Effector CD4 T cell transition to memory requires late cognate interactions that induce autocrine IL-2

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Abstract

It is unclear how CD4 T cell memory formation is regulated following pathogen challenge, and when critical mechanisms act to determine effector T cell fate. Here, we report that following influenza infection most effectors require signals from major histocompatibility complex class II molecules and CD70 during a late window well after initial priming to become memory. During this timeframe, effector cells must produce IL-2 or be exposed to high levels of paracrine or exogenously added IL-2 to survive an otherwise rapid default contraction phase. Late IL-2 promotes survival through acute down regulation of apoptotic pathways in effector T cells and by permanently upregulating their IL-7 receptor expression, enabling IL-7 to sustain them as memory T cells. This new paradigm defines a late checkpoint during the effector phase at which cognate interactions direct CD4 T cell memory generation.

Introduction

Infection with viruses and other rapidly replicating pathogens generates large numbers of effector CD4 T cells in secondary lymphoid sites, where they originate, and at sites of infection to which they migrate. As the pathogen is cleared, most effectors abruptly die, leaving a small cohort that transition to long-lived memory [1, 2]. It is unclear to what extent the contraction of effectors and transition of surviving cells to memory are programmed during early encounter with antigen presenting cells (APC) during priming [3] and/or by external factors triggered by infection acting at later stages of the response. Because the CD4 T cell response to influenza A virus (IAV) generates memory cells capable of clearing

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heterosubtypic IAV challenge [4, 5], it is a well-suited model for defining the mechanisms regulating the efficiency of CD4 T cell memory generation.

One suspected cause of T cell contraction is "cytokine withdrawal" that is triggered by inadequate access to growth and survival factors such as IL-2 that limit apoptosis [6]. After initial stimulation, naïve CD4 T cells make IL-2, which causes the cells to differentiate and may also support their survival and division. Early IL-2 can also program responding T cells to have an increased capacity for memory survival and function[7, 8, 9]. However, the effect of later IL-2 signaling is less clear. On the one hand, exposure to IL-2 during the expansion phase can drive enhanced sensitivity to apoptosis[10, 11] and to re-stimulation-induced cell death [12]. In contrast, our previous studies indicated that CD4 T cell effectors generated *in vitro* were programmed to die and that IL-2 (plus transforming growth factor beta) could block their apoptosis [13]. This mechanism of effector T cell rescue has not been thoroughly evaluated for a role in memory generation *in vivo*.

We considered that late cognate interactions might promote memory formation. This would be attractive since such regulation could link a continuing threat from a pathogen with the level of memory generated. We reasoned that differentiated effector cells responding to IAV might be induced by APC to make IL-2 that could prevent their acute apoptosis and thus provide a key late 'checkpoint' governing the efficiency with which the effectors transition to long-lived memory.

We found that late major histocompatibility complex II (MHC-II) recognition *in vivo* from 4–6 days post-infection (dpi), as CD4 T cell responses against IAV reach their peak [14], indeed promoted greater recovery of memory cells during primary and secondary responses. Autocrine IL-2 production by effectors, or high levels of exogenously administered IL-2, during this timeframe was required for the generation of almost all memory cells. This late IL-2 signaling rescued effectors from acute apoptosis and upregulated sustained CD127 expression. The level of enhanced CD127 expression correlated directly with the amount of late IL-2 available and with the size of the memory population generated. Finally, late signals from CD70, which act through CD27 expressed on effector CD4 T cells to enhance IL-2 during cognate recognition, were required for optimal memory generation. Our results define a novel late checkpoint at which CD4 T cell effectors must engage in cognate interactions to induce autocrine IL-2 that signals them to survive and is necessary for them to become long-lived memory cells.

Results

Memory is reduced by MHC- II blockade at the effector stage

To evaluate if cognate interactions of CD4 T cell effectors with MHC-II$^+$ APC are needed to promote memory generation, we asked whether blocking MHC-II with antibody (Ab) treatment [15] only at the effector stage would reduce memory cell recovery following IAV challenge. To avoid complications arising from the differential ability of cells with different T cell receptors (TcR) to form memory [16, 17], and the predicted lack of synchrony in polyclonal responses, we tracked small cohorts of adoptively transferred TcR transgenic (Tg) cells. As it is likely that even extremely large doses of monoclonal Ab would not

efficiently block MHC-II expression in wild-type (WT) mice throughout the effector stage, we utilized as hosts CD11cTg.*H2-Ab1*$^{-/-}$ mice that only express MHC-II on CD11c$^+$ cells [18]. We first transferred naïve OT-II cells to C57BL/6 or CD11cTg.*H2-Ab1*$^{-/-}$ mice and challenged with a sublethal dose of the recombinant A/PuerotRico/8/34-Ovalbumin$_{323-339}$ (PR8-OVA$_{II}$) virus that contains the OVA epitope recognized by the OT-II TcR [19]. Donor cell recovery at 7 and 28 dpi was equivalent in both hosts, indicating that MHC-II expression restricted to CD11c$^+$ cells was sufficient for optimal effector expansion and efficient memory generation (Fig 1a). Importantly, treatment of CD11cTg.*H2-Ab1*$^{-/-}$ mice with MHC-II blocking Ab from 4–6 dpi dramatically reduced MHC-II expression on CD11c$^+$ cells as assessed by flow cytometry at 7 dpi (Fig 1b, c).

We next tested the effect of MHC-II blockade from 4–6 dpi in CD11cTg.*H2-Ab1*$^{-/-}$ hosts on OT-II donor cell expansion and memory generation. We used naïve as well as *in vitro*-primed T$_H$1-polarized memory cells, that respond against IAV similarly to IAV-primed memory cells[4, 14], as donors to ask if secondary memory generation, such as following a vaccine boost, was also dependent on late MHC-II recognition. Ab treatment from 4–6 dpi did not impact viral titer detected at 7 dpi (Fig 1d). MHC-II blockade did reduce primary effector expansion in the lung at 7 dpi but did not impact secondary effector responses (Fig 1e). More importantly, late MHC-II blockade dramatically reduced donor cell recovery at 28 dpi in recipients of naïve and primed cells (Fig 1e), suggesting that cognate antigen (Ag) recognition during the effector phase is needed for efficient memory formation during primary and secondary CD4 T cell responses.

Generation of memory is impaired in the absence of IL-2

We used several strategies to determine if the effector cells require late IL-2 signals that were potentially induced by MHC-II-dependent interactions to efficiently transition to memory. First, we transferred naive WT or *Il2*$^{-/-}$ DO 11.10 cells to WT BALB/c hosts and challenged with PR8-OVA$_{II}$. Compared to WT donors, *Il2*$^{-/-}$ cells reached lower peak numbers (Fig 2a), consistent with a requirement for IL-2 for optimal primary expansion *in vivo*[20, 21, 22]. More notably the *Il2*$^{-/-}$ effectors under went more rapid and extensive contraction than WT cells, resulting in little memory cell recovery. Interestingly, the number of *Il2*$^{-/-}$ donor cells seemed to stabilize briefly between 10 and 14 dpi. Nevertheless, in the spleen and draining lymph node (dLN), *Il2*$^{-/-}$ cells became virtually non-existent by 28 dpi, while in the lung a significantly reduced population survived (Fig 2a).

Utilizing naïve *Il2*$^{-/-}$ donor cells cannot distinguish whether the need for IL-2 for memory formation is late, during the timeframe when effectors required MHC-II-restricted encounter with CD11c$^+$ cells (Fig 1), and/or during initial priming[13]. Therefore we examined responses of *Il2*$^{-/-}$ *in vitro*-primed memory cells, generated with exogenously added IL-2, reasoning that these cells would have received initial IL-2 programing required for memory competency [9], and that their effector survival might only require IL-2 late during the response against IAV. Indeed, both WT and *Il2*$^{-/-}$ *in vitro*-primed memory cells generated with exogenous IL-2 rapidly produced IFN-γ (Supplementary Fig. 1), expressed a prototypical resting memory phenotype, and survived long-term upon transfer to uninfected

WT hosts (Supplementary Fig. 2). Thus, priming with exogenous IL-2 *in vitro* generated long-lived functional *Il2*^{−/−} memory cells.

We compared responses and survival of adoptively transferred IL-2-primed WT or *Il2*^{−/−} donors following IAV challenge. Both populations generated similar peak numbers of secondary effectors (Fig 2b) that expressed an activated phenotype and produced high levels of IFN-γ and TNF (Supplementary Fig. 3). The number of *Il2*^{−/−} donor cells fell sharply between 7 and 10 dpi and, as observed with unprimed *Il2*^{−/−} donors, appeared to stabilize briefly between 10 and 14 dpi in the dLN and spleen (Fig 2b). Strikingly, the *Il2*^{−/−} donor cells contracted much more dramatically than WT cells and were barely detectable at 28 dpi in the spleen and dLN with many fewer cells surviving than WT donors in the lung (Fig 2b), reproducing the pattern seen with naïve donors (Fig 2a).

