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Regulation of CD4⁺ T-cell contraction during pathogen challenge

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Summary

Signals orchestrating productive CD4⁺ T-cell responses are well documented; however, the regulation of contraction of CD4⁺ T-cell effector populations following the resolution of primary immune responses is not well understood. While distinct mechanisms of T-cell death have been defined, the relative importance of discrete death pathways during the termination of immune responses *in vivo* remains unclear. Here we review the current understanding of cell-intrinsic and cell-extrinsic variables that regulate contraction of CD4⁺ T-cell effector populations through multiple pathways that operate both initially during T-cell priming and later during the effector phase. We discuss the relative importance of antigen-dependent and antigen-independent mechanisms of CD4⁺ T-cell contraction during *in vivo* responses, with a special emphasis on influenza virus infection. In this model, we highlight the roles of greater differentiation and presence in the lung of CD4⁺ effector T cells, as well as their polarization to particular T-helper subsets, in maximizing contraction. We also discuss the role of autocrine interleukin-2 in limiting the extent of contraction, and we point out that these same factors regulate contraction during secondary CD4⁺ T-cell responses.

Keywords

CD4⁺ T cells; immunologic memory; T-cell contraction; influenza

Introduction

During a primary immune response, naive CD4⁺ T cells recognizing antigen in the context of activated antigen-presenting cells (APCs) expand many fold and differentiate into large highly activated populations of effector cells that make their way to the site of infection and throughout tissues (1,2). Depending on the inflammatory environment and several other variables during priming, CD4⁺ T cells can become polarized into one or more distinct effector subsets with specialized functions and attributes. The best characterized of such subsets are T-helper 1 (Th1) and Th2, long associated with promoting effective cell-mediated and humoral responses, respectively (3). More recently, additional subsets have been described including Th17, T-follicular helper (Tfh), and various regulatory populations (4–6) (Table 1). However, substantial heterogeneity and plasticity, as assessed by cytokine production patterns, has been observed within subsets, especially when generated *in vivo* (7,8), and during an infection, it seems most likely that multiple ‘polarized’ CD4⁺ T-cell subsets are generated (9). These effector cells secrete large quantities of potent cytokines, chemokines, and immunoregulatory proteins and also exert cell-based effector mechanisms such as cytotoxicity in response to low doses of antigen without the need for costimulation (10). These functions make them both extremely effective when combating a pathogen but also dangerous to the host, because the inflammatory responses that they induce can damage

host tissue if unchecked as well as potentially trigger autoimmunity. Indeed, CD4⁺ T effectors play a central role in the development of several experimental models of autoimmunity including experimental autoimmune encephalitis (EAE), rheumatoid arthritis, and colitis (11–13). Thus, the clearance of the activated effector cells after the resolution of infection and the selection of an appropriate cohort of CD4⁺ T cells to transition to resting memory are of great importance.

Following the resolution of a primary immune response, usually correlating with the decline of inflammation and antigen clearance, a large majority of activated CD4⁺ T-cell effectors die via apoptosis to leave a small but relatively stable population of memory cells (14). While many of the signals and steps leading to the generation of effector CD4⁺ T-cell subsets are well defined (2,15–17), the processes and triggers involved in the contraction of activated effectors are not well understood, especially *in vivo*. Generally, apoptosis in T cells is triggered through two distinct pathways both leading to the activation of caspase cascades, DNA breaks, and ultimately cell death (18). The ‘extrinsic pathway’ is initiated through the triggering of death receptors within the tumor necrosis factor (TNF) receptor family by their specific ligands and functions through initiator caspases 8 and 10 (19–22). The ‘intrinsic’ pathway is triggered through damage to mitochondrial membranes and initiator caspase 9 (22,23). Non-apoptotic death pathways can also influence the contraction of CD4⁺ T-cell effector populations (24).

We suggest that the process of contraction is regulated by the integration of a series of complex signals that are present both during initial priming of CD4⁺ T cells and later during the effector stage. In this review, we discuss what we know about some of the factors that regulate the contraction phase of CD4⁺ T-cell subsets generated during influenza virus infection, pointing out roles from our own studies for the extent of initial antigen exposure, the stage of differentiation and anatomical location of effectors, for CD4 subset polarization, for restimulation with antigen, and for autocrine interleukin-2 (IL-2) produced during restimulation. The complexity of regulation suggests that contraction is a critical checkpoint that preferentially selects particular CD4 subsets to persist as memory and thus helps to shape the potential of secondary responses.

T-cell responses following influenza virus infection

When small numbers of naive T-cell receptor (TCR) transgenic (Tg) CD4⁺ or CD8⁺ T cells recognizing influenza, such as HNT CD4⁺ T cells recognizing an epitope of the viral hemagglutinin (25), are introduced to naive hosts shortly before infection, in our studies and those of others using adoptive transfer models (26,27), approximately 10% of the transferred cells ‘take’, enabling the dynamics of an epitope specific T-cell response to be characterized. Following intranasal infection, naive CD4⁺ and CD8⁺ T-cell responses initiate in the draining mediastinal lymph nodes (MLN) and later in the spleen (28,29). Cells divide rapidly in these sites and differentiate into a spectrum of highly activated effectors over 5–6 days, as judged by phenotypic and functional criteria. A cohort of the most differentiated effectors then migrates to the lung starting at days 5–6, leading to a peak in effector numbers of both CD4⁺ and CD8⁺ T cells in the lung at days 7–8 (Fig. 1).

