Regulation of innate CD8$^+$ T-cell activation mediated by cytokines

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Virus-specific CD8$^+$ T cells develop the ability to function in an "innate" capacity by responding to a remarkable array of cytokines in a TCR-independent manner. Although several cytokines such as IL-12 and IL-18 have been identified as key regulators of CD8$^+$ T-cell activation, the role of other cytokines and the ways in which they interact with each other remain unclear. Here, we have used an unbiased, systematic approach to examine the effects of 1,849 cytokine combinations on virus-specific CD8$^+$ T-cell activation. This study identifies several unexpected cytokine combinations that synergize to induce antigen-independent IFNγ production and CD69 up-regulation by CD8$^+$ T cells in addition to cytokines that exhibit differential regulatory functions, with the ability to either enhance or inhibit T-cell IFNγ production, depending on which cytokine partner is present. These findings underscore the complexity of cytokine interactions while also providing insight into the multifaceted regulatory network controlling virus-specific CD8$^+$ T-cell functions.

lymphocytic choriomeningitis virus | mouse | interleukin | lymphocyte

In addition to responding to peptide antigen, CD8$^+$ T cells maintain an innate capacity to be activated and produce IFNγ in response to cytokines elicited during infection (1–4). This allows CD8$^+$ T cells to act as "sentinels" for subsequent, unrelated infections even when their cognate antigen may not be present. Antigen-independent T-cell activation can be triggered under a variety of different disease conditions, and therefore CD8$^+$ T cells may be exposed to a diverse array of cytokine combinations. Viral, bacterial, fungal, and parasitic infections can each produce a unique inflammatory environment. For instance, lymphocytic choriomeningitis virus (LCMV) infection triggers IFNα and IFNβ production (5), in addition to IL-1, IL-6, IL-10, IL-15, IL-21, IL-33, and TRAIL (6). Furthermore, LCMV-specific T cells readily produce IFNγ, TNFα, IL-2, and CD40L following stimulation with viral peptide antigen (7). These results indicate that just one viral infection can trigger greater than 1/4 of all of the cytokines examined in this study. The complexity of the microenvironment is often further impacted by coinfection with other types of pathogens – with perhaps the most prominent example being influenza complicated by secondary bacterial infection (8). A number of cytokines have been identified as key T-cell activating factors, most notably IL-12 and IL-18 (3, 9, 10). Lipopolysaccharide (LPS) from Gram-negative bacteria triggers production of IL-12 and IL-18 (3, 4) and while these two cytokines are able to induce modest levels of IFNγ production by CD8$^+$ T cells on their own, they exhibit strong synergy when used in combination (3, 9, 10) or with other cytokines such as IL-7, IL-15, or IFNα/β (11–14). Together, these likely represent only a small subset of possible cytokine interactions that may regulate virus-specific T-cell functions.

Here, we describe a study examining the effects of 43 commercially available murine cytokines (Table S1) tested either individually or in pairs to determine their relative capacity to activate or repress virus-specific effector and memory CD8$^+$ T-cell responses directly ex vivo following acute lymphocytic choriomeningitis virus (LCMV) infection. T-cell activation was determined on the basis of production of IFNγ, a cytokine with direct antiviral activity (15), or CD69, a surface glycoprotein that regulates lymphocyte migration and is one of the earliest markers to be up-regulated during T-cell activation (16, 17). After this initial unbiased screen, cytokines with the ability to regulate IFNγ production or CD69 expression were tested for their ability to activate purified CD8$^+$ T cells during the acute or memory phase of LCMV infection. Interestingly, effector and memory T cells differed sharply in their responses to cytokine-induced activation, and several of the most stimulatory combinations involved either IL-12 or IL-18 paired with previously undescribed cytokine partners. This study helps define the landscape of potential T-cell:cytokine interactions that modify T-cell function during infection and provides a foundation for developing better cytokine-based therapeutics for either improving appropriate T-cell responses (6, 18) or reducing unwanted CD8$^+$ T-cell–mediated immunopathology (1, 3, 19).

Results

Differential Regulation of Cytokine-Induced IFNγ Production. CD8$^+$ T cells must integrate multiple inflammatory signals within the local microenvironment, which combine to regulate effector functions during infection. Thus, the response to individual signals is typically context dependent. Although most immunomodulatory cytokines in our study either induced or inhibited CD8$^+$ T-cell activation, a unique subset of cytokines triggered different biological outcomes depending on the partner cytokine with which they were paired (Fig. 1). For instance, IL-4 and IL-10 are traditionally considered to be cytokines that down-regulate IFNγ/TH1 responses (20, 21). IL-4 sharply reduced IFNγ production in response to IL-12 or IL-15, but had a much less dramatic effect on IL-18–induced IFNγ production (Fig. 1). Similarly, IL-10 was a potent inhibitor of IFNγ production triggered in response to IL-12 or IL-15. However, when IL-10 was paired with IL-18, an unexpectedly strong and reproducible enhancement of IFNγ production by CD8$^+$ T cells was observed (Fig. 1). Together, these results indicate that IL-4 inhibits some, but not all cytokine-mediated T-cell activation events, whereas IL-10 can either inhibit or coactivate antiviral CD8$^+$ T-cell responses depending on the context of the local cytokine microenvironment.

