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Inflammatory Cytokines as a Third Signal for T Cell Activation

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Summary

CD8 T cells require a third signal, along with Ag and costimulation, to make a productive response and avoid death and/or tolerance induction. Recent studies indicate that IL-12 and Type I IFN (IFN α/β) are the major sources of signal 3 in a variety of responses, and that the two cytokines stimulate a common regulatory program involving altered expression of about 350 genes. Signal 3-driven chromatin remodeling is likely to play a major role in this regulation. Although less well studied, there is emerging evidence that CD4 T cells may also require a 'third signal' for a productive response and that IL-1 can provide this signal. Signal 3 cytokines can replace adjuvants in supporting in vivo T cell responses to peptide and protein antigens, and a better understanding of their activities and mechanisms should contribute to more rational design of vaccines.

Introduction

T cells proliferate and differentiate in response to signals from the TCR and costimulatory receptors, predominantly CD28, and the differentiation pathway can be influenced by additional signals from the environment to result in diverse phenotypic and functional outcomes. The cytokine milieu experienced by Ag-activated CD4 T cells determines the spectrum of cytokines that will be produced by the resulting effector cells. Much is known about the identities and regulation of transcription factors that control this skewing to yield TH1, TH2 or TH17 CD4 T cells, and chromatin remodeling plays a central role in these differentiation processes. Although not as extensively studied, the differentiation fate of Ag-activated CD8 T cells is influenced in a similar manner and involves regulation of many of the same transcription factors.

For CD8 T cells, signals provided by inflammatory cytokines also have a more fundamental role in regulating responses. TCR and costimulatory signals initiate proliferation of naïve cells, but in the absence of a specific cytokine signal the cells fail to develop optimal effector functions, survive poorly, and do not form a responsive memory population [1]. Thus, inflammatory cytokines act as a 'switch' that determines whether Ag and costimulatory signals lead to tolerance in their absence (deletion/anergy), or in their presence, a productive response leading to strong effector functions, survival and memory formation (Fig. 1). Because this cytokine signal is required for a productive response, along with TCR (signal 1) and costimulatory signals (signal 2, including IL-2), it has been termed 'signal 3'. The requirement for a signal 3 inflammatory cytokine provides a means for a CD8 T cell that encounters Ag to

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determine if there is 'danger' present, and to respond accordingly. In vitro experiments initially identified IL-12 and IFN α/β as having signal 3 activity for CD8 T cells, and more recent evidence indicates that they may be the predominant sources of signal 3 for CD8 T cell responses to a variety of in vivo stimuli. The molecular mechanisms involved in the effects of IL-12 and IFN α/β are beginning to be elucidated, and appear to include cytokine-driven chromatin remodeling. Although less well studied, recent evidence suggests that like CD8 T cells, naïve CD4 T cells may also require a cytokine-dependent 'signal 3' for a productive response to Ag, and that this may be provided by IL-1.

Signal 3 cytokine requirements for CD8 T cell responses

In vitro studies using artificial APC (aAPC) provided the initial evidence that IL-12 and IFN α/β could provide a critical third signal, along with Ag and B7-1, to enhance CD8 T cell clonal expansion, and promote development of effector functions including cytolytic activity and IFN γ production [2,3]. In vivo studies examining peptide immunization models demonstrated that these cytokines could replace the need for adjuvant by acting directly on the CD8 T cells to convert tolerance induction to a productive response [4,5]. Co-administration of IL-12 with peptide increased clonal expansion, supported development of effector functions and resulted in a long-lived memory population. In the absence of cytokine or adjuvant, very few cells remained long-term following peptide immunization and those that did were anergic and could not respond to even potent stimulation with Ag and adjuvant. Sikora et.al. [6] have recently demonstrated that IFN α can effectively stimulate antitumor immunity in response to peptide vaccine by increasing the numbers, effector functions and long-term persistence of tumor-specific CD8 T cells with an effector-memory phenotype. These effects resulted from direct action of the IFN α on the tumor-specific CD8 T cells. Given these promising results, and since IFN α is already an approved therapy for melanoma, testing its role as a vaccine adjuvant in human clinical trials should be straightforward.