During the first few days following infection, virus titers in the lung increase exponentially and then level off. Once virus-specific T cells reach the lung, influenza titers of fall precipitously. When sublethal doses of virus are used to infect the animals, live virus is cleared from the lung by day 9–10. As virus is cleared, the flu-specific TCR Tg cohort of CD4⁺ T cells contracts with very similar kinetics (28). TCR Tg CD8⁺ T cells also contract, but at a slower pace, and significantly more survive long-term than corresponding CD4⁺ T cells (29). It is important to stress that although it is useful to follow the TCR Tg cohort to

visualize these events, the host polyclonal CD8⁺ and CD4⁺ T-cell dynamics are very similar (30,31). Similar differences in the survival of CD4⁺ versus CD8⁺ T cells following the resolution of infection have been described in a variety of other infectious models (32–34). Several factors might account for the differential rates of contraction between effector CD4⁺ and CD8⁺ T cells. A primary mechanism could involve access to T-cell survival factors. For example, CD8⁺ T cells may out-compete memory CD4⁺ T cells for IL-15 via expression of a higher affinity receptor (35,36), thereby causing the latter population to be less fit for long-term survival. A larger population of antigen-experienced CD4⁺ as compared to CD8⁺ T cells may also require cognate interactions with antigen (37–39), though we find memory from *in vitro*-generated CD4⁺ T-cell effectors is maintained optimally in the absence of major histocompatibility complex (MHC) class II (40), arguing this is not the case for all subsets of memory. Residual antigen depots have been detected after the resolution of primary responses in diverse models (41–44), and their gradual waning over time might constrict survival niches. In support of this hypothesis, residual antigen depots present following influenza infection seem particularly capable of generating and perhaps maintaining CD4⁺ and CD8⁺ T-cell memory populations (41,45).

T-cell contraction during influenza infection

Contraction of influenza-specific CD4⁺ T cells in the lung is generally the most marked, and attrition of virus-specific T cells in the lung continues for longer periods of time than that observed in the MLN and spleen (Fig. 2). These data are compatible with several different hypotheses that suggest a number of non-mutually exclusive mechanisms that may be responsible for contraction. First, the fact that contraction is more dramatic in the lung is compatible with the concept that re-exposure to antigen, which in the case of influenza, replicates and is detected almost exclusively in the lung (46,47), could drive an antigen-induced cell death that is either absent or more limited in other organs (48). Indeed, there is convincing evidence that CD4⁺ T cells in the lung are being restimulated by specific antigen during the contraction phase because a significant proportion secrete interferon- γ (IFN γ) and tumor necrosis factor- α (TNF α) when they are examined *ex vivo* without the need for further restimulation (28). This is not observed within T-cell populations in the MLN or spleen. It is also possible that inflammatory mediators present at high relative concentrations in the lung drive some component of contraction in synergy with TCR triggering or independently (49,50).

Another possible explanation for the increased contraction observed in the lung is that some of the effectors, presumably those that have received the highest dose of antigen stimulation for the longest (51,52), are programmed to die either autonomously following restimulation or by withdrawal of stimulation (27,53,54). The effectors that migrate to the lung could be enriched in that subset. A related hypothesis is that effectors secreting certain cytokines and other factors commit cytokine-induced suicide following induction of cytokine production (49,55). A contrasting hypothesis is that effectors, which make or respond best to survival cytokines like IL-2, are concentrated in the periphery and that when they recognize antigen they survive at higher rates (56–59). Since contraction of effector T cells is an essential component of a successful immune response, we suggest it will involve multiple mechanisms that are closely regulated to both clear effectors that are no longer necessary nor desirable and yet leave behind a small population of resting memory cells that have been ‘educated’ to respond optimally in a secondary encounter. Below, we describe these potential mechanisms in more detail and analyze whether what we know about CD4⁺ T-cell contraction fits with some or all of them in the remainder of this discussion.

Activation-induced cell death

Death of effectors may be triggered through TCR interaction with specific antigen leading to enhanced contraction relative to that due to passive or spontaneous death to be discussed further on. As is the case in passive apoptotic death due to antigen and growth factor withdrawal, the susceptibility of responding CD4⁺ T cells to activation-induced cell death (AICD) is influenced by both cell-intrinsic and cell-extrinsic variables (19–21). The relative importance of AICD versus antigen and growth factor withdrawal in the contraction of CD4⁺ T-cell populations is not yet clear and most likely differs dramatically depending on a variety of factors including the tissue environment (48), surface molecule expression of responding cells (60–63), and costimulatory ligands encountered (64–66). The major pathway of AICD involves interaction of Fas (CD95) on the target cell with Fas ligand (FasL) either on the same or different cells (19). This pathway is well established *in vitro* in murine and human systems (67–70) but seems to be less prominent in *in vivo* studies (71–74).