Direct Activation Versus Indirect Activation of Virus-Specific CD8$^+$ T Cells by Cytokines. Stimulation of T cells may occur directly, as occurs when a T cell recognizes its cognate antigen, or indirectly, as is the case when microbial products such as LPS or CpG DNA induce the production of cytokines by neighboring cells, which in turn modulate T-cell function (3, 22). In our initial screening of 1,849 cytokine combinations, we tested each cytokine pair directly ex vivo at 8 d or >60 d post-LCMV infection using splenocyte cultures containing CD8$^+$ T cells in addition to other accessory cells. The goal of this screening approach was to identify measurable regulatory cytokine combinations, regardless of whether they manipulated CD8$^+$ T cells by direct interactions or indirectly through the development of

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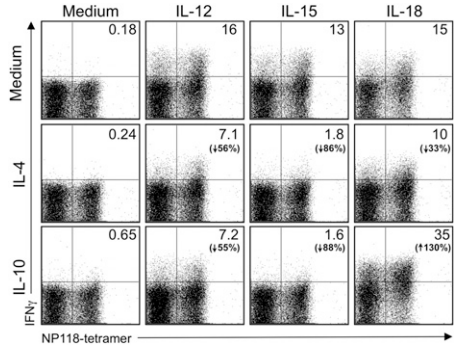

Fig. 1. Differential regulation of IFNγ production by virus-specific CD8$^+$ T cells. At 8 d post-LCMV infection, splenic CD8$^+$ T cells were stimulated in vitro with the indicated cytokines at 100 ng/mL for 6 h before intracellular staining for IFNγ and analysis by flow cytometry. Numbers in the *Upper Right* quadrant of each dot plot represent the percentage of CD11a^{high}NP118-tetramer$^+$ CD8$^+$ T cells expressing IFNγ, after background subtraction of medium controls (*Upper Left* dot plot). Numbers in parentheses represent the percent increase or decrease of IFNγ$^+$ T cells following incubation with each cytokine pair relative to incubation with IL-12, IL-15, or IL-18 alone. Data are representative of six BALB/c mice from three independent experiments.

a cytokine cascade involving other cell types. Importantly, virus-specific CD8$^+$ T cells from BALB/c and C57BL/6 mice responded similarly to a representative panel of cytokine combinations, indicating that the cytokine-mediated T-cell activation events described herein are not mouse strain or T-cell epitope specific (Fig. S1). We identified a subset of cytokines that elicited T-cell regulatory activity in mixed splenocyte cultures when paired with at least one other partner cytokine, and these cytokines were retested using magnetic activated cell sorting (MACS)-purified CD8$^+$ T cells analyzed in parallel. As expected, the prototypical T-cell–activating cytokine pair, IL-12 + IL-18, was one of the most potent combinations identified in our studies (Fig. 2 and Table 1). At 8 d postinfection, this cytokine pair induced IFNγ production in ∼73% of NP118-specific CD8$^+$ T cells (versus 11 or 5% IFNγ$^+$NP118-tetramer$^+$CD8$^+$ T cells incubated with IL-12 or IL-18 alone, respectively), with little or no loss in synergistic direct ex vivo IFNγ production observed in MACS-purified CD8$^+$ T-cell cultures. Likewise, the previously undescribed combination of IL-2 + IL-33 up-regulated IFNγ production by T cells equally well in both bulk splenocyte cultures and MACS-purified CD8$^+$ T-cell cultures (Fig. 2). In contrast, when CD8$^+$ T cells were stimulated with IL-10 + IL-18 or IL-2 + IL-15, CD8$^+$ T-cell activation was greatly reduced in MACS-purified cultures, indicating a partial (e.g., IL-10 + IL-18) to nearly absolute (e.g., IL-2 + IL-15) requirement for accessory cells to facilitate

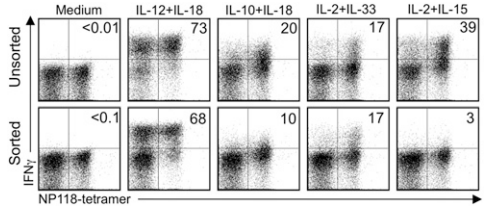

Fig. 2. Direct versus indirect activation of virus-specific CD8$^+$ T cells by cytokines. Splenocytes from BALB/c mice at 8 d after LCMV infection were stimulated with the indicated cytokines directly ex vivo, or CD8$^+$ T cells were purified by MACS (>95% purity) before direct ex vivo stimulation. Numbers in the *Upper Right* quadrant of each dot plot represent the percentage of IFNγ$^+$ CD11a^{high}NP118-tetramer$^+$ CD8$^+$ T cells. Data are representative of four mice from two independent experiments.

