Cytokine production by virus-specific CD8+ T cells varies with activation state and localization, but not with TCR avidity

Nanna Ny Kristensen, Andreas Nygaard Madsen, Allan Randrup Thomsen and Jan Pravsgaard Christensen

Institute of Medical Microbiology and Immunology, University of Copenhagen, Copenhagen, Denmark

The ability of virus-specific CD8+ T cells to produce cytokines was studied in mice infected with lymphocytic choriomeningitis virus and vesicular stomatitis virus. Intracellular staining was used to visualize cytokine-producing CD8+ and CD4+ T cells. Overall, virus-specific CD8+ T cells produce a similar range of cytokines (IFN-γ, TNF-α, IL-2, GM-CSF, RANTES, MIP-1α and MIP-1β) as CD4+ T cells, but the relative distribution of cytokine-producing subsets is different. Moreover, cytokine-producing CD8+ T cells were found to dominate numerically at all time-points tested. Co-staining for more than one cytokine revealed that while all cytokine-producing CD8+ T cells synthesized IFN-γ, additional cytokines were produced by partly overlapping subsets of this population. The frequency of cells producing more than one cytokine was higher in a tertiary site (peritoneum) and generally increased with transition into the memory phase; however, GM-CSF producing cells were only present transiently. Concerning factors predicted to influence the distribution of cytokine-producing subsets, IFN-γ and IL-12 did not play a role, nor was extensive virus replication essential. Notably, regarding the heterogeneity in cytokine production by individual cells with similar epitope specificity, variation in TCR avidity was not the cause, since in vivo-activated TCR transgene-expressing cells were as heterogeneous in cytokine expression as polyclonal cells specific for the same epitope.

INTRODUCTION

Cytokines play a pivotal role in regulating any type of adaptive immune response, and T cells are an important source of cytokines in the context of both primary and memory immune responses. It is normally assumed that CD4+ T cells are the main cytokine-producing T cell subset, and much research in this field has been focused around the division of these cells into Th1 (IL-2, IFN-γ and TNF-α) and Th2 (IL-4, IL-5, IL-6 and IL-10) cells based on their cytokine profile, and the mechanisms regulating this differentiation (Mosmann et al., 1986; Mosmann & Sad, 1996; Liew, 2002). In contrast, CD8+ T cells have traditionally been regarded as a homogeneous subset of cytolytic cells, and their potential for production of cytokines is often neglected. For this reason much of the research on antiviral CD8+ T cell responses has centred on the cytolytic abilities of these cells (Lukacher et al., 1984; Binder & Kundig, 1991; Kagi et al., 1994), proving cell killing to be an essential effector mechanism required for the control of many, but not all viral infections (Kagi & Hengartner, 1996). However, studies of long-term CD8+ T cell clones/lines revealed years ago that like CD4+ T cells, CD8+ T cells also have the potential to produce a wide variety of cytokines (Paliard et al., 1988). However, the relevance of this during

in vivo immune responses was not investigated (Kelso et al., 1982; Morris et al., 1982; Prystowsky et al., 1982). Supporting a role for CD8+ T cells as cytokine producers, fresh human CD8+ T cells treated with either PMA/ ionomycin or plate-bound α-CD3 antibody have recently been found to produce a number of cytokines and chemokines (Conlon et al., 1995). Together, these observations suggest that CD8+ T cells could be relevant producers of many cytokines in vivo. Based on these and more recent experiments aimed at studying CD8+ T cell effector cell differentiation in vitro, it has been suggested that activated CD8+ T cells can also be divided into two functionally distinct subsets producing either the Th1 or Th2 types of cytokines; by analogy to their CD4+ counterparts these subsets have been named Tc1 and Tc2 cells, respectively (Croft et al., 1994; Maggi et al., 1994; Le Gros & Erard, 1994; Sad et al., 1995). Existing data also suggest that these phenotypes are relatively stable: in vitro-generated effector CD8+ T cells of either Tc1 or Tc2 phenotype have been shown to survive in vivo for at least 90 days after adoptive transfer and still maintain their characteristic cytokine profile (Cerwenka et al., 1998). However, whether a similar stability applies to in vivo-activated polyclonal CD8+ T cells is presently unknown.
Despite the fact that it is becoming increasingly evident that CD8\(^+\) T cells may be relevant as producers of cytokines, there are still a number of questions that need to be addressed. What is the range of cytokines – at the population level as well as at the level of the individual cell – which may be produced by in vivo-primed antigen-specific CD8\(^+\) T cells? Is this profile fixed or does it change as a function of time and the state of activation (effector→memory cell)? How do cytokine-producing CD8\(^+\) T cells compare qualitatively and quantitatively to antigen-specific CD4\(^+\) T cells primed in parallel? The primary aim of this study was therefore to determine the ability of virus-specific CD8\(^+\) T cells to produce cytokines as a function of their activation state, and to define some of the parameters influencing the spectrum of cytokines produced. Cells were analysed \textit{ex vivo} using primarily intracellular staining for cytokines and flow cytometry. This type of analysis makes it possible not only to determine the spectrum of cytokines, which can be produced at the population level, but also allows a precise evaluation of the number of cells involved as well as the relationship between subset of cells producing different cytokines.