Availability of antigen is obviously one critical factor in determining the extent of AICD. When T-cell effectors generated in the periphery reach the lung of influenza-infected mice, they are exposed to high levels of viral antigen on both classical APCs (75–77) and on infected cells, mainly epithelial cells, which become MHC class II positive (78, Hernandez, Brown and Swain, unpublished data). This antigen-specific restimulation of effectors in the lung can be seen to trigger cytokine production, which is clearly observed (28), and to elicit other effector functions that contribute to viral clearance (79). This same encounter is also likely to trigger the AICD of those effectors that are susceptible and hence contribute to the contraction of the responding population. In unpublished studies, we find little or no role for Fas in the contraction of CD4⁺ T cells that have become effectors *in vivo* and are in the lung, after influenza infection. This could be related to the high levels of CD43 expressed on these cells (80), as high CD43 expression has been shown to confer resistance of CD4⁺ T cells to Fas-mediated death signals (63). Similarly, recent observations in an influenza model point towards engagement of CD44 as a critical signal in protecting highly activated Th1-polarized CD4⁺ T cells from Fas-dependent apoptosis and facilitating development of memory populations (81). Several other studies have also found significant AICD in the absence of Fas signaling, and it is possible that a wide variety of death pathways including perforin-dependent (82), TCR proximal kinase-mediated (20,83), and exposure to reactive oxygen species (ROS) can play a role (84).

Regulation of AICD by Th polarization

Levels of susceptibility to AICD differ dramatically between differentially polarized Th subsets. It is well established that Th1-polarized effectors are more susceptible to AICD than Th2 cells (53,85,86). More recently, it has been suggested that Th17-polarized populations generated *in vitro* are also more resistant than Th1 populations to AICD (87). Most studies evaluate AICD *in vitro*, and few *in vivo* studies of AICD, especially in infectious models, have been reported. The enhanced susceptibility to AICD observed in Th1 populations is likely due to differential expression of regulation of several molecules including cytotoxic T-lymphocyte antigen-4 (CTLA-4), CD43, FasL, and granzyme B (53,82,85–88). Interestingly, in our and other studies (28,80), each of these factors is upregulated significantly on lung effectors relative to those in the peripheral lymphoid organs, correlating with the greater contraction seen in the lung. Similarly, enhanced susceptibility of Th1-populations compared to Th17 populations to AICD has been correlated with reduced levels of cellular FADD-like interleukin-1 β -converting enzyme inhibitory protein (c-FLIP) (89), a well-established anti-apoptotic molecule that can inhibit death receptor signaling (90,91). Our studies indicate that the CD4 effectors in the lung of influenza-

infected mice are strongly Th1-polarized, while effectors in the periphery are less strongly polarized, as indicated by the fact they make less IFN γ and more IL-2 (28). If AICD is an important contributor to contraction, the polarization of effectors in the lung could help to explain why the rate of contraction is greater there than in the peripheral lymphoid organs. Antigen and cytokine withdrawal-mediated programmed cell death of effectors, to be discussed below, is also influenced by Th subset polarization.

To what extent AICD actually influences contraction of CD4⁺ T cells following influenza infection has not been determined. To evaluate if effectors from the lung are indeed more susceptible to AICD than those in the periphery and to confirm that peak susceptibility is seen at day 7 just as contraction begins and is reduced at day 14 when contraction has largely finished, we transferred naive HNT cells to Thy-disparate hosts that were subsequently infected with influenza and harvested donor-enriched T cells from lung and MLN at 7 and 14 days after infection. We restimulated the isolated populations with anti-CD3 and then added 7-AAD and gated on the donor CD4⁺ T cells to evaluate whether the restimulation induced AICD (Fig. 3). Indeed, *ex vivo* restimulation induced substantial death in the 7 day donor CD4⁺ effector population from the lung but less in those cells isolated from the MLN, and susceptibility to AICD was much less in both donor cell populations recovered after 14 days. This result supports the concept that effectors in the infected tissue do have a higher susceptibility to AICD at the peak of the anti-influenza response, when they are most activated. AICD is likely to play a less prominent role in contraction following resolution of those infections in which antigen levels are low at the time effectors are most numerous or in the site where they reside (19). Instead, it would seem that AICD may play the most prominent role in regulating contraction during situations in which high amounts of antigen are present for prolonged periods, such as chronic infection or situations of autoimmunity where T-cell exhaustion must also be taken into consideration (92,93). We are currently evaluating the extent to which specific antigen is necessary for contraction of CD4⁺ effectors in influenza.

