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B cells promote CD8 T cell primary and memory responses to subunit vaccines

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SUMMARY

The relationship between B cells and CD4 T cells has been carefully studied, revealing a collaborative effort in which B cells promote the activation, differentiation, and expansion of CD4 T cells while the so-called “helper” cells provide signals to B cells, influencing their class switching and fate. Interactions between B cells and CD8 T cells are not as well studied, although CD8 T cells exhibit an accelerated contraction after certain infections in B-cell-deficient mice. Here, we find that B cells significantly enhance primary CD8 T cell responses after vaccination. Moreover, memory CD8 numbers and function are impaired in B-cell-deficient animals, leading to increased susceptibility to bacterial challenge. We also show that interleukin-27 production by B cells contributes to their impact on primary, but not memory, CD8 responses. Better understanding of the interactions between CD8 T cells and B cells may aid in the design of more effective future vaccine strategies.

In brief

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AUTHOR CONTRIBUTIONS

Research conceptualization: J.K., E.W.C., Z.X., C.A.H., A.G., and R.M.K. Performed research: J.K., E.W.C., S.B.T., B.W., D.L.A., A.K.C.-C., A.G., and R.M.K. Data analysis: J.K. and R.M.K. Statistical analysis: J.K. and R.M.K. Writing: J.K. and R.M.K.

DECLARATION OF INTERESTS

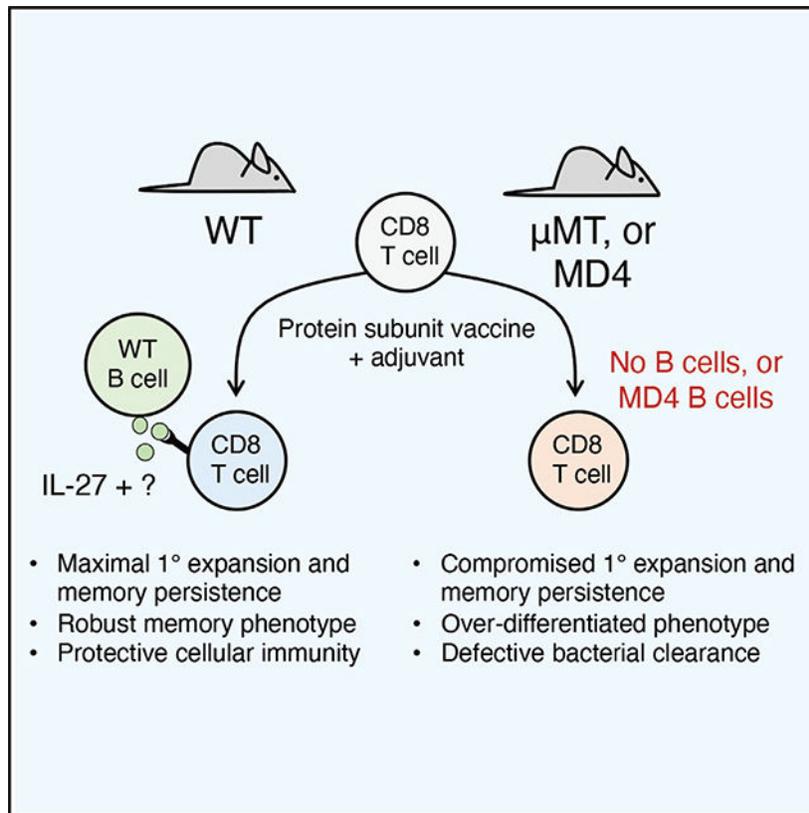
R.M.K. is a founder of ImmuRx Inc., a vaccine company for which intellectual property is based on the combined TLR agonist/anti-CD40 immunization platform. All other authors declare no competing interests.

SUPPLEMENTAL INFORMATION

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Generating cytotoxic CD8 T cell responses with vaccines can greatly improve their efficacy, but inducing adequate numbers of these cells can be challenging. Klarquist et al. reveal that the magnitude, persistence, and function of CD8 T cell vaccine responses depend on B cells.

Graphical abstract



INTRODUCTION

Studies in mice utilizing experimental infectious agents have helped outline the mechanisms driving T cell immunity. Given the similarly robust cellular responses they elicit, it is often assumed that protein subunit vaccine formulations function via the factors that govern responses to infections. In contrast to these assumptions, numerous mechanisms of adjuvant-elicited CD8 T cell immunity that are not shared by their infection-elicited T cell counterparts have been identified. We recently documented the amount of interleukin (IL)-27 produced by conventional type 1 dendritic cells (cDC1s) as an innate correlate of CD8 T cell protective memory after adjuvant administration (Kilgore et al., 2018, 2020). This correlation was sufficiently robust so as to facilitate the predictive stratification of adjuvants for their CD8 T-cell-eliciting potential (Kilgore et al., 2020). The predictive value of this relationship fit well with the dependency of adjuvant-elicited, but not infection-elicited, CD4 and CD8 T cell responses on T-cell-intrinsic IL-27 signaling (Klarquist et al., 2018; Pennock et al., 2014). Indeed, maximal T cell expansion to adjuvant administration requires multiple cytokines (IL-27, IL-15) and transcription factors (Tbet, Eomesodermin

[Eomes]) (Klarquist et al., 2018) that are not required by T cells responding to infectious challenges (Banerjee et al., 2010; Becker et al., 2002; Intlekofer et al., 2008; Liu et al., 2014; Mayer et al., 2008; Obar et al., 2004; Wehrens et al., 2018; Wherry et al., 2002). After vaccine adjuvant delivery, these cytokines are necessary for enabling a unique metabolic program in subunit vaccine-elicited T cells (Klarquist et al., 2018). Whereas T cells responding to infection depend on glycolysis to fuel clonal expansion, mitochondrial function is overwhelmingly favored during adjuvant-elicited clonal expansion, and the proliferating T cells are largely indifferent to glycolytic blockade. Thus, there are major differences in the factors relied upon by infection-elicited and adjuvant-elicited CD8 T cell responses to support clonal expansion, survival, and memory formation.

Whereas IL-15 and IL-27 are produced by cDC1s (Kilgore et al., 2018; Sosinowski et al., 2013), other antigen (Ag)-presenting cells (APCs) including B cells, also have the capacity to make one or both cytokines. Indeed, one member of the IL-27 heterodimer, Ebi3 (Epstein-Barr virus [EBV] induced gene 3), was originally isolated from EBV-infected B cells, and B cells produce IL-15 and IL-27 upon stimulation with Toll-like receptor (TLR) ligands or CD40L (Schneider et al., 2011; Yan et al., 2020). Furthermore, B cell expression of IL-27 has been linked to CD8 T cell recovery after lymphoablation (Ayasoufi et al., 2019), and T cell memory in IL-27R $\alpha^{-/-}$ hosts is functionally compromised (Pennock et al., 2014). Similarly, IL-15 production by B cells has been observed in mouse models of multiple sclerosis (MS) and systemic lupus erythematosus (SLE), as well as in human MS patients (Ma et al., 2014; Schneider et al., 2011). In addition to cytokine production, B cells may also be competent for cross-presentation to CD8 T cells. Although still controversial (Keller et al., 2009), multiple groups have demonstrated B cell cross-presentation to CD8 T cells using a variety of model systems (Castiglioni et al., 2005; Hon et al., 2005; Ke and Kapp, 1996; Robson et al., 2008; Zanetti et al., 2004). CD8 T cells are not classically thought to traffic into or reside within B cell zones, legitimately calling into question the *in vivo* applicability of *in vitro* demonstrations of B cell cross-presentation. However, a large portion of CD8 T cells actually do migrate into the marginal and B cell zones of the spleen within the first 3 days after lymphocytic choriomeningitis virus (LCMV) challenge (Hu et al., 2011; Jung et al., 2010), and a substantial proportion of memory CD8 T cells can be found within the B cell zone at late time points after *Listeria monocytogenes* (LM) challenge (Khanna et al., 2007). These data suggest that there may be ample opportunity for CD8 T-cell:B-cell interactions over the course of adaptive immune generation.

Previous work in various infection models has broadly documented an indifference of primary CD8 T cell responses to the presence of B cells, including LM (Shen et al., 2003), influenza (León et al., 2014), LCMV (Asano and Ahmed, 1996; Homann et al., 1998), vaccinia virus (Bründler et al., 1996), and vesicular stomatitis virus (Bründler et al., 1996). Several of these studies detailed an accelerated contraction that yielded either low (Shen et al., 2003) or normal (Asano and Ahmed, 1996; León et al., 2014) numbers of Ag-specific memory CD8 T cells; however, this finding was not universal, with B cells dispensable for CD8 T cell memory in other studies (Bründler et al., 1996; Di Rosa and Matzinger, 1996; Whitmire et al., 2009). Indeed, in some model systems, CD8 T cells actually limit the B cell response as a result of Ag-mediated recognition and killing of Ag-specific B cells (Barnaba et al., 1990). These data do not necessarily contradict, but rather underscore, the

idea that immune processes learned from one infectious model do not define immunity at large. In one study, antibodies were found to play a role in capturing and stabilizing Ag after influenza infection, thereby enhancing Ag presentation by DCs (León et al., 2014). However, in all other examples where B cells influenced CD8 T cell contraction (Asano and Ahmed, 1996; Bründler et al., 1996; Homann et al., 1998; Shen et al., 2003), underlying mechanisms have been left largely unexplained.

In exploring a potential role for B cells in the generation of CD8 T cell responses, we found that B cells impact both the primary and memory Ag-specific CD8 T cell responses to subunit vaccines. These results were independent of CD4 T cells or antibody production and were instead due to B-cell-intrinsic factors that were absent in mice deficient in B cells or with a fixed B cell receptor (BCR). Collectively, our results challenge the conventional paradigm of how B cells influence cellular immunity and suggest that some form of CD8 T cell:B cell collaboration is critical for elicitation of maximal CD8 T cell responses after subunit vaccination.

