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CD70 and IFN-1 selectively induce Eomesodermin or T-bet and synergize to promote CD8+ T cell responses

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Abstract

CD70-mediated stimulation of CD27 is an important cofactor of $CD4^+$ T cell licensed dendritic cells. However, it is unclear how CD70-mediated stimulation of T cells is integrated with signals that emanate from Signal 3 pathways, such as type-1 interferon (IFN-1) and IL-12. We find that while stimulation of CD27 in isolation drives weak $Eomesodermin^{hi}T\text{-}bet^{lo}$ $CD8^+$ T cell responses to OVA immunization, profound synergistic expansion is achieved by co-targeting TLR. This cooperativity can substantially boost anti-viral $CD8^+$ T cell responses during acute infection. Concomitant stimulation of TLR significantly increases per-cell IFNγ-production and the proportion of the population with characteristics of short-lived effector cells, yet also promotes the ability to form long-lived memory. Notably, while IFN-1 contributes to the expression of CD70 on dendritic cells, the synergy between CD27 and TLR stimulation is dependent upon IFN-1's effect directly on $CD8^+$ T cells, and is associated with the increased expression of T-bet. Surprisingly, we find that IL-12 fails to synergize with CD27 stimulation to promote $CD8^+$ T cell expansion, despite its capacity in driving effector differentiation. Together these data identify complex interactions between Signal 3 and costimulatory pathways, and identify opportunities to influence the differentiation of $CD8^+$ T cell responses.

Keywords

$CD8^+$ T cells; CD27; CD70; IFN-1; IL-12

Introduction

Effective therapeutic vaccination strategies against viral infections and tumors will likely require the development of potent $CD8^+$ T cells. By defining the cellular and molecular determinants of naïve $CD8^+$ T cell differentiation into either effectors or long-lived poly-

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Conflict of interest

The authors declare no commercial or financial conflict of interest.

functional memory cells, it can be anticipated that vaccine efficacy will be improved, and that vaccines may be tailored according to the type of protection that is needed.

Initial $CD8^+$ T cell activation by TCR engagement (Signal 1) and CD28 stimulation (Signal 2) is followed by a program of proliferation [1;2] and differentiation into short-lived effector cells (SLECS; characterized by $CD127^{lo}$ $KLRG1^{hi}$) or memory precursor effector cells (MPECs; $CD127^{hi}KLRG1^{lo}$) [3] /id;Marzo, 2005 12214 /id;Angelosanto, 2010 12793 / id;Sarkar, 2008 12803 /id}. During and after initial activation, $CD8^+$ T cells receive a variety of stimuli that influence fate decisions, dictating the expression of transcription factors such as T-bet, Eomesodermin (Eomes), and B lymphocyte-induced maturation protein-1 (BLIMP1) that regulate $CD8^+$ T cell proliferation, differentiation and survival (Signal 3), and subsequent differentiation into effector and memory cells [4]. Some Signal 3 stimuli are normally elicited by the response to pathogenic infection, sensed by TLR and other PRR, and are manifest in the form of inflammatory cytokines such as IL-12, type I IFN (IFN-1), IFN-γ and IL-6. IL-12 has been shown to direct $CD8^+$ T cells to take the characteristics of SLECs by the induction of T-bet [5–7]. IFN-1 has been shown to elicit DC activation [8] and also contribute to both the expansion and differentiation of T cell responses [9;10]. Its contribution to the expression of the transcription factors that regulate SLEC/MPEC differentiation *in vivo* is less well characterized. Other stimuli that influence T cell fate are derived from components of adaptive immunity, and are primarily orchestrated by helper $CD4^+$ T cells.

Evidence from our lab and others has demonstrated that a major consequence of $CD4^+$ T cell-mediated licensing of DC via CD40 is the induction of the TNF-superfamily member CD70 [11–16]. CD70 stimulates CD27, which, among other functions, reduces activation-induced [17] and Fas-L mediated cell death [18]. Prolonged survival is in part by inducing sustained expression of IL-2 [19] in peripheral $CD8^+$ T cells, and CD27 stimulation supports effector cell generation against viral infections [20–23], and subsequent differentiation to memory cells [20;24–27]. While the expression of CD70 on DC is primarily induced by stimulation of CD40, it is strongly enhanced by concurrent stimulation of TLR and signaling via IFNαβ receptors, leading to the potent activation of $CD8^+$ T cell responses and a strategy for subunit vaccination[12–14;28;29]. However, IFNαβR-independent, IL-12R-dependent, activation of $CD8^+$ T cells can occur, particularly to IL-12-inducing TLR agonists [30;31]. Whether IL-12 can synergize with CD40 to induce CD70 is not known. Thus, on the one hand, the contribution of TLR/IL-12/IFN-1 to $CD8^+$ T cell expansion and differentiation could be to sensitize DC to enhance CD70 expression [31]. On the other hand, as recent studies have implicated a role for IL-12 and IFN-1 in the direct stimulation of $CD8^+$ T cells [9;10;32–37], stimulation by CD27 and IFN-1/IL-12 may co-operate to induce transcription factors that regulate the expansion, survival and differentiation of $CD8^+$ T cells. This raises the question whether CD27 stimulation can drive $CD8^+$ T cell proliferation and differentiation alone, as suggested by studies using transgenic expression of CD70 by DC and recombinant CD70 [38–40], or whether concomitant IFN-1 or other Signal 3 co-factors are also required [28;41].

Results

Co-targeting CD40 and TLR results in CD70-dependent, helper CD4+ T cell independent primary and memory CD8+ T cell responses

Concurrent stimulation of CD40 and TLR has been shown to bypass the necessity for $CD4^+$ T cell help in the generation of primary $CD8^+$ T cell responses to OVA protein immunization [30]. It is not certain whether this combination of stimulations is sufficient for the formation of functional memory $CD8^+$ T cells in the absence of $CD4^+$ T cell help and if so, whether memory formation is dependent upon CD70. In agreement with the previous studies [28;42], we found that mice deficient of $CD4^+$ T cells (MHC class II-knockout) generated substantial primary $CD8^+$ T cell responses to OVA protein when given both agonistic αCD40 and polyI:C (pIC, TLR3 agonist) (Figure 1A). These responses were equivalent in magnitude to those generated in mice with an intact $CD4^+$ T cell population (Supporting Information Figure 1A). In the absence of either αCD40 or pIC, minimal primary $CD8^+$ T cell responses were detected in either WT or MHC class II-knockout animals (data not shown). Consistent with previous findings in CD4-intact animals [28], the primary $CD8^+$ T cell response in CD4-deficient mice, or mice depleted of $CD4^+$ T cells (not shown) elicited by immunization of OVA and combined αCD40/pIC was ~75% dependent upon CD70 (Figure 1A, B). Notably, CD70 blockade resulted in a greater loss of KLRG1-expressing SLECs (95% reduction) than of CD127-expressing MPECs (65% reduction) (Figure 1B).

We next assessed whether immunization with combined αCD40/pIC resulted in the generation of functional memory in the absence of $CD4^+$ T cells. Mice that had been rested for 35d after protein immunization were challenged with recombinant adenovirus expressing OVA (OVA-adeno). MHC-tetramer staining revealed that the secondary $CD8^+$ T cell response elicited in CD4-deficient mice approached ~60% of the level achieved in WT mice (Figure 1C and Supporting Information Figure 1B). Further, short term *in vitro* culture of secondary effector cells with $OVA_{257\text{-}264}$ peptide-pulsed APC demonstrated an equivalent degree of both CD107a expression and IFNγ production (data not shown). Notably, as predicted by the reduction in MPECs after CD70 blockade, CD27 stimulation during the primary response also substantially contributes to the magnitude of $OVA_{257\text{-}264}$-specific secondary $CD8^+$ T cell response, though to a lesser extent (50%, Figure 1C) than seen in the primary response (75%, Figure 1A).