To control for artifacts that could result from the deletion of IL-2 throughout CD4 T cell development, we transferred WT *in vitro*-generated memory cells and treated recipients with IL-2 neutralizing Abs from 1–7 dpi. Blocking IL-2 did not affect donor cell recovery at 7 dpi but it significantly reduced memory recovery in the lung and nearly eliminated memory in the spleen and dLN, phenocopying results obtained with *Il2*^{−/−} donor cells (Fig 2c). Moreover, when IL-2 neutralizing Ab treatment was restricted to 4–7 dpi, memory was impaired to a nearly equal extent as with longer treatment from 1–7 dpi (Fig 2c). Importantly, no differences in viral titer were detected on 7 dpi between isotype and IL-2 neutralizing Ab treated mice (Fig 2d). These data confirm that late IL-2 signals during the effector phase of the CD4 T cell response are required for optimal memory generation.

IL-2 neutralization also reduced long-term survival of *in vivo*-generated polyclonal memory CD4 T cells responding to IAV. We transferred bulk CFSE-labeled CD4 T cells from IAV-primed WT mice to unprimed Thy-disparate hosts and infected with IAV. We identified IAV-specific donor cells using an IAV-specific MHC-II-tetramer (Fig 2e). IL-2 neutralization significantly reduced donor CFSE[low] tetramer-positive polyclonal donor cells at 28 dpi (Fig 2f). Together these results suggest that following IAV infection CD4 T cell-produced IL-2 is required to limit contraction of effectors and support their survival as memory cells.

High levels of IL-2 are needed to induce memory CD4 T cells

The experiments summarized in Figure 2 suggest that autocrine IL-2 signals are needed to rescue effector CD4 T cells from enhanced contraction as the number of *Il2*^{−/−} donor cells present at 28 dpi in untreated hosts was similar to the number of WT donor cells present in hosts treated with anti-IL-2 Abs. To formally test whether autocrine IL-2 signaling is required to rescue effector cell survival we co-transferred equal numbers of WT and *Il2*^{−/−} DO11.10 donor cells distinguishable by allelic markers (Fig 3a). Co-transfer only slightly enhanced the poor survival of *Il2*^{−/−} cells(Fig 3b). The simplest interpretation of these results is that memory generation is directly dependent on availability to effector cells of high levels of IL-2 that are likely present during physiological immune responses only when there is autocrine production. However, the slight improvement in survival of *Il2*^{−/−} donors upon co-transfer with WT cells suggests that high levels of paracrine IL-2 can enhance the long-term survival of *Il2*^{−/−} CD4 T cells responding against IAV.

To test if the reduced survival of *Il2*^{−/−} versus WT donor cells would result in an impaired recall response on the population level, or if the lower number of surviving *Il2*^{−/−} donor cells was still sufficient to mount a robust secondary response, we challenged PR8-OVA$_{II}$-primed recipients of WT and *Il2*^{−/−} donor cells with a heterosubtypic IAV virus expressing the OVA$_{323–339}$ peptide (X31-OVA$_{II}$) 30 days post-priming. Both donor populations in the lung increased in forward scatter indicating an increase in size and upregulated CD69 at 2 dpi (Fig 3c, d), but by 6 dpi WT cells out numbered *Il2*^{−/−} donors by about 11-fold in the lung (Fig 3e), while in the spleen and dLN WT cells were recovered but virtually no *Il2*^{−/−} cells were detected (not shown). Thus, the impaired memory CD4 T cell formation in the absence of autocrine IL-2 signaling resulted in are call response of dramatically reduced magnitude.

Late administration of IL-2 substitutes for autocrine IL-2

To confirm that the lack of late IL-2 was responsible for the poor survival of *Il2*^{−/−} donor cells, we administered IL-2: anti-IL-2 Ab complexes [23] (IL-2C) to recipients of primed donor cells, which at high doses should mimic autocrine IL-2. We injected IL-2C from 5–7 dpi, corresponding to the timeframe when MHC-II interactions were required for optimum memory formation (Fig 1). The IL-2C effectively signaled *Il2*^{−/−} effector cells as indicated by increased *ex vivo* STAT5 but not STAT3 phosphorylation at 7 dpi (Fig 4a), and enhanced CD25 and CD122 expression comparable to levels on WT donors (Fig 4b).

Strikingly, IL-2C treatment restored *Il2*^{−/−} memory at 28 dpi to WT levels but did not impact the peak expansion of *Il2*^{−/−} cells at 7 dpi (Fig 4c). The effect of IL-2C treatment was long-lived as *Il2*^{−/−} donor cell recovery in IL-2C-treated mice was still equivalent to WT donor populations at over 100 dpi (Fig 4d). Optimal rescue required a high dose of 2 μg of IL-2, while 1 μg led to reduced but still improved *Il2*^{−/−} donor cell survival (Fig 4e). Late IL-2C treatment also enhanced the survival of WT donor cells (Fig 4f), suggesting that IL-2 production by effectors is normally limiting for optimum memory generation.

In contrast to its ability to restore survival of primed donor cells, IL-2C given at 5–7 dpi did not enhance recovery of naïve *Il2*^{−/−} donor cells at 28 dpi (Fig 4g). Since naïve cells may require early IL-2 signals to 'program' the capacity to form memory [9], we treated recipients of naïve *Il2*^{−/−} donors at 1–3 dpi or at both 1–3 and 5–7 dpi with IL-2C. Only treatment at both 1–3 and 5–7 dpi restored memory generation (Fig 4g). We conclude that naïve CD4 T cells require both early IL-2-dependent programming and later IL-2 signals to efficiently become memory following IAV challenge. In contrast, IL-2-primed cells no longer require early IL-2 programming but areas dependent on late IL-2 at the effector stage for effective memory generation.

Late IL-2-induced CD127 on effectors correlates with memory

To search for late IL-2-dependent changes that might lead to better memory generation, we compared gene expression from WT and *Il2*^{−/−} effectors at 7 dpi by microarray. We found only 88 genes differentially expressed at a threshold of > 1.5 and *P*< 0.05 (Supplementary Dataset I). DAVID functional annotation clustering revealed 5 pathways with *P* values < 0.05 and > 1.3 fold enrichment (Fig 5a). *Il7r,* encoding the IL-7 receptor α chain (CD127),

was decreased in $Il2^{-/-}$ effectors and was common to 3 of the 5 pathways (Fig 5b). When a more conservative 2-fold threshold was applied, only 27 genes, including $Il7r$, were differentially expressed by the WT and $Il2^{-/-}$ effector cells (Fig 5c). Since IL-7 is a critical and limiting factor regulating T cell homeostasis and memory T cell survival [24, 25, 26] decreased CD127 expression could be a major factor limiting the ability of $Il2^{-/-}$ effector cells to successfully transition to memory.

To directly evaluate the impact of late IL-2 on effector cell CD127 expression, we analyzed donor cells at 7 dpi by flow cytometry. $Il2^{-/-}$ effectors expressed lower CD127 than WT cells and IL-2C treatment at 5–7 dpi restored CD127 on $Il2^{-/-}$ cells to WT levels (Fig 5d). Importantly, enhanced CD127 expression by $Il2^{-/-}$ cells following IL-2C treatment at 5–7 dpi was maintained at 28 dpi (Fig 5d), and the level of CD127 expression was proportional to the dose of IL-2 administered (Fig 5e, f). Furthermore, treatment of recipients of WT DO11.10 donor cells with IL-2 neutralizing Ab reduced their CD127 expression at 7 and 28 dpi (Fig 5g). IL-2 neutralization during the first week of IAV infection also reduced CD127 expression on polyclonal donor CD4 T cells present at 28 dpi (Fig 5h). Finally, IL-2C treatment of recipients of naïve $Il2^{-/-}$ donor cells at both 1–3 and 5–7 dpi, but not at either time point alone, induced enhanced CD127 expression (Fig 5i). These results indicate that the level of CD127 expression on both primary and secondary effector CD4 T cells is directly linked to IL-2 exposure at 5–7 dpi and closely correlated memory cell recovery.

Enhanced access to IL-7 rescues survival of $Il2^{-/-}$ memory

We treated recipients of $Il2^{-/-}$ donors with IL-7: anti-IL-7 complexes (IL-7C)[27] to determine if enhanced IL-7 signals could overcome the need for late IL-2 to facilitate memory generation. IL-7C treatment from 5–7 dpi failed to rescue donor cell survival (not shown). However, when administered every other day starting at 14 dpi [following viral clearance[4]] through 26 dpi, IL-7C treatment restored $Il2^{-/-}$ donor cell recovery to levels comparable to those achieved by IL-2C treatment from 5–7 dpi (Fig 6a). These results suggest that late IL-2 induces permanent upregulation of CD127 on effector cells thus increasing their ability to compete for ambient IL-7 that is required for their long-term persistence.

In nude mice access to IL-7 is greater because it is not limited by competition from host T cells, but survival of transferred CD4 T cells is still IL-7 dependent [26]. Therefore, we predicted that in nude hosts the $Il2^{-/-}$ donor cells would have greater access to IL-7 and would perform more like WT donor cells. Indeed, we found equivalent long-term survival of WT and $Il2^{-/-}$ donor cells in IAV-challenged nude hosts (Fig 6b), suggesting that when ambient IL-7 levels are higher, a substantial level of memory generation is seen without late IL-2 production by effector CD4 T cells.