RESULTS

B cells augment primary CD8 T cell responses to subunit vaccination

After adjuvanted subunit immunization, total B cell numbers increase as much as 4-fold within 4 days, in a manner dependent on the adjuvant. The largest increase seen was predictably with a vaccine that utilizes whole ovalbumin protein (OVA) with poly(I:C) and agonistic CD40 antibody (combined-adjuvant), but all single adjuvants evaluated also induced substantial B cell expansion after vaccination (Figure 1A). The considerable size of the B cell pool at these early time points led us to investigate whether B cells contributed to the CD8 T cell response. To explore this, we compared the CD8 T cell response to combined-adjuvant immunization in C57BL/6 wild-type (WT) and $\mu\text{MT}^{-/-}$ B-cell-deficient mice. To mitigate any possible confounding effects on the T cell repertoire of having no B cells present during T cell development, 200 T cell receptor (TCR) transgenic OT1 T cells were transferred 1 day before immunization, a number known to respond to immunological challenge but not completely outcompete an endogenous CD8 T cell response (Badovinac et al., 2007). The number and frequency of endogenous and transferred Ag-specific CD8 T cells were significantly reduced in B-cell-deficient mice at the peak of the response, 7 days after combined-adjuvant immunization (Figures 1B and 1C). This was not simply due to the lower number of total CD8 T cells typically found in $\mu\text{MT}^{-/-}$ mice, because (1) the total number of transferred OT1 T cells was significantly lower, and (2) endogenous tetramer+ cells were significantly reduced as a percentage of total CD8 T cells in $\mu\text{MT}^{-/-}$ mice compared to WT controls (Figure 1D).

A trivial explanation for these results might be that the complete absence of B cells indirectly impacted T cell priming simply because of the disruption of secondary lymphoid tissue architecture (Ngo et al., 2001). Therefore, we used the MD4 transgenic mouse, in which >90% of the B cells have a fixed specificity toward hen egg lysozyme (HEL) (Goodnow et al., 1988). Unlike the $\mu\text{MT}^{-/-}$ mice, the splenic architecture in MD4 mice is relatively normal (Figure 1E), and the MD4 mouse has a normal frequency of CD4 T cells, cDC1s, and Tregs (Figure S1). Compared to WT, MD4 mice have slightly fewer CD8 T cells

and B cells overall (Figure S1) but exhibited a similar early expansion and contraction of total B cells after combined-adjuvant subunit vaccination compared to WT controls (Figure 1F). As in the B-cell-deficient host, CD8 T cell responses to combined-adjuvant subunit vaccination in the MD4 mice were substantially reduced. This was true for combined-adjuvant immunization administered intravenously (i.v.) or subcutaneously (s.c.) (Figure 1G), as well as for single adjuvants delivered as soluble (Figure 1H) or alum-precipitated (Figure 1I) formulations. Again, the CD8 T cell response was reduced in both numbers (Figures 1G–1I, top) and frequency (Figures 1G–1I, bottom). In contrast to these various forms of subunit vaccination, there was no difference in the CD8 T cell response between MD4 and WT controls after infection with OVA-expressing LM (LM-OVA), (Figure 1J), in line with previous reports of LM infections in $\mu\text{MT}^{-/-}$ mice (Shen et al., 2003). Finally, the quantitative difference in primary CD8 T cell responses between WT and MD4 mice was independent of Ag dose (Figure S2A) and was not restricted to the use of OVA; we obtained similar data when immunizing with herpes simplex virus glycoprotein B (HSVgB) or severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike receptor-binding domain (RBD) proteins (Figures S2B–S2D). CD8 T cell responses to SARS-CoV-2 RBD were analyzed by intracellular cytokine staining after restimulation with the peptide VVLSFELL, a peptide within the RBD known to elicit CD8 T cell responses to SARS-CoV (Zhao et al., 2010), and conserved in SARS-CoV-2 (Davenport et al., 2021). Collectively, we concluded that B cells influence the CD8 T cell response to subunit vaccination, but not infection, and that this influence is not limited to a specific adjuvant formulation, Ag, or route of administration.

Primary CD8 T cell responses to subunit vaccines are not dependent on Ag-antibody (Ag-Ab) complexes or CD4 T cell responses

Since the presence of B cells specific for an irrelevant Ag did not rescue the CD8 T cell response, it was possible that the production of Ag-specific antibody was required for maximal primary CD8 T cell responses to vaccines. Indeed, the formation of Ag-specific Ag-Ab complexes have been shown to facilitate CD8 T cell immunity by enhancing DC Ag capture, retention, and presentation (León et al., 2014). Therefore, we investigated whether mice deficient in the capacity to make Ag-Ab complexes, formed either by secreted natural immunoglobulin (Ig)M or Ag-specific IgG, would also show reduced CD8 response to vaccination. However, no defects in CD8 T cell primary cell numbers were observed after vaccination of secreted IgM-deficient mice (sIgM^{-}) or activation-induced cytidine deaminase-knockout ($\text{AID}^{-/-}$) hosts (Figures 2A and 2B), effectively ruling out these possibilities. We did not specifically test a possible role for secreted, circulating IgD, although this seems highly unlikely, given that detectable sIgD largely depends on class-switched $\text{IgD}^{+}\text{IgM}^{-}$ cells (Chen et al., 2009; Choi et al., 2017), a process that requires AID (Choi et al., 2017) and was therefore tested in our $\text{AID}^{-/-}$ experiments. We also eliminated the possibility that defective CD4 T cell responses were responsible for the diminished primary response in both $\mu\text{MT}^{-/-}$ and MD4 mice by depleting CD4 T cells 2–5 days before immunization (Figure S3). CD4 depletion did not significantly change the Ag-specific CD8 T cell numbers at day 7 (Figure 2C), consistent with our published data showing no role for CD4 T cells in the primary expansion of CD8 T cells to subunit vaccination (Ahonen et al., 2004; Edwards et al., 2013).

Cytokine and transcription factor analyses indicate that B cells promote vaccine-elicited CD8 T cell memory skewing

To determine whether there were any differences in the quality of the response, we performed *ex vivo* cytokine restimulation assays at the peak of the response to combined-adjuvant immunization. In line with our tetramer data, there was a significant difference in the frequency of cytokine-producing, Ag-specific CD8 T cells between WT and MD4 mice (Figures 3A–3C). Additionally, more detailed analyses revealed striking differences in the quality of the response. Although there were no differences observed in the percentage of cells producing 2 or more cytokines (Figures 3D and 3E), a significantly higher percentage of cytokine+ cells in WT mice produced IL-2, compared to those in MD4 mice (Figure 3F). Moreover, WT cells produced higher levels of interferon gamma (IFN γ) and IL-2 on a per-cell basis (Figure 3G). These data were potentially highly significant, given that IL-2 is uniquely capable of driving T cell memory development (Feau et al., 2011; Sarkar et al., 2008; Williams et al., 2006), and suggested a skewing of the response toward effectors in the MD4 host. Consistent with this, Ag-specific T cells in MD4 mice substantially increased their percentage of granzyme B+CD127 $^-$ T cells (Figures 3H and 3I). In contrast, CD8 T cells in WT mice skewed more toward a memory phenotype, with a reduced percentage of granzyme B+ cells (Figure 3I) that also produced less granzyme B on a per-cell basis (Figure 3J).

These results were highly informative, given that the primary goal of prophylactic vaccination is the development of long-lasting, protective immunity. Therefore, we examined more closely the memory/terminal effector phenotypes of CD8 T cells 7 days after vaccination in WT and MD4 mice, including their expression of transcription factors known to influence these cell-fate decisions. Eomes, although dispensable for the primary response to infectious challenge, is critical to the primary response to subunit vaccination (Klarquist et al., 2018). Endogenous CD8 T cells responding in the MD4 host had reduced levels of Eomes compared to those in the WT (Figures 4A and 4B). The biological impact of this was apparent in the reduction in CD122 (IL-2R β) expression, a shared component of the heterotrimeric IL-15 and IL-2 receptors that Eomes is known to regulate (Intlekofer et al., 2005). Both of these factors are downstream of IL-27, which is essential for promoting the necessary metabolic support for the CD8 T cell responses to subunit vaccination (Klarquist et al., 2018). Indeed, the CD8 T cells in the MD4 phenocopied (Eomes^{low}CD122^{low}) vaccine responses generated in the absence of IL-27 (Klarquist et al., 2018; Pennock et al., 2014). Additionally, the percentage of cells maintaining high TCF1, which correlates with the self-renewal capacity of pre-memory T cell populations (Im et al., 2016; Kratchmarov et al., 2018; Lin et al., 2016), was significantly reduced in Ag-specific CD8 T cells from MD4 mice. Reciprocally, those T cells exhibited higher IRF4, which can inhibit TCF1 and impair memory development (Man et al., 2017) (Figures 4A and 4B). These results, along with the IL-2 and granzyme B data noted earlier, suggested that CD8 T cells from the MD4 host would display increased phenotypic characteristics of effector cells (high KLRG1 and/or low CD127) and decreased characteristics of memory precursors (high CD127). Indeed, and in contrast to previous reports on infection in B-cell-deficient hosts (León et al., 2014), we observed significant changes in the memory precursor versus terminally differentiated effector phenotype of the cells after immunization. In fact, the entire defect in the MD4

primary response was limited to a loss of CD127^{high} cells (Figures 4C and 4D). Taken together, the cytokine profile, transcriptional program, and surface protein phenotype of Ag-specific CD8 T cells in MD4 animals demonstrated a skewing away from the memory phenotype that usually predominates subunit vaccination.