Synergy between CD27 and TLR stimulation to generate primary CD8+ T cell response

Previous studies have indicated that the synergy between CD40 and TLR stimulation in the generation of primary $CD8^+$ T cell responses is dependent upon CD70 and IFN-1[13;30]. Further, IFN-1 has been shown to synergize with CD40 stimulation to induce CD70 expression [31], leading us to hypothesize that the major contribution of pIC in the primary $CD8^+$ T cell response is to support CD40-mediated up-regulation of CD70 expression via IFN-1 induction. Indeed, in mice lacking the IFNαβ receptor, the ability to elicit CD70 expression on DC after infusion with combined αCD40/pIC is significantly reduced. In contrast, IL-12 deficiency has negligible impact on CD70 expression (Supporting Information Figure 2A).

However, IFN-1 has also been shown to directly support $CD8^+$ T cell expansion and differentiation [9;10;33;34;43]. Therefore, to begin to dissect how αCD40 and pIC drive $CD8^+$ T cell responses, we examined the effect of directly stimulating CD27. If the role of IFN-1 is primarily to synergize with αCD40 in inducing CD70 expression, then CD27 stimulation should be sufficient to elicit primary $CD8^+$ T cell responses to OVA protein in the absence of $CD4^+$ helper T cells. Surprisingly, when applied as a sole adjuvant, endotoxin-free αCD27 only drove weak $OVA_{257-264}$-specific $CD8^+$ T cell responses (Figure 2). Thus, while CD27-mediated costimulation is necessary for generation of $CD8^+$ T cell responses to OVA with combined αCD40/pIC, alone it is inefficient at stimulating a robust primary response, at least not from an endogenous population where the precursor frequency is low.

As the combination of pIC and αCD40, but neither alone, had driven a CD70-dependent $CD8^+$ T cell response to OVA immunization, we then sought to determine whether either of these adjuvants would synergize with CD27 stimulation. We found that co-treatment with αCD40 did not increase the αCD27-driven response (Supporting Information Figure 2B). In contrast, co-targeting CD27 and TLR3 synergized, substantially enhancing the $OVA_{257-264}$-specific endogenous primary $CD8^+$ T cell response in the absence of $CD4^+$ T cell-mediated help (Figure 2). Thus, together these data indicate that the inclusion of TLR stimulation promotes primary $CD8^+$ T cell responses independently from their effects on inducing CD70 expression.

IFN-1 but not IL-12 supports the ability of CD27 stimulation to promote $CD8^+$ T cell responses

The preceding data indicated that additional stimulatory cofactor(s) cooperate with CD27 stimulation to elicit primary $CD8^+$ T cell responses to OVA protein in the absence of helper $CD4^+$ T cells. Both IFN-1 and IL-12 have been implicated as additional Signal 3 molecules that are capable of driving the expansion of primary $CD8^+$ T cell responses [44] and are produced by pIC stimulation [45]. To address the role of these cytokines in supporting CD27-mediated $CD8^+$ T cell expansion, we immunized $CD4^+$ T cell-depleted mice with OVA and αCD27, with or without endotoxin-free recombinant IFNα or IL-12. We found that IFNα synergized with CD27 stimulation, resulting in a >3-fold greater $CD8^+$ T cell response compared to either IFNα or CD27 stimulation alone (Figure 3A). In contrast, IL-12 not only failed to synergize with CD27 stimulation, but reduced the limited response driven by CD27 stimulation alone (Figure 3B). Despite its failure to drive effector expansion, inclusion of IL-12 successfully promoted KLRG1 expression and SLEC differentiation (Figure 3B inset). Minimal $OVA_{257-264}$-specific $CD8^+$ T cell responses were achieved when either recombinant cytokine was applied as a sole adjuvant (Figure 3A, B), consistent with the observation of limited responses driven by pIC alone (Figure 2). Thus, the capacity of CD27 to synergize with pIC to augment $CD8^+$ T cell responses is dictated by the elaboration of IFN-1 by pIC. The synergy between IFN-1 and CD27 stimulation was further confirmed by immunizing $CD4^+$ T cell-depleted IFNαβRKO mice with OVA and combined αCD27/pIC. We found that the primary $CD8^+$ T cell response to OVA was substantially reduced (~75%) in the absence of IFNαβR signaling (Supporting Information Figure 3A). In contrast to the results with the IFNαβRKO mice, the $OVA_{257-264}$-specific primary $CD8^+$ T cell

response was unimpeded if not enhanced after IL-12 blockade or in IL-12p40KO mice (Supporting Information Figure 3B-D), and that the requirement for IFNαβR signaling was not overcome by blocking IL-12 (Supporting Information Figure 3D). These data together demonstrate that the synergy achieved by co-targeting CD27 and TLR3 is dependent on IFN-1, and IFNα is sufficient to replicate the majority of the synergy with αCD27, while IL-12 surprisingly does not promote CD27-driven responses.

CD27 and IFNα augment the functional activity of primary CD8$^+$ T cell responses

We next sought to understand whether the synergy between CD27 and IFNα at driving the expansion of CD8$^+$ T cells had an impact on their functional activity. We found that mice immunized with the combination of αCD27 and IFNα had the greatest ability to clear antigen loaded target cells *in vivo* (Figure 3A, bottom plots). As this enhanced *in vivo* cytotoxicity could simply reflect the increase in magnitude of the cytotoxic T cell response, we examined effector functions on a per-cell basis. In general the magnitude of the CD8$^+$ T cell populations that could de-granulate or produce IFNγ or IL-2 followed the response size as gauged by MHC-dextramer staining (Figure 3C, left). However, CD8$^+$ T cells that respond to αCD27+IFNα produce IFNγ at a greater amount per cell (3-fold increase compared to IFNα and 60% increase compared to CD27 alone; Figure 3C, right). Interestingly, this increased functional activity per cell was not evident in degranulation or IL-2 production, suggesting the combination specifically targets IFNγ production. Thus, not only does the combination of CD27 and IFNα synergize in the expansion of CD8$^+$ T cells, the responding CD8$^+$ T cells are poly-functional and have enhanced ability to secrete IFNγ relative to controls.

The physiological relevance of this immunization strategy is demonstrated by an increase in the magnitude of the $OVA_{257-264}$-specific CD8$^+$ T cell population achieved by OVA+αCD27+pIC super-imposed upon the vaccinia infection that was not achieved by immunizations that used OVA+αCD27, OVA+pIC (Figure 3D). The magnitude of the CD8$^+$ T cell response to $OVA_{257-264}$ correlates closely with the viral titer in the spleen at day 7 (Figure 3D). Therefore, these data highlight the potential of co-targeting CD27 and IFNα along with antigen delivery to augment a functional CD8$^+$ T cell response that constrains acute viral infections.