A role for signal 3 cytokines in programming for memory has also been demonstrated in experiments employing in vitro stimulation of highly purified naïve CD8 T cells under well-defined conditions, followed by transfer into normal mice to monitor the transition to memory. Malek and coworkers [7,8] showed that CD8 T cells stimulated for 3 days in vitro, under conditions where signal 3 was likely to be present, could rapidly transition to a memory phenotype and persist long term upon transfer into naïve mice. Using this approach, we found that OT-I CD8 T cells expanded during 3 days of in vitro stimulation with aAPC having Ag and B7-1 on the surface, but the cells died within a few days following transfer into mice [9]. In contrast, if IL-12 was present during the in vitro stimulation the cells continued to expand for several days following transfer and then underwent a characteristic contraction phase, and 15 – 20% of the peak number of cells remained long-term. The surviving cells had a memory phenotype, included both central (T_{cm}) and effector (T_{em}) memory cells, and rapidly re-expanded and protected against challenge with *Listeria monocytogenes* (LM) that expressed OVA. Provision of IFN α during the in vitro stimulation has a similar effect, although the size of the memory pool is not as great as with IL-12 stimulation (Xiao et.al., unpublished). These results strongly suggest that signals delivered by IL-12 or IFN α/β during the initial response to Ag not only support development of effector functions but also program cells to subsequently develop a responsive memory population.

Adjuvant activity in immunization models demonstrated that IL-12 and IFN α could provide signals to support responses, but did not address the question of whether productive CD8 T cell responses to physiological stimuli normally depended upon these cytokines. In fact, results of numerous studies employing mice deficient for one or the other of the cytokines, or their receptors, raised the possibility that they might not be required in many cases. Interpretation of such studies is not straightforward, however, given the redundant effects of IL-12 and

IFN α/β , and is made further complicated by the fact that IFN α/β can inhibit IL-12 production [10]. Definitive determination of their roles has therefore required the use of models employing adoptive transfer of cytokine receptor-deficient CD8 T cells into wild type recipients. Using this approach, it was shown that CD4-dependent activation of CD8 T cells to mediate rapid rejection of an ectopic allogeneic heart transplant depended upon the CD4 T cells conditioning DC to produce IL-12 which then provided the third signal needed to support development of graft-specific CD8 T cell effector function [11].

The first direct evidence for a signal 3 cytokine role in response to a virus came from the demonstration that the CD8 T cell response to LCMV requires IFN α signaling directly to the T cells [12,13]. When TCR transgenic P14 CD8 T cells specific for the GP33-41 epitope were adoptively transferred and the recipient mice infected with LCMV, clonal expansion of the cells was reduced by over 99% if they did not express the Type I IFNR receptor (IFN-1R). Because primary expansion was almost completely eliminated, these results could not directly address whether IFN α had a role in memory programming independent of initial clonal expansion. The very small number of IFN-1R-deficient cells that expanded during the primary response appeared to undergo a normal transition to memory. It is not clear whether these represent a small subpopulation that is independent of a signal 3 cytokine for memory, or a small number of cells that received a sufficient IL-12 signal to program for memory.

In contrast to LCMV, IFN-1R-deficient P14 cells responded strongly to vaccinia virus (VV), vesicular stomatitis virus (VSV), or LM that expressed the GP33-41 epitope [12,14]. Using cytokine-receptor deficient OT-I CD8 T cells and pathogens expressing the OVA epitope, we have found that responses to VV and LM by IL-12R-deficient cells are substantially impaired, and further impaired when both the IL-12R and IFN-1R are absent [9]. Lack of the receptors had a modest effect on clonal expansion at the peak of the responses, at most a 3-fold reduction, and function was only partially impaired. However, development of memory was completely dependent on either IL-12 or IFN α/β signals. IL-12 signaling played the major role for generation of memory in response to VV infection, while both IL-12 and IFN α/β made substantial contributions to the generation of memory in response to LM.