**METHODS**

**Mice.** Female BALB/c and C57BL/6 (B6) mice were purchased from M & B (Ry, Denmark). RAG-2-deficient mice expressing a transgenic TCR for np\(_{52-59}\) of vesicular stomatitis virus (VSV) [B6, 129S6-Rag2\(^{-/-}\)-TgN(N15)] were obtained via Taconic (Germantown, NY, USA). Transgenic mice (TCR-318) expressing a TCR for lymphocytic choriomeningitis virus (LCMV) gp\(_{33-41}\) on about 60\% of their CD8\(^+\) T cells were the progeny of breeding pairs kindly provided by H. Pircher and R. M. Zinkernagel, University of Zurich, Switzerland. These mice (TCR-318) were backcrossed onto the B6 background, by which time they had been bred for at least 1 week before entering into experiments, at which time the animals usually were 7–9 weeks old. Animals were housed under specific pathogen-free conditions, and all experiments were conducted in compliance with national regulations.

**Viruses and virus infections.** The visceral tropic Traub strain of LCMV was administered i.v. or i.p. in a dose of approx. 200 p.f.u.; inoculation by these routes results in non-lethal, immunizing infections. VSV was administered i.v. or i.p. in a dose of approx. 200 p.f.u.; inoculation by these routes results in non-lethal, immunizing infections. LCMV was administered i.v. or i.p. in a dose of approx. 200 p.f.u.; inoculation by these routes results in non-lethal, immunizing infections. LCMV was administered i.v. or i.p. in a dose of approx. 200 p.f.u.; inoculation by these routes results in non-lethal, immunizing infections.

**Cell preparation.** Spleens were removed from mice killed by cervical dislocation. Single-cell suspensions of spleen cells were obtained by pressing the organs through a fine steel mesh. Peritoneal cells were obtained by lavage with 5 ml of ice-cold Hanks’ balanced salts solution.

**Adoptive transfer of TCR transgenic CD8\(^+\) T cells.** Single-cell suspensions of spleen cells from TCR-318 or TgN(N15) mice were obtained as described above, and 1–3 \times 10^8 splenocytes were transferred i.v. to B6 mice. TCR\(^+\)CD8\(^+\) T cells from TCR-318 donor mice were tracked using a combination of anti-V\(_\alpha\)2 and -V\(_\beta\)8 antibodies; <2\% of wild-type cells express this combination, and transgenic cells were only analysed functionally when >20\% of splenic CD8\(^+\) T cells expressed this receptor combination. Since no Ab was commercially available for the V\(\gamma\)1 chain expressed by TCR\(^+\)CD8\(^+\) T cells from TgN(N15) donor mice, these cells were tracked using anti-V\(_\alpha\)8 only. However, V\(_\alpha\)8\(^+\) cells specific for np\(_{52-59}\) comprise <1\% of the CD8\(^+\) T cells in untransplanted mice, while only recipients in which np\(_{52-59}\) specific V\(_\alpha\)8\(^+\) cells had expanded to >8\% of splenic CD8\(^+\) T cells were accepted for analysis of donor cell cytokine phenotype. Thus, in both cases contamination with recipient cells represented ~10\% or less of the cells analysed.