CD8$^+$ T cell expression of IFNαβR and CD27 is necessary for TLR synergy with CD27 stimulation

IFN-1 can influence both DC and T cell function, while CD27 can stimulate multiple subsets of lymphocytes. To determine the cellular target of IFN-1, we transferred ~1000 IFNαβRKO or WT OT-I into CD4-depleted, IFNαβR-competent mice and challenged with OVA and combined αCD27/pIC. The magnitude of the primary OT-I response at day 7 after immunization was ~3-fold lower when the responding OT-I cells did not express the IFNαβR, indicating that the ability of IFN-1 to synergize with CD27 stimulation is highly dependent on the expression of IFNαβR on CD8$^+$ T cells (Figure 4A). Conversely, the magnitude of a WT OT-I response was not impeded in IFNαβRKO hosts compared to WT hosts (Figure 4B), indicating that the expression of IFNαβR on host population (e.g. DC) is dispensable. Likewise, the expansion of WT OT-1 cells is unimpeded in CD27-deficient

hosts (Supporting Information Figure 4A), and the absence of either $CD4^+$ T cells or NK cells (Supporting Information Figure 4B) did not reduce the response to the vaccine. Thus, CD27 and IFN-1 synergize directly on $CD8^+$ T cells to generate primary $CD8^+$ T cell responses to protein immunization.

Induction of transcription factors T-bet and Eomesodermin by stimulation of IFN-1 and CD27

The T-box transcription factors T-bet and Eomes have been well defined as two key drivers governing $CD8^+$ T cell differentiation and cytolytic function. We previously reported CD27-mediated Eomes up-regulation during acute viral infection [26]. Consistent with this, here we found that the few $CD8^+$ T cells that respond to OVA alone has little expression of T-bet or Eomes, while those responding to pIC+OVA mostly induced T-bet expression with a subset also expressing Eomes (Figure 5A). Stimulation of CD27 during priming with OVA resulted in both a ~2-fold increase in frequency of Eomes-expressing $CD8^+$ T cells and a >1.5-fold increase in the level of Eomes expression (within Eomes-expressing $CD8^+$ T cells) when compared to mice primed with OVA and either IFNα or IL-12 (Figure 5B). In contrast, αCD27 only moderately induces T-bet (Figure 5B), which is commonly required for effector cell expansion [46]. Notably, while IL-12 and IFNα have both been described as good inducers of T-bet in other settings [5;47], in this protein-based vaccination system we observed higher levels of T-bet with IFNα compared to IL-12 treatment (Figure 5B).

We then sought to determine how CD27 and the cytokine (IFN-1/IL-12) stimulation, when applied in combination, would impact Eomes and T-bet expression in effector $CD8^+$ T cells. We found that the αCD27-dependent Eomes up-regulation was not impeded by inclusion of either IFNα or IL-12 (or pIC) (Figure 5C, left). Interestingly, while adding IFNα (or pIC) with αCD27 led to a 2-fold increase of T-bet in $OVA_{257\text{-}264}$-specific $CD8^+$ T cells, no significant increase in T-bet was identified by adding IL-12 with αCD27 (Figure 5C, middle). On a per cell basis, the inclusion of pIC or IFNα doubled the ratio of T-bet:Eomes (Figure 5C, right). Conversely, T-bet levels were 25% lower in response to combined αCD27/pIC in IFNαβRKO mice, yet remained stable in IL-12p40KO (Figure 5D). These data correlate with the failure of IL-12 to synergize with αCD27 in promoting primary expansion of $CD8^+$ T cells. Consistent with the notion that T-bet levels are a critical regulator of the expansion of $CD8^+$ T cells in response to combined αCD27/pIC immunization, the magnitude of the $OVA_{257\text{-}264}$-specific $CD8^+$ T cell response positively correlated with their T-bet levels (Figure 5E). Together our data suggested that the synergy between αCD27 and IFN-1 (or pIC) is a product of optimized regulation of transcription factors, including αCD27-mediated induction of Eomes and IFN-1-mediated induction of T-bet.

Co-targeting CD27 and TLR stimulation generates functional memory $CD8^+$ T cells

CD70 blockade had a more profound impact on effector $CD8^+$ T cell expansion than the generation of memory (Figure 1B). This result, together with the ability of CD27 to promote Eomes expression, which is known to support memory cell development, led us to question whether CD27 stimulation is sufficient to promote memory $CD8^+$ T cells in the context of protein immunization. We found that although CD27 stimulation increased the proportion of

SLECs compared to OVA immunization alone (despite modestly reducing the T-bet:Eomes ratio), there was nonetheless a clearly discernable MPEC population (Figure 6A). Remarkably, however, mice primed with OVA+αCD27 made very limited secondary responses after rechallenge (Figure 6B). The addition of pIC to OVA+αCD27 further increased the proportion of SLECs in the primary response (in accordance with the increase with the T-bet:Eomes ratio) yet did not increase the number of MPECs (Figure 6A). Despite this, mice primed with OVA+combined αCD27/pIC generated a ~10-fold greater secondary response than those primed with αCD27 (Figure 6B). These data indicate that TLR and CD27 stimulation not only synergize to generate primary $CD8^+$ T cell responses to protein immunization, but also that the addition of TLR stimulation provides a critical co-factor that promotes the development of memory $CD8^+$ T cells.

Discussion

We demonstrate that direct stimulation of CD27 in conjunction with TLR stimulation can drive the expansion and differentiation of the $CD8^+$ T cell response and that CD27 stimulation is an obligatory component of the activity of CD40 stimulation. Blocking CD70 dramatically reduced the frequency of KLRG1-expressing SLECs, a phenotype that also occurs in the absence of $CD4^+$ T cells [26]. Yet in contrast, we find that CD27 stimulation by itself is a weak agonist for $CD8^+$ T cell responses to protein immunization, and the majority of the response is equally distributed between SLECs ($CD127^{lo}KLRG1^{hi}$), MPECs ($CD127^{hi}KLRG1^{lo}$) and $CD127^{hi}KLRG1^{hi}$ cells. Thus, CD27 promotes the expansion of primary effectors, but is insufficient to drive SLEC expansion in isolation. Profound synergistic expansion of the primary KLRG1-expressing SLEC component of the $CD8^+$ T cell response after CD27 stimulation was achieved by the inclusion of TLR stimulation. Notably, therapeutic vaccination substantially increased the magnitude of the $CD8^+$ T cell response during an acute viral infection, and this correlated strongly with reduced viral titer, suggesting an approach for curtailing viral infection prior to the establishment of protective antibodies. Pointedly, we don't find that CD27 stimulation leads to reduced staining with MHC-multimers (data not shown), arguing against the notion that CD27 stimulation increases the magnitude of the primary response by recruiting low affinity $CD8^+$ T cells [48]. Underscoring the interaction between CD27 and TLR stimulation, we have found that if endotoxin is not removed from the agonistic αCD27, then it can initiate substantial $CD8^+$ T cell responses independently from further TLR stimulation. This could potentially explain the difference in CD27-driven response sizes in this study compared to those in a recent publication describing differences in the influence of CD27 and 4-1BB driven on $CD8^+$ T cell differentiation [49]. Additionally, previous studies using soluble recombinant CD70 [42] did not require TLR stimulation to support $CD8^+$ T cell responses to $OVA_{257\text{-}264}$ peptide; however, these studies were performed in chimeric mice containing large numbers of OT-I TCR transgenic T cells, a situation which we find to be independent of either CD27 or TLR stimulation (not shown).