B cells, not CD4 T cells, are critical to the persistence and function of CD8 T cell memory after subunit vaccination

To determine the long-term consequences of the functional and phenotypic differences we observed earlier, we tracked Ag-specific CD8 T cell memory for 12 weeks after immunization. To control for the requirement of CD4 help during priming, we depleted CD4 T cells 2–5 days before vaccination (Figure S3). As seen in the spleens 7 days after immunization (Figure 2), CD4 T cell depletion actually resulted in an overall increase in tetramer+ CD8 T cell frequency at all time points (Figure 5A). This increase was independent of a polyclonal B cell repertoire, as it also occurred in the MD4 host (Figure 5A). However, circulating Ag-specific CD8 T cell survival to memory was dramatically reduced in MD4 mice compared to WT controls in both the intact and CD4-depleted conditions. Analysis of these results by a two-way ANOVA yielded a significant interaction effect ($p < 0.001$), indicating that the MD4 defect in CD8 memory was exaggerated compared to what would have been predicted simply by the reduced primary response; that is, the slope of the decay in Ag-specific CD8 T cells was significantly increased in MD4 mice compared to that in WT mice. Analysis of the Ag-specific CD8 T cell responses in WT and MD4 mice depleted of CD4 T cells before vaccination also resulted in a statistically significant interaction effect, indicating that the accelerated decay of CD8 T cells in the MD4 host was not CD4 dependent. Moreover, there was no significant interaction effect when comparing the decay of memory T cells over time between isotype-control-treated and CD4-depleted WT mice; thus, while initially depleting CD4 T cells changed the peak magnitude of the CD8 T cell response, it had no impact on the persistence of memory CD8 T cells. This memory T cell defect was also not dependent on the use of the combined-adjuvant, as MD4 mice also had significantly reduced splenic tetramer+ cell numbers after vaccination with single adjuvants (Figure 5B). Furthermore, within the splenic memory CD8 T cells, there were significantly lower proportions of CD62L+ central memory cells at 3 months post-vaccination, and fewer memory CD8 T cells expressed TCF1, which regulates the differentiation and longevity of CD8 memory (Figure 5C). Thus, we conclude that B cells influence the maintenance of CD8 T cell memory after vaccination in a manner independent of CD4 T cells.

The data thus far indicates increased effector differentiation, decreased memory fate commitment, and accelerated loss of memory in the CD8 T cells from immunized MD4 hosts. Our next goal was to determine whether these factors ultimately result in decreased memory T cell function. Using an *in vivo* killing assay, we found that CD8 T cell memory function was indeed compromised on a per-cell basis in MD4 mice. Transferring differentially CTV-labeled targets bearing control or vaccine-specific Ag into WT or MD4 mice 3 months post-immunization revealed reduced killing of the Ag-pulsed targets in MD4 hosts (Figure 6A). This defect in the MD4 CD8 T cells remained even when accounting for the differences in memory T cell frequency between WT and MD4 hosts (Figure

6B), indicating reduced lytic function of memory CD8 T cells in the MD4 host on a per-cell basis. Interestingly, this was unaffected by CD4 depletion (Figure 6B), once again identifying this as a B-cell-dependent phenomenon.

These results described the functional capacity of resting memory T cells 3 months post-vaccination. To assess the capacity of these cells for a secondary response, the mice were challenged with a lethal dose of LM-OVA at this same time point post-vaccination. Four days after LM-OVA challenge, the frequency and function of Ag-specific T cells and bacterial load were determined in the spleen and liver, respectively. Consistent with the decreased per-cell killing observed in the resting memory T cells (Figure 6B), 9/10 MD4 mice were unable to clear the infection, whereas WT mice had no detectable bacterial burden (Figure 6C). Unlike previous results in the context of infection-elicited immunity (Shen et al., 2003), bacterial challenge yielded fewer secondary effector CD8 T cells post-challenge in MD4 mice (Figure 6D), although MD4 mice exhibited a largely unimpaired secondary expansion compared to WT controls (Figure 6E). Secondary responses were unaffected in sIgM⁻ and AID^{-/-} mice (Figure S4A), demonstrating that capture and stabilization of Ag (León et al., 2014) by secreted natural IgM or Ag-specific IgG also does not play a role in the development of CD8 memory after subunit vaccination. Notably, μ MT^{-/-} mice exhibited poor CD8 T cell memory persistence after immunization, similar to MD4 mice, although their secondary expansion was significantly impaired, in stark contrast with previous data (Shen et al., 2003). Those same factors in sIgM⁻ and AID^{-/-} mice mirrored that of WT mice (Figures S4A–S4E). Since antibodies do not naturally afford protection against LM (Matsuzaki et al., 1999; Shen et al., 2003), the lack of naive, Ag-specific B cells in μ MT^{-/-} and MD4 mice is not a confounding variable when assessing bacterial load. In MD4 mice, bacterial titers negatively correlated with numbers of secondary effectors (Figure 6F). Notably, several mice had high bacterial titers despite similar numbers of secondary effectors to WT mice, again indicating a deficit in effector function on a per-cell basis. This could not be accounted for by reductions in cytokine or granzyme production, as, somewhat counterintuitively, T cells from MD4 mice were highly polyfunctional and produced more IFN γ , tumor necrosis factor alpha (TNF- α), and granzyme B on a per-cell basis than their WT counterparts in *ex vivo* restimulation assays (Figures 6G–6N). However, the ongoing bacterial load in these mice (Figure 6C) and the propensity of these CD8 T cells toward effector differentiation (Figure 3) collectively suggest an elevated degree of terminal effector differentiation. The reduced capacity of the MD4 T cells for persistence into memory (Figure 5) further indicates a reduced capacity for self-renewal, a critical factor for T-cell-mediated host protection (Wherry et al., 2003). Substantial cytokine production was detected in control wells lacking cognate peptide (Figure 6J), evidence in further support of over-differentiation driven by continued antigenic stimulation *in vivo*. Taken together, these results indicate that B cells, not CD4 T cells, influence the magnitude of the primary CD8 T cell response to vaccination; its transition to, and persistence as a memory T cell pool; and its capacity to enact host-protective functions.

B cells can cross-present Ag to CD8 T cells, but B cell Ag presentation is not required for maximal CD8 responses to subunit vaccines

The data thus far suggest that a restricted B cell repertoire is detrimental to the generation and maintenance of vaccine-elicited CD8 T cell responses. Immunofluorescence evaluation of spleen sections after immunization revealed that, as B cells expand early after vaccination, a high percentage of Ag-specific T cells are found within B cell zones on days 2–4 post-vaccination (Figure S5). This localization of CD8 T cells into the B cell zone has been previously observed (Hu et al., 2011; Jung et al., 2010) and could conceivably facilitate direct CD8 T cell:B cell interactions at these early time points.

Given previous conflicting reports on whether B cells can cross-present Ag (Castiglioni et al., 2005; Hon et al., 2005; Ke and Kapp, 1996; Keller et al., 2009; Robson et al., 2008; Zanetti et al., 2004), we began by assessing their abilities in an *in vitro* experimental system. We compared the capacity of highly purified B cells from WT or MD4 spleens (Figure S6A) to cross-present Ag to proliferation-dye-labeled OT1 T cells. APCs were cultured with fast protein liquid chromatography (FPLC)-purified whole protein HEL-OVA conjugates or OVA. As positive controls, we included WT splenocytes enriched for DCs as well as SIINFEKL-peptide-pulsed DCs/B cells. Analysis of OT1 proliferation 2–3 days later demonstrated that, whereas DCs induced OT1 proliferation in all conditions, WT B cells only did so in the presence of peptide, and MD4 B cells did so only in the context of HEL-OVA conjugates or peptide (Figure 7A). These results confirm published data that B cells can indeed cross-present Ag to CD8 T cells *in vitro* and that they do so in a BCR-restricted manner.

However, whether Ag presentation to CD8 T cells by B cells occurs *in vivo* and is necessary for B cell promotion of CD8 T cell responses to vaccines remained open questions. To test these, bone marrow chimeras were generated so that the B cell compartment was selectively limited in its ability to present the SIINFEKL peptide. Bone marrow from $\mu\text{MT}^{-/-}$ mice was mixed with bone marrow from either H-2K^{bm8} mice (that express a mutant form of H-2K^b that cannot present the SIINFEKL peptide to CD8 T cells) or WT H-2K^b mice, at a ratio of 9:1. This ratio ensures that although all B cells express either the mutant or WT H-2K^b allele, ~90% of all other cells express WT H-2K^b derived from the $\mu\text{MT}^{-/-}$ background. H-2K^b 3 H-2K^{bm8} F1 mice were used as recipients to ensure acceptance of bone marrow grafts. Ten weeks after lethal irradiation and bone marrow reconstitution, mice were vaccinated with combined-adjuvant + OVA, and the T cell responses were measured at the peak of the response, 7 days later. The chimeric mice mounted similar Ag-specific CD8 T cell responses regardless of whether B cells were (K^b) or were not (K^{bm8}) capable of presenting cognate SIINFEKL peptide (Figures 7B and 7C). The CD127^{high} memory phenotype was also equal between these groups (Figure 7D), consistent with the conclusion that Ag presentation by B cells is not responsible for the promotion of CD8 T cell responses after immunization.

IL-27 produced by B cells contributes to the primary CD8 T cell vaccine response

As noted earlier, CD8 T cell responses to adjuvanted subunit vaccination are uniquely dependent on IL-27 (Klarquist et al., 2018; Pennock et al., 2014), which is not required

for the primary CD8 T cell response to infections (Pennock et al., 2014; Wehrens et al., 2018) or, in some cases, even inhibits the response (Liu et al., 2014). We have characterized the IL-27 production by DC subsets after vaccination and its unique capacity to predict the magnitude of protective CD8 T cell memory. Given the importance of IL-27 to the vaccine-elicited response, it seemed possible that the influence of B cells on the CD8 T cells might be through their production of this cytokine. We used our recently described IL-27p28-GFP reporter mouse (Kilgore et al., 2018) to examine B cell production of IL-27, specifically over the time frame in which we had observed Ag-specific CD8 T cells within the B cell zone early after vaccination (Figure S5). Although the percentage of B cells producing IL-27p28 was considerably lower than that seen in XCR1+ cDCs (Figures S6B and S6C), a significant number of GFP+ B cells were observed 15 days after combined-adjuvant vaccination (Figure 7E). We again utilized the bone marrow from $\mu\text{MT}^{-/-}$ mice in mixed bone marrow chimeras, this time in combination with bone marrow derived from $\text{Ebi3}^{-/-}$, $\text{IL-27p28}^{-/-}$, and WT controls. We used the 9:1 ratio in favor of $\mu\text{MT}^{-/-}$, ensuring that all B cells were the desired cytokine-deficient phenotype, whereas the majority of non-B cells were WT (Figure S6D). In contrast to the chimeras in which B cells were deficient in Ag presentation, selective B cell deficiency in either of the heterodimeric IL-27 subunits significantly compromised the primary vaccine-elicited CD8 T cell response (Figure 7F). The *Ebi3* subunit can be shared with either IL-35 and the newly described IL-39, whereas p28, either alone or in concert with cytokine-like factor 1 (CLF), can exert signaling as IL-30. Thus, the fact that we observed similar results in mice with B cells deficient in either p28 or *Ebi3* effectively identifies B cell production of IL-27 as the cytokine necessary for maximal vaccine-elicited CD8 responses. Intriguingly, however, this defect in CD8 T cell expansion was not associated with the characteristic skewing away from memory phenotype seen in the MD4 hosts. Indeed, significantly more of the Ag-specific CD8 T cells from the B-cell-selective IL-27-deficient bone marrow chimeras were $\text{CD127}^{\text{high}}$, although similar percentages of tetramer+ cells were seen 3 months post-vaccination (Figures 7G and 7H). Furthermore, bacterial challenge of chimeric mice resulted in similar bacterial burden and secondary expansion (Figures 7I and 7J). Thus, although the production of IL-27 by B cells does not fully account for the effect of B cells on CD8 memory, their production of this cytokine promotes the primary CD8 T cell response to subunit vaccines.