Table 1. Summary of the most potent cytokine combinations capable of triggering IFNγ production by virus-specific effector and memory T cells

	Effector		Memory	
Cytokine combination	% IFNγ$^+$ unsorted	% IFNγ$^+$ CD8 sorted	% IFNγ$^+$ unsorted	% IFNγ$^+$ CD8 sorted
IL-12 + IL-18	76.6	77.5	31.6	41.3
IL-12 + TNFα	67.4	47.9	29.7	24.5
IL-12 + IL-33	53.3	36.9	9.3	12.2
IL-2 + IL-18	52.7	46.9	4.9	10.0
IL-2 + IL-12	52.0	28.1	8.5	12.3
IL-12 + IL-15	44.0	8.9	12.6	7.6
IL-10 + IL-18	36.9	32.6	1.3	2.6
IL-18 + IL-21	34.7	33.7	2.8	5.9
IL-18 + IFNβ	31.6	28.5	13.7	17.1
IL-15 + IL-18	29.8	25.4	1.8	3.1
IL-12 + TL1A	27.7	6.2	7.8	8.2
IL-2 + IL-33	23.7	15.7	1.6	2.6
IL-7 + IL-12	22.0	3.3	2.7	3.0
IL-2 + TNFα	21.8	5.9	<1	<1
IL-15 + IL-33	18.5	13.0	1.8	2.3
IL-21 + IL-33	16.1	10.6	1.0	2.7
IL-10 + IL-33	14.0	8.8	<1	<1
IL-18 + IFNα	13.4	6.7	2.1	2.4
IL-33 + IFNβ	13.1	8.9	3.0	4.6
IL-2 + IL-15	11.9	3.5	1.4	1.0
IL-12	14.9	2.1	<1	<1
IL-18	6.2	1.7	<1	<1
IL-2	3.5	1.7	<1	<1
IL-33	2.2	<1	<1	<1
IL-15	1.4	<1	<1	<1
IL-7	<1	<1	<1	<1
IL-10	<1	<1	<1	<1
IL-21	<1	<1	<1	<1
TNFα	<1	<1	<1	<1
TL1A	<1	<1	<1	<1
IFNα	<1	<1	<1	<1
IFNβ	<1	<1	<1	<1

The top 20 cytokine combinations that triggered IFNγ production by virus-specific CD8$^+$ T cells at 8 d (effector) or >60 d postinfection (memory) were tested at 10 ng/mL and ranked according to the percentage of effector NP118-tetramer$^+$CD11a^{high}CD8$^+$ T cells producing IFNγ. Spontaneous production of IFNγ in medium-only controls was typically <0.2% for effector T cells and <0.1% for memory T cells and this background was subtracted before preparing the table. Results represent the average of two to six mice per group.

cytokine-mediated T-cell activation and IFNγ production. Interestingly, the levels of IFNγ in MACS-purified CD8$^+$ T cells measured by intracellular cytokine staining correlated with the levels of secreted IFNγ measured by ELISA (R^2 = 0.59, P < 0.01; Fig. S2).

Modulation of IFNγ Production by Virus-Specific Effector T Cells During Acute LCMV Infection. To determine the responsiveness of virus-specific effector T cells to direct cytokine-mediated activation, we used MACS-purified CD8$^+$ T cells from LCMV-infected mice at 8 d postinfection and stimulated them for 6 h with the indicated cytokines, either alone or in pairwise combinations before determining IFNγ production by NP118-tetramer$^+$CD8$^+$ T cells (Fig. 3).

IL-2 has long been recognized as a growth factor involved with T-cell proliferation and survival (23), but the full scope of T-cell activation elicited by this cytokine has not been comprehensively examined. We found that IL-2 was able to synergistically enhance IFNγ production by virus-specific T cells in combination with