In another recent study, Keppler et.al. [15] examined responses of P14 cells deficient in the IL-12R to VSV, VV, and LM. The response to LM was strongly decreased for the IL-12R-deficient P14 cells, consistent with the results of Xiao et.al. described above [9]. In contrast, responses of the IL-12R-deficient P14 cells to VV and VSV did not differ significantly from those of WT P14 cells [15]. Since responses to both of these pathogens are also little impaired for IFN-1R-deficient P14 cells [12,14] this could suggest that neither IL-12 nor IFN α/β are involved. Alternatively, it may be the case that both cytokines are present at sufficient levels in these infections to provide a third signal, so that eliminating the response to either one alone has no effect. That the latter may be the case is suggested by the results of Xiao et.al. [9] demonstrating that OT-I cells lacking both receptors fail to form a memory population in response to VV. OT-I cells lacking just the IL-12R also had a substantially reduced response to VV [9], in contrast to the strong response of IL-12R-deficient P14 cells [15]. The basis for this discrepancy is unclear, but may stem from the use of different TCR transgenic T cells and/or from differing levels of IL-12 and or IFN α/β production depending on the recombinant virus that is used.

In addition to the transplant, virus and bacterial models described above, direct signaling to CD8 T cells by IFN α/β and IL-12 has also been shown to be important for responses to tumor [16] and *Toxoplasma gondii* infection [17], respectively. We have also found that OT-I cells lacking both IL-12R and IFN-1R fail to form memory in response to adjuvant-dependent immunization with OVA peptide or protein, indicating that a signal from one or the other of these cytokines is necessary (Casey and Mescher, unpublished results). Finally, Hamilton and

Jameson [18] have demonstrated that CD4 T cell help is required for generation of protective memory cells by Ag-independent homeostatic expansion of CD8 T cells, and that exogenous IL-12 could substitute for the CD4 T cells. Thus, accumulating evidence is suggesting that IL-12 and/or IFN α/β may be the major sources of signal 3 in many CD8 T cell responses. There is also evidence, however, that they are not the only source of signal 3 under all circumstances. Orgun et.al [19] showed that mice lacking both IL-12p40 and IFN-1R developed a CD8 T cell memory population comparable to that of WT mice in response to LM infection, i.e. in the absence of any IL-12 or IFN α/β signal to support CD8 memory programming. Similarly, Xiao et.al. [9] found that OT-I cells deficient in both IL-12R and IFN-1R developed memory comparable to that of WT cells upon transfer into IL-12-deficient hosts and challenge with VV expressing the OVA epitope. This suggests that the altered cytokine milieu in the IL-12-deficient host may result in the presence of an alternate signal 3 cytokine that is not present in sufficient amounts in the normal host to support CD8 T cell responses. IL-21 has signal 3-like activity in vitro [20], and during chronic viral infection it can provide a signal to CD8 T cells to prevent their deletion [21-23]. Whether naïve cells making an initial response to Ag can be programmed by IL-21 to develop memory remains to be determined.

During CD8 T cell responses to pathogens a population of relatively short-lived effector cells (SLEC) is generated that is characterized by high KLRG1 and low CD127 expression [24, 25]. Whether this population expands at the expense of the long-lived memory population is not clear. The percent of activated cells that survive long-term as memory cells is decreased when the effector pool has a high proportion of SLEC, but the absolute size of the memory pool may be relatively unaffected. IL-12 signaling can promote expansion of the SLEC pool in a T-bet-dependent manner [24,25]. At the same time, it is clear that IL-12 can provide a critical signal needed for memory development under conditions where it does not promote generation of SLEC. Thus, when cells are stimulated in vitro with Ag and B7 on aAPC and then transferred into normal mice, the cells only survive and transition to memory if IL-12 is also present during the in vitro activation period, but very few if any of the cells express detectable KLRG1 under these conditions [9]. Conversely, neither IL-12 nor IFN α signaling is required for formation of KLRG1+ cells, since OT-I cells deficient for both cytokine receptors developed a KLRG1+ population comparable to that of WT OT-I cells (about 30%) in response to VV infection [9]. Consistent with this, Sarkar et.al. [26] have shown that prolonged Ag signaling can also lead to increased numbers of SLEC in CD8 T cells responding to virus infection. Taken together, these observations suggest that IL-12 can provide an early signal necessary to program development of a long lived population of central and effector memory cells, but prolonged signaling by IL-12, and possibly Ag, drives the formation of SLEC [25-27].