Late IL-2 limits acute apoptosis of effector cells

To analyze if additional IL-2-dependent mechanisms improve the capacity of effector cells to form memory, or if enhanced fitness to access to IL-7 is the dominant factor, we treated recipients of WT donor cells with blocking Ab against CD127 every other day from 7 to 28 dpi to disrupt IL-7 signaling [28]. Blocking IL-7 access substantially reduced donor cell

recovery at 28 dpi (Fig 6c), confirming that sustained access to IL-7 is necessary for optimal memory survival. However, CD127 blockade did not reduce donor recovery at 14 dpi, when the effect of IL-2-deficiency was already striking (see Figure 2), and the reduction in donor cell recovery at 28 dpi was not as dramatic as that seen following IL-2 neutralization. Thus autocrine IL-2 signals during 5–7 dpi must block acute apoptosis of effector cells before 14 dpi by an IL-7-independent pro-survival mechanism [13] resulting in a larger pool of effectors able to compete for survival as memory.

We analyzed expression of the pro-apoptotic molecule Bim vs. the anti-apoptotic molecule Bcl-2, which play key opposing roles in regulating T cell lifespan [29], from 7–14 dpi to ask if late IL-2 signals impacted their expression by effector CD4 T cells responding against IAV. The Bim/Bcl-2 ratio was higher in $Il2^{-/-}$ than in WT donors at 7 dpi and when the *in situ* survival of $Il2^{-/-}$ effectors was enhanced by IL-2C treatment, the Bim/Bcl-2 ratio was reduced (Fig 6d, e) from 7 through 14 dpi (Fig 6f). $Il2^{-/-}$ effectors also expressed higher levels of TNF-related apoptosis inducing ligand (TRAIL), which can potentiate effector CD4 T cell apoptosis[30] (Supplementary Fig 4). These findings suggest that late IL-2 signals also enhance the efficiency of memory generation by directly limiting acute apoptosis of effector cells.

Late CD27:CD70 signals enhance memory through IL-2 induction

CD27:CD70 interactions enhance IL-2 production by and CD127 expression on CD8 T cells[31, 32, 33, 34], and IL-2 production by CD4 T cells [35]. To determine a connection between late cognate interactions between effectors with APC and resulting autocrine IL-2 induction, we asked if CD27:CD70 interactions enhanced memory CD4 T cell generation following IAV challenge. We analyzed expression of CD27 on WT CD4 T cells and CD70 on CD11c$^+$ cells to determine if a potential interaction was plausible during the late memory checkpoint identified here. Donor CD27 expression fell sharply during 5–7 dpi while a reciprocal increase in CD70$^+$ CD11c$^+$ cells was observed (Fig 7a), in agreement with previous analysis [36], and confirming the potential for CD27:CD70 signaling during 4–7 dpi. We could not detect CD70 on T cells (not shown).

We then treated recipients of primed WT donor cells with anti-CD70 or isotype control Ab from 1–6 dpi and found enhanced donor CD27 expression at 7 dpi after CD70 blockade (Fig 7b, c), suggesting that the donor cells do interact with CD70 expressed by APC during the memory checkpoint [37]. Anti-CD70 Ab treatment did not impact viral titers detected at 7 dpi (Fig 7d) nor did treatment have an impact on the peak expansion of donor cells (Fig 7e). Strikingly however, anti-CD70 Ab treatment significantly reduced donor cell recovery at 28 dpi (Fig 7f) and treatment reduced CD127 expression on surviving cells (Fig 7g). Importantly, when we restricted anti-CD70 Ab treatment to 4–7 dpi, donor cell survival was comparably reduced to that observed with treatment from 1–7 dpi (Fig 7f). This implies that the key CD70-dependent signals regulating memory CD4 T cell generation occur late during the effector phase instead of during initial interactions with APC.

As expected[35], anti-CD70 Ab treatment reduced effector cell IL-2 production (Fig 7h). When we treated recipients of WT donor cells with both anti-CD70 Ab from 4–7 dpi and with IL-2C from 5–7 dpi, donor cell survival at 28 dpi was restored to that observed in

isotype Ab treated mice (Fig 7i), implying that CD70 blockade impaired memory generation primarily by reducing late IL-2 production by effector cells. Donor recovery was actually slightly enhanced in mice receiving IL-2C and anti-CD70 Ab versus IL-2C and control Ab. This finding may reflect that CD70 delivers pro-apoptotic signals to some of the effector cells [38]. We conclude that late CD27:CD70 signals improve memory generation by enhancing IL-2 production by effector CD4 T cells during interactions with APC. This is further evidence that key fate decisions governing CD4 T cell survival leading to memory are made at the effector phase during late, presumably cognate, interactions with APC.

Discussion

Our studies identify a novel and discrete checkpoint during the CD4 T cell response against IAV that determines the generation of long-lived memory. Efficient transition to memory requires both MHC-II and CD27:CD70 interactions between 4–7 dpi, implying that the effectors must engage in a late cognate interaction, and we find that CD11c$^+$ APC are sufficient for this implied interaction. We show that the majority of effectors responding against the virus express a default program for death unless this interaction with APC causes them to produce high levels of IL-2. Autocrine IL-2, or high levels of exogenous IL-2 delivered in the form of IL-2C, directly rescues some effectors from acute apoptotic death, and also acts in a dose-dependent manner to upregulate CD127 expression. Once upregulated by IL-2, higher expression of CD127 is maintained and increases the capacity of cells to compete for ambient IL-7 that sustains their long-term persistence.

The fact that IL-2 production by CD4 T cells requires TcR stimulation [39], and that both late CD70 and MHC-II expression are required for optimal long-term survival of effector CD4 T cells following IAV challenge, strongly supports the concept that the effector CD4 T cell : APC interaction is the first step in the memory checkpoint. Expression of CD70 and MHC-II are tightly linked [40], and levels of CD70 on APC and of CD27 on T cells are quickly down regulated following their interaction, resulting in a potent negative feedback loop[37]. This and the rapid kinetics of default apoptosis of effector CD4 T cells likely help define the timeframe during which late cognate interaction must occur to rescue a cohort of cells to survive and transition to memory. Other signals could also be involved as we note that CD70 blockade did not have as dramatic of an impact on reducing memory generation as did blockade of IL-2.

We found similar kinetics of effector contraction in the lung, spleen, and dLN, consistent with uniform T cell effector apoptosis in diverse organs following peptide immunization [41]. Effectors responding against IAV in different organs are marked by wide differences in phenotype, cytokine profile, and gene expression [14, 42], and thus the late IL-2-dependent memory checkpoint defined here appears to act broadly rather than in a subset-specific manner. We did, however, consistently identify a population of long-lived IL-2-independent memory cells in the lung. Unique mechanisms may govern the generation and persistence of tissue-resident memory CD4 T cells in the lung, as has been reported with brain-resident memory CD8 T cells[43].

Infection with live IAV results in high levels of Ag presentation and inflammation throughout the period of CD4 T cell priming and expansion, followed by rapid viral clearance. This may be required to drive effectors to the state in which they die by default unless they re-encounter APC, and make and bind IL-2. Non-replicating, shorter-term Ag exposure may result in less differentiated effectors [44] and it remains to be determined if such effectors have the same requirements for the memory checkpoint identified here. Studies describing 'stem cell memory' [45] suggest that some less-differentiated effectors that may not yet have become programmed to undergo default apoptosis can efficiently enter the memory pool. However, we point out that during vigorous responses against pathogens the number of effector T cells at the peak of response far exceed those at earlier time points, and so such effectors would appear to have a greater potential to dominate the memory pool. Furthermore, the more differentiated effector populations are comprised of many functionally specialized subsets. For example, IAV infection generates T_{FH} in secondary lymphoid organs, and cytotoxic CD4 T cells in the lung by 7–8 dpi [46]. Since virtually no memory cells develop in secondary lymphoid organs without late IL-2 signals, even though there are less divided donors with central memory phenotypes in these organs[42], it appears that in this model effector cells at various states of differentiation require late IL-2 to generate memory.

Memory CD4 T cells provide broad spectrum immunity and robust protection against IAV[4, 47, 48] and other viral infections [47] and are not as readily evaded by new variants that rapidly mutate to avoid neutralizing Abs. Thus, the case for developing vaccines that induce strong CD4+ as well as CD8+ T cell and long-term B cell memory including Ab, is compelling. Our results suggest a new paradigm for when and how CD4 T cell memory formation is regulated following infection. Effective cognate interaction at the effector phase is necessary to induce optimal autocrine IL-2 signaling that is required to limit apoptosis of effectors and to sustain their transition to and persistence as memory cells. Moreover, boosting CD4 T cell memory is equally dependent on this late memory checkpoint. Thus, we predict that vaccines that provide both Ag and adjuvant effects that span the CD4 T cell effector phase and hence better mirror natural infection will be better able to induce robust memory that includes multiple functional subsets[47].

Methods

Mice

Naïve CD4+ T cells were obtained from 5–8 week old male or female DO11.10. Thy1.2, DO11.10.Thy1.2/Thy1.1, and *Il2*−/− DO11.10.Thy1.2 mice originally provided by A. Abbas (UCSF). Recipients of cell transfers were male BALB/c.Thy1.2 or BALB/c.Thy1.1 or nude mice that were at least 8 weeks old. OT-II.Thy1.1, CD11c Tg.*H2-Ab1*−/−, and C57Bl/6 Thy1.1 or Thy1.2 mice were used in certain experiments. T cell-deficient nude mice were purchased Charles River Laboratories. All other mice were obtained from the breeding facility at Trudeau Institute, the University of Massachusetts Medical School, or Jackson Laboratories. The Animal Care and Use Committee at Trudeau Institute (Saranac Lake, NY) or the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School (Worcester, MA) approved all experimental animal procedures.