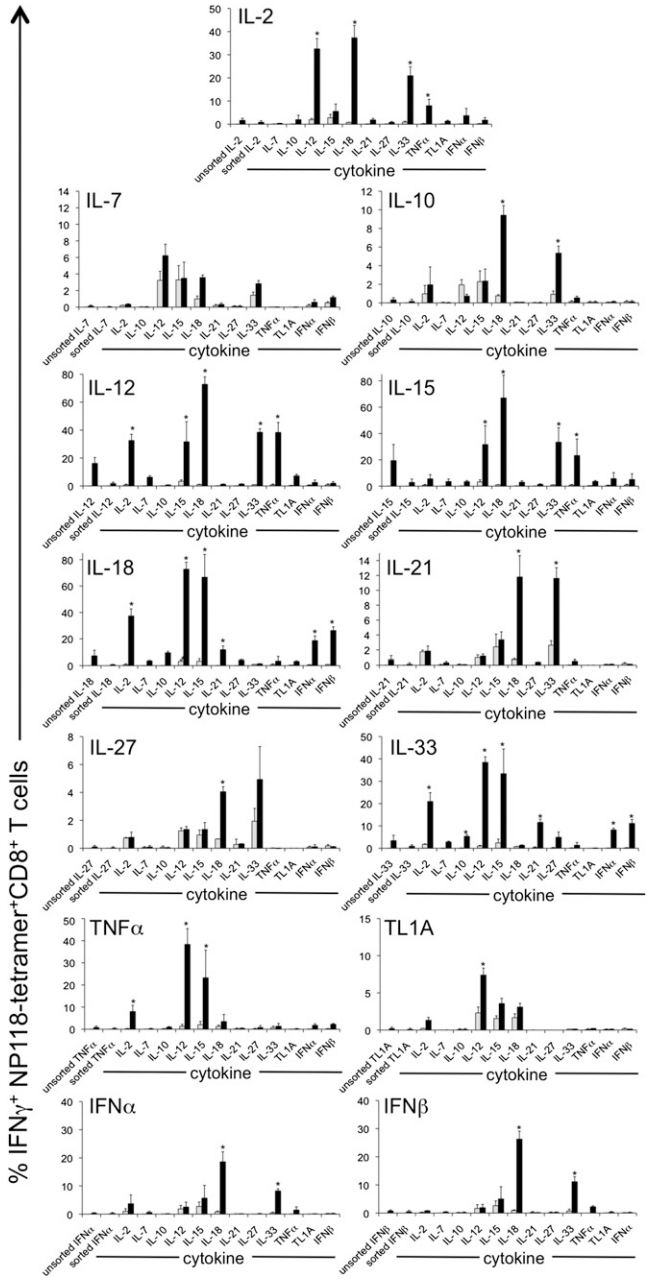

Fig. 3. Cytokine-mediated IFNγ production by effector T cells during acute LCMV infection. At 8 d postinfection with LCMV, MACS-purified $CD8^+$ T cells from BALB/c mice were stimulated with the indicated cytokine combinations at 100 ng/mL. Bars labeled "unsorted" represent the IFNγ response of NP118-tetramer$^+$ $CD8^+$ T cells to the indicated single cytokine in a population of bulk splenocytes. All other responses represent the results observed with purified $CD8^+$ T-cell populations. Open bars represent IFNγ responses to the unpartnered individual cytokines on the *x* axis, and the corresponding solid bars represent IFNγ responses to each cytokine in combination with the cytokine labeled at the *Top* of each panel. Spontaneous production of IFNγ in medium-only controls was typically <0.2%, which was subtracted before preparing the graphs. Data represent the mean ± SD of four to eight mice per group. IFNγ responses to each cytokine pair were compared with responses after stimulation with the individual cytokines using an unpaired two-tailed *t* test. Cytokine pairs that induced T-cell responses that were significantly different ($P < 0.05$) from both responses to the individual cytokines within the pair are marked with an asterisk (*). Note that different *y* axis scales are used for each cytokine.

IL-12, IL-18, IL-33, and TNFα (Fig. 3). Similar to IL-2, IL-7 is a member of the common γ chain family of cytokines and plays a central role in the regulation of naïve and memory $CD8^+$ T-cell homeostasis and survival (24). However, IL-7 did not synergize with other cytokines as dramatically as IL-2 to trigger IFNγ production by effector T cells. This result is not unexpected because IL-7R (CD127) is expressed by only a small subset of LCMV-specific T cells at the peak of the primary $CD8^+$ T-cell response (25).

IL-10 emerged as a cytokine of particular interest, as it exhibited differential regulatory capabilities depending on the cytokine with which it was paired. IL-10 inhibited $CD8^+$ T-cell activation by IL-12, but enhanced activation by IL-18 in both unsorted (Fig. 1) and sorted (Fig. 3) populations. In addition to its striking synergy with IL-18, IL-10 also synergized with IL-33. These results represent an unexpected and previously unrecognized role for IL-10 in regulating antigen-independent activation of virus-specific T cells.