Regulation of AICD by cytokines

Another critical factor regulating AICD in T-cell populations is activation status. *In vitro*, only highly activated and cycling effectors are susceptible to AICD, and even short periods of rest dramatically reduce the amount of AICD observed upon restimulation (20,53,94,95) (Fig. 4). Since effectors *in vivo* quickly become resting in the absence of antigen (54,96), we predict AICD susceptibility is also transient *in vivo* and only applies to activated effectors, not resting memory cells. Several cytokines can influence the relative susceptibility of effectors to AICD; however, the roles of each individual cytokine may depend on the cytokine milieu present during priming in addition to conditions present during antigen restimulation. Somewhat paradoxically, IL-2, which is a potent growth factor of effectors, has been shown to play a major role during priming in sensitizing cells to AICD (97–99). Triggering of the IL-2R upregulates FasL via a pathway involving the IL-2 inducible T-cell kinase (Itk) (99–101). Again, somewhat paradoxically given the reduced susceptibility of Th2 cells to AICD (53,85,86), the Th2-associated cytokine IL-4 has been shown to enhance AICD (102), sensitizing cells in an IL-2-dependent manner also involving upregulation and triggering of the IL-2R (CD25). Furthermore, both IL-2 and IL-4 can enhance AICD of effectors by initiating the degradation of the apoptosis inhibitor c-FLIP (20,102). *In vitro*, IL-2 and TGF β can synergize to protect Th1 but not Th2 effectors from AICD (86,103), but it has not yet been established whether TGF β plays a similar role *in vivo*. TNF, a cytokine that is produced by most T-cell subsets, has also been shown to enhance susceptibility to AICD (50,104–106), but depending on the signaling pathway engaged by ligation of the TNF receptor, different biological responses such as resistance to AICD may ensue (107,108). This in conjunction with many non-redundant AICD-inducing pathways may

explain why, in some instances, TNF receptor-deficient mice have levels of AICD comparable to wildtype mice (109). Hence, the contribution of TNF to AICD may be dependent upon both the cytokine milieu in which responding effectors are present in addition to the model system employed, both of which may result in the triggering of unique intrinsic and extrinsic signaling pathways.

IL-10 has also been demonstrated to enhance Fas-dependent T-cell death through IL-10-dependent upregulation of Fas ligand (110). During influenza infection, we observe that IL-10 levels are maximal in the lung and peak during the contraction phase at around day 7–8 (111). Furthermore, our results show that highly activated CD4⁺ T-cell effectors represent a major source of IL-10. To test if IL-10 contributes to contraction of CD4⁺ T cells responding against influenza, we transferred WT HNT donor cells to WT hosts or IL-10-deficient HNT donors to IL-10-deficient hosts infected with a sublethal dose of A/PR8, and enumerated donor cells at the peak of the response (day 7) and on day 14. We observed no difference in the number of donor cells responding in the presence or absence of IL-10 at either timepoint, suggesting a minimal role for IL-10 in the contraction of influenza-specific CD4⁺ T-cell effectors (Strutt, McKinstry, and Swain, unpublished observations).

Lack of role for IFN γ in CD4⁺ T-cell contraction in the influenza model

In addition to having a significant role in models of antigen-independent cell death of CD4⁺ T cells, to be discussed, several studies have implicated IFN γ as a major sensitizing factor to AICD (49,112,113). Both *in vitro* and *in vivo* models have shown IFN γ to impact susceptibility of CD4⁺ T cells to AICD. Given its central role in orchestrating apoptosis in responding CD4⁺ T cells, it is not surprising that aberrant contraction has been observed *in vivo* in the absence of IFN γ both in situations of chronic and acute infections, IFN γ -deficient cells appear resistant to contraction (49,113). This is likely due to the fact that IFN γ globally regulates expression of a tremendous number of genes including several involved in intracellular apoptotic machinery, including signal transducer and activator of transcription-1 (STAT-1)-dependent upregulation of caspase-8 as well as several extracellular apoptosis-associated ligands (49,112,114,115). Furthermore, given the early kinetics of IFN γ receptor expression on responding CD4⁺ T cells (116,117), it is likely that the impact of IFN γ on contraction of CD4⁺ T-cell effectors occurs during the early priming phases of the response in a similar manner, as has been proposed for the action of IL-2. This mode of action might also help to explain why IFN γ -producing CD4⁺ T-cell effectors clearly accumulate during responses against many pathogens including influenza.

However, a pro-apoptotic role for IFN γ during CD4⁺ T-cell responses *in vivo* is not universal. In fact, studies employing LCMV show that IFN γ signaling is required for maximal antiviral CD4⁺ T-cell memory (118). Our studies with influenza suggest that IFN γ also plays a minimal role in coordinating the contraction of effector CD4⁺ T cells. To assess such a role, we transferred WT naive HNT CD4 T cells into WT hosts or IFN γ -deficient HNT CD4⁺ T cells into IFN γ -deficient hosts and infected with influenza. Both donor CD4⁺ T-cell populations reached similar peak numbers in both hosts on day 7 of the response in the lung as well as the spleen and draining lymph nodes. When assessed on day 14 post-infection, both populations were again present at similar numbers, suggesting a similar contraction program (Fig. 5) and its independence from autocrine or paracrine IFN γ . Thus, the impact of IFN γ on contraction of responding CD4⁺ T cells can be positive, negative, or negligible depending on the nature of the challenge, but certainly dramatic CD4⁺ T-cell contraction can occur without IFN γ .