Molecular basis of signal 3-dependent differentiation

There are numerous reports of IL-12 influencing the expression of a variety of proteins in CD8 T cells. As an initial approach to understanding the molecular basis of signal 3 cytokine effects on naïve cells, global cytokine-dependent regulation of gene expression was determined over the course of a 3 day in vitro response by naïve CD8 T cells [28]. Stimulation of naïve cells for 72hr with aAPC having Ag and B7-1 on the surface (2 signals), resulted in altered expression of about 2,300 genes. When IL-12 was also present the expression levels of about 730 genes were altered in comparison to levels expressed in response to 2 signals. Similarly, IFN α altered the expression of about 610 genes in comparison to 2 signals. Since the functional outcome is similar for both cytokines the set of genes regulated in common by both was of greatest interest, and included about 350 genes. This common set of genes included many whose products are involved in effector functions (granzymes, IFN γ , FasL), proliferation and costimulation (CD25, Ox-40, 4-1BB), survival (serine protease inhibitor 6, Bcl-3) and trafficking and migration. Both cytokines also regulated expression levels of mRNA for a number of

transcription factors, several of which are known to play important roles in CD8 T cell differentiation including T-bet [29,30], eomesodermin [31] and Blimp-1 [32-35]. Examination of the kinetics of signal 3-cytokine-dependent gene regulation over the 3-day course of differentiation revealed that a large subset of the 350 commonly regulated genes were upregulated by 24 hr in response to just 2 signals. However, expression then declined to near baseline (naïve) levels by 72 hr, but was increased and sustained when either IL-12 or Type I IFN were present.

The finding that much of the signal 3-dependent gene regulation program was initiated by TCR and CD28 signals early but was rapidly extinguished unless IL-12 or IFN α signals were available suggested that chromatin remodeling mechanisms might be involved. Support for this was provided by the finding that stimulating naïve cells with Ag and B7-1 in the presence of histone deacetylase inhibitors mimicked the effects of IL-12 and IFN α , suggesting that the cytokines might act, at least in part, to relieve repression by promoting increased histone acetylation to allow continued gene expression [28]. This was directly shown for granzyme B and eomesodermin, where IL-12 and IFN α were found to stimulate increased association of acetylated histones with the promoters of these genes [28]. IL-12 also causes long-range hyperacetylation in the promoter and exon regions of the IFN γ gene when naïve CD8 T cells are stimulated with anti-TCR and anti-CD28 mAbs [36].

There is accumulating evidence that the more rapid and robust responses of memory CD8 T cells in comparison to naïve cells may result at least in part from epigenetic changes in critical genes, involving changes in histone acetylation [37,38] and methylation [39] as well as DNA methylation [40,41]. Histone acetylation modifications that would promote greater transcriptional accessibility have been demonstrated in memory cells for several genes including eomesodermin, perforin and granzyme B and IFN γ [37,41] and DNA methylation changes for the perforin [40], IL-2 and IFN γ loci [41]. In some models, CD4 T cell help is necessary during priming for the generation of responsive memory CD8 T cells, and Northrop et.al. [42] have recently shown that this requirement for CD4 T cells is bypassed if priming of the CD8 T cells is done in the presence of Trichostatin A to inhibit histone deacetylation. Thus, inhibiting histone deacetylation during the initial 3 days of response to TCR and CD28 signals not only promotes signal 3-independent development of effector functions [28], it also promotes CD4 helper-independent programming for memory [42]. One way by which CD4 T cells can provide help for CD8 priming is through stimulation of DC to produce signal 3 cytokines [11]. It seems reasonable to speculate that chromatin remodeling events driven by IL-12 and/or IFN α during initial priming not only contribute to development of functions but also contribute to the ability of these cytokines to program the cells to survive and become memory cells.

Finally, while IL-12 and IFN α each support function and memory and regulate expression of a common set of genes, they also differentially regulate a relatively large number of genes. IL-12 regulates expression of about 375 genes, and IFN α about 255 genes, which are not included in the common set. Many of these additional genes have important functions in T cells, and their differential regulation raises the possibility that the properties of effector and memory CD8 T cell populations may differ depending on which signal 3 cytokine supported their initial response to Ag. This is illustrated in Table I that shows the changes in expression levels in effector CD8 T cells (72 hr stimulation) of genes that encode molecules involved in adhesion and migration functions. Four distinct classes are apparent; those regulated in common by IL-12 and IFN α , those regulated by only one or the other of the cytokines, and those regulated in opposite directions by the cytokines. Assuming that this differential mRNA expression reflects differences in protein expression, then the effector CD8 T cells resulting from IL-12-dependent versus IFN α -dependent differentiation may differ in functionally important ways with respect to their migration, localization and ability to recruit other cells to

the site of Ag. Determining if this is the case, and if the differential expression persists as the effector cells transition to memory, may have important implications for vaccine design.