CD4 T cell isolation and in vitro-primed memory generation

Naïve CD4$^+$ T cells were obtained from pooled spleen and peripheral lymph nodes as previously described [42]. Briefly, cells were purified through nylon wool and per coll gradient separation. CD4 T cells were isolated by positive CD4 MACS selection (Miltenyi). Resulting CD4$^+$ cells routinely expressed a characteristic naive phenotype (small size, CD62L^{hi}, CD44^{lo} and CD25^{lo}) >97%TcR$^+$. T_H1-polarized effectors were generated *in vitro* as described [49]. Briefly, naïve WT or *Il2*$^{-/-}$ CD4 T cells were cultured with an equal number of irradiated APC (2×10^5 per mL) in the presence of exogenous IL-2 (20 ng per mL), 2 ng per mL IL-12 (Peprotech), 10 μg per mL anti-IL-4 Ab (11B11; Bioxcell), and 5 μM OVA$_{II}$ peptide. *In vitro*-primed memory cells were obtained by thoroughly washing effector cultures at 4 days and re-culturing the cells in fresh media for at least 3 days in the absence of Ag and exogenous cytokines. Live cells were isolated by lympholyte separation (Cedarlane). In some experiments, total polyclonal CD4 T cells were isolated from IAV-primed C57BL/6. Thy1.1 mice by positive selection with CD4 MACS beads (Miltenyi), and 6-Carboxyfluorescein succinimidyl ester (CFSE) labeled as previously described [50]. 1×10^6 TcR Tg donor cells (naïve or memory) were adoptively transferred in all experiments, a number found previously to be required to reliably detect donor cells following IAV challenge in the memory phase [4]. Alternatively, 1×10^7 polyclonal memory cells were transferred to adoptive hosts, as in previous studies [14]. All donor CD4 T cells were adoptively transferred in 200 μl phosphate buffered saline (PBS) by intravenous (i.v.) injection.

Virus stocks and infections

Influenza A/PR8-OVA$_{II}$ (H1N1) and A/HKx31-OVA$_{II}$ (H3N2) viruses were produced in the allantoic cavity of embryonated hen eggs from stock obtained from P. Doherty. Mice were infected intranasally under light isoflurane anesthesia (Webster Veterinary Supply) with a sublethal 0.2 LD$_{50}$ dose of A/PR8-OVA$_{II}$ virus or 25 Hemagglutination Units of A/HKx31-OVA$_{II}$ in 50 μl PBS. Donor cell injection and viral infection occurred on the same day.

Detection of IAV titer

Pulmonary viral titer was determined by quantitation of viral RNA. RNA was prepared from whole lung homogenates using TRIzol (Sigma-Aldrich), and 2.5 μg of RNA was reverse transcribed into cDNA using random hexamer primers and Superscript II Reverse Transcriptase (Invitrogen). Quantitative PCR was performed to amplify the acidic polymerase (PA) gene of A/PR8-OVA$_{II}$ using an ABI Prism 7700 Sequence Detector (Applied Biosystems) with 50 ng of cDNA per reaction and the following primers and probe: forward primer, 5′-CGGTCCAAATTCCTGCTGA-3′; reverse primer, 5′CATTGGGGTTCCTTCCATCCA-3′; probe, 5′-6-FAM-CCAAGTCATGAAGGAGAGGGAATACCGCT-3′. Data were analyzed with Sequence Detector v 1.7a (Applied Biosystems). The copy number of the PA gene per 50 ng of cDNA was calculated using a PA-containing plasmid of known concentration as a standard. The number of copies of PA gene per lung is presented.

Cytokine complex and neutralizing Ab treatments

For some experiments, mice were treated on indicated days with injections of cytokine: anti-cytokine monoclonal Ab complexes. For IL-2 complexes (IL-2C), mice received 2 μg per day of recombinant IL-2 (eBioscience) premixed with 20 μg of anti-mouse IL-2 monoclonal (m) Abclone S4B6-1 (S4B6) (BD Pharmingen). In certain experiments, the amount of IL-2 complexes was varied, as indicated. For IL-7 complexes, (IL-7C), mice received 1.5 μg of recombinant mouse IL-7 (eBioscience) premixed with 15 μg of anti-IL-7 mAb clone M25 (BioXcell). Complexes were incubated at room temperature for 20 minutes (min.) before intraperitoneal (i.p.) injection in 200 μL of PBS.

In other experiments mice were treated as indicated with 0.25 mg per day of both anti-IL-2 Abs S4B6 and JES6-1A12 (Rat IgG2a), 0.5 mg per day of anti-IL-7 receptor blocking Ab (A7R34; Rat IgG2a), 0.5 mg per day of anti-CD70 Ab (FR-70), 1 mg per day of anti-MHC-II (M5/114) or appropriate isotype control Ab (all BioXcell) on stated days. Ab was delivered by i.p. injection in 200 μL of PBS, with the exception of anti-MHC-II Ab that was delivered i.v.

Flow cytometry and intracellular cytokine staining

Lung, dLN, and spleen were prepared into single cell suspensions by mechanical disruption and passage through a nylon membrane. Flow cytometry was performed as described [42], using fluorochrome-labeled Abs at manufacturer's recommended 1:50 to 1:100 dilutions for surface staining including anti-Thy1.1 (OX-7), anti-Thy1.2 (53-2.1), anti-CD4 (RM4.5), anti-CD62L (MEL14), anti-CD69 (H12F3), anti-CD44 (1M7), anti-CD27 (LG.3A10), anti-CD25 (PC61), anti-CD127 (IL-7R, A7R34), anti-CD278 (ICOS, 7E.17G9), anti-CD134 (OX40, OX-86), anti-CD70 (FR70), anti-CD253 (TRAIL, N2B2), and anti-MHC-II (M5/114) (Pharmingen, eBioscience, or BioLegend). To detect IAV-specific donor polyclonal CD4 T cells in adoptive hosts, cells were stained for 1 hour (hr.) at 37°C with a 1:100 dilution of I-A^b/NP$_{311-325}$-fluorochrome-labelled tetramer obtained from Trudeau Institute prior to surface marker staining. Tetramer-positive cells in the CD90.1$^+$ CD90.2$^-$ CD4$^+$ CFSE^{low} gate were enumerated.

Intracellular cytokine staining was performed as previously described [42]. Briefly, cells were stimulated for 16 hrs. with OVA peptide-pulsed APC. After 2hrs., 10 μg per ml Brefeldin A (Sigma) was added. Cells were then surface stained, fixed for 20 min. in 4% paraformaldehyde, and permeablized by 10 min. incubation in 0.1% saponin before staining for cytokine by the addition of a 1:100 dilution of anti-IFN-γ, -TNF, and –IL-2, fluorescently labeled Abs. Intracellular staining for Bim (Cell Signaling Technology) and Bcl-2 (BD Pharmingen) was performed as per manufacturers' instructions. Analysis was performed using LSRII instruments (BD Biosciences) and Flow Jo (Tree Star) analysis software.

Detection of Secreted Cytokines

Naïve or primed DO11.10 CD4 T cells, or donor DO11.10 populations isolated from IAV-challenged hosts, and syngeneic irradiated APC were cultured together at a 1:1 ratio with or without various concentrations of OVA$_{323-339}$ peptide for 18 hrs. or with 1 μg of OVA

peptide for various periods of time. Culture supernatants were harvested from triplicate wells and ELISA assays performed as per manufacturer's instructions to determine levels of IFN-γ and IL-2 (eBioscience).

Isolation of donor memory cells for microarray data analysis

WT or $Il2^{-/-}$ D011.10 memory cells, 1×10^6, were CFSE labeled and transferred to naïve hosts then infected with PR8-OVA$_{II}$. Donor cells that had undergone > 5 divisions were sort-purified from lungs at 7 dpi and total mRNA isolated (Qiagen) and amplified (MessageAmp; Ambion). cRNAs were labeled and hybridized to M430 2.0 chips according to Affymetrix protocols. Data were normalized with the Plier algorithm, Log transformed, and analyzed with GeneSpring GX 11.0 (Agilent Technologies Inc.). Significant genes were selected based on P values < 0.05 and fold change >1.5 or 2 after application of the Benjamini and Hochberg correction for false discovery. Heat maps were generated using Gene-E (The Broad Institute).