Other studies have indicated the synergy achieved between αCD40 and TLR agonists [30;31;50] is dependent upon IFN-1 and associated with its ability to upregulate CD70 expression on DC. However, by using agonist antibodies to CD27, consistent with previous reports [9;10;34;37;43;51], we reveal an additional critical role for IFN-1 as a major

cofactor with CD27 stimulation at the level of the T cell. This is demonstrated by the reduced expansion of $CD8^+$ T cells deficient in IFNαβR expression in response to OVA with combined αCD27/pIC, and reciprocally the unimpeded expansion of WT OT-I in IFNαβRKO mice in which only the OT-I cells are responsive to IFN-1. IFN-1-mediated signaling in host populations is dispensable in this priming system – indeed, the magnitude of OT-I responses were even elevated in IFNαβRKO hosts, probably due to a reduced competition from the deficient endogenous $CD8^+$ T cell responses. The competitive advantage of IFNαβR-expressing OT1 could also be as a consequence of increased IFN-1 availability to OT-I cells due to the reduced consumption by the host, or the ability of IFN-1 to impart resistance of $CD8^+$ T cells to NK-mediated lysis via the expression of NRC-1 [52;53]. Although we have determined that NK cells are not required for αCD27/pIC-mediated expansion of primary $CD8^+$ T cell responses, we are currently assessing whether NK cells are deleterious in the absence of either CD27 or IFN-1. Similarly, CD27 expression on the responding $CD8^+$ T cells was found to be sufficient for the agonistic activity of αCD27. Though unlikely not to be the case, we have not currently been able to definitively show that CD27 expression on the responding $CD8^+$ T cells is necessary for the effects of αCD27 as $CD27^{-/-}$ OT-1 cells do not expand in response to this immunization system.

Our data argue that the varied ability of costimulatory/Signal 3 molecules to induce T-bet or Eomes dictates their contribution to the primary $CD8^+$ T cell response after protein immunization. CD27 stimulation weakly induces T-bet expression in primary effectors. Rather, T-bet is more strongly promoted by either TLR or IFN-1, and the level of T-bet, and the ratio of T-bet:Eomes, positively correlates with the magnitude of $CD8^+$ T cell response, providing a probable explanation for the ability of TLR to augment CD27-stimulated responses. If T-bet induction is sufficient to drive primary $CD8^+$ T cell responses, it raises the question why CD27 stimulation is necessary to support TLR agonists and IFN-1. Consistent with our previous studies with vaccinia virus, and recent reports studying 4-1BB [49;54](a related TNF-superfamily member) we find that CD27 stimulation significantly increases the expression of Eomes compared to either TLR or cytokine stimulation. In viral infection models, Eomes has been demonstrated to play a critical role in effector $CD8^+$ T cell differentiation and function, yet is not thought to play a significant role in promoting the expansion or survival of well-differentiated primary effectors, perhaps due to some redundancy between Eomes and T-bet [46;55]. We hypothesize that the $CD8^+$ T cell response to protein immunization might be enhanced by CD27-derived Eomes expression as the level of T-bet expression induced by soluble TLR agonists/IFN-1 may be low compared to that achieved by viral infections, reducing the potential redundancy between these molecules [46]. Arguing against this hypothesis, however, is the observation that immunization in the context of a vaccinia infection leads to a greater magnitude response and better control of virus. We are currently generating the appropriate knockouts to directly test this hypothesis.

How CD27 increases Eomes expression is not currently clear. CD27 is known to support the expression of CD25, the high affinity receptor for IL-2 ([19]; and our data not shown), and STAT5 augments Eomes expression [56]. Thus, the enhanced expansion achieved with CD27 stimulation may be a consequence of its ability to regulate IL-2 signals. Previous

studies have demonstrated that receptivity to IL-2 is critical for sustained CD8+ T cell expansion ([57]; [58]), but sustained IL-2R expression has been attributed to IFN-1 ([59]. Preliminary studies (unpublished results) suggest that, in the current system, IFNα (in contrast to CD27) is not able to support CD25 expression at d5. Notably, in these preliminary data sets CD27 (or CD27 and IFNα) stimulated cells continue to expand from d5 to d7, while IFNα-alone stimulated cells contract. Thus, while the static/decreasing magnitude of the IFNα-driven response that occurs between d5 and d7 could be accounted for by a shift in the balance between proliferation and survival, the increasing magnitude of the responses induced by CD27 can only be accounted for by sustained proliferation, not prevention of cell death as this latter possibility would also result in a stagnant response size between d5 and d7. Based on these observations, our current hypothesis is that IFN-1 is needed for the initial expansion of primary $CD8^+$ T cells, perhaps by inducing T-bet or preserving them from NK-mediated lysis [52;53], while signals emanating from CD27 support their sustained expansion, perhaps by activating an IL-2-Eomes node. We are currently directly measuring T cell proliferation at these time-points to directly test this conclusion.

Aside from influencing the magnitude of the $CD8^+$ T cell response, CD27 augmented IFNγ production on a per cell basis. CD27-mediated potentiation of $CD8^+$ T cell function was not found for degranulation (CD107a expression) or IL-2 production. The capacity for CD27 stimulation to augment IFNγ production in $CD8^+$ T cells is under-appreciated [49;60], and important for potential therapeutic interventions based on CD27 stimulation (tumor control [61];[62]; viral infection [63];[64]), and may be a function of the increased Eomes expression [55] achieved by CD27 stimulation.

It is currently unclear why IL-12 does not support the effects of CD27 stimulation in a manner similar to IFN-1. In the context of protein immunization used in the current study we found that IFN-1 is a stronger inducer of T-bet than IL-12, and that IL-12 is in fact dispensable for T-bet induction in the presence of pIC. We have confidence that recombinant IL-12 is bio-active in our system as it induced higher KLRG1 expression in conjunction with CD27 stimulation. It is noteworthy that IL-12 has been reported to be a negative regulator of Eomes, and IFN-1 an inducer of T-bet [37;65]. However, we found no detrimental effect of IL-12 on Eomes expression in the context of CD27 immunization, suggesting that this is not the mechanism in play here. Of interest, we previously reported that CD27 stimulation can antagonize the polarizing effects of IL-12 on $CD8^+$ T cells [26]. While we do not know the mechanism by which this occurs, an antagonistic effect of CD27 stimulation on IL-12 signaling could explain their inability to cooperate in the context of protein immunization.

Surprisingly, mice stimulated by αCD27 alone did not mount strong secondary responses upon antigen re-challenge, despite the expansion of CD127-expressing primary effector $CD8^+$ T cells, indicating that an additional signal is necessary for the programming of fully-functional memory precursors. Given the synergy between IFN-1 and CD27 stimulation during primary response we hypothesize that IFN-1 is also promoting memory cell differentiation or capacity to re-expand as the addition of IFN-1 to CD27 stimulation did not increase the absolute number of MPECs. This may be related to the increase in T-bet