The classic TH1-promoting cytokine, IL-12, displayed strong synergies with several cytokine partners. In addition to its most widely recognized partner, IL-18, cytokines that synergized with IL-12 to induce IFNγ production included IL-2, IL-7, IL-15, IL-33, TNFα, and TL1A (TNF-like ligand 1A/TNF superfamily 15. Interestingly, IL-12 itself triggered ∼15% of virus-specific $CD8^+$ T cells to produce IFNγ in the presence of other splenic accessory cells, but only ∼2% of purified $CD8^+$ T cells produced IFNγ in response to IL-12 alone (Fig. 3). This indicates that IL-12 is relatively ineffective at directly stimulating $CD8^+$ T cells and instead, the stimulatory activity of this important cytokine appears to be largely dependent on the presence of other cell types and/or the induction of a cytokine cascade.

IL-15, like IL-2 and IL-7, is a member of the common γ chain cytokine family and although it can enhance IFNγ production in $CD8^+$ T cells (13), it is primarily known for its role in T-cell proliferation and homeostasis (24, 26). Our data demonstrate the ability of IL-15 to enhance antigen-independent effector T-cell activation in response to a variety of cytokines (e.g., IL-12, IL-18, IL-33, and TNFα), in addition to its functions as a homeostatic regulator. As with IL-12, IL-15 was heavily reliant upon the presence of accessory cells to exhibit stimulatory activity. At 100 ng/mL, IL-15 induced ∼20% of virus-specific $CD8^+$ T cells to produce IFNγ in a population of unsorted spleen cells, whereas <5% of NP118-specific $CD8^+$ T cells produced IFNγ in response to this cytokine after MACS purification (Fig. 3). At 10 ng/mL, IL-15 stimulated <2% of $CD8^+$ T cells to produce IFNγ (Table 1), indicating the importance of cytokine concentration on observed regulatory function.

IL-18 synergized with a wide array of cytokines, but it differed substantially from IL-12 in several notable aspects. Although IL-18 synergized with IL-2 and IL-15 to promote IFNγ production (similar to IL-12), it did not enhance IFNγ responses when paired with IL-33 or TNFα. Moreover, IL-18 enhanced T-cell responses to pairwise combinations including IL-10, IL-21, IFNα, and IFNβ, whereas IL-12 did not elicit enhanced IFNγ production under these conditions.

IL-21 is another member of the common γ chain cytokine family. It is produced primarily by $CD4^+$ T cells and has been shown to have an important role in sustaining functional $CD8^+$ T-cell responses to chronic LCMV infection (27, 28). Little is known about the array of cytokines with which IL-21 can interact to manipulate $CD8^+$ T-cell function, but we found that IL-21 reproducibly synergized with IL-18 and IL-33 to promote IFNγ production by virus-specific $CD8^+$ T cells.

IL-27 is an IL-12 family member and shares closely related ligand and receptor subunits with IL-12 (29). However, these cytokines are clearly different in terms of their ability to activate virus-specific $CD8^+$ T cells when paired with other regulatory cytokines. Similar to IL-12, IL-27 synergized with IL-18 and IL-33, but these combinations elicited IFNγ production in only 4–8% of virus-specific $CD8^+$ T cells—a much lower percentage than that observed following stimulation with IL-12 + IL-18 (∼70% IFNγ$^+$) or IL-12 + IL-33 (∼40% IFNγ$^+$). In addition, IL-27 did not

demonstrate any measurable synergy with IL-2 or IL-15, again indicating functional differences between IL-12 and IL-27.

IL-33, a member of the IL-1 family, has previously been classified as a TH2-promoting cytokine (30) and been implicated in a number of TH2-mediated diseases (31). More recently, IL-33 has been found to be important in driving antiviral $CD8^+$ T-cell responses during LCMV infection (6). Here, we found a new role for IL-33 in enhancing antigen-independent IFNγ responses of virus-specific $CD8^+$ T cells, including synergies with IL-2, IL-10, IL-12, IL-15, IL-21, IL-27, IFNα, and IFNβ. Induction of T-cell–mediated IFNγ production by these IL-33 combinations represents a previously unrecognized function for this recently identified cytokine.

TNFα proved to be the most stimulatory of the TNF superfamily members that we examined, particularly when paired with IL-12. These two cytokines exhibited potent synergy, triggering IFNγ production by about 40% of virus-specific $CD8^+$ T cells at 8 d after LCMV infection (Fig. 3). In contrast, another TNF superfamily member, TL1A, demonstrated only modest synergy with IL-12 for inducing IFNγ production by virus-specific $CD8^+$ T cells. TL1A was also marginally capable of enhancing IFNγ responses to IL-2, IL-15, and IL-18 but in only about 1–4% of virus-specific effector T cells.

