated with BLyS (27, 28). In addition to the more ubiquitously present myeloid cell types, specialized cell subsets in the RT such as airway epithelial cells and alveolar macrophages could allow for local regulation of APRIL and BlyS production (26–28, 33, 34, 71, 72). Geurtsvankessel at al. recently demonstrated that local depletion of CD11c$^+$ DCs in the lungs of influenza-infected mice coincided with decreased IgA titers in airway secretions and ASCs in the lung (73). This treatment could have removed a source of cells that are able to produce ASC survival factors. Indeed, we show in this study that CD11b$^+$CD11c$^+$ cells (including DCs and alveolar macrophages) isolated from lungs exhibited high levels of *April* mRNA (Figure 7D). Moreover, we demonstrate that CD11b$^+$CD11c$^-$ cells included subsets with increased BLyS and APRIL expression as compared with cells that were CD11b$^-$CD11c$^-$. Importantly, we show that these CD11b$^+$CD11c$^-$ cells were reduced in *TACI*$^{-/-}$ mice (Figure 7, A and B, Supplemental Figure 8A), suggesting a model in which TACI signaling in innate cell subsets indirectly regulates ASC differentiation and survival. Of note, whole body irradiation used in this study to determine the lifespan of ASCs may have ablated these cells, thus indirectly affecting ASC survival in the RT (Figure 2, B and C). Moreover, it has been demonstrated that TACI may be involved in human monocyte maturation and differentiation (39, 62). A reduction in macrophages was also observed in APRIL-deficient mice infected with influenza virus (65). Our results support the possibility, therefore, that the predisposition to lung infections in TACI-deficient individuals is mediated through a failure to maintain isotype-switched ASCs in the lungs by a mechanism involving CD11b$^+$ cells. We are currently investigating CD11b$^+$ cell subsets in lungs (Supplemental Figure 8B) as well as lymphoid organs during influenza infection, with particular attention to eosinophils (74) and basophils (75), which have been implicated in controlling ASC survival.

Finally, we demonstrate that reduction of virus-specific ASCs in the lungs and BM correlates with significantly lower virus-specific Ab titers in the airways and serum. The importance of antiviral Abs for protection against reinfection is well documented (1, 76). There are several mechanisms for antiviral Abs to control influenza infection; the most efficient is through viral neutralization mediated predominantly by Abs against hemagglutinin (77). We show that decreased serum Ab-titers in *TACI*$^{-/-}$ mice translated into a significant reduction in HI (Figure 8A) and less efficient cross-reactive binding of serum Abs to another H1N1 virus (Figure 8B). Importantly, this abrogates the protection of mice against secondary viral infection with an antigenic variant virus (Figure 8D), such as those that arise during antigenic drift of influenza strains.

Taken together, our observations reveal a critical role for TACI in generating optimal humoral immunity and maintaining protection against secondary viral infection. Targeting TACI on both B cells and innate cells for enhanced antiviral ASC survival could lead to improved Ab titer maintenance and protection and may inform future vaccine strategies.

Methods

Supplemental Methods are available online with this article; doi:10.1172/JCI57362DS1.

Mice. BLIMP-1–YFP (53) and *TACI*$^{-/-}$ mice (46) on a C57BL/6 background were housed and bred at the Wistar Institute under specific pathogen–free conditions. C57BL/6 WT and congenic CD45.1 (Ly5.2/Cr) mice were purchased from the National Cancer Institute. Male and female mice used in studies were 8–12 weeks old, and all experiments were performed under a protocol approved by the Wistar Institute Animal Care and Use Committee.

Influenza viruses and infection. Influenza viruses were grown in the allantoic fluid of 10-day embryonated chicken eggs (B&E Eggs) and stored at –80°C. Mice were immobilized with ketamine/xylazine (70 mg/10 mg/kg) and infected intranasally with a dose of 500 $TCID_{50}$/30 μl mouse-adapted PR8 virus (influenza virus A/Puerto Rico/8/34; H1N1; Mount Sinai strain) as described previously (78). SW virus (influenza virus A/Swine/31; H1N1) for rechallenge was given at a dose of 2,000 $TCID_{50}$/30 μl.

Cell preparations. Tissues were collected and cells prepared as described previously (78). In brief, blood was collected through cardiac puncture. BAL was harvested by flushing the airway compartment with 0.8 ml PBS with 1% FBS (PBS/FBS) 3 times. Perfused lungs were digested in HBSS with 400 U/ml Collagenase D (Roche) for 30 minutes at 37°C. Tissues were passed through metal wire mesh, and after rbc lysis, cells were resuspended in Iscove's complete medium containing 10% FBS for ELISPOT assays or in PBS/FBS for flow cytometric analysis.

Flow cytometry. For surface staining, cells were incubated first with Fc-block (anti-CD32/anti-CD16), then with various Abs. The following antibodies were purchased from eBioscience if not indicated otherwise: CD3ε-FITC, CD4-PerCPcy5.5, CD8-PerCPcy5.5, CD11b-PerCPcy5.5, CD19-PEcy7, IgD-eFluor450, Fas-PE, PNA-FITC (Sigma-Aldrich), CD45R/B220-FITC (BioLegend), Gr-1–PE, CD138-PE and -APC, and CD11c-APC (BD). Analysis was performed on a FACSCalibur or LSR II (BD). Data were processed using FlowJo software (Tree Star).