The impact of IFN γ on contraction could be differentially regulated in different experimental models in several ways. For example, it is possible that signals through the

IFN γ receptor must synergize with other signals to induce maximal apoptosis in effectors, and the presence of or levels of these signals is likely to vary considerably depending on the nature of the challenge. It is also possible that factors produced by responding CD4⁺ T cells that counteract the IFN γ -mediated cell death, such as the immunity related GTPase Irgm1 (119), are differentially induced in responding IFN γ -producing populations.

Growth factor and antigen withdrawal in contraction of CD4⁺ T cells

Changes in the environment in which CD4⁺ T cells are responding can play a central role in orchestrating contraction. In contrast to mechanisms of AICD that are induced by antigen recognition, just discussed, withdrawal of antigen and the coincident removal of growth and survival factors may induce contraction especially in activated effector cells that already express substantial levels pro-apoptotic proteins and caspases (21,23,71). Both TCR signaling and signals from IL-7 and other IL-2R γ -binding cytokines can induce the upregulation of anti-apoptotic proteins such as Bcl-2 and Bcl-XL (120–124) and the downregulation of proapoptotic components such as Bim and Puma (125–127), which prevent the Bcl-2 family members from blocking Bad and Bax that directly induce caspases (128,129). Studies in several different models show that Bim plays a central role coordinating cell death and that its expression level is a critical regulator of T-cell contraction (73,74,130). Since the pro- and anti-apoptotic molecules exist in equilibrium, the expressed level in an effector is critical to its fate. Those levels are often determined during initial antigen encounter and effector differentiation (27). Below we briefly discuss how antigen withdrawal and changes in the inflammatory environment, often referred to as 'death by neglect' or 'passive cell death', can impact the contraction of effector CD4⁺ T cells. How distinct effector populations respond to these signals can differ dramatically.

Perhaps the most obvious factor that influences contraction of effector T cells *in vitro* is the removal of antigen. After antigen withdrawal from highly activated *in vitro*-generated CD4⁺ T-cell effector cultures, a significant number of cells undergo apoptosis within 2–3 days (53,54,96). Similar kinetics of contraction occur when highly activated *in vivo*-generated effectors are isolated and plated *in vitro* without antigen (131). Similar kinetics of contraction are observed when antigen levels wane *in vivo*; as shown in Fig. 1 and discussed above, numbers of CD4⁺ T-cell effectors drop dramatically during the period of viral clearance and thereafter following influenza challenge. Such similar patterns of contraction within effector populations both *in vitro* and *in vivo* suggest that some common programmed cell death pathways are initiated upon antigen withdrawal, although growth factors are removed as well in most models.

IL-2 signaling during antigen-specific priming of CD4⁺ T cells both *in vitro* and *in vivo* (132,133) has been shown to significantly enhance the long-term survival of effectors after antigen withdrawal (134,135). A similar impact on CD4⁺ T-cell survival after antigen clearance has also been reported for CD28 signaling during priming (64,136–138). The enhanced survival of effectors primed in the presence of IL-2 signals has been shown to be due in some cases to IL-2-dependent upregulation of IL-7 receptor (CD127) (139). This mechanism is consistent with earlier findings showing that access to IL-7 promotes optimal survival of effector CD4⁺ T cells during the transition to memory, when growth factor signals such as those delivered through CD25 are no longer available (124,140). However, other factors must also come into play, as recent studies have shown IL-7 is not likely to be the limiting factor during the transition CD4⁺ T-cell contraction in a response to vaccinia virus (141). IL-15 could represent such a factor, as studies suggest that IL-15 signaling can rescue responding T cells from AICD (142) and promote survival of CD4⁺ and CD8⁺ T-cell memory (36,143).

Paradoxically, other studies have suggested that IL-2 drives susceptibility to programmed death as well as AICD (97–99). This apparent discrepancy can be resolved by considering the status of the cells in question. IL-2 is critical *in vitro* for the full differentiation of both Th1 and Th2 subsets (51,144), including the induction of many of the components of death pathways (99–101). This explains, at least in part, why it is those differentiated effectors that are susceptible to programmed death. Layered on top of this susceptibility due to expression of death pathway components is induction of expression of receptors for survival factors that will prevent death, if these factors are available (139). Thus, IL-2-dependent mechanisms can both enhance and moderate the degree of programmed death seen.

To test if IL-2 production by CD4⁺ T cells regulates contraction during responses against influenza, we transferred naive WT or IL-2-deficient CD4⁺ T cells specific for OVA to unprimed WT hosts and infected with a sublethal dose of A/PR8-OVA. Similar to observations in numerous other models (133), WT and IL-2-deficient expanded to a similar degree, with similar kinetics in all organs tested by day 7 post-infection (McKinstry, Strutt, and Swain, unpublished observations). However, when donor populations were assessed at day 14 post-infection, significantly fewer IL-2-deficient donor cells were detected compared to WT donors. Given the fact that IL-2-deficient donor CD4⁺ T cells were responding in WT mice, these results suggest that autocrine IL-2 production by responding CD4⁺ T cells during influenza challenge has an important role in regulating contraction.