Statistical analysis

Group sizes of n = 3–5 were employed. Unpaired, two-tailed, Students t-tests, ∞= 0.05, were used to assess whether the means of two normally distributed groups differed significantly. One-way ANOVA analysis with Bonferroni's multiple comparison post-test was employed to compare multiple means. All error bars represent the standard deviation. Significance is indicated as * P< 0.05, ** P< 0.005, *** P< 0.001, **** P< 0.0001.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Kaech SM, Wherry EJ, Ahmed R. Effector and memory T-cell differentiation: implications for vaccine development. Nat Rev Immunol. 2002; 2:251–262. [PubMed: 12001996]

2. McKinstry KK, Strutt TM, Swain SL. Regulation of CD4+ T-cell contraction during pathogen challenge. Immunol Rev. 2010; 236:110–124. [PubMed: 20636812]

3. Masopust D, Kaech SM, Wherry EJ, Ahmed R. The role of programming in memory T-cell development. Curr Opin Immunol. 2004; 16:217–225. [PubMed: 15023416]

4. McKinstry KK, et al. Memory CD4+ T cells protect against influenza through multiple synergizing mechanisms. J Clin Invest. 2012; 122:2847–2856. [PubMed: 22820287]

5. Teijaro JR, Verhoeven D, Page CA, Turner D, Farber DL. Memory CD4 T cells direct protective responses to influenza virus in the lungs through helper-independent mechanisms. J Virol. 2010; 84:9217–9226. [PubMed: 20592069]

6. Lenardo M, et al. Mature T lymphocyte apoptosis--immune regulation in a dynamic and unpredictable antigenic environment. Annu Rev Immunol. 1999; 17:221–253. [PubMed: 10358758]

7. Williams MA, Tyznik AJ, Bevan MJ. Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells. Nature. 2006; 441:890–893. [PubMed: 16778891]

8. Feau S, Arens R, Togher S, Schoenberger SP. Autocrine IL-2 is required for secondary population expansion of CD8(+) memory T cells. Nat Immunol. 2011; 12:908–913. [PubMed: 21804558]

9. Dooms H, Wolslegel K, Lin P, Abbas AK. Interleukin-2 enhances CD4+ T cell memory by promoting the generation of IL-7R alpha-expressing cells. J Exp Med. 2007; 204:547–557. [PubMed: 17312008]

10. Kalia V, Sarkar S, Subramaniam S, Haining WN, Smith KA, Ahmed R. Prolonged interleukin-2R alpha expression on virus-specific CD8+ T cells favors terminal-effector differentiation in vivo. Immunity. 2010; 32:91–103. [PubMed: 20096608]

11. Blattman JN, Grayson JM, Wherry EJ, Kaech SM, Smith KA, Ahmed R. Therapeutic use of IL-2 to enhance antiviral T-cell responses in vivo. Nat Med. 2003; 9:540–547. [PubMed: 12692546]

12. Snow AL, Pandiyan P, Zheng L, Krummey SM, Lenardo MJ. The power and the promise of restimulation-induced cell death in human immune diseases. Immunol Rev. 2010; 236:68–82. [PubMed: 20636809]

13. Zhang X, Giangreco L, Broome HE, Dargan CM, Swain SL. Control of CD4 effector fate: transforming growth factor beta 1 and interleukin 2 synergize to prevent apoptosis and promote effector expansion. J Exp Med. 1995; 182:699–709. [PubMed: 7650478]

14. Strutt TM, McKinstry KK, Kuang Y, Bradley LM, Swain SL. Memory CD4+ T-cell-mediated protection depends on secondary effectors that are distinct from and superior to primary effectors. Proc Natl Acad Sci U S A. 2012

15. Mizuochi T, Tentori L, Sharrow SO, Kruisbeek AM, Singer A. Differentiation of Ia-reactive CD8+ murine T cells does not require Ia engagement. Implications for the role of CD4 and CD8 accessory molecules in T cell differentiation. J Exp Med. 1988; 168:437–442. [PubMed: 2840481]

16. Teixeiro E, et al. Different T cell receptor signals determine CD8+ memory versus effector development. Science. 2009; 323:502–505. [PubMed: 19164748]

17. Zehn D, Lee SY, Bevan MJ. Complete but curtailed T-cell response to very low-affinity antigen. Nature. 2009; 458:211–214. [PubMed: 19182777]

18. Lemos MP, Fan L, Lo D, Laufer TM. CD8alpha+ and CD11b+ dendritic cell-restricted MHC class II controls Th1 CD4+ T cell immunity. J Immunol. 2003; 171:5077–5084. [PubMed: 14607905]

19. Chapman TJ, Castrucci MR, Padrick RC, Bradley LM, Topham DJ. Antigen-specific and non-specific CD4+ T cell recruitment and proliferation during influenza infection. Virology. 2005; 340:296–306. [PubMed: 16054188]

20. Malek TR. The biology of interleukin-2. Annu Rev Immunol. 2008; 26:453–479. [PubMed: 18062768]

21. Boyman O, Sprent J. The role of interleukin-2 during homeostasis and activation of the immune system. Nat Rev Immunol. 2012; 12:180–190. [PubMed: 22343569]

22. Smith KA, Popmihajlov Z. The quantal theory of immunity and the interleukin-2-dependent negative feedback regulation of the immune response. Immunol Rev. 2008; 224:124–140. [PubMed: 18759924]

23. Boyman O, Kovar M, Rubinstein MP, Surh CD, Sprent J. Selective stimulation of T cell subsets with antibody-cytokine immune complexes. Science. 2006; 311:1924–1927. [PubMed: 16484453]

24. Surh CD, Boyman O, Purton JF, Sprent J. Homeostasis of memory T cells. Immunol Rev. 2006; 211:154–163. [PubMed: 16824125]

25. Li J, Huston G, Swain SL. IL-7 promotes the transition of CD4 effectors to persistent memory cells. J Exp Med. 2003; 198:1807–1815. [PubMed: 14676295]

26. Kondrack RM, Harbertson J, Tan JT, McBreen ME, Surh CD, Bradley LM. Interleukin 7 regulates the survival and generation of memory CD4 cells. J Exp Med. 2003; 198:1797–1806. [PubMed: 14662907]

27. Boyman O, Ramsey C, Kim DM, Sprent J, Surh CD. IL-7/anti-IL-7 mAb complexes restore T cell development and induce homeostatic T Cell expansion without lymphopenia. J Immunol. 2008; 180:7265–7275. [PubMed: 18490726]

28. Grabstein KH, et al. Inhibition of murine B and T lymphopoiesis in vivo by an anti-interleukin 7 monoclonal antibody. J Exp Med. 1993; 178:257–264. [PubMed: 8315381]

29. Marrack P, Kappler J. Control of T cell viability. Annu Rev Immunol. 2004; 22:765–787. [PubMed: 15032596]

30. Li X, McKinstry KK, Swain SL, Dalton DK. IFN-gamma acts directly on activated CD4+ T cells during mycobacterial infection to promote apoptosis by inducing components of the intracellular apoptosis machinery and by inducing extracellular proapoptotic signals. J Immunol. 2007; 179:939–949. [PubMed: 17617585]

31. Peperzak V, Xiao Y, Veraar EA, Borst J. CD27 sustains survival of CTLs in virus-infected nonlymphoid tissue in mice by inducing autocrine IL-2 production. J Clin Invest. 2010; 120:168–178. [PubMed: 19955658]

32. Matter MS, Claus C, Ochsenbein AF. CD4+ T cell help improves CD8+ T cell memory by retained CD27 expression. Eur J Immunol. 2008; 38:1847–1856. [PubMed: 18506879]

33. Dolfi DV, Boesteanu AC, Petrovas C, Xia D, Butz EA, Katsikis PD. Late signals from CD27 prevent Fas-dependent apoptosis of primary CD8+ T cells. J Immunol. 2008; 180:2912–2921. [PubMed: 18292513]

34. Penaloza-MacMaster P, Ur Rasheed A, Iyer SS, Yagita H, Blazar BR, Ahmed R. Opposing effects of CD70 costimulation during acute and chronic lymphocytic choriomening it is virus infection of mice. J Virol. 2011; 85:6168–6174. [PubMed: 21507976]

35. Xiao Y, Peperzak V, Keller AM, Borst J. CD27 instructs CD4+ T cells to provide help for the memory CD8+ T cell response after protein immunization. J Immunol. 2008; 181:1071–1082. [PubMed: 18606659]

36. Ballesteros-Tato A, Leon B, Lund FE, Randall TD. Temporal changes in dendritic cell subsets, cross-priming and costimulation via CD70 control CD8(+) T cell responses to influenza. Nat Immunol. 2010; 11:216–224. [PubMed: 20098442]

37. Kuka M, Munitic I, Giardino Torchia ML, Ashwell JD. CD70 is downregulated by interaction with CD27. J Immunol. 2013; 191:2282–2289. [PubMed: 23913967]

38. van Gisbergen KP, et al. The costimulatory molecule CD27 maintains clonally diverse CD8(+) T cell responses of low antigen affinity to protect against viral variants. Immunity. 2011; 35:97–108. [PubMed: 21763160]

39. Sojka DK, Bruniquel D, Schwartz RH, Singh NJ. IL-2 secretion by CD4+ T cells in vivo is rapid, transient, and influenced by TCR-specific competition. J Immunol. 2004; 172:6136–6143. [PubMed: 15128800]

40. Keller AM, et al. Costimulatory ligand CD70 is delivered to the immunological synapse by shared intracellular trafficking with MHC class II molecules. Proc Natl Acad Sci U S A. 2007; 104:5989–5994. [PubMed: 17389361]

41. Garrod KR, et al. Dissecting T cell contraction in vivo using a genetically encoded reporter of apoptosis. Cell Rep. 2012; 2:1438–1447. [PubMed: 23159042]

42. Roman E, et al. CD4 effector T cell subsets in the response to influenza: heterogeneity, migration, and function. J Exp Med. 2002; 196:957–968. [PubMed: 12370257]

43. Wakim LM, Woodward-Davis A, Bevan MJ. Memory T cells persisting within the brain after local infection show functional adaptations to their tissue of residence. Proc Natl Acad Sci U S A. 2010; 107:17872–17879. [PubMed: 20923878]

44. Divekar AA, et al. Protein vaccines induce uncommitted IL-2-secreting human and mouse CD4 T cells, whereas infections induce more IFN-gamma-secreting cells. J Immunol. 2006; 176:1465–1473. [PubMed: 16424174]