Histology. Tissues were fixed in 4% formaldehyde (Ricca Chemical) with 10% sucrose (Fluka) for 6–8 hours to preserve YFP expression and frozen in OCT medium (Tissue-Tek). Sections from organs of BLIMP-1–YFP mice were stained with B220-PE or CD4-PE (BD). All histology was examined and recorded on an upright Nikon E600 Microscope with the image software Image-Pro (Media Cybernetics).

Irradiation of influenza-virus–infected mice. Influenza virus–infected BLIMP-1–YFP mice received whole-body irradiation (7.5 Gy, cesium source) at indicated times after infection. Within 24 hours, mice were injected intravenously with 1×10^6 T cell–depleted BM cells from congenic CD45.1 mice.

Neutralization of BLyS and APRIL and blockade of CD40L in vivo. To neutralize BLyS and APRIL, we injected BLIMP-1–YFP mice i.p. with 100 μg TACI-Fc or human IgG control Ab starting at day 35 p.i. every 2–3 days for 2 weeks.

To block CD40L, we injected mice i.p. with 150 μg MR1 or hamster IgG control Ab (BioXCell) every 3 days between days 3 and 15 p.i. or 3 times starting on day 38 p.i.

ELISA and ELISPOT assay. Antiviral Ab concentrations in serum and BAL were determined by ELISA as described previously (79). To determine virus-specific or total IgG and IgA, we coated plates with purified virus or anti-mouse $Ig_{(H+L)}$ and anti-mouse IgA (R5-140). Concentrations of Abs were calculated based on purified anti-HA Ab standards of IgG (H36-4-5.2), IgA (H37-66-7), or IgM (H35-C7-2R1) isotypes and graphed as Ab equivalents. Bound Ab was detected with biotinylated Cκ (clone 187.1) and ExtrAvidin-coupled alkaline phosphatase (AP) (Sigma-Aldrich), or anti-IgG, IgM, and IgA conjugated to AP (Sigma-Aldrich) and developed with *p*-nitrophenyl phosphate (pNPP) (Sigma-Aldrich). The optical intensity ($OD_{405–750\ nm}$) was determined with an ELISA reader (Molecular Devices) and calculated using SoftMax Pro software (Molecular Devices).

To detect PR8-specific ASCs, we coated ELISPOT plates (Millipore) with 100 hemagglutination units (HAU) purified PR8 per well in 100 μl PBS the day before and kept them at 4°C. Plates were washed and blocked with Iscove's medium containing 10% FBS (Atlanta Biologicals) prior to plating of cells. Cell suspensions in various dilutions were plated and incubated for 6–7 hours at 37°C in humidified air/7% CO_2. Ab spots were detected with anti-IgM, anti-IgG, and anti-IgA conjugated to AP (Sigma-Aldrich) and developed with NBT/BCIP substrate (Sigma-Aldrich). Automated spot counts were performed with an ImmunoSpot Reader (CTL) and ImmunoSpot satellite software (CTL). Unless stated otherwise, PR8-specific ASCs represent a combination of virus-specific IgM-, IgG-, and IgA-secreting cells.

Real-time PCR. Cell populations from lungs were sorted based on CD11b, CD11c, and Gr-1 RNA, extracted with the RNeasy Kit according to the manufacturer's instructions (QIAGEN) and reverse transcribed by Superscript II Reverse Transcriptase (Invitrogen). Real-time PCR was performed as previously described (36). Master Mix and primer and probe sets for mouse *Gapdh*, *Il6*, *BLyS*, and *April* were from Applied Biosystems. The ABI 7300 system (Applied Biosystems) was used for real-time PCR, and the cycling threshold method ($2^{-(\Delta\Delta Ct)}$) was used for relative quantification of the gene of interest to *Gapdh* (80). In some experiments, expression levels were normalized based on control RNA (Zyagen).

HI assay. The HI assay was performed as described previously (64). Serum or BAL samples were serially diluted and mixed in equal volumes with purified PR8 virus. After 1 hour, chicken rbc (B&E Eggs) were added, and the pattern of agglutination was recorded. The HI titer is expressed as reciprocal of the highest serum dilution at which virus was inhibited from agglutinating rbc.

Determination of infectious virus titers in lungs. The concentration of infectious virus in lungs was determined by titration of homogenized tissues in Madin-Darby canine kidney cell (MDCK) microcultures as described previously (81). Lung titers are expressed as dilution of lung extract at which 50% of the MDCK cultures revealed virus growth ($TCID_{50}$/ml).

Statistics. Data are presented as mean ± SEM. Statistical significance between 2 groups was calculated using an unpaired 2-tailed Student's *t* test. Multiple groups were compared using a 1-way ANOVA. All statistical tests were performed using Prism software (GraphPad Software).

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