Do naïve CD4 T cells require a third signal?

Earlier work had suggested that IL-1 might provide a third signal to support CD4 T cell responses. CD4 T cells respond minimally to immunization with peptide Ag in the absence of adjuvant, but co-administration of IL-1 substantially enhanced proliferation and differentiation in response to the Ag [43]. IL-12 did not have this effect, despite its important role in skewing CD4 T cell responses. Thus, IL-12 could replace the need for adjuvant in supporting a CD8 T cell response to peptide Ag [5] but IL-1 could not, while IL-1 could replace the need for adjuvant for a CD4 T cell response while IL-12 could not. A subsequent study provided evidence that IL-1 was acting at least in part on the Ag-presenting cells [44]. That IL-1 might also act directly on the CD4 T cells was suggested by in vitro experiments examining responses of highly purified naïve CD4 T cells to aAPC presenting Ag and B7-1 [2]. IL-1 was found to enhance clonal expansion in response to the Ag, while IL-12 did not.

Ben-Sasson et.al. [45] have now shown that in fact IL-1 can act directly on CD4 T cells in vivo to enhance Ag-driven expansion and differentiation. Administration of IL- α or IL- β strongly increased primary responses of functionally active polyclonal or TCR transgenic CD4 T cells to peptide epitope or protein Ags and there was a corresponding increase in size of the resulting memory populations, while no memory cells were detectable when immunization was with Ag alone. Experiments employing adoptive transfer of OT-II cells into IL-1R-deficient hosts, and IL-1R-deficient OT-II cells into WT hosts demonstrated that the major effect of IL-1 resulted from its direct action on the responding Ag-specific CD4 T cells. In addition, LPS-dependent enhancement of clonal expansion was reduced by over 50% in the presence of IL-1 receptor antagonist, indicating that a large portion of the adjuvant effect is due to IL-1.

Thus, the direct effects of IL-1 on CD4 T cells responding to Ag exhibit a striking parallel to the effects of the signal 3 cytokines IL-12 and IFN α/β acting on CD8 T cells. These results strongly suggest that IL-1 can provide a third signal to CD4 T cells that is needed to support a productive response and establish a memory population. Comparison of the molecular basis of the effects of IL-1 on CD4 T cells to those of IL-12 and IFN α/β on CD8 T cells is likely to provide considerable insight into the requirements for promoting T cell survival and differentiation as the cells respond to Ag and transition to memory. Eliminating IL-1 or blocking its action has been found to have variable effects on in vivo CD4 T cell responses ranging from minimal to dramatic, depending upon the response being examined. This suggests that there may be some redundancy in signal 3 for CD4 T cells, as is the case for CD8 T cells. At the same time, there is clearly a high degree of specificity; different cytokines are needed to provide signal 3 to CD8 versus CD4 T cells, and a large number of other proinflammatory cytokines have failed to exhibit signal 3-like activity for either CD8 [3] or CD4 [43,45] T cells.

Conclusions

There is now considerable evidence that CD8 T cells need a third signal, along with Ag and costimulation, to undergo robust clonal expansion, develop strong effector functions and form a responsive memory population. Recent studies are indicating that IL-12 and Type I IFN are the predominant sources of this third signal for a variety of responses to transplanted tissue, tumors, pathogens and adjuvant-based vaccines. Either cytokine can support responses, and which plays the dominant role for a particular challenge is likely to be determined by the relative amounts of each that are available during the early period of Ag recognition. The signal 3 cytokines act, at least in part, by promoting chromatin remodeling to maintain transcription of numerous genes needed for differentiation and effector functions. Epigenetic memory of chromatin remodeling contributes to the more rapid and robust response of memory cells upon