45. Farber DL, Yudanin NA, Restifo NP. Human memory T cells: generation, compartmentalization and homeostasis. Nat Rev Immunol. 2014; 14:24–35. [PubMed: 24336101]

46. Strutt TM, McKinstry KK, Marshall NB, Vong AM, Dutton RW, Swain SL. Multipronged CD4(+) T-cell effector and memory responses cooperate to provide potent immunity against respiratory virus. Immunol Rev. 2013; 255:149–164. [PubMed: 23947353]

47. Swain SL, McKinstry KK, Strutt TM. Expanding roles for CD4(+) T cells in immunity to viruses. Nat Rev Immunol. 2012; 12:136–148. [PubMed: 22266691]

48. Strutt TM, et al. Memory CD4+ T cells induce innate responses independently of pathogen. Nat Med. 2010; 16:558–564. 551p following 564. [PubMed: 20436484]
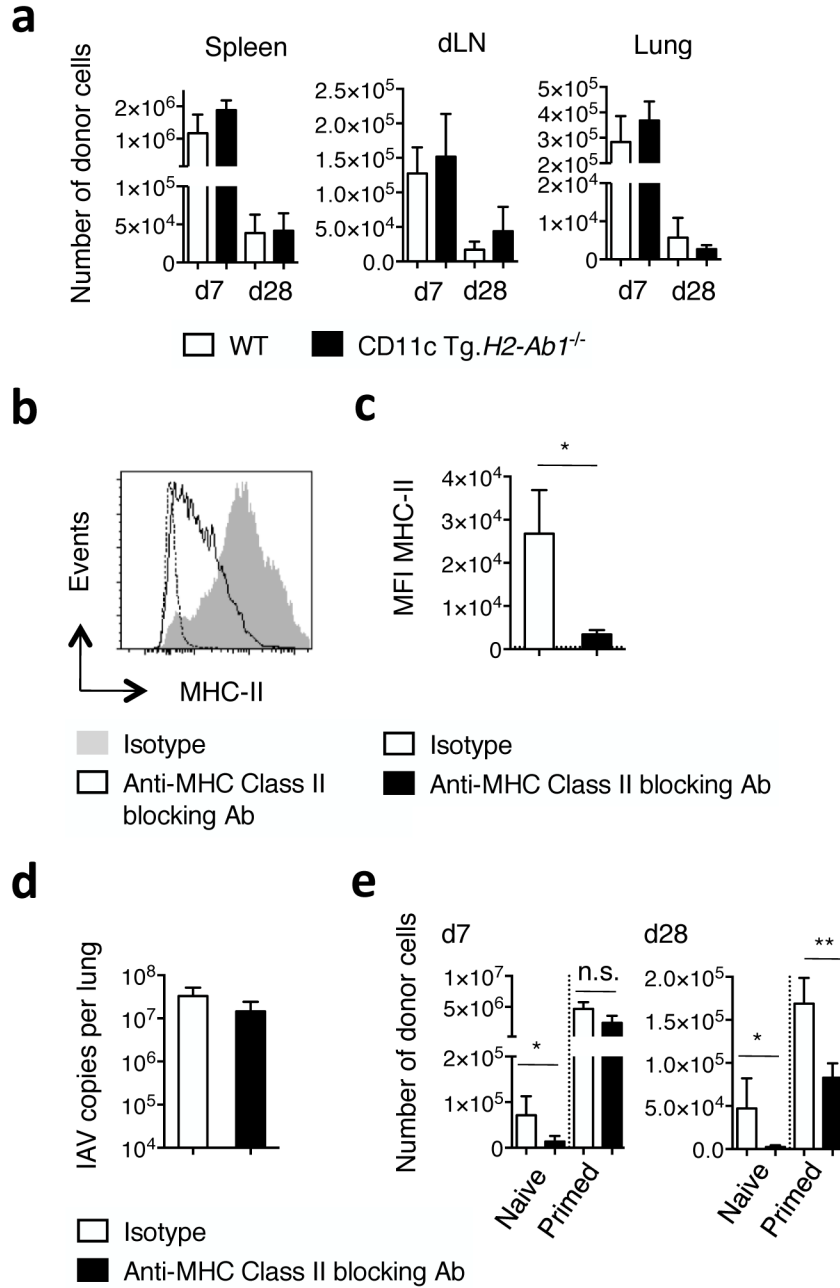
49. McKinstry KK, Golech S, Lee WH, Huston G, Weng NP, Swain SL. Rapid default transition of CD4 T cell effectors to functional memory cells. J Exp Med. 2007; 204:2199–2211. [PubMed: 17724126]

50. Lyons AB, Parish CR. Determination of lymphocyte division by flow cytometry. J Immunol Methods. 1994; 171:131–137. [PubMed: 8176234]

Figure 1. Blocking interactions with MHC-II$^+$ APC at the effector stage reduces memory CD4 T cell generation following IAV challenge

(**a**) Recovery of WT donor cells in spleen, draining lymph node (dLN) and lung at 7 dpi, the peak of effector response, and 28 dpi, a memory time-point, in WT C57BL/6 and CD11cTg. *H2-Ab1$^{-/-}$* hosts challenged with IAV. CD11c Tg. *H2-Ab1$^{-/-}$* recipients of WT donor cells challenged with IAV were treated with isotype or anti-MHC-II blocking Ab on 4–6 dpi (4 mice per group; 1 of 2 experiments). (**b, c**) Representative staining and mean fluorescent intensity (MFI) of MHC-II on CD11c$^+$ cells on 7 dpi. Negative MHC-II staining of CD11c$^-$ cells is shown as a dashed line. (**d**) Viral titer on 7 dpi and (**e**) recovery of naïve and *in vitro-*
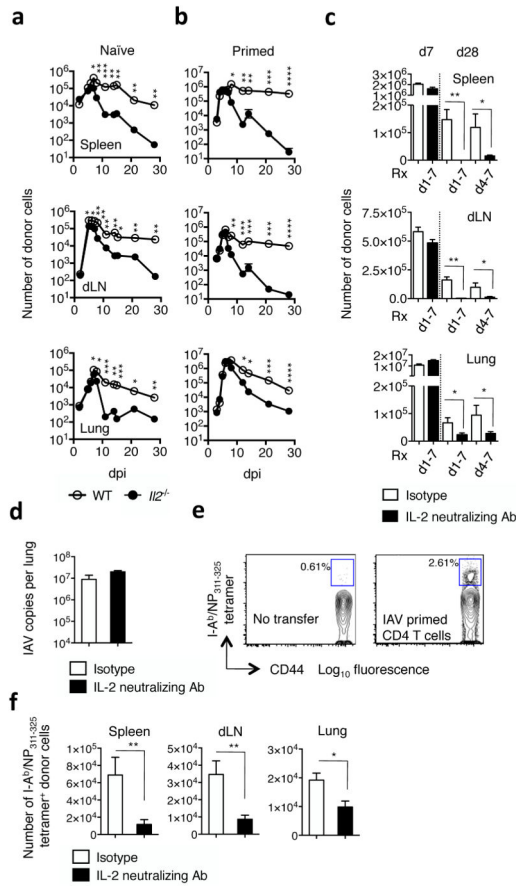
primed Th1-polarized memory (primed) donor cells at 7 and 28 dpi following isotype or anti-MHC-II Ab treatment from 4–6 dpi (4 mice per group; 1 of 2 experiments). All error bars represent the standard deviation and significant differences determined with unpaired, two-tailed, students *t*-tests (α = 0.05, and * *P*< 0.05, ** *P*< 0.005).