expression in response to IFN-1, as T-bet is known to support memory $CD8^+$ T cell development ([66], and our unpublished results). Thus, there may be a "Goldilocks" level of T-bet that is necessary for effector cell expansion and differentiation yet also engenders memory cell survival, without driving to terminally differentiated effector cells [67]. Interestingly, the ratio of T-bet:Eomes is equivalent in both KLRG1-expressing SLECs and IL-7R-expressing MPECs (data not shown), suggesting that the ratio of these transcription factors is not decisive in their fate decisions. The necessity of IFN-1 during the primary response to protein immunization complicates directly dissecting its role in memory cell differentiation. However, preliminary data (not shown) using adenoviral immunization indicates a critical role for IFN-1 in CD27-dependent memory cell generation, independent from its role in supporting primary $CD8^+$ T cell expansion described here. It should also be noted that although priming with αCD27/pIC produced 30-fold less $CD127^{hi}KLRG1^{lo}$ MPECs than achieved with αCD40/pIC, there was only a 3-fold difference in the magnitude of the secondary response between these cohorts. Thus, αCD27/pIC immunization may generate either more memory $CD8^+$ T cells or memory $CD8^+$ T cells with greater expansion potential compared to those that are generated with αCD40/pIC. Pertaining to this, we note that αCD27/pIC results in a large increase in $CD127^{hi}KLRG1^{hi}$ cells. As these cells have previously been characterized as having a better ability to become long-term memory cells than $CD127^{lo}KLRG1^{hi}$ SLECs [3], it is possible that the expansion of this population contributes to the increased secondary responses. Future studies that focus on these subpopulations (dissected by CD127/KLRG1 expression) will help define how the addition of IFN-1 to stimulation augments memory cell development. Together, these data indicate that the combinatorial targeting of CD27 with TLR stimulation can be a potent mechanism for eliciting the expansion of effector and memory $CD8^+$ T cells to helper-dependent antigens. IFN-1 is critical for the efficacy of this approach. Thus, coordinate targeting of CD27 and IFNαβR defines a potentially effective therapeutic avenue, circumventing the need for potentially dangerous pathogen-derived vectors.

Materials and Methods

Animals

C57Bl/6 mice were obtained from National Cancer Institute (Frederick, MD). MHC class II-deficient mice were obtained from Taconic (B6.129-*H2-Ab1*^{tm1Gru} N12, model # ABBN12). IL-12p40KO mice (B6.129S1-*Il12b*^{tm1Jm}/J, stock # 002693) were purchased from the Jackson Laboratory (Bar Habor, ME). IFNαβRKO mice were provided by Dr. Ross Kedl (University of Colorado). OT-I mice, expressing TCRs specific for $OVA_{257\text{-}264}$ peptide in complex with $H\text{-}2K^b$, were purchased from Taconic (B6.129S7-*Rag1*^{tm1Mom} Tg(TcraTcrb)1100Mjb N9+N1, model # 4175), and crossed on $Thy1.1^+$ (B6.PL-*Thy1*^{a}/CyJ stock # 000406) mice obtained from the Jackson Laboratory. Mice were maintained in specific pathogen-free facilities and were treated in accordance with the guidelines established by the Animal Care and Use Committee at the University of Virginia.

Peptide, protein and virus

Synthetic peptide $OVA_{257\text{-}264}$ (SIINFEKL) was purchased from GenScript (Piscataway, NJ). Endotoxin was removed by Detoxi-Gel endotoxin-removal kit (Pierce, Rockford, IL).

OVA protein was purchased from Sigma (St Louis, MO) and endotoxin was removed as above. OVA-adeno was either purchased from Gene Transfer Vector Core (University of Iowa) or provided by Dr. Young Hahn (University of Virginia), and was propagated on 293A fibroblasts.

Virus and viral titer assay

Recombinant vaccinia virus expressing OVA (OVA-vac) was provided by J. Yewdell (National Institute of Allergy and Infectious Diseases), and was propagated on $HuTK^-$ cells. Virus titers from infected mice were determined 7 d after i.p. priming with 1×10^7 PFU OVA-vac. Ovaries were excised and then homogenized. Homogenate was subject to three cycles of freezing and thawing and then sonicated. Sonicate was cleared of particulate matter by a centrifugation, and the supernatant was used to infect $HuTK^-$ cells. Virus plaques were revealed 48 h later by crystal violet staining.

Antibodies and other reagents

GK1.5-depleting anti-CD4 was obtained from ATCC. Agonistic FGK45 anti-CD40 was provided by Dr. Steven Schoenberger. Agonistic AT124.1 anti-CD27 has been described [68] and endotoxin was removed as above. FR70-blocking anti-CD70 has been described before [12]. TLR3 agonist PolyI:C was purchased from Invivogen (San Diego, CA). Mouse IL-12 p70 recombinant protein was purchased from eBioscience (San Diego, CA). Recombinant IFNα was provided by Dr. Ross Kedl (University of Colorado). Control Ig was purchased from Sigma.

Immunization

Unless indicated otherwise, mice were depleted of peripheral $CD4^+$ T cells by i.p. injection of 200 μg GK1.5 7 and 3 d before primary immunization and confirmed by tail vain bleed. For the generation of primary $CD8^+$ T cell responses, 500 μg OVA protein was injected i.p. alone or in combination with the indicated adjuvants in 200 μL total volume. CD40 stimulation was performed by injecting 50 μg FGK45 i.p. on d0. CD27 stimulation was performed by injecting 50 μg AT124.1 i.p. on d0, 3 and 6. CD70 blockade was performed by injecting 500 μg FR70 i.p. on d0, 2, 4 and 6. TLR stimulation was performed by injecting 50 μg the indicated agonist i.p. on d0. For recombinant cytokine administration, approximately 1×10^7 units of IFNα was given i.p. on d0, or 1ug IL-12was given i.p. on d0, 1 and 2 [69]. For the generation of secondary $CD8^+$ T cell responses, 2×10^8 p.f.u. OVA-adeno was injected i.p.

In vivo cytotoxicity assay

Splenocytes from CD45.1 B6 mice were either pulsed with $OVA_{257\text{-}264}$ peptide and labeled with high levels of CFSE (5 μM) as targeting cells, or labeled with low levels of CFSE (0.5 μM) without peptide pulse as control cells. 3 X 10^6 targeting cells and 3 X 10^6 control cells were co-transferred into CD45.2 pre-immunized mice at d6, and the cytotoxicity was assessed 16 hr later.

Flow cytometry

Lymphocytes were isolated from blood or homogenized spleens 7d after primary immunization or 5d after secondary challenge. Cells were first stained with Aqua vital dye (Invitrogen; Carlsbad, CA) for 20 min at 4°C, and pre-incubated with $OVA_{257-264}$-specific H2-K^b dextramer-APC (Immudex, Denmark) for 20 min at 4°C before other antibodies were applied. After Fc blockade, surface markers were stained for 30 min at 4°C, with antibodies anti-CD8-ef450 (53–6.7), anti-CD44-FITC (IM7), anti-KLRG1-PE (2F1), anti-CD127-PerCP-Cy5.5 (A7R34) from eBioscience. In some cases, anti-Thy1.1-FITC (HIS51) was applied to identify adoptively transferred OT-I population. In some early experiments, $OVA_{257-264}$-specific H2-K^b tetramer-APC (Dr. Vic Engelhard, University of Virginia) was co-stained with surface markers as an alternative for the dextramer. For transcriptional factor staining, lymphocytes were then permeabilized (eBioscience Fixation/Permeabilization) and stained with T-bet-PE (4B10) and Eomes-PercP-eFlour710 (Dan11mag) from eBioscience for 30 min at 4°C. Functional assays for the production of intracellular IFNγ, IL-2 and expression of CD107a were performed as previously described with $OVA_{257-264}$-peptide-pulsed or unpulsed LB15.13 hybridoma [12].Staining was assessed by flow cytometry on a FACS Canto II (Becton Dickinson; Franklin Lakes, NJ) and analyzed using FlowJo 7.6.5 Software (Treestar, OR).