Type I interferons, IFNα and IFNβ, can have diverse effects on $CD8^+$ T cells, either promoting IFNγ production or inhibiting it via the suppression of IL-12–mediated pathways (32). When paired with other cytokine partners under these defined experimental conditions, IFNα and IFNβ exhibited similar patterns of T-cell activation, as might be expected for these closely related cytokines (Fig. 3). Both cytokines triggered enhanced IFNγ production in response to IL-18 and IL-33 in sorted effector T-cell populations. IFNγ production was especially strong when these cytokines were paired with IL-18, which is in line with previous studies describing IFNα-induced up-regulation of the IL-18 receptor (33). Interestingly, type II IFN, IFNγ, did not elicit a measurable autocrine feedback loop of activation or inhibition and did not synergize with any of the other 42 cytokines that were tested in this study (Table S1).

Cytokine-Mediated IFNγ Production by Memory T Cells. The activation/maturation state of a $CD8^+$ T-cell can strongly influence how the cell responds to various stimuli. For this reason, we also examined memory T-cell responses at >60 d post-LCMV infection to determine how cytokines may regulate virus-specific memory T-cell activation after resolution of acute viral infection. Several of the T-cell response patterns observed at 8 d postinfection were recapitulated during the memory phase, with some notable exceptions (Fig. 4 and Table 1). IL-2 still synergized with either IL-12 or IL-18 to induce IFNγ production by memory T cells, but they were largely unresponsive to other cytokine combinations such as IL-2 + IL-33 or IL-2 + TNFα. IL-10 inhibited the relatively weak IFNγ response elicited by IL-12 and enhanced IFNγ production in response to IL-18 or IL-33, albeit in only 1–2% of NP118-specific memory T cells. These observations were substantially enhanced in the presence of accessory cells (Fig. 1). IL-12–induced IFNγ responses by memory T cells closely mirrored the responses observed in effector T cells at 8 d postinfection, as did IL-15–induced IFNγ responses. $CD8^+$ T-cell activation by IL-15 + IL-18 was somewhat lower in memory T cells compared with effectors, but T-cell activation by IL-15 + TL1A, IL-15 + IFNα and IL-15 + IFNβ was higher in memory T-cell populations. Memory T-cell responses to IL-18 combinations were also similar to that observed with effector T cells (Fig. 4), whereas memory T cells were largely unresponsive to cytokine combinations containing IL-21 or IL-27. Memory T cells also responded to IL-33 + IL-12 and IL-33 + IL-15, but the other IL-33 combinations that induced IFNγ responses in effector T cells were not effective at eliciting IFNγ responses by memory T cells. Likewise, although TNFα paired with several cytokines to induce IFNγ in effector T cells, only the combination of TNFα + IL-12 triggered IFNγ production by a large