DISCUSSION

The data presented here establish that B cells promote the primary and memory responses of CD8 T cells to subunit vaccines. Our data suggest that perhaps the role of B cells may have been underappreciated in earlier studies of infection that demonstrated accelerated CD8 T cell contraction in B-cell-deficient mice (Asano and Ahmed, 1996; Bründler et al., 1996; Homann et al., 1998). Given the numerous differences cited earlier between vaccine- and infection-elicited CD8 T cell responses, it is likely that the participation of B cells in CD8 T cell immunity also has functions unique to subunit vaccination. Our documented role for B cell IL-27 production is consistent with this prediction, as IL-27 clearly has a role in vaccine-elicited cellular immunity not shared with T cells responding to infectious challenge. Although we have yet to fully appreciate the reciprocal nature of this relationship, recent reports that describe the production of IL-21 by CD8 T cells in

the context of influenza virus infection (Yang et al., 2016) and experimental autoimmune disease (Valentine et al., 2018) suggest that it needs reconsideration. In these studies, CD8 T cells were also found to colocalize with B cells and, in particular, with germinal center B cells, where they promoted class switching and the production of antibodies. With the data we present here, these studies collectively raise the question as to whether a B cell-CD8 T cell bidirectional collaboration has been similarly underappreciated.

The function of B cells as APCs presenting Ag to CD4 T cells and promoting their activation, differentiation, and expansion is well documented (Constant, 1999; Molnarfi et al., 2013). Although B cells are clearly capable of Ag cross-presentation to CD8 T cells *in vitro*, we found that this was not the mechanism by which they help CD8 T cell responses to vaccines *in vivo*. Similarly, we found that B cells do not indirectly impact the magnitude of the CD8 T cell response via CD4 T cells. Indeed, antibody depletion of CD4 T cells before vaccination actually increased the primary and memory CD8 responses, indicating a more direct action of B cells, independent of CD4 cells, in augmenting CD8 T cell responses. CD4 T cells seem instead to play an inhibitory role, possibly through the action of Tregs or by competing for interactions with APCs. We previously showed that memory but not primary CD8 cell numbers were reduced in CD4-deficient, major histocompatibility complex class II knockout (MHCII^{-/-}) mice (Edwards et al., 2013). In the context of the data presented here, the poor memory survival in our MHCII^{-/-} mouse studies is more likely to have been caused by B cell dysfunction rather than deriving from a lack of CD4 T cells. Indeed, MHCII^{-/-} mice have no germinal centers (Madsen et al., 1999), and even B-cell-specific MHCII deletion prevents activated B cell differentiation and proliferation (Giles et al., 2015). This fits well with the data we provide here, where B cells were critical for maintaining CD8 memory after vaccination. The role of CD4 T cells in suppressing CD8 T cell responses to subunit vaccines remains an open question.

Our observed defect in the CD8 T cell responses to vaccination in mice with a limited or absent B cell repertoire has numerous practical implications. Rituximab, an anti-CD20, B-cell-depleting therapy, has been used since 1997, when it was first approved by the Food and Drug Administration to treat non-Hodgkin's lymphoma. It has since been approved for additional diseases, and its off-label use has increased in recent years to more than half of patients receiving the drug (Delate et al., 2020). As almost all of the uses of the various anti-CD20 monoclonal antibody treatments involve prolonged B cell depletion, our data from μ MT hosts are directly relevant. Consistent with this, B cell depletion in a mouse model of immune thrombocytopenia (ITP) showed reduced CD8 T cell proliferation in response to TCR stimulation (Guo et al., 2016). This is additionally applicable to disease settings in which limiting the function of CD8 T cells by depleting B cells may be beneficial. Rituximab use in patients with antineutrophil cytoplasmic antibody-associated vasculitis (AAV) alters the CD8 T cell memory compartment, reducing the frequency of effector memory cells re-expressing CD45RA (TEMRA) (Néel et al., 2019). B cells isolated from these patients increased the cytokine output of CD8 T cells in *ex vivo* co-cultures, providing evidence for a direct, positive effect of B cells on CD8 T cells in this disease setting, where CD8 T cells are pathogenic. A contemporary implication of our data is that of next-generation vaccines deployed to combat emerging viruses, such as SARS-CoV-2. Necessary to consider are the negative consequences of B cell deficiency in the generation

of the T cell responses, which might otherwise help compensate for impaired humoral immunity.

The data presented here should also inform our understanding of common variable immunodeficiency (CVID), a heterogeneous group of disorders characterized by hypogammaglobulinemia of unknown cause. Multiple CD8 T cell abnormalities have been documented in CVID (Wong and Huissoon, 2016), including decreased numbers of naive CD8 T cells (Bateman et al., 2012; Giovannetti et al., 2007), an increase in markers of exhaustion (Klopperk et al., 2020; Paquin-Proulx et al., 2013), dysregulated cytokine production (Giovannetti et al., 2007; North et al., 1998; Thon et al., 1997), and impaired proliferation after TCR engagement (Fischer et al., 1994; Jaffe et al., 1993). The reasons for these CD8-related sequelae have not been described, but our data suggest that it is reasonable to postulate a causal relationship between the B cell and CD8 T cell dysfunctions. Consistent with what we observed for T cells in MD4 compared to WT mice 7 days after immunization and after bacteria challenge, CD8 T cells from patients with CVID (Klopperk et al., 2020) exhibit higher granzyme B than controls. This is similarly true of CD8 T cells after B cell depletion in an experimental model for ITP (Guo et al., 2016). Further studies to uncover the B cell deficiencies in CVID may aid in better understanding the accompanying cellular defects and, in so doing, provide new avenues for therapeutic intervention for the frequent infections experienced by these patients.

Last, it is worth noting that these studies have unveiled an unexpected role for B cell production of IL-27 in vaccine-elicited CD8 T cell immunity. Despite the fact that Ebi3 was originally discovered in B cells, there are surprisingly few reports on IL-27 in B cells and its influence on adaptive immunity, humoral or cellular. Moreover, while canonically pleotropic, the influence of IL-27 on cellular immunity, outside of subunit vaccination, is overwhelmingly suppressive, so that any T-cell-centric elimination of IL-27 signaling typically results in elevated lymphoproliferation and its related pathology. In addition to the findings we report here, we and our colleagues recently documented the extent of B-cell-produced IL-27 in virus-infected and TLR-agonist-treated mice (Yan et al., 2020). In these studies, IL-27 was highly expressed in mice treated with a range of TLR agonists often used as adjuvants. Further, in mice with a B-cell-specific deletion of IL-27, there was substantially reduced vaccinia-specific IgG as compared to WT virus challenge mice. The immune defects in vaccinia-challenged mice with IL-27-deficient B cells were substantial enough so as to significantly delay virus clearance. Thus, the importance of IL-27 in the normal functions of B cells responding to adjuvant and virus challenges is also underappreciated. Collectively, our results reveal functions for B cell production of IL-27 in vaccine-elicited CD8 T cell immunity. Future studies will be necessary to determine the specific B cells involved in producing IL-27 and when and exactly how this IL-27 shapes primary CD8 T cell responses, particularly in the context of DC-produced IL-27. It remains to be determined whether new avenues of therapeutic application for this cytokine are possible and in what clinical areas these data will find their most influential application. Finally, unraveling the additional mechanism(s) by which B cells contribute to CD8 T cell memory responses to vaccination remains the subject of ongoing investigation, which will hopefully yield further avenues for improving vaccine-elicited immunity.

STAR★METHODS

RESOURCE AVAILABILITY

Lead contact—Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ross Kedl (Ross.Kedl@cuanschutz.edu).

Materials availability—This study did not generate unique reagents.

Data and code availability—Microscopy data reported in this paper will be shared by the lead contact upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice—All experiments involving mice were conducted following protocols approved by the University of Colorado Institutional Animal Care and Use Committee (IACUC) according to guidelines provided by the Association for Assessment and Accreditation of Laboratory Animal Care. WT (C57BL/6J), congenic CD45.1 B6 (B6.SJL-*Ptprca*^a *Pepcb*^b/BoyJ), OVA-specific TCR-transgenic OT-1 (C57BL/6-Tg(TcraTcrb)1100Mjb/J), μ MT^{-/-} (B6.129S2-*Ighm*^{tm1Cgn}/J) and MD4 (C57BL/6-Tg(IghelMD4)4Ccg/J) mice were originally obtained from the Jackson Laboratory and subsequently bred inhouse at the University of Colorado. MD4 mice were bred as hemizygous MD4 x C57BL/6J, and hemizygous MD4 mice were compared to WT littermate controls. Secreted-IgM-deficient (Boes et al., 1998), AID^{-/-} (Muramatsu et al., 2000), and H-2K^{bm8} (B6(C)-H2-Kbm8/LrpJ) mice were provided by Drs. Claudia Jakubzick, Jing H. Wang, and Larry Pease, respectively. IL-27p28-GFP mice were generated by our lab as previously described (Kilgore et al., 2018) and used as hemizygotes. IL-27p28^{-/-} mice, originally provided by Janssen Research & Development (Spring House, PA), and Ebi3^{-/-} mice (B6.129X1-Ebi3tm1Rsb/J), from Jackson laboratory, were bred in house the University of Pennsylvania. Experiments were performed in 6–10-week-old male and female mice.