**Monoclonal antibodies for flow cytometry.** The following mAbs were purchased from BD PharMingen as rat anti-mouse mAbs: CyChrome (Cy)-conjugated anti-CD4 and anti-CD8a, FITC-conjugated anti-CD49d [common \(\alpha\)-chain of lymphocyte Peyer’s patch adherence molecule-1 and very late Ag-4 (VLA-4)], FITC-conjugated anti-CD44, PE-conjugated anti-V\(\beta\)8, FITC-conjugated anti-V\(_\alpha\)2, FITC-conjugated anti-V\(_\alpha\)8, FITC- and PE-conjugated anti-IFN-\(\gamma\), PE-conjugated anti-IL-3, anti-IL-4, anti-IL-5, anti-IL-10, anti-GM-CSF, PE- and APC-conjugated anti-IL-2, PE- and FITC-conjugated anti-TNF-\(\alpha\) and matched isotype controls.

**MHC/peptide tetramers (tet) for flow cytometry.** H-2K\(^b\)/np\(_{52-59}\) tetramers were obtained through the National Institute of Allergy and Infectious Disease Tetramer facility and the National Institutes of Health AIDS Research and Reference Reagent Program.

**Flow cytometric analysis.** To detect intracellular cytokines, splenocytes were cultured for 5 h at 37°C in complete RPMI 1640 medium supplemented with murine recombinant IL-2 (50 U ml\(^{-1}\)), monensin (3 \(\mu\)M) and relevant peptides at a concentration of 0·1 \(\mu\)g ml\(^{-1}\) (LCMV gp\(_{33-41}\) and np\(_{118-126}\)) or 1 \(\mu\)g ml\(^{-1}\) (LCMV gp\(_{61-80}\) and VSV np\(_{52-59}\)). After stimulation the cells were stained with relevant antibodies as described previously (Kristensen \textit{et al}, 2002). For tetramer staining, cells were incubated with tetramers for 30 min at 4°C, at which time mAbs for surface labelling were added; the cells were then incubated for a further 30 min at 4°C, before washing and fixation (Christensen \textit{et al}, 2001). Sample data were acquired using a FACSCalibur (BD Biosciences), and results were analysed using CellQuest software (BD Biosciences). Isotype control mAbs and in some cases also unstimulated cells were included to define appropriate cut-off levels.

**Quantification of chemokine production.** Supernatants harvested from peptide (1 \(\mu\)g ml\(^{-1}\)) and unstimulated cultures of splenic cells incubated for 6 h \textit{in vitro} were assayed for chemotactic cytokines using commercially available sandwich ELISA kits (R&D systems).

**RESULTS**

**LCMV-specific cytokine-producing CD8\(^+\) T cells outnumber CD4\(^+\) T cells both during the primary immune response and in the memory phase**

To determine the range of cytokines produced by a polyclonal population of virus-specific CD8\(^+\) T cells, B6 mice were infected i.v. with LCMV of the Traub strain, and cytokine production by CD8\(^+\) T cells specific to the class I-restricted viral peptide gp\(_{33-41}\) (an immunodominant epitope in H-2\(^b\) mice) was determined by staining for intracellular cytokine or – in the case of chemokines – by ELISA. For comparison cytokine production by CD4\(^+\) T cells specific to the class II-restricted viral peptide gp\(_{61-80}\)
As shown previously (Butz & Bevan, 1998; Murali-Krishna et al., 1998; Sifka & Whitton, 2000; Varga & Welsh, 2000; Kristensen et al., 2002), primary virus-specific CD4<sup>+</sup> and CD8<sup>+</sup> effector T cells produce the cytokines IFN-γ, TNF-α and IL-2 (Fig. 1A). A substantial subset of primary effector T cells also produced GM-CSF. Besides producing pro-inflammatory cytokines, MHC class I-restricted T cells were also found to be the predominant source of the chemotactic cytokines RANTES, MIP (macrophage inflammatory protein)-1α and β (Fig. 2). In contrast, T cells producing type 2 cytokines (IL-4, IL-5 and IL-10) or IL-3 were either absent or found at marginal frequencies within both T cell compartments (Fig. 1B and C and data not shown).