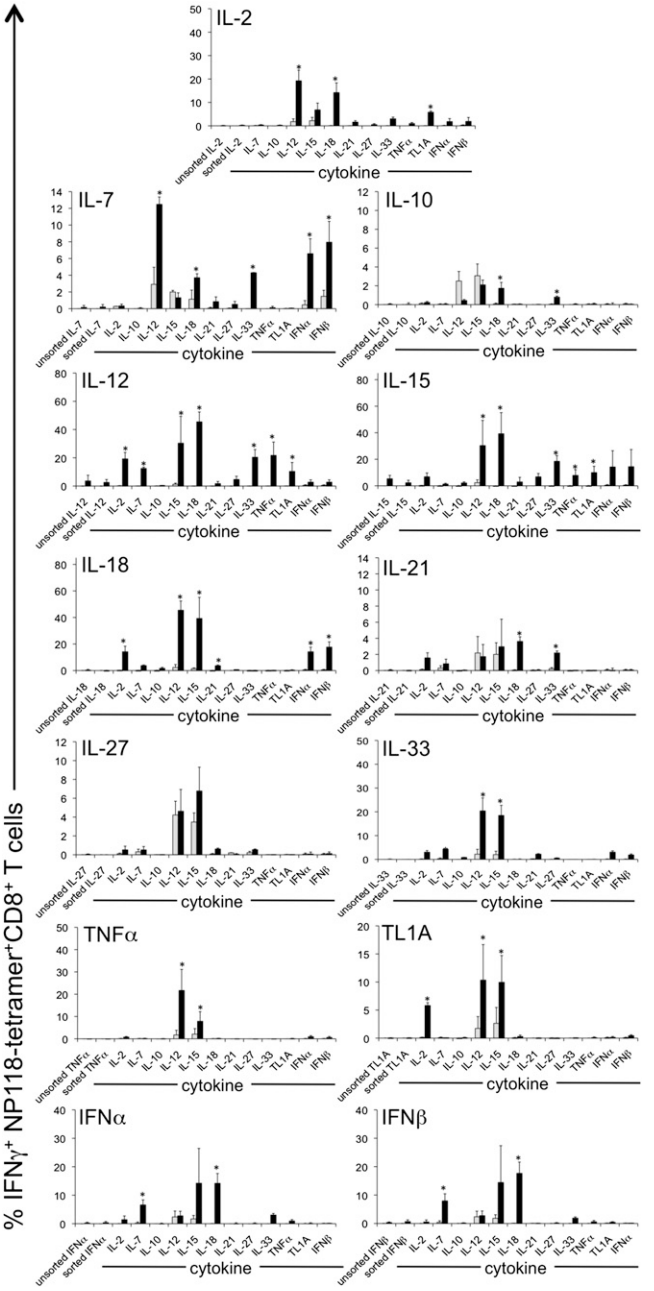

Fig. 4. IFNγ production by memory T cells following exposure to defined cytokine combinations. MACS-purified $CD8^+$ T cells from LCMV-immune BALB/c mice (>60 d postinfection) were stimulated as described in Fig. 3. Spontaneous production of IFNγ in medium-only controls was typically <0.1%, which was subtracted before preparing the graphs. Data represent mean ±SD of four to eight mice. Note that different *y* axis scales are used for each cytokine.

proportion of virus-specific memory T cells. Similar to effector $CD8^+$ T cells, type I interferons, IFNα and IFNβ, induced relatively strong IFNγ production in memory T cells when paired with IL-18. However, in contrast to effector T cells, memory T cells showed a reduced ability to respond to the combinations of IFNα/β + IL-33, whereas a small subset of memory T cells gained the ability to respond to IFNα/β + IL-7.

Cytokine-Induced CD69 Expression. IFNγ production represents only one outcome of virus-specific $CD8^+$ T-cell activation, and it is unlikely that all forms of cytokine-mediated stimulation will

result in production of this one specific cytokine. Therefore, as an alternative approach to measuring basic T-cell activation, we examined CD69 expression on virus-specific effector $CD8^+$ T cells (Fig. 5 and Fig. S3) and memory T cells (Figs. S4 and S5) following cytokine stimulation. At 8 d postinfection, stimulation with IL-12 + IL-18 led to nearly equivalent levels of IFNγ production and CD69 up-regulation (around 70%), which was a dramatic increase over the frequency of $IFN\gamma^+$ or $CD69^+$ T cells observed following stimulation with either of these cytokines alone (Figs. 3 and 5 and Fig. S3). On the other hand, there were several cytokine combinations that led to enhanced CD69 expression without a concomitant increase in the number of $IFN\gamma^+$ T cells. Interestingly, IL-15 potently induces CD69 up-regulation in both effector and memory T cells, but IL-7 blocked this outcome in memory T cells (Fig. S4). Given the role that CD69 has in lymphocyte migration (17), this highly specific interaction may be an intriguing mechanism by which cytokines which are not considered inherently chemotactic are able to influence the trafficking of virus-specific T cells at various time points after infection. The most dramatic dichotomy between cytokine-mediated CD69 expression and IFNγ production was observed with combinations containing type I interferons. This finding indicates that although virus-specific $CD8^+$ T cells may recognize and respond to a specific cytokine combination via up-regulation of CD69, in some cases only a subpopulation of these antiviral T cells are programmed to produce IFNγ.

Discussion

Virus-specific $CD8^+$ T cells integrate multiple inflammatory signals, which are not limited simply to viral peptides, but also include antigen-independent stimuli such as cytokines. The ability of virus-specific $CD8^+$ T cells to respond to cytokines independently from their cognate antigen allows them to play a role beyond the clearance of their specific virus and function in an innate capacity as sentinels for potentially unrelated infections (2, 4). In this study, we discovered several unexpected cytokine combinations capable of modulating the activation of virus-specific $CD8^+$ T cells in the absence of further stimulation through the T-cell receptor and noted several differences in the responses of effector vs. memory $CD8^+$ T cells following cytokine exposure. These differences in responsiveness to cytokine stimulation likely contribute to the variations in susceptibility to endotoxic shock that are observed during the acute and convalescent phases of viral infection (1, 3) and may impact treatment efficacy. Moreover, understanding which cytokines interact to regulate innate functions of $CD8^+$ T cells will be of central importance when developing targeted therapeutic strategies for specific infections, which may each induce a unique inflammatory microenvironment.

In vivo, cytokine-mediated T-cell activation is in many ways a double-edged sword. In some cases, bystander T-cell activation can be beneficial—as is the case when $CD8^+$ T cells produce IFNγ in response to cytokines triggered by infection with *Listeria monocytogenes* and provide innate protection in a non–antigen-specific manner (2). On the other hand, endotoxic shock associated with Gram-negative bacteria can be exacerbated by a cytokine storm that includes IFNγ-mediated immunopathology due to $CD8^+$ T cells and NK cells (1). $CD8^+$ T cells do not respond directly to LPS stimulation, but are responsive to cytokines (e.g., IL-12 and IL-18) elicited by LPS (3). Direct administration of cytokines provides another example of the double-edged sword of cytokine-mediated activation. Some cytokines, such as IFNβ and IL-2, have shown therapeutic efficacy, whereas administration of other cytokines such as IL-12 or TNFα to human subjects have been shown to elicit serious, sometimes fatal consequences (19). Indeed, synergy of some cytokine combinations, such as IL-2 + IL-18 (Fig. 3 and Table 1), result in lethal lung injury (34) and indicates that systemic administration of cytokine mixtures should be pursued cautiously.