Regulation of programmed cell death by Th polarization

While some factors, such as decreased Bmi1 expression (145), seem to negatively regulate survival of effectors upon antigen withdrawal independently of Th-polarization, several observations demonstrate that the degree of programmed contraction varies significantly between different effector populations specific for the same antigen. For example, well-polarized Th1, Th2, Th17, and unpolarized effectors generated from naive TCR transgenic cells *in vitro* in the presence of IL-2 have different rates of apoptotic death following antigen withdrawal. Compared to Th1 and especially unpolarized (Th0) populations, significantly more Th2 and Th17 survive 3 days post antigen withdrawal (Fig. 6). Thus, availability of polarizing cytokines and APC populations during priming that impact Th-polarization contribute to the sensitivity of CD4⁺ T-cell effectors to apoptotic death following resolution of infection. This may be an important consideration in optimizing the design of non-replicating vaccination strategies in which antigen is cleared rather rapidly, since the number of memory T cells may be significantly influenced by routes of immunization (146) in addition to adjuvants that favor particular Th subset survival (147). These same considerations also influence the susceptibility to AICD, as already discussed.

Cytokines produced by effectors and other Th-specific effectors can also significantly affect the degree of contraction observed upon antigen withdrawal. For example, *in vitro* Th1-polarized effector cultures generated from IFN γ -deficient TCR transgenic CD4⁺ T cells, while reaching similar peak numbers, undergo significantly less contraction following antigen withdrawal compared to those from WT cultured cells (Fig. 7). The enhanced survival of IFN γ -deficient cells is observed even when high concentrations of exogenous IFN γ are added to the priming milieu, suggesting that IFN γ signals during initial priming do not substantially impact passive cell death upon antigen withdrawal. Similarly, Th1-polarized effectors generated from perforin-deficient cells display a distinct survival advantage compared to WT cells of the same specificity upon antigen withdrawal.

These findings show that beyond signals experienced during priming, autocrine or paracrine signals experienced during the expansion phase can directly impact an effector cell's fitness after the removal of antigen and growth factors. The strong impact of IFN γ on programmed

cell death *in vitro* (Fig. 7) is seemingly at odds with the negligible role for IFN γ on the contraction of effectors during influenza infection (Fig. 5). These results might suggest that passive cell death pathways are not as critical as antigen-dependent mechanisms in regulating death of CD4⁺ T cells responding to influenza. More importantly, these results again highlight the difficulty of applying *in vitro* observations of contraction, where levels of antigen and inflammatory constituents can be controlled, to cell death *in vivo* during pathogen challenge, where the relative impact of individual pro-apoptotic signals are more difficult to define and are likely to flux.

Programmed cell death and residual antigen

Antigen clearance following the resolution of a primary immune response can set in motion the contraction of effector CD4⁺ T cells that is influenced both by imprinting during priming by signals that ultimately generate different subsets of CD4⁺ effectors, and also by growth and survival factors present at the effector stage. Since different sites, such as the site of infection and peripheral non-infected secondary lymphoid sites, are populated by distinct effector populations (28,148) and since different sites likely have different antigen loads (46,47) and cytokine environments, it is not surprising that contraction of effectors is more dramatic in the infected site than in secondary lymphoid tissues. While control of antigen is easily manipulated *in vitro*, its availability to T-cell populations in different sites *in vivo* is complex and less well understood. For example, residual antigen presentation has been observed following the resolution of several acute infections (41–44), including our own studies with influenza (41,45). One unresolved question is whether there is sufficient antigen available to trigger autocrine cytokine production or only to generate cytokine-independent division? Another issue regarding persisting antigen is whether access to residual antigen is restricted to certain anatomical or cellular sites and if only certain T-cell subsets are able to migrate to those sites (76). Clearly, more analysis is needed to evaluate whether persisting antigen not associated with live pathogen plays a role in some aspects of T-cell contraction.

In most infectious models, it is difficult to ascertain the contribution of antigen withdrawal to contraction of effector CD4⁺ T-cell populations *in vivo*, because there is a coordinated downregulation of inflammation coincident with clearance (71). As many elements of inflammation can promote T-cell proliferation and survival (149–152), an unknown fraction of contraction may be dependent on growth factor and/or survival factor withdrawal (27,73). Indeed, as mentioned earlier, responding effectors and the resting memory cells derived from them depend on IL-7R and IL-2R γ -mediated survival signals so the level of CD127 expression influences the extent of contraction (122,124,139), illustrating the importance of access to proper cytokine signals. It will be important to determine whether any other cytokines or chemokines associated with inflammatory sites influence effector and/or memory survival, as there is some evidence that bystander inflammatory environments can prolong the survival of effector T-cell populations (153).