METHOD DETAILS

Immunizations and infections—Mice were immunized via tail-vein injection (I.V.), subcutaneous injection in the ear (S.C.), or intraperitoneal injection (I.P.) with the indicated adjuvant(s) with 150 μ g (only 25 μ g in the case of SC immunization) of whole chicken OVA (Sigma) or 100 μ g of SARS-CoV-2 spike protein receptor binding domain (RBD) protein plus adjuvant. OVA (Sigma) and HSVgB (produced inhouse by baculovirus expression) proteins were detoxified by phase separation (Aida and Pabst, 1990) and were lipopolysaccharide-free as determined by a limulus assay. SARS-CoV-2 spike RBD protein (GenBank: MT380724.1) was generated by transfection of HEK293 T cells with a His-tagged vector (a gift from F. Krammer, Icahn School of Medicine at Mount Sinai, New York, NY) (Amanat et al., 2020) and subsequent purification over an ATKA nickel column (Cytiva) by the University of Colorado Cell Technologies Shared Resource. The following adjuvant doses were used for immunizations: 40 μ g poly(I:C) (5 μ g for S.C., Invivogen), 40 μ g α CD40 (5 μ g for S.C., clone FGK4.5, BioXCell), 40 μ g Pam3Cys, or

100 μ L Alu-Gel-S (1.3% aluminum hydroxide, SERVA Electrophoresis GmbH). Vaccines were made immediately prior to immunization with the exception of alum; OVA was emulsified in alum by gentle rocking at 4C overnight. Unless otherwise specified, mice were immunized I.V. with combined-adjuvant subunit vaccine (anti-CD40, poly(I:C), and OVA). Mice were infected with 2,000 CFU of OVA-expressing *Listeria monocytogenes* (LM-OVA) or challenged with $1-2 \times 10^5$ CFU of LM-OVA by I.V. injection. Four days after challenge, spleens, blood, and liver were harvested from euthanized mice. Livers were massed then processed in 5 mL of 0.02% NP40 using sterile plastic tissue homogenizers (Miltenyi). Bacterial counts were performed after incubating multiple dilutions of liver homogenates for 48 hours at 37C on BHI agar plates with 5 μ g/ml erythromycin (Sigma).

Bone marrow chimeras—Recipient BoyJ mice (CD45.1/1) were lethally irradiated with 2×600 rads 3 hours apart and reconstituted 18 hours later with T cell-depleted bone marrow from sex-matched μ MT^{-/-} (CD45.1/2) and either C57BL/6, H-2K^{bm8}, IL-27p28^{-/-}, or Ebi3^{-/-} (CD45.2/2) bone marrow at a 9:1 ratio. T cells were depleted using CD3 (clone 145-2C11), CD4 (GK1.5), CD8 (53.67) and CD90.2 (30-H12) APC-conjugated antibodies and anti-APC magnetic beads (BioLegend). At 10 weeks post-reconstitution, mice were immunized as described above.

Flow cytometry—Spleen single cell suspensions or whole blood (collected by tail vein puncture into 5mM EDTA in HBSS), as indicated, were subjected to ACK red blood cell lysis and counted using a Vi-Cell automated cell counter (Beckman Coulter). Cells were then incubated with α CD16/32 (clone 2.4G2; hybridoma supernatant) and tetramer stained as previously described at 37C for 30 minutes, or in the case of OT-1 transfer, cells were stained for CD45.1 (clone A20; BioLegend) at room temperature (RT) for 10 minutes. Kb-SIINFELK tetramers were provided by the NIH Tetramer Core. Following tetramer staining, surface antibodies were added for 10 min. at 37C, including CD3e (145-2C11), B220 (RA3-6B2), CD8a (53-6.7), CD44 (IM7; Tonbo), CD62L (MEL-14), CD122 (TM- β 1), CD127 (A7R34; Tonbo), and KLRG1 (2F1/KLRG1) (all from BioLegend, unless otherwise noted). For transcription factor analysis, cells were surface stained, then fixed and permeabilized using Tonbo Foxp3 fixation/permeabilization buffers (Tonbo) and stained for Eomes (Dan1 1mag; eBioscience), IRF4 (3E4; eBioscience), and TCF1 (C63D9; Cell Signaling Technology). Intracellular cytokine staining was performed after a 5-hour stimulation with either 1 μ g/ml peptide of SIINFELK for OVA immunizations, or VVLSFELL for SARS-CoV-2 RBD immunizations (ChinaPeptides) and 3 μ g/ml brefeldin A. After fixation and permeabilization, CD8 T cells were stained for intracellular IFN γ (XMG1.2), TNF α (MP6-XT22), and IL-2 (JES6-5H4); granzyme B (GB11; all from BioLegend) was stained directly *ex vivo* and analyzed on tetramer-positive cells. Flow cytometry data were acquired on a four-laser (405, 488, 561, 638 nm) CytoFLEX S flow cytometer (Beckman Coulter) and analysis was performed using FlowJo (version 10.7.1; BD Biosciences).

OT1 adoptive transfers and CD4-depletion—CD8 T cells were magnetically purified (BioLegend) from OT1 mice to > 95% purity. Cells were counted and 200–500 (as indicated) OT1s were transferred to recipient mice by tail vein injection 1 day prior to

immunization. CD4 T cell-depletion was achieved by IV injection of 50 µg αCD4 antibody (clone GK1.5, BioXCell) 2–5 days prior to immunization.

Immunofluorescence—Whole spleens were frozen in OCT compound at –80C until cutting 7 µm sections with a cryostat followed by acetone fixation. Sections were dried, then rehydrated with PBS. Non-specific staining was blocked using PBS + 10% FBS and 2.5% anti-CD16/32 (2.4G2) hybridoma supernatant, and an Avidin/Biotin blocking system (BioLegend). Sections were then stained using CD45.1-PE (clone A20; BioLegend), CD169-AlexaFluor488 (3D6.112; BioLegend), B220-DyLight650 (RA3–6B2; made inhouse with hybridoma), F480-AlexaFluor488 (BM8; BioLegend), CD3e-biotin (145–2C11; BioLegend) followed by streptavidin-AlexaFluor750 (ThermoFisher), and/or IgM-Cy5 (JacksonImmunoResearch), followed by incubation with 300nM DAPI (ThermoFisher) and coverslipping with ProLong Gold anti-fade reagent (ThermoFisher). Images were captured by whole-slide scanning with a Vectra Polaris (Akoya Biosciences) and analyzed using inForm software (Akoya).

In vitro cross-presentation assay—B cells were highly purified by staining with CD43-APC (clone S11) and CD11c-APC (clone N418; BioLegend) followed by anti-APC bead separation over magnetic LS columns (Miltenyi), where the supernatant consisted of ~99% B220+CD19+ B cells. DCs were enriched to ~30% by CD11c-APC staining and magnetic bead separation. CD8 T cells from OT1 mice were enriched to > 95% as described above and stained with 2 µM CellTrace Violet proliferation dye (ThermoFisher). HEL-OVA conjugates were created by glutaraldehyde crosslinking essentially as previously described (Allen et al., 2007). Whole OVA and HEL (Sigma) were combined at a 1:1 molar ratio in KPO4 (pH 7.2) with 0.023% glutaraldehyde (Sigma). Crude protein conjugates were dialyzed overnight with 20kD dialysis cassettes (ThermoFisher) in PBS. Precipitates were removed by 0.2 µm filtration. Conjugated HEL-OVA was concentrated with 30kD MWCO spin concentrators (Sigma), then purified by FPLC (AKTA). B cells or DCs were incubated with 10 µg/ml FPLC-purified HEL-OVA, OVA, or SIINFEKL peptide and 10 µg/ml anti-CD40 for 1 h at 37C, then washed 3 times with RPMI 1640 containing 10% FBS, 10 mM HEPES, 0.1 mM β-ME, 0.1 mM non-essential amino acids, 0.1 mM sodium pyruvate, 2 mM L-glutamine and penicillin-streptomycin (complete media). B cells or DCs were plated at 100,000 cells/well together with 50,000 dye-labeled OT1 cells/well and incubated for 48–72 hours in 200 µL complete media.

In vivo killing assay—Splenocytes were peptide-pulsed for 1 h in complete media with 1 µg/ml of OVA_{257–264} (target cells) or HSVgB_{498–505} (control cells), washed, then labeled with 0.05 µM or 0.5 µM CellTrace Violet (ThermoFisher) dye, respectively. Recipient WT or MD4 mice were immunized with anti-CD40, poly(I:C), and OVA 1 day after adoptive transfer of 500 OT1 T cells. After 3 months, 8.5 million target splenocytes combined with 1.5 million control splenocytes were transferred into mice by tail vein injection. Mice were euthanized after 2 h and the ratio of target:control was used to determine the percent killing compared to the same ratio observed for non-immunized control mice. Percent killing was plotted against the total number of splenic Ag-specific CD8 T cells as determined by tetramer staining.

QUANTIFICATION AND STATISTICAL ANALYSIS

GraphPad Prism (version 9.1.2, GraphPad) was used for all statistical analyses. Figure legends detail the number of experimental replicates and n-values. Unless noted, data shown are means \pm SEM. Significance was defined by a two-tailed, unpaired Student's t tests unless otherwise specified, where * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Primary and memory CD8 T responses to vaccines are hampered in B-cell-deficient mice
- B cells support a self-renewal program in vaccine-elicited CD8 T cells
- IL-27 from B cells drives the magnitude of the primary CD8 T cell vaccine response

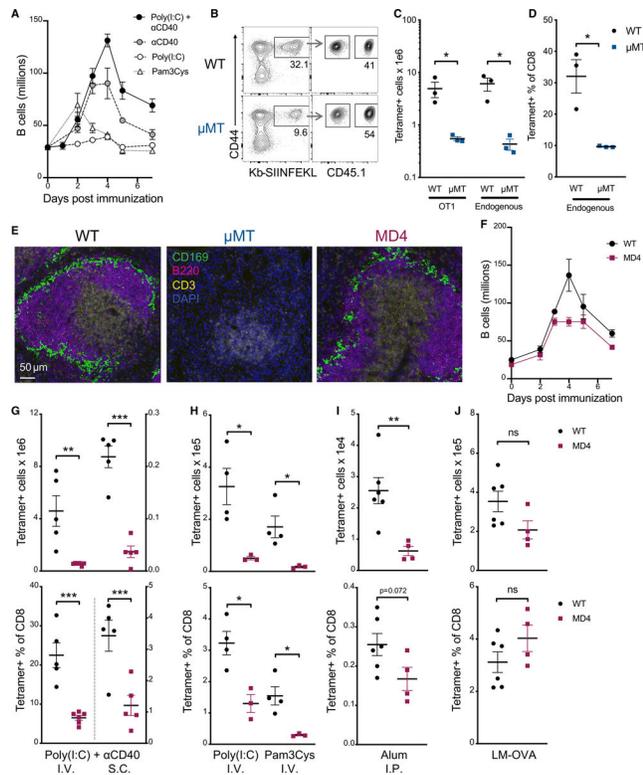


Figure 1. B cells promote primary CD8 T cell responses to subunit vaccines

(A) Total splenic B220+CD19+ B cells from WT C57BL/6 mice 0–7 days after i.v. administration of combined-adjuvant vaccine (anti-CD40, poly(I:C), and OVA).