With progression towards the memory state a higher proportion of antigen-specific CD8<sup>+</sup> T cells produce TNF-α and IL-2 (Fig. 1B). Interestingly, the kinetics of GM-CSF-producing cells is different. In the acute phase [day 9–11 post infection (p.i.)] the number of GM-CSF-producing CD8<sup>+</sup> T cells make up 25–30% of the number of IFN-γ-producing cells and exceed the number of IL-2-producing cells. However, during the contraction phase there is a

![Fig. 1](http://vir.sgmjournals.org)

**Fig. 1.** Cytokine production by antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells during primary LCMV infection. B6 mice were infected i.v. with 200 p.f.u. of LCMV Traub, and on the indicated days spleen cells were stimulated with gp33–41 (CD8<sup>+</sup> T cells) or gp61–80 (CD4<sup>+</sup> T cells) for 5 h in vitro. Following stimulation, cells were co-stained with anti-CD8 or anti-CD4 and either anti-VLA-4 followed by staining for intracellular cytokines (IFN-γ, IL-2 and GM-CSF) or IFN-γ and TNF-α, as indicated; staining with isotype-matched mAbs was used to set cut-off limits. (A) Representative plots for primary effector cells (days 9–10 p.i.). The number in the upper-right quadrant represents percentage cytokine<sup>+</sup> and either VLA-4 hi or IFN-γ<sup>+</sup> cells of the indicated T-cell subset. (B) and (C) Total numbers of cytokine-producing CD8<sup>+</sup> and CD4<sup>+</sup> T-cells in the spleen as a function of time. Columns represent means ± SD of six animals analysed individually in two independent experiments.

* = Not determined
preferential loss of GM-CSF-producing CD8+ T cells, and the ratio of IL-2- to GM-CSF-producing cells is inverted 28 and 70 days after infection (Fig. 1B).

It should be noted that although both virus-specific CD4+ and CD8+ T cells are clearly type 1 cells, the distribution of cytokine-producing subsets differs. Similar numbers of CD4+ T cells produce TNF-α, IL-2 or GM-CSF during the primary response whereas TNF-α-producing cells are more prevalent amongst CD8+ effector T cells. Following contraction of the T cell population, equal numbers of IFN-γ-, TNF-α- and IL-2-producing CD4+ T cells were observed (Fig. 1C). As with CD8+ T cells, GM-CSF-producing cells were less prevalent in the memory phase.

Virus-specific CD8+ T cells producing TNF-α, GM-CSF or IL-2 constitute overlapping subpopulations of cells also producing IFN-γ

To study the relationship between CD8+ T cells producing different cytokines, gp33–41 stimulated CD8+ effector T cells were co-stained for two of the following cytokines: IFN-γ, TNF-α, GM-CSF and IL-2 (Fig. 3). Confirming earlier observations (Slifka & Whitton, 2000; Kristensen et al., 2002), we found that TNF-α and IL-2 are produced by cells, which also synthesize high amounts of IFN-γ; a similar pattern was found for GM-CSF-producing cells (Fig. 3, upper panel). With regard to co-production of the less frequently produced cytokines, TNF-α, GM-CSF and IL-2, a more heterogeneous pattern was observed (Fig. 3, lower panel). However, statistical analysis revealed that all pairs of cytokines were co-expressed at frequencies higher than expected by chance (P < 0.05; χ² contingency table testing) indicating that the ability to produce these cytokines is partially linked.

Cytokine profile of virus-specific CD8+ T cells generated during primary VSV infection

To investigate whether the same cytokines would be produced by virus-specific CD8+ T cells generated in response to another virus, B6 mice were inoculated i.v. with VSV. Infectious VSV can only be isolated briefly from the spleen, and this CD8 T cell response therefore represents programmed expansion and differentiation of cells in the absence of actual infection. On day 7 (the peak of the primary response) and day 21 (memory state) after infection, we analysed the capacity of np52–59 specific (immunodominant epitope in H-2b mice) CD8+ T cells to produce cytokines (Fig. 4).

Except for a failure to produce GM-CSF, VSV-specific CD8+ T cells produced the same range of cytokines as did LCMV-specific cells, and virus-specific CD8+ T cells producing the cytokines IL-4, IL-5 and IL-10 were not detected in either case (data not shown). Interestingly, at the peak of the primary response, TNF-α-producing cells made up only about one-fifth to one-third of IFN-γ- producing VSV-specific cells compared with ≥ one-half of primary LCMV-specific cells. Furthermore, both these cytokines was produced at a lower level by VSV-specific cells compared with LCMV-specific cells (Fig. 4B).

Cytokine phenotype of virus-specific CD8+ T cells as a function of localization

Besides splenic T cells, we evaluated the ability of LCMV- and VSV-specific CD8+ T cells from the peritoneum to...
produce cytokines (Table 1 and 2). The peritoneum represents a tertiary organ site, and the cells present here are likely to represent a more differentiated phenotype compared with cells obtained from secondary lymphoid tissues (Masopust et al., 2001).