The effector to memory transition of CD4⁺ T cells

Coincident with the contraction phase of activated CD4⁺ T cells is the initiation of the transition of surviving effectors to resting memory cells. Both our laboratory and others have shown that antigen withdrawal marks a critical starting point in this transition process and that few other signals are required (53,54,96). A remarkable feature of the effector to memory transition is its rapid kinetics. Employing *in vitro*-generated populations, we found that effectors rested for 3 days in the absence of antigen and cytokine, closely resemble long-term memory cells as judged by cell-surface phenotype, key functional attributes, and by gene expression profiles (53). A similar rapid transition is seen when we transfer effectors to antigen-free hosts (53,96,140). More recently, we have also shown that 3 day

rested effectors respond similarly to influenza challenge, as virus-specific memory cells generated through heterosubtypic priming (111, our unpublished observations). Another striking element of this transition program is that Th1-, Th2-, Th0-, and Th17-polarized effector populations all give rise, with identical kinetics, to corresponding memory populations. Similarly, we have shown that 3 days rest *in vitro* is sufficient for highly activated CD4⁺ T-cell effectors responding to influenza in the lung to assume phenotypic and functional criteria associated with long-term memory cells (131). Thus, beyond the rapid contraction of large activated effector populations that can pose a risk to the host, surviving effector cells rapidly transition to rest and the more benign memory mode during the termination of immune responses.

As the effector to memory transition often occurs during the period of contraction, it is important to ask which effector cells survive. The answer to this question remains unclear. Our own results and those of others (53,154,155) suggest that memory cells arise stochastically from effectors progressing along a linear differentiation pathway. However, it seems clear that effectors can reach a terminally differentiated state (156) and that certain molecules capable of influencing cell death, such as IFN γ R, can be differentially expressed on dividing T cells at very early phases of the response (157).

Contraction during secondary responses: role of IL-2

While contraction following primary CD4⁺ T-cell responses has been analyzed in several different model systems, contraction of T cells during recall responses is less well studied. As phenotypic, functional, and gene expression differences distinguish memory from naive CD4⁺ T cells (131), the signals impacting the contraction phase could also be distinct. Furthermore, significant heterogeneity has been observed in both human and mouse memory T-cell populations, as defined by phenotypic and functional criteria (158–160). In particular, several recent studies have found differences in the ability of memory CD4⁺ T cells to produce IL-2 in addition to other effector cytokines (56,161–165), and memory CD4⁺ T cells producing IL-2 in addition to IFN γ have been correlated with improved protection against a variety of pathogens compared to memory cells producing only IFN γ (166). Given the central role of IL-2 in both sensitizing responding CD4⁺ T cells to AICD and in rescuing them from antigen-independent as well as dependent apoptosis, it is possible that IL-2-positive versus -deficient memory populations specific for the same antigen would have different survival characteristics following *in vivo* antigen restimulation. This could have important consequences for vaccine design, especially when involving prime-boost regimens.

To test this hypothesis, we generated memory CD4⁺ T cells specific for influenza *in vitro* from wildtype or IL-2-deficient TCR Tg CD4⁺ T cells. We generated effectors in the presence of high amounts of exogenous IL-2, so that both populations were exposed to IL-2 during priming. Indeed, we observed similar expansion of both populations after 4 days of culture and also similar numbers of memory cells following a period of *in vitro* rest (authors' unpublished observations). We transferred equal numbers of wildtype or IL-2-deficient memory CD4⁺ T cells to naive wildtype mice. We then infected with influenza and observed, surprisingly, that both populations reached similar peak numbers in the lung, at levels significantly above those observed in primary responses. This finding suggests that autocrine IL-2 production is not involved in driving enhanced memory CD4⁺ T-cell responses *in vivo*. After expansion, the wildtype memory effector cells underwent extensive contraction, confirming that secondary effectors contract much like those generated during primary responses. However, mirroring our observations with naive IL-2-deficient responders (authors' unpublished observations), IL-2-deficient memory CD4⁺ T cells underwent significantly enhanced contraction (Fig. 8). This finding suggests that not all

memory CD4⁺ T cells are equivalent in terms of resistance to apoptosis during the contraction phase of recall responses and that ability to produce IL-2 may substantially enhance resistance to multiple mechanisms of cell death in responding memory CD4⁺ T-cell populations. Whether memory populations respond to other signals impacting antigen-dependent and -independent apoptosis pathways in a similar or different manner than during primary responses will require further study.

Concluding remarks

How the death of activated T cells is regulated *in vivo* remains unclear. It is evident that signals impacting the contraction of CD4⁺ T cells occur both during the effector phase of immune responses and also during priming, where cells are programmed to different fates. The evidence so far suggests that both antigen-dependent and antigen-independent pathways drive cell-death during the termination of productive CD4⁺ T-cell responses (Fig. 9). Thus, multiple mechanisms have the potential to shape contraction of CD4⁺ T-cell populations, but determining the relative contribution of discrete pathways during *in vivo* responses against pathogens is difficult. This is compounded by the fact that the influence of signals impacting contraction can differ dramatically depending on the pathogen challenge.