Figure 2. IL-2 production is needed for IAV-specific memory CD4 T cell generation
Unprimed BALB/c hosts received 1×10^6 congenically marked WT or $Il2^{-/-}$ (**a**) naïve or (**b**) primed DO11.10 cells. Donor cells were enumerated on the indicated days following IAV challenge (summary of at least 2 experiments per time-point with at least 3 mice per group). Recipients of WT primed donors were treated with 250 µg of each IL-2 neutralizing Ab from 1–7 dpi or with 500 µg of each from 4–7 dpi and (**c**) donors enumerated on 7 and 28 dpi and (**d**) viral titers assessed on 7 dpi (3–5 mice per group per day; 1 of 3 experiments). Polyclonal CD4 T cells from IAV-primed C57BL/6 mice were CFSE-labeled and 1×10^7 transferred to Thy-disparate hosts then challenged with IAV and treated with IL-2 neutralizing Ab from 1–7 dpi. At 28 dpi, tetramer-positive cells (**e**), within the CFSE^{low} donor population were enumerated in the spleen, dLN, and lung (**f**) (summary of at least 2 experiments with at least 3 mice per group). All error bars represent the standard deviation and significant differences determined with unpaired, two-tailed, students t-tests ($\alpha = 0.05$, and * $P< 0.05$, ** $P< 0.005$, *** $P< 0.001$, **** $P< 0.0001$).
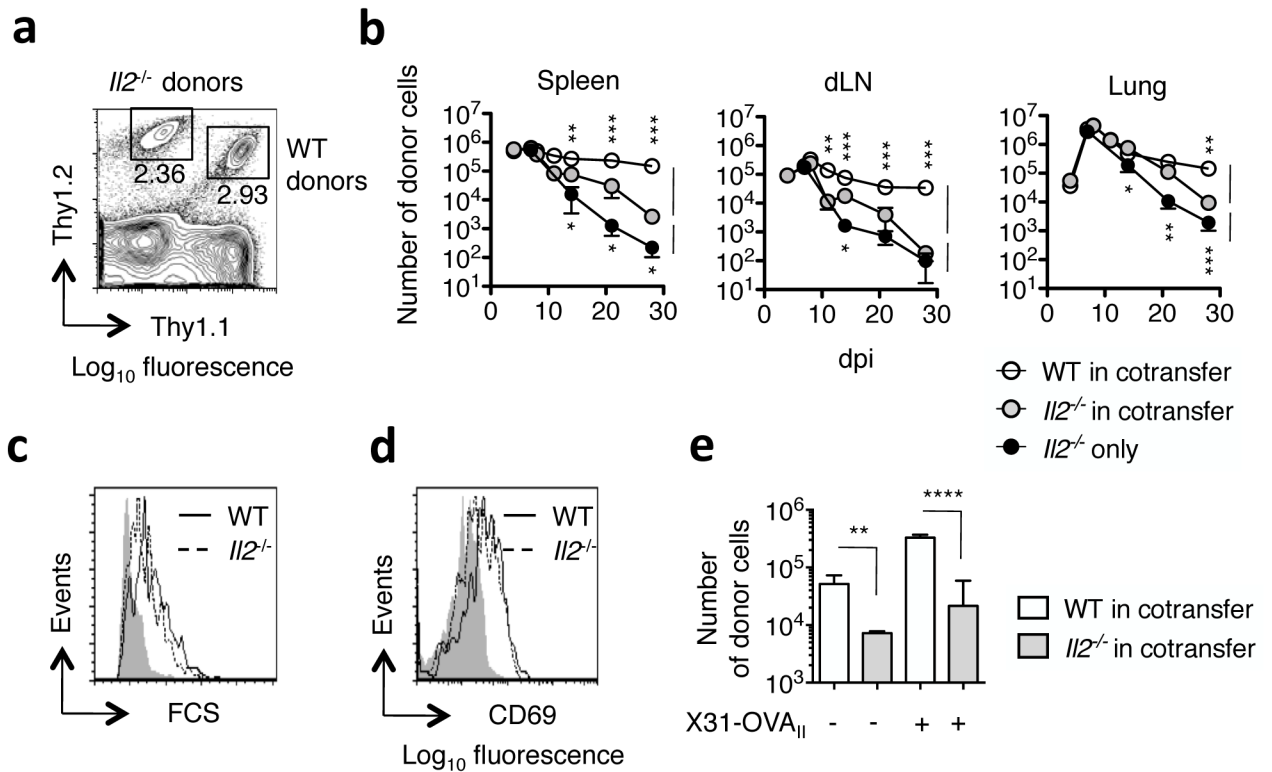
Figure 3. High levels of IL-2 are required for optimal memory CD4 T cell survival

Primed $Il2^{-/-}$ DO11.10 donors, 1×10^6, were transferred to unprimed hosts either alone or together with 1×10^6 primed WT D011.10 cells and subsequently challenged with IAV. **(a)** In cotransferred mice, donors could be distinguished by Thy expression. **(b)** Donor populations were enumerated on stated dpi: WT vs. $Il2^{-/-}$ comparisons shown above and $Il2^{-/-}$ donors in single vs. cotransfer shown below (4 mice per day per group; 1 of 3 experiments). Significant differences were determined following one-way ANOVA analysis with Bonferroni's multiple comparison post-test (* $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$, **** $P < 0.0001$). At 30 dpi, mice receiving cotransfer of WT and $Il2^{-/-}$ donor cells were infected with heterosubtypic virus ($X31\text{-}OVA_{II}$). **(c, d)** Representative histograms of forward scatter (FCS) and CD69 expression on donor populations on day 2 post-$X31\text{-}OVA_{II}$ challenge. Grey histograms represent donor staining at 30 dpi prior to heterosubtypic challenge. **(e)** Number of donor cells in lungs of primed mice before and 4 days post-heterosubtypic challenge (n=4 mice per group per time point; 1 of 2 experiments). Significant differences determined with unpaired, two-tailed, students t-tests ($\alpha = 0.05$, and * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$, **** $P < 0.0001$). All error bars represent the standard deviation.
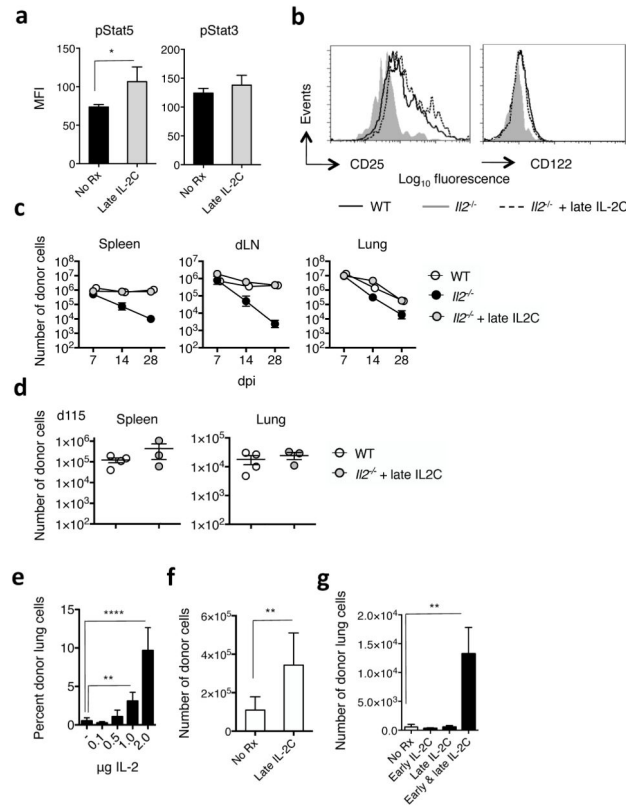
Figure 4. A late IL-2 dependent checkpoint controls memory CD4 T cell survival
Unprimed BALB/c mice receiving primed WT or $Il2^{-/-}$ donor cells were challenged with
IAV and treated with IL-2C on 5–7 dpi (late IL-2C). **(a)** pSTAT5 and pSTAT3 mean
fluorescent intensity (MFI) of $Il2^{-/-}$ donor cells *ex vivo* and (**b**) CD25 and CD122
expression on donor cells, with and without late IL-2C at 7 dpi (1 of 3 experiments).
Significant differences determined with unpaired, two-tailed, students *t*-tests (α = 0.05, and
* *P*< 0.05). **(c)** Longitudinal analysis of recipients of WT or $Il2^{-/-}$ donors with and without
late IL-2C (4 mice per day per group; 1 of 2 experiments). **(d)** Recovery of donor cells 115
dpi (1 of 2 experiments). **(e)** Recipients of $Il2^{-/-}$ cells were treated from 5–7 dpi with IL-2C
containing stated amounts of IL-2 and donor cells detected in the lung at 28 dpi (4 mice per
group). Significant differences in 1 of 2 replicate experiments were determined following
one-way ANOVA analysis with Bonferroni's multiple comparison post-test (** *P*< 0.005,
**** *P*< 0.0001).**(f)** Recovery of WT donor cells from hosts treated with or without late
IL-2C (1 of 2 experiments). Significant differences were determined with unpaired, two-
tailed, students *t*-tests (α = 0.05, and (** *P*< 0.005). **(g)** Recipients of naïve $Il2^{-/-}$ cells were
treated on stated dpi with IL-2C and donor cells detected in the lung at 28 dpi (4 mice per
group; (1 of 3 experiments)). Significant differences determined following one-way
ANOVA analysis with Bonferroni's multiple comparison post-test (** *P*< 0.005). All error
bars represent the standard deviation.
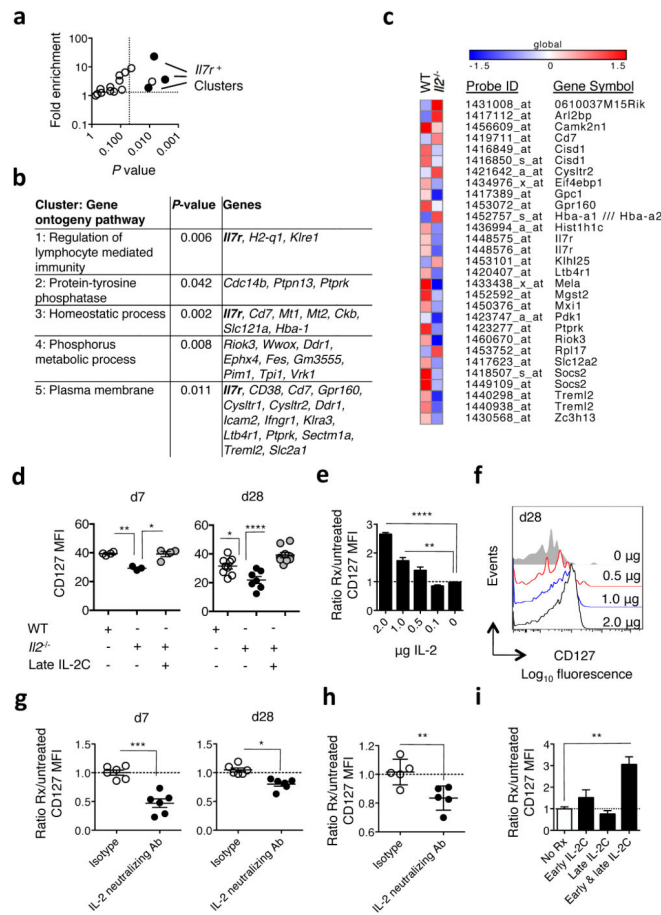
Figure 5. IL-2-dependent CD127 induction at the effector phase is associated with memory generation