Ten days after LCMV infection i.p., at the peak of the local inflammatory response, we found (Table 1) that while the frequency of IFN-γ-producing cells was only slightly increased in peritoneum, the frequencies of TNF-α-, GM-CSF- and IL-2-producing cells were all markedly (2- to 3-fold) higher in this site compared to the spleen. As TNF-α, GM-CSF and IL-2 in both organ sites were produced by cells, which also synthesized IFN-γ (Fig. 3 and data not shown), this pattern suggested that co-producing cells are preferentially attracted to and/or sustained in tertiary organ sites. However, since i.p. inoculation of LCMV results in peritonitis, the explanation could also be that virus-specific CD8+ T cells locally receive inflammatory signals, which may influence their differentiation/maturation.

To investigate whether such signals were essential, we analysed virus-specific cells harvested from the peritoneum of mice infected with VSV i.v.; in this case no inflammatory response is observed (no increase in cell numbers).

At the height of the primary response to VSV, not only the frequency of IFN-γ producers (Table 2), but also the level of per-cell expression was significantly higher for peritoneal

Fig. 3. Co-expression of cytokines by antigen-specific CD8+ T cells during acute LCMV infection. B6 mice were infected with 200 p.f.u. of LCMV Traub, and 10 or 11 days after infection gp33–41-stimulated splenic CD8+ T cells were co-stained for two of the following cytokines: IFN-γ, TNF-α, GM-CSF and IL-2. Numbers refer to the percentage of CD8+ T cells that produce the indicated cytokines either alone or in combination. Each plot is representative of at least six animals analysed individually in two independent experiments. Numbers in parentheses represent expected frequencies if cytokine production is independently regulated; all observed values are significantly higher (P<0.05; χ² contingency table testing).

Fig. 4. Cytokine expression by antigen-specific CD8+ T cells during acute VSV infection. (A) B6 mice were infected with 10⁶ p.f.u. of VSV, and on day 7 and 21 after infection the frequencies of splenic CD8+ T cells producing cytokines following peptide (np 52–59) stimulation were determined. Columns represent means±SD of six to nine animals analysed individually in three independent experiments. (B) Representative plots of primary effector CD8+ T cells in the spleens of mice infected with VSV (day 7 after infection) compared with LCMV-specific primary effector CD8+ T cells (day 10 after infection). Peptides used for stimulation were np52–59 and gp33–41 for VSV and LCMV, respectively. Each plot is representative of at least four animals analysed individually in two independent experiments. Numbers refer to mean fluorescence intensity (MFI) of antigen-specific (IFN-γ+) CD8+ T cells.
Table 1. Comparison of cytokine expression in virus-specific CD8+ T cells harvested from spleen and peritoneum on day 10 after LCMV virus-specific Traub infection

B6 mice were infected on day 0 with 200 p.f.u. of LCMV Traub. On day 10 after infection gp33-41-specific CD8+ T cells were analysed as indicated. Data represent means ± SD of six mice from two independent experiments.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Percentage*</th>
<th>Ratio†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IFN-γ</td>
<td>TNF-α</td>
</tr>
<tr>
<td>Spleen</td>
<td>17-48±3-34</td>
<td>8-47±0-99</td>
</tr>
<tr>
<td>Peritoneum</td>
<td>24-62±2-70</td>
<td>19-35±2-77</td>
</tr>
</tbody>
</table>

*% Cytokine-producing cells of CD8+ T cells.
†Ratio of cytokine-producing cells within the CD8+ T cell subset.

cells than for splenocytes (data not shown). In addition, the ratio of cytokine+/tetramer (tet+) cells was significantly higher in peritoneum, and, as in LCMV-infected mice, a higher frequency of peritoneal CD8+ T cells produced more than one cytokine (all TNF-α-producing cells also produced IFN-γ) (Table 2). Twenty one days after infection the majority of VSV-specific (tet+) CD8+ T cells both in the spleen and in the peritoneum produced IFN-γ. About half the virus-specific CD8+ T cells in the spleen were also TNF-α+ whereas nearly all virus-specific CD8+ T cells harvested from the peritoneum co-produced TNF-α. Thus, the proportion of co-producing cells increased with time in both spleen and peritoneum. Moreover, cells with this phenotype were found at a higher frequency in the peritoneum independently of local inflammatory signals (Table 2).