Statistical analysis

Statistical analysis was performed with Prism 5 (GraphPad Software, Inc., La Jolla, CA) and data were presented as the Mean ± SD. Comparisons between groups were performed by unpaired two-tailed Student's *t* test or one-way ANOVA. Statistical significance was determined for 95% confidence interval. Synergism of two treatment arms was identified by a significant interaction determined in two-way ANOVA as previously described [70].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

DC	dendritic cells
cIg	control Ig
Eomes	Eomesodermin
IFN-1	type-1 IFN

MPEC	memory precursor effector cells
OVA-adeno	recombinant adenovirus expressing OVA
pIC	polyI:C
SLEC	short-lived effector cells
T-bet	T-box expressed in T cells

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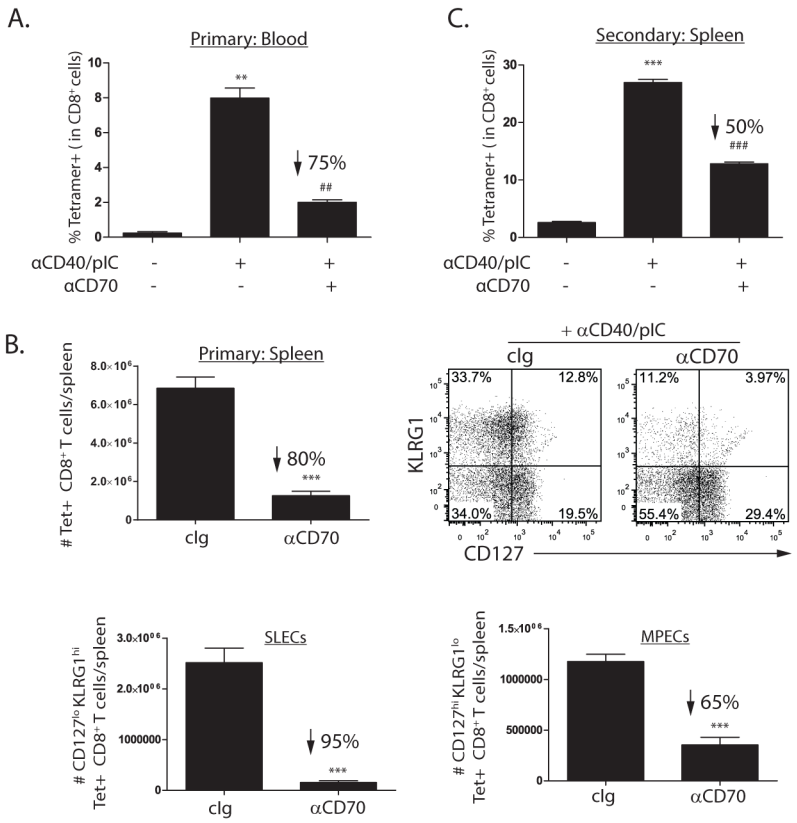

Figure 1. Impact of CD70 induction on primary and secondary CD8$^+$ T cell responses

Cohorts of MHC class II-deficient mice (n=3/cohort) were immunized with OVA alone, or concurrently with combined αCD40/pIC and treated with either αCD70 or cIg. Secondary responses were initiated by challenging with OVA-adeno >35 d post primary immunization. Data are representative of two-three similar, independent experiments. **(A)** Frequency of primary $OVA_{257-264}$-specific CD8$^+$ T cells 7d after immunization. One-way ANOVA with Tukey's post test, **p<0.01 compared to the first column, ##p<0.01 compared to the second column. **(B)** Top plots: representative data showing CD127/KLRG1 expression on $OVA_{257-264}$-specific CD8$^+$ T cells; regions are based on FMO stains. Top histogram: enumeration of $OVA_{257-264}$-specific CD8$^+$ T cells. Bottom histograms: enumeration of SLECs and MPECs. Student t test, ***p<0.001. **(C)** Frequency of secondary $OVA_{257-264}$-specific CD8$^+$ T cells 5d after OVA-adeno challenge. One-way ANOVA with Tukey's post test, ***p<0.001 compared to the first column, ###p<0.001 compared to the second column.

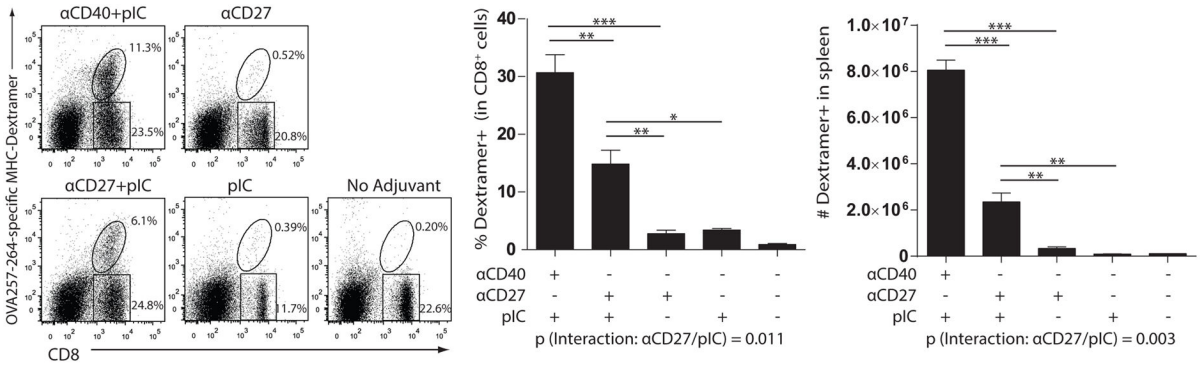

Figure 2. Synergy between CD27 and TLR stimulation to promote CD8$^+$ T cell responses

Frequency and absolute number of $OVA_{257-264}$-specific CD8$^+$ T cells in spleens of CD4-depleted mice 7d after immunization with OVA and indicated adjuvant(s). Dot plots show representative mice from each cohort. Histogram shows quantification of a representative experiment from at least four independent experiments with three mice per group. One-way ANOVA with Tukey's post test, *p<0.05, **p<0.01, ***p<0.001. p(Interaction) was determined by two-way ANOVA of the last four columns.