In this study, two quantitative outcomes of $CD8^+$ T-cell activation were examined: IFNγ and CD69. However, cytokine production and migratory potential are only two aspects of a multifaceted T-cell response and the biological relevance of cytokines elicited by heterologous infection can also be ascertained by their influence on other parameters such as proliferation and cytolytic activity. Naïve T-cell proliferation may be inhibited by heterologous infection and/or type I interferons (35), whereas LCMV-specific memory $CD8^+$ T cells may undergo up to three rounds of proliferation in response to heterologous infection with vesicular stomatitis virus (36) and become actively cytolytic during heterologous vaccinia virus infection (37). Likewise, virus-specific $CD4^+$ T cells initiate their proliferative program more rapidly when exposed to an inflammatory in vivo environment (38). These studies suggest an important role for host factors such as cytokines in determining the rapidity of antiviral T-cell responses. It remains to be determined which of the newly identified cytokine combinations unveiled here are capable of modulating proliferation, cytotoxic activity, and the production of other cytokines besides IFNγ. Indeed, several cytokine combinations led to the CD69 up-regulation in a subset of NP118-tetramer$^+$ $CD8^+$ T cells in the absence of IFNγ production, suggesting that although these cells are not producing IFNγ, they are still becoming activated. During infection, T cells will likely be exposed to more than one or two cytokines at any given point in time, but by determining the synergies of pairwise combinations, we have begun to lay a foundation for examining more complex cytokine interactions. We presume that exposure to multiple cytokines will result in additive effects or potentially higher-order synergies, but this remains to be examined experimentally and represents an area worthy of further investigation.

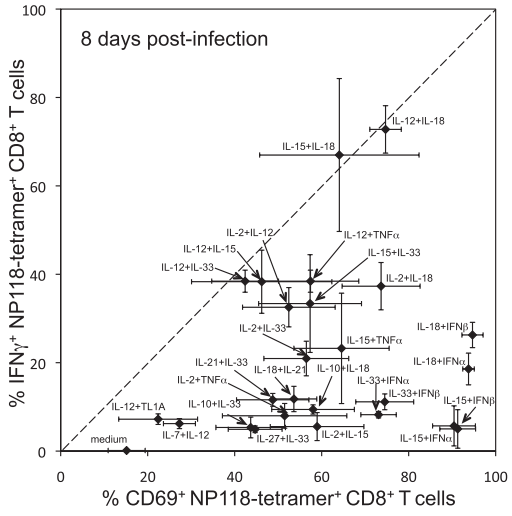

Fig. 5. Differential cytokine-mediated induction of CD69 and IFNγ expression. To determine whether virus-specific $CD8^+$ T cells can be activated without producing IFNγ, MACS-purified $CD8^+$ T cells from BALB/c mice were stimulated directly ex vivo with the indicated cytokine combinations at 100 ng/mL. Each data point represents the percentage of NP118-tetramer$^+$ effector T cells that up-regulated CD69 or produced IFNγ in response to the indicated cytokine combinations. Spontaneous production of IFNγ in medium-only controls was typically <0.2%, and CD69 was typically expressed on ~15–20% of NP118-tetramer$^+$ $CD8^+$ T cells directly ex vivo. Data represent the average ±SD of three to six mice per group.

Materials and Methods

Mice and Viral Infections. Female BALB/c and C57BL/6 mice were purchased from The Jackson Laboratory. Mice were infected at 6–12 wk of age via i.p. injection of 2×10^5 pfu LCMV-Armstrong. All animal experiments were reviewed and approved by the Oregon Health and Science University Institutional Animal Care and Use Committee.

Reagents and Stimulation Conditions. $CD8^+$ T-cell purification was performed by MACS in accordance with the manufacturer's directions (Miltenyi Biotec). Cytokines (n = 43 individual cytokines, certified endotoxin-free; Table S1)

were purchased from R&D Systems and used at a final concentration of 100 ng/mL or 10 ng/mL as indicated. IFNγ ELISA kit was purchased from BD Biosciences. Surface and intracellular cytokine staining was performed as previously described after 6 h of cytokine stimulation, with brefeldin A (2 μg/mL; Sigma Aldrich) added for the final hour of stimulation (3).

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