Co-infection with LCMV augments the VSV-specific CD8+ T cell response, but does not induce GM-CSF production

The state of the APC is known to critically influence T cell differentiation. To see whether the cytokine production by VSV-specific CD8+ T cells would be affected if the APCs simultaneously were activated by LCMV, B6 mice were co-infected with LCMV and VSV (there is no cross-reactivity between the two viruses) (Andreasen et al., 2000), and on day 7 after infection the number of cytokine-producing cells in the spleen was determined using intracellular staining and FACS analysis (Fig. 5). In co-infected mice the number of splenic VSV-specific CD8+ T cells producing IFN-γ and in particular TNF-α was significantly increased (2-6- and 3-2-fold, respectively). Furthermore, per-cell expression of both cytokines was also increased (not shown) so that the fluorescence distribution of cytokine-producing specific cells resembled that seen in LCMV-infected mice (cf. Fig. 4). Together these findings strongly indicate that signals induced during LCMV infection are able to augment the generation/differentiation of VSV-specific CD8+ effector T cells. However, the latter cells did not begin to produce GM-CSF indicating that other regulatory mechanisms control this differentiation.

Table 2. Comparison of ratios between cytokine producing and virus-specific CD8+ T cells in spleen and peritoneum on day 7 and 21 after primary VSV infection

B6 mice were infected on day 0 with 106 p.f.u. of VSV Indiana. On the selected days after infection np52-59-specific CD8+ T cells were analysed as indicated. Data represent means ± SD of six mice from two independent experiments.

<table>
<thead>
<tr>
<th>Day</th>
<th>Tissue</th>
<th>IFN-γ/Tet*</th>
<th>TNF-α/Tet*</th>
<th>TNF-α/IFN-γ†</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>Spleen</td>
<td>0-64±0-06</td>
<td>0-12±0-03</td>
<td>0-18±0-02</td>
</tr>
<tr>
<td></td>
<td>Peritoneum</td>
<td>1-40±0-19</td>
<td>0-82±0-09</td>
<td>0-60±0-14</td>
</tr>
<tr>
<td>21</td>
<td>Spleen</td>
<td>0-82±0-08</td>
<td>0-44±0-12</td>
<td>0-53±0-11</td>
</tr>
<tr>
<td></td>
<td>Peritoneum</td>
<td>1-08±0-32</td>
<td>0-88±0-22</td>
<td>0-82±0-06</td>
</tr>
</tbody>
</table>

*Ratio between cytokine- and tetramer-positive CD8+ T cells.
†Ratio between TNF-α- and IFN-γ-positive CD8+ T cells.

Fig. 5. Co-infection with LCMV augments cytokine production in VSV-infected mice. B6 mice were infected with 106 p.f.u. of VSV Indiana plus 5000 p.f.u. LCMV (Armstrong), and on day 7 after infection the number of splenic CD8+ T cells producing IFN-γ, TNF-α and GM-CSF following VSV peptide (np52-59) stimulation was determined. For comparison mice infected with 106 p.f.u. of VSV Indiana only were also analysed. Columns represent means ± SD of six animals analysed individually in two independent experiments; asterisk (*) denotes statistical significance (P<0-05, Mann–Whitney rank test) relative to mice infected with VSV only.
Heterogeneity in cytokine production by virus-specific CD8+ T cells does not require TCR diversity

The degree of TCR occupancy required for activation of different effector responses is known to vary (Valitutti et al., 1996; Itoh & Germain, 1997). The heterogeneity in cytokine phenotypes induced during an antiviral CD8+ T cell response could therefore reflect the range of avidities found for a normal, polyclonal response involving a spectrum of TCR clonotypes. To test this assumption, we analysed the cytokine production from in vivo activated T cells expressing a single TCR clonotype.

Naive B6 mice were given spleen cells from TCR transgenic mice (TCR-318) expressing a Vß2/V°8-2 T cell receptor specific for LCMV gp33-41. The day after cell transfer, recipients were infected with LCMV, and 9 days after infection TCR+ CD8+ T cells were analysed for the ability to produce cytokines (Fig. 6). Despite the uniform expression of a single clonotype, in vivo activated TCR+ cells not only produced the same spectrum of cytokines (IFN-γ, TNF-α, IL-2 and GM-CSF) (Fig. 6) as did gp33-41-specific cells from normal mice (see Fig. 1 for comparison), but the distribution of cytokine-producing cells also matched that observed for polyclonal cells with the same specificity.