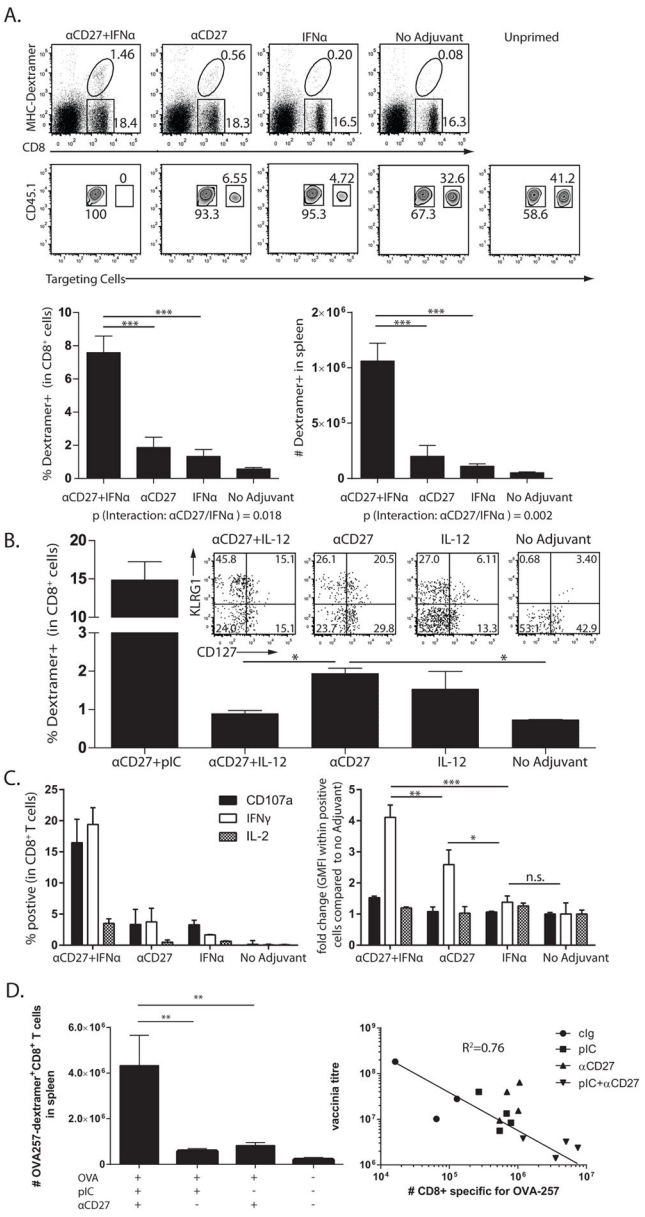

Figure 3. IFNα but not IL-12 synergizes with CD27 stimulation

Analysis of spleens from CD4-depleted mice 7d after immunization with OVA and indicated adjuvant(s). **(A)** Top dot plots show $OVA_{257-264}$-specific $CD8^+$ T cells from representative mice in each cohort where αCD27 and/or rIFNα were applied as adjuvant(s); bottom plots show target cell killing in representative mice. Histograms show quantification of a representative experiment from four independent experiments, with three-four mice in each cohort. One-way ANOVA with Tukey's post test, ***$p<0.001$. p(Interaction) was determined by two-way ANOVA. **(B)** A representative experiment from at least three independent experiments where αCD27 and/or rIL-12 were applied; inset dot plots show representative data of CD127/KLRG1 expression on $OVA_{257-264}$-specific $CD8^+$ T cells. One-way ANOVA with Tukey's post test, *$p<0.05$. (**C**) Degranulation and cytokine

production induced by $OVA_{257-264}$-peptide pulse *in vitro*. Left histogram shows population frequency while right histogram shows per-cell expression levels compared to no adjuvant control. Data are derived from a representative experiment with three mice per group, which independently recapitulated three times. One-way ANOVA with Tukey's post test, *p<0.05, **p<0.01, ***p<0.001. (D) Therapeutic control induced by immunization during vaccinia infection. Left histogram shows magnitude of MHC-multimer identified d7 $CD8^+$ T cell response in spleens of mice infected with 1×10^7 p.f.u. OVA-vac and immunized with the indicated combinations 24h later. One-way ANOVA with Tukey's post-test. **p<0.01. Right scatterplot shows linear regression of viral titer in the ovaries of mice correlated with the magnitude of the primary $CD8^+$ T cell response.

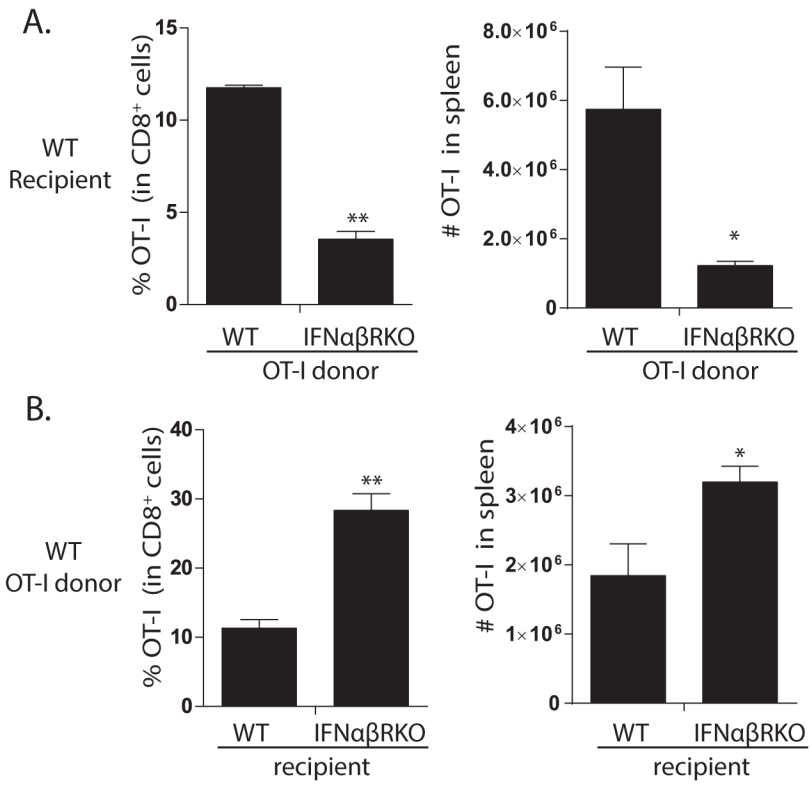

Figure 4. CD8$^+$ T cell expression of IFNαβR is necessary for IFN-1 synergy with CD27 stimulation

(A) ~1000 CD45.2$^+$ OT-I (WT or IFNαβRKO) CD8$^+$ T cells were transferred into CD4-depleted CD45.1$^+$ C57Bl/6 (WT) and challenged with OVA and combined αCD27/pIC. The frequency and absolute number of CD8$^+$CD45.2$^+$OVA$_{257-264}$-specific tetramer$^+$ cells were measured in spleens 7d later. **(B)** ~1000 Thy1.1$^+$OT-I (WT) CD8$^+$ T cells were transferred into CD4-depleted Thy1.2$^+$C57Bl/6 (WT or IFNαβRKO) mice, and challenged with OVA and combined αCD27/pIC. The frequency and absolute number of CD8$^+$Thy1.1$^+$OVA$_{257-264}$-specific tetramer$^+$ cells were measured in spleens 7d later. Bar graphs show combined data with three mice per cohort, which are derived from one of two identical, independent experiments. Student t test, *p<0.05, **p<0.01.