Primed WT or $Il2^{-/-}$ DO11.10 cells were sort purified from the lungs of IAV-challenged recipients at 7 dpi in 2 replicate experiments. (**a**) Genes differentially expressed by microarray segregated into 16 clusters by David functional annotation analysis. The fold enrichment and *P*-value of the most significant pathways in each cluster is shown (>1.3 and < 0.05, respectively). (**b**) Gene ontogeny pathway and enriched genes within clusters of interest represented in (a). (**c**) Summary of differentially expressed genes between WT and $Il2^{-/-}$ donors with a threshold of 2-fold and *P*< 0.05. (**d**) Surface expression of CD127 at 7 and 28 dpi on WT and $Il2^{-/-}$ donors from individual mice treated as indicated (1 of 3 experiments). (**e** and **f**) Summary and representative CD127 staining on $Il2^{-/-}$ donors at 28 dpi from mice treated with stated amounts of IL-2C from 5–7 dpi, summary from 5 mice/ group (1 of 2 experiments). (**g**) Ratio of CD127 expression on WT donor cells in the lung at 7 and 28 dpi from mice either treated with IL-2 neutralizing or control Ab on 1–7 dpi (1 of 2 experiments). (**h**) Ratio of CD127 expression on polyclonal donor CD4 T cells from IAV-primed C57BL/6 mice on 28 dpi from host mice either treated with IL-2 neutralizing or control Ab on 1–7 dpi (1 of 2 experiments). (**i**) Ratio of CD127 expression on naïve $Il2^{-/-}$ donors treated on stated dpi with IL-2C (4 mice per group; 1 of 3 experiments). Significant differences were determined on raw data following one-way ANOVA analysis with Bonferroni's multiple comparison post-test (**d**, **e**, and **i**) or determined with unpaired, two-
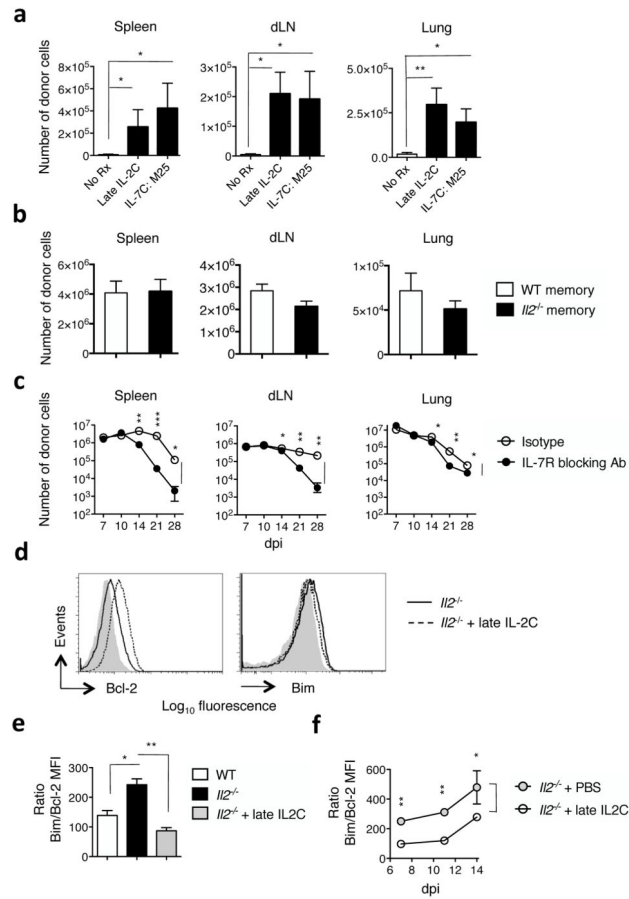
tailed, students *t*-tests (**g** and **h,** with α = 0.05, and * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$, **** $P < 0.0001$). All error bars represent the standard deviation.

Figure 6. IL-2-mediated rescue limits acute apoptosis of effector cells
WT recipients of primed *Il2*$^{-/-}$ DO11.10 cells challenged with IAV were treated with IL-2C from 5–7 dpi or with IL-7C (using anti-IL-7 Ab clone M25) every other day from 14–26 dpi. (**a**) The number of donors at 28 dpi is shown (5 mice per group; 1 of 2 experiments). (**b**) The number of WT or *Il2*$^{-/-}$ donors at 28 dpi in nude recipients challenged with IAV (5 mice per group; 1 of 3 experiments). WT recipients of primed WT DO11.10 cells challenged with IAV were treated with isotype control or IL-7 receptor blocking Ab every other day from 7 to 26 dpi. (**c**) Number of donors detected at the stated dpi (4 mice per group; 1 of 2 experiments). WT recipients of primed *Il2*$^{-/-}$ DO11.10 cells were infected with IAV and were untreated or treated with late IL-2C. (**d**) Representative donor cell Bcl-2 (left) and Bim (right) expression at 7 dpi, from 4 mice per group. Isotype staining is shown as a grey histogram. (**e**) The Bim/Bcl-2 MFI ratio was determined for groups described in (d) versus WT donors (4 mice/group; 1 of 2 separate experiments). (**f**) Kinetics of Bim/Bcl-2 MFI ratio in *Il2*$^{-/-}$ donors treated as described on stated days following IAV infection (4 mice per group; 1 of 2 separate experiments). Significant differences were determined following one-way ANOVA analysis with Bonferroni's multiple comparison post-test (**a** and **e**) or determined with unpaired, two-tailed, students *t*-tests (**c** and **f,** with $\alpha = 0.05$, and * $P< 0.05$, ** $P< 0.005$, *** $P< 0.001$, **** $P< 0.0001$) on raw data. All error bars represent the standard deviation.
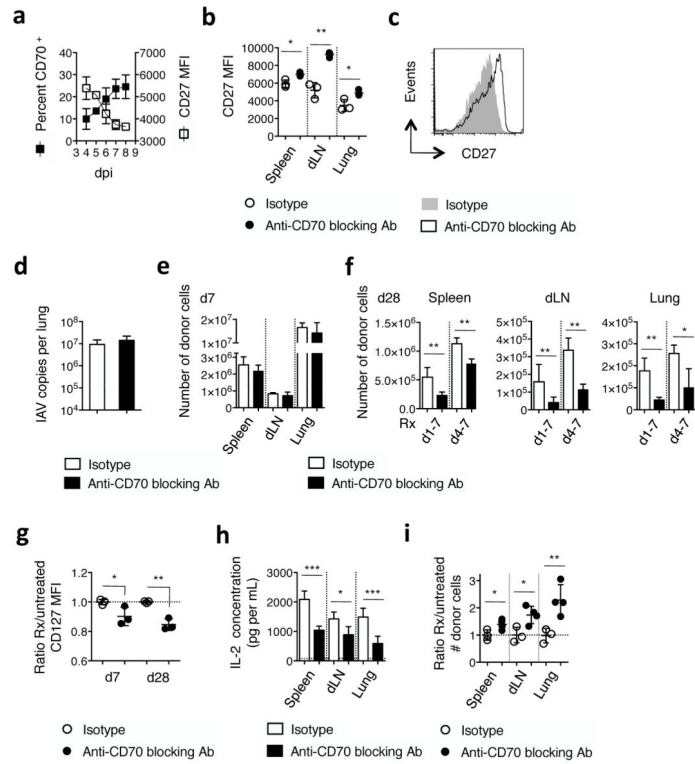
Figure 7. *Late* CD27:CD70 signals contribute to memory CD4 T cell survival by enhancing IL-2 production

Unprimed BALB/c mice receiving primed WT DO11.10 donor cells were challenged with IAV. (**a**) Expression of CD27 on donor cells (MFI) and the % CD70$^+$ CD11c$^+$ MHC-II$^+$ APC in the dLN on the stated dpi (3 mice per group; 1 of 3 experiments). Recipients of WT donor cells challenged with IAV were treated with isotype or anti-CD70 blocking Ab on 1–7 dpi. (**b**) Donor cell CD27 MFI, (**c**) representative CD27 surface expression, (**d**) viral titer, and (**e**) donor cell recovery at 7 dpi with and without blockade on 1–7 dpi. (**f**) Donor cell recovery on 28 dpi with CD70 blockade on the indicated days. (**g**) Ratio of CD127 expression on donor cells on d7 and d28 in isotype and anti-CD70 blocking Ab treated animals (3 mice per group; 1 of 3 experiments). (**h**) IL-2 production of donor cells isolated at 7 dpi from isotype and anti-CD70 blocking Ab treated animals stimulated *ex vivo* with peptide for 18 hrs (1 of 2 experiments). (**i**) Recovery of donor cells in isotype and anti-CD70 blocking Ab treated animals that were also treated with late IL-2C (4 mice per group; 1 of 2 experiments). All error bars represent the standard deviation and significant differences determined with unpaired, two-tailed, students *t*-tests (α = 0.05, and * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$) on raw data.