In parallel experiments naive B6 mice were given TCR transgenic cells specific for VSV np52-59 and recipients were subsequently infected with VSV. Again the cytokine profile of in vivo-activated TCR+ cells (Fig. 6) mimicked that of polyclonal cells with the same specificity (in this case TCR+ cells were only recovered from half the transplanted recipients, possible due to incompatibilities at minor histocompatibility loci, but the pattern observed in all animals in which the TCR transgenic cells had expanded was highly reproducible). Together these observations strongly indicate that the normal diversity in TCR avidity is not the explanation for the cellular heterogeneity in cytokine production.

IFN-γ and IL-12 is not required to inhibit the generation of Tc2 cells during primary VSV infection

From in vitro studies it is known that CD8+ T cells cultured with antigen in the presence of anti-IFN-γ (and IL-4) will develop a T12-like phenotype (Tc2 cells) (Croft et al., 1994; Sad et al., 1995). To see if Tc2 cells would be generated in vivo in the absence of IFN-γ, IFN-γ−/− mice were infected with VSV (failure to produce IFN-γ does not influence the course of VSV infection) (Andersen et al., 1999), and on day 7 p.i. we looked for np52-59 specific cells producing typical Tc2 cytokines (IL-4, IL-5 and IL-10) as well as TNF-α (positive control). No VSV-specific CD8+ Tc2 cells from IFN-γ−/− mice (n=3) produced typical Tc2 cytokines (not shown) indicating that IFN-γ is not required to block Tc2 differentiation in vivo. It should be stressed that absence of IFN-γ does not impair the generation of VSV-specific cells since (i) the generation of TNF-α-producing cells was unimpaired and (ii) other experiments revealed that the number of splenic tet+ CD8+ cells was similar in IFN-γ−/− and wild-type mice (data not shown). Similar to the findings in IFN-γ−/− mice absence of IL-12β did not influence the cytokine profile (data not shown).

DISCUSSION

In this report we have studied the ability of in vivo-primed virus-specific CD8+ T cells to produce cytokines directly ex vivo. At the peak of the primary response CD8+ T cells have the ability to produce a broad spectrum of cytokines: IFN-γ, TNF-α, IL-2, GM-CSF, RANTES, MIP-1α and β. Since CD8+ T cells appear to be pre-programmed to undergo much more extensive proliferation and to be sustained at a higher level than CD4+ T cells (Christensen et al., 1996a,b; Homann et al., 2001; Foulds et al., 2002), our analysis strongly suggests that CD8+ T cells are the major source of T-cell derived cytokines throughout most viral infections. Certainly, our results readily explain why CD4+ T cells are not essential for LCMV-induced T cell-mediated inflammation (Marker et al., 1995).

CD8+ T cells can be divided into Tc1 and Tc2 subsets producing the same range of cytokines as the corresponding T11 subsets (Croft et al., 1994; Maggi et al., 1994; Le Gros & Erard, 1994; Sad et al., 1995). However, virus-specific CD8+ T cells seem to have a strong propensity to develop
a Tc1 cytokine profile: IL-4-, IL-5- and IL-10-producing cells were not found in either LCMV-infected B6 or BALB/c (data not shown) mice or following VSV infection. IFN-γ and IL-12 is known to regulate the in vitro differentiation of CD8+ T cells into a type 1 cytokine-producing phenotype. However, similar to the situation for Tc1 cells (Schijns et al., 1994; Oxeniou et al., 1999), we did not find type 2 Tc cells in IFN-γ or IL-12/β deficient mice. Notably, this type 1 profile was observed even following inoculation of a virus (VSV), which undergo very limited replication in the host (no infectious VSV can be detected in spleen beyond day 2 p.i.). Taken together these observations suggest that CD8+ T cells intrinsically are hardwired towards type 1 differentiation. Consistent with this assumption, and unlike the situation for CD4+ T cells, very few instances of in vivo-generated type 2 CD8+ T cells have been described (Salgame et al., 1991; Maggi et al., 1994).