(B–D) 200 CD45.1+ OT1 T cells were transferred into WT or $\mu\text{MT}^{-/-}$ mice 1 day before combined-adjuvant vaccination. Spleens were harvested 7 days later. (B) Representative tetramer and CD45.1 staining. (C) Total OT1 cells (left) and tetramer+ endogenous CD8 T cells (right). (D) Tetramer as a percentage of endogenous CD8 T cells.

(E) Representative immunofluorescent staining of 2 naive mice per group with DAPI (blue), CD3 (yellow), B220 (magenta), and CD169 (green). Scale bar represents 50 μm for all images.

(F) Total splenic B220+CD19+ B cells from WT or MD4 mice 0–7 days after combined-adjuvant vaccine.

(G and H) Total splenic tetramer+ CD8 T cells (top) or tetramer+ cells as a percentage of CD8 T cells (bottom) were measured 7 days after subunit vaccination or infection. (G) Ag-specific CD8 T cell responses after i.v. (left) or s.c. (right) administration of combined-adjuvant + OVA vaccine. (H) Responses after i.v. subunit immunization with the single adjuvants poly(I:C) (left) or Pam3Cys (right).

(I) Responses after i.p. administration of OVA emulsified in alum.

(J) Responses after infection with LM-OVA.

Data shown are means \pm SEM; $n = 3$ mice per group, representative of 2 experiments, except for (F) and (H), which are from one experiment each. Significance was defined by two-tailed, unpaired Student's *t* tests, where * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. See also Figure S2.

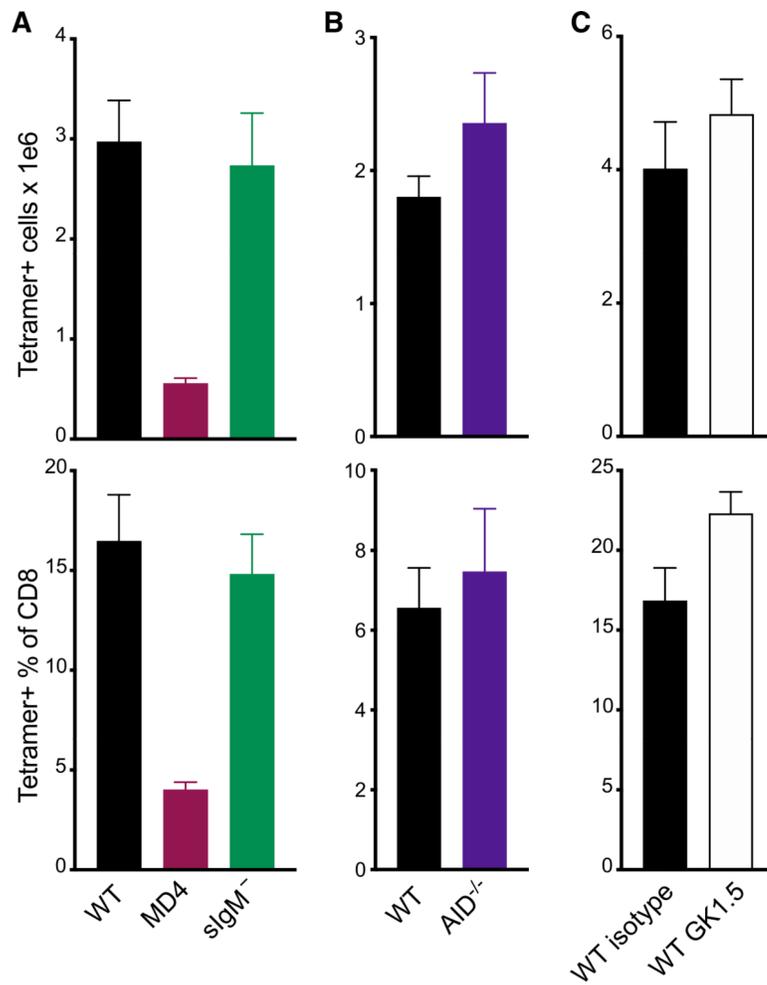


Figure 2. Primary CD8 T cell responses are not dependent on Ag-Ab complexes or CD4 T cell responses

(A–C) Mice were immunized with the combined-adjuvant vaccine. Total splenic tetramer+ CD8 T cells (top) or tetramer+ cells as a percentage of CD8 T cells (bottom) were measured 7 days after subunit vaccination in: (A) WT, MD4, or sIgM⁻ mice; (B) WT or AID^{-/-} mice; or (C) WT mice treated with control antibodies (isotype) or CD4-depleting antibodies (GK1.5). Data shown are means ± SEM; 3–5 mice per group, representative of 2 experiments.

See also Figure S3.

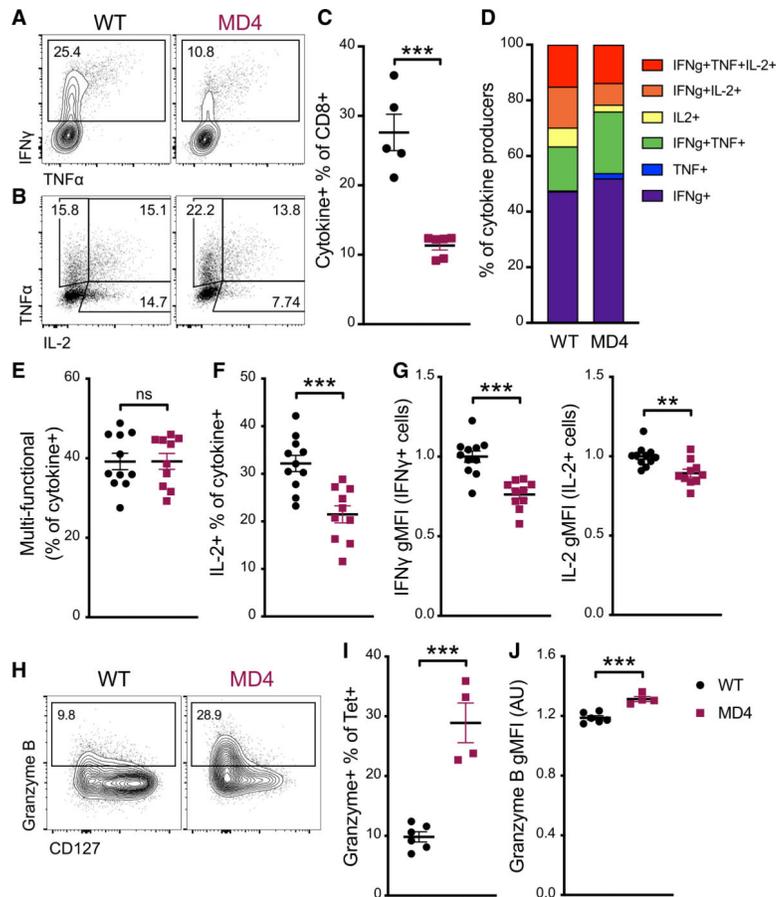


Figure 3. Cytokine and granzyme production by effector cells demonstrates greater terminal differentiation in mice with a fixed B cell repertoire

(A–G) Seven days after immunization with the combined-adjunct vaccine, splenocytes were incubated with SIINFEKL peptide *ex vivo* and assessed for cytokine production. (A) Representative flow cytometry plots of total CD8 T cells and (B) IFN γ + CD8 T cells, showing gating for IFN γ , TNF- α , and IL-2. (C) The percentage of CD8 T cells positive for one or more of the cytokines IFN γ , TNF- α , and IL-2. (D) The distribution of single-, double-, and triple-positive cells within all cytokine-producing CD8 T cells. (E) Multi-functional CD8 T cells, defined as producing more than one cytokine, as a percentage of all cytokine-producing CD8 T cells. (F) The percentage of cytokine-producing cells making IL-2. (G) The gMFI for IFN γ (left) and IL-2 (right) of the respective cytokine+ cells.

(H and I) Ag-specific CD8 T cells were identified by tetramer-staining splenocytes 7 days after mice received combined-adjunct vaccine. (H) Representative plots displaying CD127 and granzyme B staining of tetramer+ cells. (I) Granzyme-B-positive cells as a percentage of tetramer+ CD8 T cells.

(J) The gMFI of granzyme-B-positive tetramer+ cells.

Data shown are means \pm SEM; n = 3 mice per group, representative of 2 experiments. Significance was defined by two-tailed, unpaired Student's t tests, where *p < 0.05, **p < 0.01, and ***p < 0.001.