re-challenge, and it appears likely that at least some of this remodeling occurs in response to the signal 3 cytokines during the early phase of Ag recognition and differentiation. While IL-12 and IFN α regulate a common set of about 350 genes in cells responding to Ag and costimulation they also uniquely regulate similar numbers of genes, many of which may influence the functional properties of the effector populations. Determining if this is in fact the case, and how programming by the cytokines differs, should contribute to the more rational design of vaccine strategies. The effects of IL-1 on CD4 T cells responding to Ag parallel many of the effects of IL-12 and IFN α on CD8 T cells, suggesting that it may provide a 'third signal' needed to support a productive CD4 T cell response. If so, it will be important to determine if other cytokines can also provide this signal, and to compare the molecular basis for signal 3 effects in CD4 and CD8 T cells, as this is likely to provide considerable insight into the requirements for promoting T cell survival and differentiation as the cells respond to Ag and transition to memory.

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Figure 1. Activation of naïve CD8 T cells requires three signals: Ag, costimulation and either IL-12 or IFN α/β

Stimulation with Ag and B7-1 results in extensive proliferation, but survival is compromised and development of effector functions is suboptimal. The small numbers of cells that survive long term are anergic. When either IL-12 or IFN α/β are present, proliferation is comparable but survival is increased, the cells develop strong effector functions, and a protective memory population is formed.

Table I
Differential regulation of gene expression by IL-12 and IFN α

Oligonucleotide microarray analysis was used to determine mRNA expression levels for naïve CD8 T cells stimulated with Ag and B7-1 in the absence or presence of IL-12 or IFN α . Expression of about 350 genes is regulated in common by the two cytokines, and each cytokine also uniquely regulates 200 to 300 genes, as illustrated here. Values shown for selected genes are the fold change at 72hr in mRNA expression level in the presence of the indicated cytokine in comparison to the level without cytokine added. Increases in expression are shown in red, decreases in green, and nc indicates no change in comparison to cells stimulated with just Ag and B7-1. Data are from Agarwal et.al. [28].

Adhesion & Migration Differences				
	IL-12	IFN α		
	72h	72h		
	Fold Change Relative to		Function	
Gene	2 signal stimulation			
			Common to Both	
Xcl1	1.7	2.1	CD4, DC recruit	chemokine (C motif) ligand 1
Cxcr3	-3.5	-2.1	integrin activation, chemotactic migration	chemokine (C-X-C motif) receptor 3
Itgb7	-1.7	-2.3	mucosal migration	integrin beta 7
Sell	-3.0	-2.3	L-selectin	selectin, lymphocyte
Ccr5	10.6	2.0	migration to inflamm	chemokine (C-C motif) receptor 5
Nptn	-1.9	-1.7	cell-cell, cell-substrate interactions	neuroplastin
			IL-12 Only	
Ccl3	2.5	nc	MIP-1a granulocyte activation, inflam cytokine	chemokine (C-C motif) ligand 3
Ccl4	2.8	nc	MIP-1b granulocyte activation, inflam cytokine	chemokine (C-C motif) ligand 4
Ccr2	12.1	nc	migration to inflamm	chemokine (C-C motif) receptor 2
Cxcr4	1.7	nc	chemotactic receptor (ligand is SDF-1 (CXCL12)	chemokine (C-X-C motif) receptor 4
Cmtm7	-2.0	nc	chemokine-like super family	CKLF-like MARVEL
Itga4	-2.0	nc	alpha 4 integrin (with b1) homing receptor	integrin alpha 4
Itga6	-2.3	nc	alpha 6 integrin	integrin alpha 6
Lgals3	3.0	nc	galectin-3 adhesion	lectin, galactose binding, soluble 3
Nrp1	-2.1	nc	coreceptor of VEGF receptor, migration	neuropilin 1
			IFN α only	
Cd83	nc	2.6	unknown function (on DC)	CD83 antigen
Cd83	nc	3.5	unknown function (on DC)	CD83 antigen
Cxcl10	nc	2.8	NK, B, neutrophil, TH1 attractant	chemokine (C-X-C motif) ligand 10
Crtam	nc	-4.3	on activated T's, adhesion??	cytotoxic and regulatory T cell molecule
			Opposite	
Lgals9	-2.0	1.9	eosinophil attractant, galectin-9	lectin, galactose binding, soluble 9