Virtually all virus-specific CD8+ T cells generated during primary LCMV infection produce IFN-γ (Butz & Bevan, 1998; Murali-Krishna et al., 1998). Part of these cells also produce TNF-α and IL-2 (Slioka & Whitton, 2000; Kristensen et al., 2002). Here, we extend these observations by showing that a subpopulation of IFN-γ-producing CD8+ T cells may also produce GM-CSF. More important, we find that although virus-specific CD8+ T cells are quite heterogeneous in their ability to produce cytokines, the ability to produce more than one cytokine is not a random occurrence. Indeed there is a significant trend for co-production of several cytokines. The frequency of co-producing cells was significantly higher in the peritoneum than in the spleen independently of local inflammatory signals. This may indicate that co-producing CD8+ T cells are to be found mainly among fully differentiated cells with the potential to leave the secondary lymphoid organs and migrate into tertiary tissues. Notably, there also seems to be a preferential survival of these multipurpose cells as the relative frequency of this phenotype increases following contraction of the CD8+ T cell population. These observations expand on existing data (Slioka & Whitton, 2000; Kristensen et al., 2002) suggesting that there is a preferential maintenance of a T cell phenotype characterized by the potential to produce several cytokines. Interestingly, the ability to produce all cytokines is not maintained; LCMV-specific CD8+ T cells producing GM-CSF are only abundant during the effector phase and preferentially disappear with transition into the memory phase.

Interestingly, few GM-CSF-producing cells are generated during VSV infection, and overall VSV-specific CD8+ T cells appear to be less functionally differentiated during the primary response than do LCMV-specific cells: while the ratio of IFN-γ+/Tet+ is close to one for LCMV at the time of peak effector cell numbers (Butz & Bevan, 1998; Murali-Krishna et al., 1998), this ratio is only about 1:2 in VSV infected mice (present report). Furthermore, per-cell IFN-γ production is lower [lower mean fluorescence intensity (MFI)], and fewer VSV-specific, IFN-γ+ CD8+ T cells co-produce TNF-α. With time these differences tend to diminish. One explanation for this pattern could be that during acute VSV infection, many antigen-specific CD8+ T cells are generated, which are arrested at an early stage of differentiation and therefore preferentially lost during the contraction phase. Part of the reason why functional differentiation may be less complete following VSV infection as compared with LCMV infection, could be a difference in the state of the APCs during the two infections (Ruedl et al., 1999). Consistent with this assumption we find that numbers and per-cell capacity of VSV-specific cells to produce IFN-γ and TNF-α increase in co-infected animals. However, no induction of GM-CSF-producing cells was observed, indicating that additional factors influence cytokine production – perhaps the transient nature of antigen presentation in VSV infected mice (Andreasen et al., 2000; Christensen et al., 2002) also plays a role. Supporting that the environment in which antigen presentation takes place may influence the overall cytokine profile, we found that naive TCR+ cells specific for immunodominant epitopes of either virus differentiate into cytokine-expression patterns which reproduce the differences seen for polyclonal cells of the same specificity (cf. production of TNF-α and GM-CSF).

From other studies it appears that different thresholds exist for induction of different cytokines (Valitutti et al., 1996; Itoh & Germain, 1997). Thus, a likely assumption would be that much of the heterogeneity in cytokine production by cells with the same epitope specificity reflected the normal diversity of the involved TCRs, i.e. cells with high avidity for their antigen might be activated to produce a wider range of cytokines than low avidity cells. However, in vivo-primed TCR transgenic cells were as heterogeneous in their cytokine profile as were a normal polyclonal T cell population with the same epitope specificity. Thus, a diverse avidity spectrum does not suffice to explain the heterogeneity in cytokine phenotypes. This conclusion is consistent with previous findings demonstrating intraclonal heterogeneity in cytokine production by long-term CD4+ T cells in culture (Itoh & Germain, 1997).

In conclusion, our results underscore the extensive range of cytokines that may be produced by in vivo activated CD8+ T cells. Interestingly, almost similar cytokine profiles are generated whether or not extensive virus replication takes place. This may suggest a deeply founded propensity of CD8+ T cells for type 1 differentiation, which may be linked to their evolutionary role as effector cells controlling parasites with a cytoplasmic habitat.

ACKNOWLEDGEMENTS

This study was supported in part by the Danish Medical Research Council, the Biotechnology Center for Cellular Communication and the Novo Nordisk Foundation. N. N. K was the recipient of a scholarship from the Leo Nielsen and Wife Foundation and J. P. C is the recipient of a Senior Research Fellowship from the Benzon
Foundation, Denmark. We acknowledge the National Institute of Allergy and Infectious Disease Tetramer facility and the National Institutes of Health AIDS Research and Reference Reagent Program for providing relevant tetramers.

REFERENCES