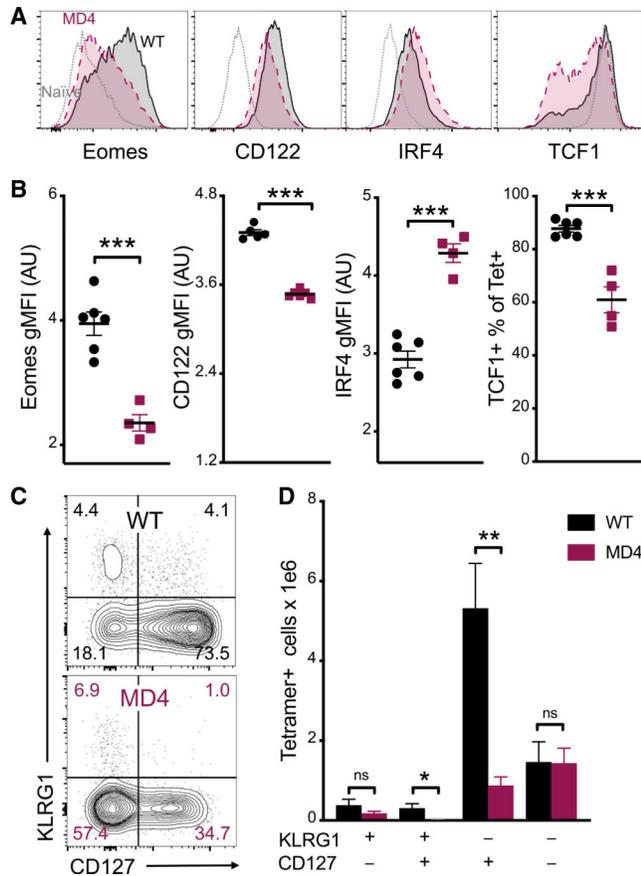


Figure 4. B cells promote CD8 T cell memory programming following subunit vaccination

(A) Representative histograms for Eomes, CD122, IRF4, and TCF1 staining of tetramer+ cells from WT (black) or MD4 (maroon) mice 7 days after combined-adjuvant vaccination or for CD44⁻ naive control CD8 T cells (gray).

(B) The gMFI of tetramer+ cells for Eomes, CD122, and IRF4, where the x axis intersects the y axis at the average gMFI value for CD44⁻ naive controls, and the percentage of tetramer+ cells that stained positive for TCF1.

(C and D) Representative KLRG1 versus CD127 staining (C) and numbers of tetramer+ splenocytes in the respective quadrants (D), 7 days after immunization.

Data shown are means \pm SEM; n : mice per group, representative of 3 experiments.

Significance was defined by two-tailed, unpaired Student's t tests, where *p < 0.05, **p < 0.01, and ***p < 0.001.

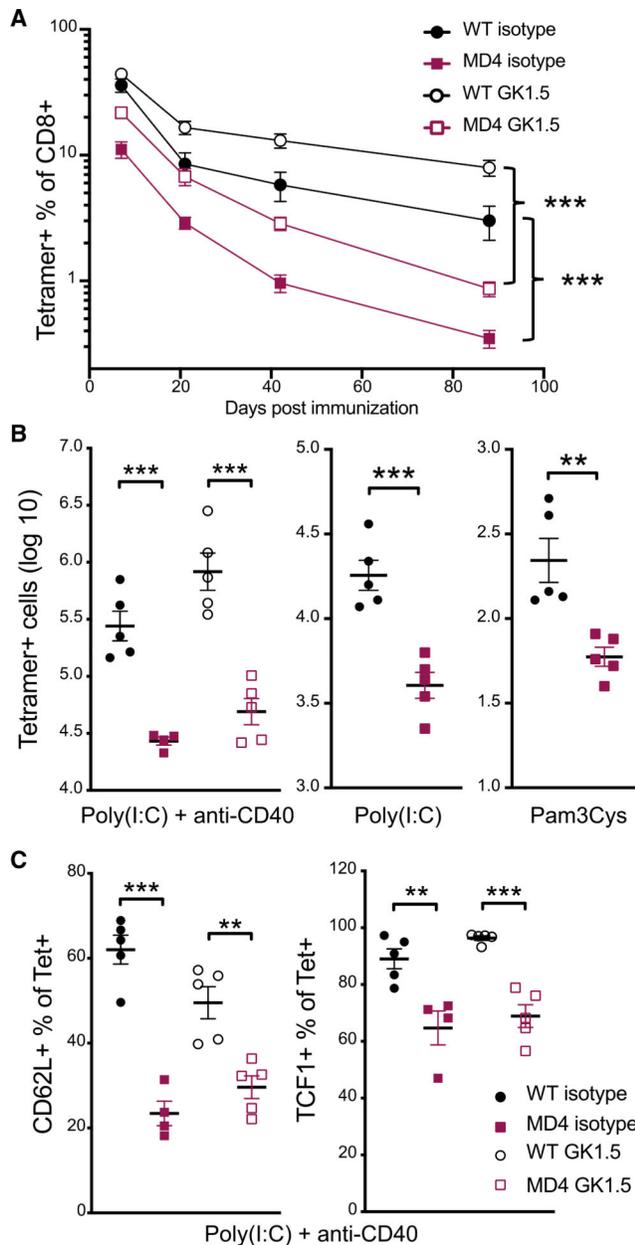


Figure 5. Defective persistence of CD8 T cell memory cells in the

Figure 5. Defective persistence of CD8 T cell memory cells in the absence of a normal B cell repertoire

(A) Endogenous tetramer+ cells as a percentage of CD8 T cells in the blood over time after combined-adjutant immunization of CD4-depleted mice (GK1.5) or controls (isotype).

(B) The absolute number of tetramer+ CD8 T cells 3–5 months after combined-adjutant (left) or single-adjutant subunit vaccinations (middle and right).

(C) The percentage of tetramer+ splenocytes positive for CD62L (left) or TCF1 (right) 3 months after combined-adjutant vaccination.

Data shown are means \pm SEM; n = 3 mice per group, representative of 2 experiments for (A) and one experiment each for (B) and (C). Significance was defined by 2-way ANOVA

interaction effects (A) or by two-tailed, unpaired Student's t tests (B and C), where * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. See also Figure S3.

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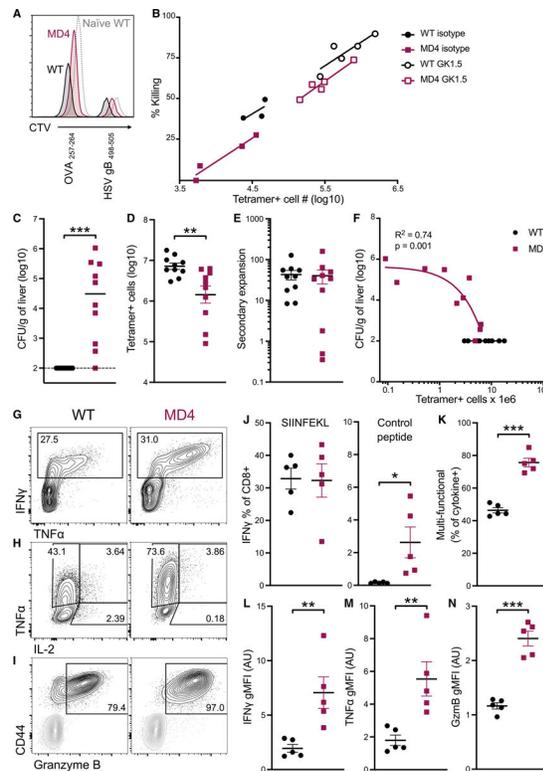


Figure 6. Dysfunctional CD8 memory in the absence of a normal B cell repertoire

(A and B) Mice that were either depleted of CD4 T cells 5 days before vaccination or kept intact received 500 OT1 cells immediately before combined-adjunct vaccination. The function of resting memory cells was assessed by an *in vivo* killing assay 3 months later. SIINFEKL-loaded splenocytes and HSVgB-peptide-loaded splenocytes were labeled with low and high levels of CTV, respectively; mixed; and injected intravenously into mice. Two hours later, splenic target and effector numbers were quantified. (A) Representative raw data are shown with staggered histograms. (B) Data are plotted as percentage of total SIINFEKL-loaded targets killed versus total number of effector cells.

(C–N) Separate WT and MD4 mice were administered the i.v. combined-adjunct subunit vaccine. After 3 months, they were challenged with 10^5 colony-forming units (CFUs) of LM-OVA and analyzed 4 days later. (C) LM CFUs in the liver of mice 4 days post-challenge. (D) Number of splenic tetramer+ cells 4 days post-challenge. (E) Fold secondary expansion of Ag-specific CD8 T cells in the blood as determined by dividing the frequency of tetramer+ (out of a total CD8 T cells) 3 months after immunization, i.e., pre-challenge, by the frequency of tetramer+ cells 4 days post-challenge. (F) LM CFUs from (C) were correlated with Ag-specific T cell numbers in (B), and analysis of the MD4 data yielded a significant linear regression with an R^2 of 0.74. (G–I) Representative flow cytometry plots of memory CD8 T cells from WT and MD4 4 days post-LM challenge. (G) IFN γ and TNF- α intracellular cytokine staining of CD8 T cells after 5-h stimulation with SIINFEKL (OVA_{257–264}) peptide. (H) TNF- α and IL-2 staining of IFN γ + T cells from (E). (I) Granzyme B staining directly *ex vivo* 4 days post-challenge, showing tetramer+ CD8 T cells (black) overlaid with CD44– naive control cells (gray). (J) IFN γ + as a percentage of CD8 T cells after 5-h incubation with SIINFEKL (left) or an irrelevant control peptide (HSV

gB498–505, right). (K) Multi-functional cells—double- or triple-positive for IFN γ , TNF- α , and/or IL-2—as a percentage of total cytokine producers. (L) IFN γ and (M) TNF- α gMFI of respective cytokine+ cells. (N) Granzyme B gMFI of tetramer+granzyme+ CD8 T cells. Data shown are means \pm SEM; n = 3 mice per group, representative of 2 experiments. Significance was defined by two-tailed, unpaired Student's t tests, where *p < 0.05, **p < 0.01, and ***p < 0.001.