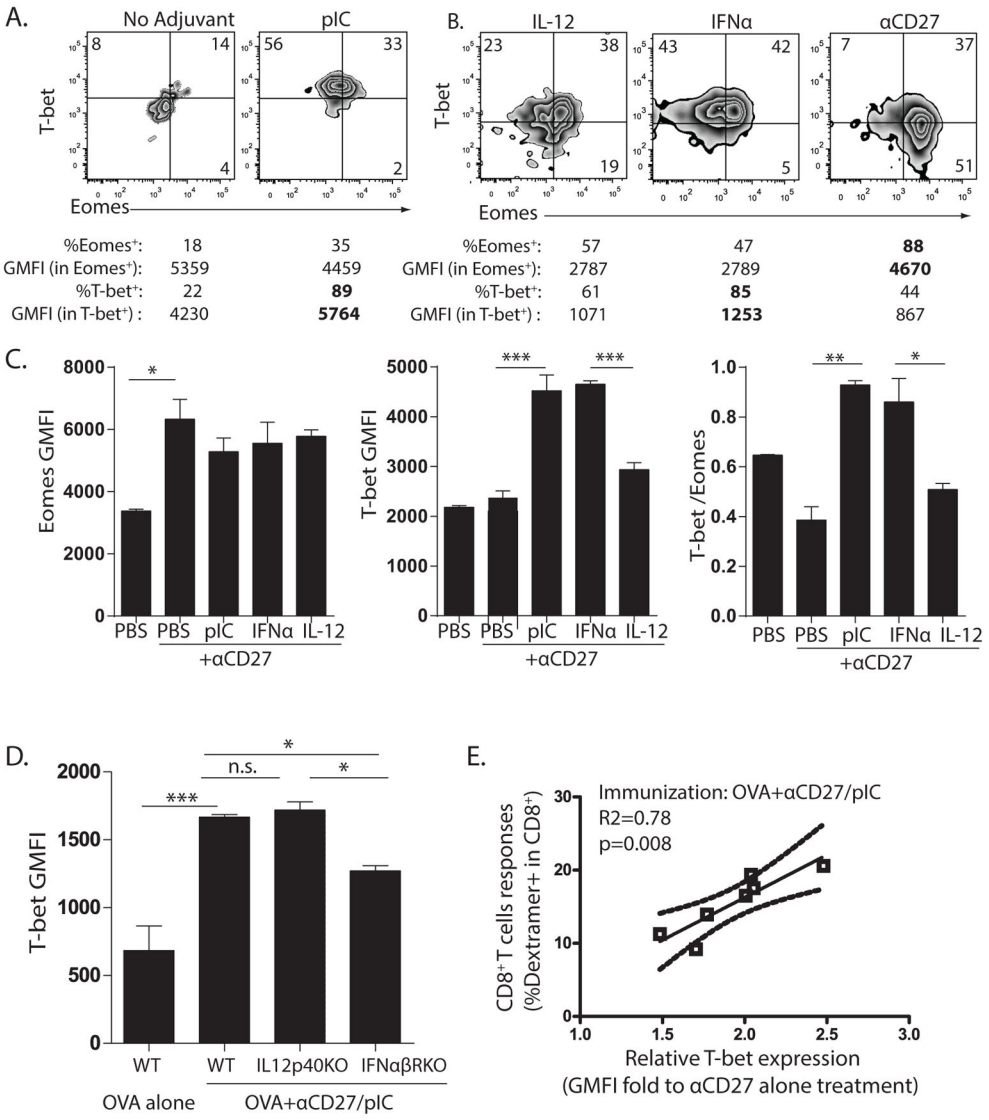

Figure 5. CD27 stimulation induces Eomes while IFN-1 induces T-bet

Eomes/T-bet expression in $OVA_{257-264}$-specific dextramer$^+$ $CD8^+$ T cells from spleens 7d after immunization with OVA and indicated adjuvant(s). Representative plots from two separate experiments where mice were immunized with either OVA alone or in combination with pIC **(A)**; or IL-12, IFNα or αCD27 **(B)**. Regions are based on FMO stains. Percentage of positive and geometric mean of fluorescence intensity (GMFI) for the positive subsets are listed below. **(C)** Expression of Eomes and T-bet (gated on positively expressing cells), and T-bet/Eomes ratio from mice treated with αCD27 alone, or in combination with pIC, IFNα or IL-12, respectively. Histograms show combined data for three mice per group from representative experiments, which were repeated twice independently. **(D)** T-bet expression from WT, IL12p40KO and IFNαβRKO mice. Data are representative of two independent repeats. **(E)** Correlation between T-bet protein level and the magnitude of $CD8^+$ T cells responses. Each dot presents an individual mouse from three independent experiments. For

(C) (D), one-way ANOVA with Tukey's post-test, *p<0.05, **p<0.01, ***p<0.001, n.s.= not significant.

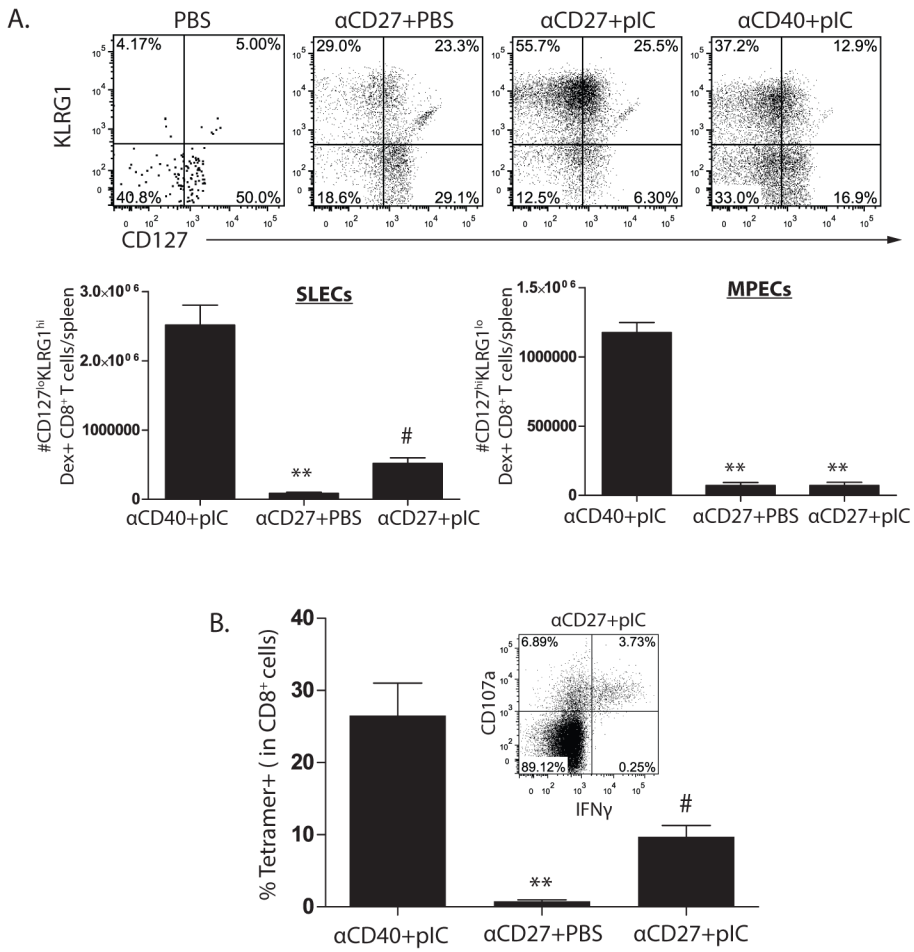

Figure 6. Functional secondary CD8$^+$ T cell responses from αCD27/TLR-primed mice

(A) Top plots: representative data showing CD127/KLRG1 expression on OVA$_{257-264}$-specific dextramer$^+$ CD8$^+$ T cells from spleens 7d after immunization with OVA and indicated adjuvant(s); regions are based on FMO stains. Bottom histograms: enumeration of SLECs and MPECs (n=3/cohorts) in a representative experiment, which were repeated at least twice independently. **(B)** Frequency of secondary OVA$_{257-264}$-specific CD8$^+$ T cells in spleens of mice with indicated priming was measured 5d after OVA-adeno challenge. Data shown are from one of two similar experiments with three mice per group. Inset: representative data showing CD107a/IFNγ expression after 5h in *vitro* OVA$_{257-264}$ peptide stimulation of secondary effectors (identified by CD44^{hi}CD8$^+$ live cells) from spleen of mice primed with OVA+αCD27/pIC; gatings are based on unstimulated controls. One-way ANOVA with Tukey's post-test for all histograms, **p<0.01 compared to the first column, #p<0.05 compared to the second column.