We feel that influenza infection provides an excellent model in which to study the regulation of contraction of CD4⁺ T cells for several reasons. First, a tremendous degree of contraction occurs during the period of just a few days. Second, antigen is confined largely to the lung, and antigen levels fall precipitously during the period of contraction. Third, contraction can be assessed in different organs in which cohorts of activated CD4⁺ T cells are exposed to very different stimuli in terms of levels of antigen and inflammation. Finally, the mouse model of influenza infection allows the implementation of powerful tools for the manipulation of critical signals impacting cell death.

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Fig. 1. T-cell contraction following influenza infection

Following maximal effector responses in the lung, an acute contraction phase (yellow highlighted) occurs in the lung that is coincident with viral clearance.

Fig. 2. CD4⁺ T-cell contraction following influenza infection

Naive TCR Tg CD4⁺ T cells recognizing influenza (HNT) were transferred to BALB/c hosts then infected with a sublethal dose of virus. Donor CD4⁺ T-cell numbers were determined between 7 and 30 days post infection. Data presented on a Log₂ scale to emphasize the early acute contraction in all sites, but more prolonged contraction seen in the lung.

Fig. 3. Lung-resident effector CD4⁺ T cells are most susceptible to AICD during influenza infection

Naive HNT CD4⁺ T cells were transferred to naive hosts, which were then infected with influenza. At 7 (shaded) and 14 (red line) days post infection, donor CD4⁺ T cells were recovered from the lung or draining lymph nodes (DLN). Cells were then restimulated with anti-CD3 for 20 hours and stained with 7-AAD to detect apoptotic cells.

Fig. 4. Effector CD4⁺ T cells rapidly lose susceptibility to AICD

(A) *In vitro*-generated Th1 effectors or effectors that rested for 3 days *in vivo* were either left unstimulated or restimulated for 20 h with peptide-pulsed antigen-presenting cells (APCs). Significantly fewer rested effectors underwent AICD, as determined by assessing a variety of markers associated with apoptotic death. (B) Naive HNT CD4⁺ T cells were compared to activated Th1 and Th2 effectors (generated *in vitro*), as well as memory cells (generated following adoptive transfer of *in vitro* effectors into antigen-free hosts). All populations were restimulated *in vitro* with antigen and APC, and CD4⁺ T-cell apoptosis in responding cultures was assessed by TUNEL staining.

Fig. 5. IFN γ does not appreciably impact contraction of responding CD4⁺ T cells during influenza infection

Naive wildtype (WT) HNT CD4⁺ T cells were transferred to WT BALB/c hosts or naive IFN γ KO HNT cells transferred to IFN γ KO hosts. Mice were then infected with a sublethal dose of influenza. Donor CD4⁺ T-cell numbers in the lung were determined at 7 and 14 days post-infection.

Polarized T cell subset

Fig. 6. Th-polarization impacts antigen-independent contraction of CD4⁺ T-cell effectors
Naive HNT cells were cultured with antigen and antigen presenting cells under Th0, Th1, Th2, Th17, or Treg polarizing conditions *in vitro*. After 4 days, well-polarized effectors were rested for three days in the absence of antigen and cytokines and the number of live cells determined (depicted as percentage of effector cells).

Fig. 7. *In vitro* contraction of Th1-polarized effector populations is regulated by effector molecules

Naive WT, IFN γ KO, or perforin KO HNT CD4⁺ T cells cultured under Th1-polarizing conditions *in vitro*. After 4 days, resulting effectors were rested for three days in the absence of antigen and cytokines and the number of live cells determined (depicted as percentage of effector cells).

Fig. 8. IL-2 regulates contraction of memory-effectors

Naive WT or IL-2 KO CD4⁺ T cells were cultured for 4 days under Th1-polarizing, including exogenous IL-2 conditions and then rested for 3 days in the absence of antigen and cytokines to generate memory. WT and IL-2 KO memory cells were transferred to WT hosts, then infected with influenza, and donor CD4⁺ T-cell numbers were determined between.

Fig. 9. Signals impacting contraction of CD4⁺ T cells *in vivo*

In the schematic, we point out key roles for factors that influence contraction at different stages of response (red arrows denote increased contraction, and blue arrows denote reduced contraction). First, in regulating the programming of effectors to susceptibility to contraction by inducing maximal differentiation and migration to sites of infection (to the lung in the influenza model). Second, at the effector stage where availability of antigen, and autocrine and paracrine cytokines can further modulate the extent of death and degree of contraction, determining what fraction of the population survives to memory.

Table 1

CD4 T cell effector subsets

Th Subset	Polarizing Factors	Transcription Factors	Signature Cytokines	Susceptibility to AICD
Th1	IL-12, IFN- γ	T-bet	IFN- γ , TNF, IL-2	++++
Th2	IL-4	GATA-3	IL-4, IL-5, IL-13	+
Th17	TGF- β , IL-6	ROR- γ t	IL-17, IL-21, IL-22	++
TFh	IL-6, IL-21	Bcl-6	IL-4, IL-21	?
Treg	TGF- β , RA	Foxp3	IL-10	?