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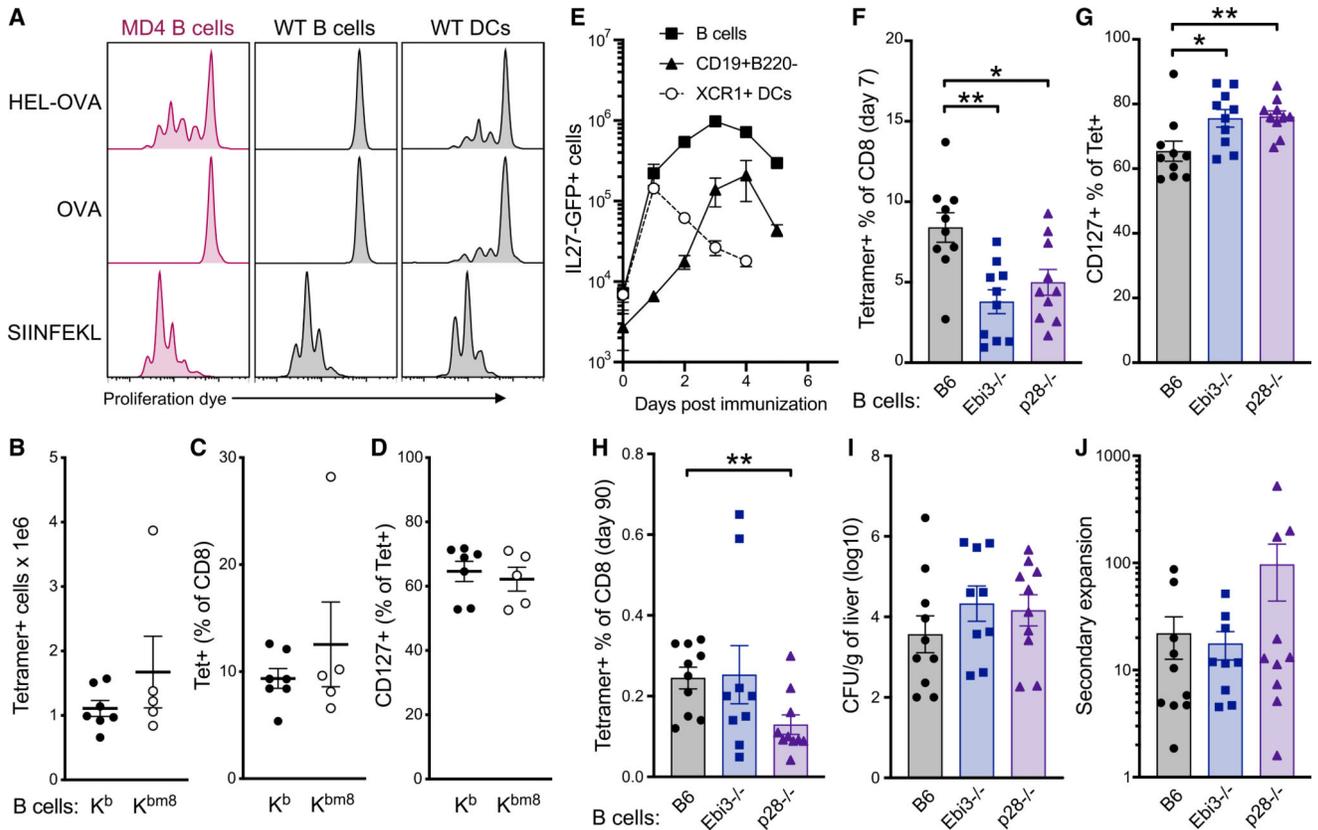


Figure 7. B cell production of IL-27, not cross-presentation, contributes to CD8 T cell vaccine responses

(A) B cells or splenocytes enriched for DCs were pre-incubated with protein or peptide for 1 h and then washed thoroughly before combining with proliferation dye-labeled OT1s. OT1 proliferation was determined by proliferation dye dilution 72 h after incubation with the indicated cell type.

(B–D) Bone marrow chimeras were generated by reconstituting H-2K^b/K^{bm8} CD45.1/2 F1 mice with a mixture of μ MT^{-/-} CD45.1/2 + WT^{CD45.2/2}, or H-2K^{bm8} CD45.2/2 bone marrow. Chimeric mice were immunized 10 weeks after reconstitution and analyzed for Ag-specific CD8 T cell responses 7 days later. (B) Total splenic tetramer+ CD8 T cells. (C) Tetramer+ cells as a percentage of CD8 T cells. (D) CD127+ cells as a percentage of tetramer+ cells.

(E) The percentage of cells producing IL-27 was tracked over time in B cells (CD19+B220+), CD19+B220– cells, and XCR1+ DCs (B220–CD11c^{high}XCR1+) using IL-27p28-GFP reporter mice after combined-adjuvant immunization.

(F–J) Bone marrow chimeras were generated by reconstituting WT CD45.1/2 mice with a mixture of μ MT^{-/-} CD45.1/2 + WT^{CD45.2/2}, Ebi3^{-/-} CD45.2/2, or p28^{-/-} CD45.2/2 bone marrow. Chimeric mice were immunized 10 weeks after reconstitution. (F) Tetramer+ cells as a percentage of grafted CD8 T cells in the blood 7 days after vaccination. (G) CD127+ cells as a percentage of tetramer+ cells on day 7. (H) Tetramer+ cells as a percentage of grafted CD8 T cells 3 months after vaccination. (I) Secondary expansion of Ag-specific CD8 T cells in the blood defined as tetramer+ percentage of CD8 (pre-challenge) divided by tetramer+ percentage of CD8 (post-challenge). (J) LM CFUs in the liver of mice 4 days post-challenge.

Data in (A) are representative of 3 experiments. Data shown in (B)–(D) and (F)–(J) are means \pm SEM from 2 combined experiments. Data shown in (E) are means \pm SEM; n 3 mice per group, from 3 experiments combined. Significance was defined by two-tailed, unpaired Student's t tests, where *p < 0.05, **p < 0.01, and ***p < 0.001. See also Figure S6.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
APC anti-mouse CD45.1	BioLegend	Cat# 110714
Brilliant Violet 421 anti-mouse CD3e	BioLegend	Cat# 155617
PerCP anti-mouse B220	BioLegend	Cat# 103234
Brilliant Violet 605 anti-mouse CD8a	BioLegend	Cat# 100744
redFluor 710 anti-Human/Mouse CD44	Tonbo Biosciences	Cat# 80-0441-U100
FITC anti-mouse CD62L	BioLegend	Cat# 104406
FITC anti-mouse CD122 (IL-2R β)	BioLegend	Cat# 123208
PE anti-Mouse CD127 (IL-7Ra)	Tonbo Biosciences	Cat# 50-1271-U100
PE/Cyanine7 anti-mouse/human KLRG1	BioLegend	Cat# 138416
Foxp3 / Transcription Factor Staining Buffer Kit	Tonbo Biosciences	Cat# TNB-0607-KIT
PE/Cyanine7 anti-mouse/human Eomes	ThermoFisher Scientific	Cat# 25-4875-82
PE anti-mouse/human IRF4	ThermoFisher Scientific	Cat# 12-9858-82
Alexa Fluor 488 anti-mouse/human TCF1	Cell Signaling	Cat# 6444S
APC anti-mouse IFN- γ	BioLegend	Cat# 505810
PE anti-mouse IL-2	BioLegend	Cat# 503808
PE/Cyanine 7 anti-mouse TNF- α	BioLegend	Cat# 506324
FITC anti-human/mouse Granzyme B	BioLegend	Cat# 372206
MojoSort Mouse anti-APC Nanobeads	BioLegend	Cat# 480072
<i>In VivomAb</i> anti-mouse CD4 (GK1.5)	BioXCell	Cat# BE0003-1
PE anti-mouse CD45.1	BioLegend	Cat# 110708
Alexa Fluor® 488 anti-mouse CD169 (Siglec-1)	BioLegend	Cat# 142419
Alexa Fluor® 488 anti-mouse F4/80	BioLegend	Cat# 123120
Biotin anti-mouse CD3e	BioLegend	Cat# 100304
Cy5 AffiniPure Goat Anti-Mouse IgM, μ chain specific	Jackson ImmunoResearch	Cat# 115-175-075
APC anti-mouse CD43	BioLegend	Cat# 143208
APC anti-mouse CD11c	BioLegend	Cat# 117310
<i>InVivoMAB</i> anti-mouse CD40 (FGK4.5)	BioXCell	Cat# BE0016-2
Chemicals, peptides, and recombinant proteins		
Streptavidin, Alexa Fluor 750 conjugate	ThermoFisher Scientific	Cat# S21384
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)	ThermoFisher Scientific	Cat# D1306
ProLong Gold Antifade Mountant	ThermoFisher Scientific	Cat# P36934
CellTrace Violet Cell Proliferation Kit	ThermoFisher Scientific	Cat# C34557
Albumin from chicken egg white (ovalbumin)	Millipore Sigma	Cat# A5503
Avidin/Biotin Blocking System (Previously Covance catalog# SIG-31126)	BioLegend	Cat# 927301
Alu-Gel-S	Serva	Cat# 12261.01
Ghost Dye Red 780	Tonbo	Cat# 13-0865-T500
Critical commercial assays		

REAGENT or RESOURCE	SOURCE	IDENTIFIER
DyLight 650 Antibody Labeling Kit	ThermoFisher Scientific	Cat# 84535
Experimental models: Organisms/strains		
Mouse: C57BL/6J	The Jackson Laboratory	Cat# 000664
Mouse: B6 CD45.1	The Jackson Laboratory	Cat# 002014
Mouse: OT-1	The Jackson Laboratory	Cat# 003831
Mouse: μ MT ^{-/-}	The Jackson Laboratory	Cat# 002288
Mouse: MD4	The Jackson Laboratory	Cat# 002595
Mouse: sIgM ⁻	Boes et al., 1998	N/A
Mouse: AID ^{-/-}	Muramatsu et al., 2000	N/A
Mouse: H-2K ^{bm8}	The Jackson Laboratory	Cat# 023029
Mouse: IL-27p28-GFP	Kilgore et al., 2018	N/A
Mouse: IL-27p28 ^{-/-}	Janssen Research and Development	N/A
Mouse: Ebi3 ^{-/-}	The Jackson Laboratory	Cat# 008691
Oligonucleotides		
Poly(I:C) HMW (long synthetic analog of dsRNA)	Invivogen	Cat# tlr1-pic-5
Recombinant DNA		
SARS-CoV-2 spike vector	Amanat et al., 2020	N/A
Software and algorithms		
Prism version 9.1.2	GraphPad	N/A
inForm version 2.5.1	Akoya Biosciences	N/A
FlowJo version 10.7.1	BD Biosciences	N/A