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Differential Kinetics of Antigen-Specific CD4⁺ and CD8⁺ T Cell Responses in the Regression of Retrovirus-Induced Sarcomas

Koen Schepers,* Mireille Toebes,* Gitte Sotthewes,* Florry A. Vyth-Dreese,* Trees A. M. Dellemijn,* Cornelis J. M. Melief,† Ferry Ossendorp,‡ and Ton N. M. Schumacher²*

Despite the accepted role for CD4⁺ T cells in immune control, little is known about the development of Ag-specific CD4⁺ T cell immunity upon primary infection. Here we use MHC class II tetramer technology to directly visualize the Ag-specific CD4⁺ T cell response upon infection of mice with Moloney murine sarcoma and leukemia virus complex (MoMSV). Significant numbers of Ag-specific CD4⁺ T cells are detected both in lymphoid organs and in retrovirus-induced lesions early during infection, and they express the 1B11-reactive activation-induced isoform of CD43 that was recently shown to define effector CD8⁺ T cell populations. Comparison of the kinetics of the MoMSV-specific CD4⁺ and CD8⁺ T cell responses reveals a pronounced shift toward CD8⁺ T cell immunity at the site of MoMSV infection during progression of the immune response. Consistent with an important early role of Ag-specific CD4⁺ T cell immunity during MoMSV infection, CD4⁺ T cells contribute to the generation of virus-specific CD8⁺ T cell immunity within the lymphoid organs and are required to promote an inflammatory environment within the virus-infected tissue. The Journal of Immunology, 2002, 169: 3191–3199.

The development of protective T cell immunity against intracellular pathogens involves MHC class I-restricted cytotoxic CD8⁺ T cells and MHC class II-restricted helper CD4⁺ T cells. Whereas the activity of Ag-specific CD8⁺ T cell immunity primarily involves direct inactivation of Ag-expressing cells, the actions of Ag-specific CD4⁺ T cell populations are more diverse. Traditionally, CD4⁺ T cells are considered to provide a setting for optimal CD8⁺ T cell and B cell activation. Although originally this T cell help was thought to be mediated by Th-secreted cytokines, more recent studies have indicated that CD4⁺ T cell help is at least in part mediated through the “licensing” of APCs for CD8⁺ T cell activation (1–3). In addition to this role of CD4⁺ T cell immunity in the initiation of cytotoxic T cell responses, CD4⁺ T cells can also mediate other orchestrating functions, as well as direct effector functions in the control of (retro)viral infections (4) and tumors (5, 6). Despite these accepted roles for Ag-specific CD4⁺ T cells in disease control, comparatively little is known about the development of CD4⁺ T cell immunity.

In the past few years, MHC class II multimers have been extensively used to study CD8⁺ T cell responses in a number of tumor and virus models (7, 8). These studies have provided insight into fundamental characteristics of CD8⁺ T cell immunity, such as the magnitude, distribution, and kinetics of CD8⁺ T cell responses during pathogen encounter. More recently, MHC class II multimers have been used to study the presence of Ag-specific CD4⁺ T cells in different models. These studies showed the presence of small but demonstrable numbers of Ag-specific CD4⁺ T cells in mice vaccinated with model Ags and in the synovial fluid of individuals suffering from chronic Lyme arthritis and rheumatoid arthritis (9–12). In addition, virus-specific CD4⁺ T cells have been detected in the blood of patients exposed to HSV or influenza A virus, after specific in vitro expansion of PBMCs (13, 14). Recently, Homann et al. (15) used MHC class II and class I tetramers to address the formation and stability of CD4⁺ and CD8⁺ T cell memory upon infection of mice with lymphocytic choriomeningitis virus (LCMV) and demonstrated that, whereas CD8⁺ T cell memory is stably maintained for life, CD4⁺ T cell memory declines gradually.

Here, we use an onco-retrovirus infection model to study the development of primary Ag-specific T cell immunity. Upon infection of mice with Moloney murine sarcoma and leukemia virus complex (MoMSV), animals rapidly develop pronounced virally-induced sarcomas. These lesions are characterized by a mixture of virus-infected myocytes and a large infiltrate of lymphocytes, granulocytes, and macrophages (16–18). In immunocompetent mice, the virus-induced lesions regress in a T cell-dependent manner over a period of 4–5 wk. In contrast, immunodeficient mice succumb as a consequence of uncontrolled viral spreading/cellular transformation by the mos oncogene that is encoded by this oncoretrovirus (19–21). Importantly, CD4⁺ T cell immunity is essential for successful viral clearance upon MoMSV infection, as shown by the fact that CD4-depleted mice develop progressive and

*Department of Immunology, The Netherlands Cancer Institute, Amsterdam, The Netherlands; and †Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands

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2 Address correspondence and reprint requests to Dr. Ton N. M. Schumacher, Department of Immunology, The Netherlands Cancer Institute, Plesmanalaan 121, 1066 CX, Amsterdam, The Netherlands. E-mail address: tschum@nki.nl

3 Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; MoMSV, Moloney murine sarcoma and leukemia virus complex; H19-Env, I-A b-restricted MoMSV-envelope epitope; NP, nucleoprotein; GagL*, the GagL85–93 peptide variant Abu-Abu-Leu-Thr-Val-Phc-Leu; D9-GagL* tetramer, H-2D b tetramer containing the GagL* peptide; FMR, Friend/Moloney/Rauscher; I-A b·-GagL* tetramer, H-2D b·-GagL* tetramer containing the H19-Env peptide; DLN, draining lymph node; HAu, hemagglutinating unit; IHC, immunohistochemistry.
lethal lesions (Refs. 22 and 23 and see Results). Furthermore, vaccination with a Moloney-derived T helper epitope protects mice from retrovirus-induced tumors (24). Consequently, analysis of the MoMV-specific CD4+ T cell response may provide insights into the characteristics of a successful retrovirus-specific immune response and the development and function of Ag-specific CD4+ T cell immunity in general.

Materials and Methods

Mice, viruses, and Abs

C57BL/6 mice were bred at the animal experimental department of the Netherlands Cancer Institute (Amsterdam, The Netherlands) and at the Leiden University Medical center animal facility (Leiden, The Netherlands). Mice were kept under specified pathogen-free conditions. Mice were handled at all times in accordance with institutional guidelines.

MoMSV was prepared and injected (10^5 focus-forming units) in the thigh muscle as described (25). Purified recombinant influenza A virus strain A/NT/60/68 virus was used.

The following primers were used for PCR amplification:

Forward primer: 5'-CTGCTTTCACCGGTAACACCTACG-3'

Reverse primer: 5'-GATCCGTTCAGCGTAACCTGCCGTTCGACAAACCGACCATCATGG-3'

The resulting pMT2 plasmids were transiently transfected (5 μg of each plasmid) into 293T cells by standard DEAE transfection or DMSO shock, and cells were cultured for 72 h at 37 °C in 1% Hybridoma medium NS (Boehringer Mannheim, Mannheim, Germany) in IMDM (Life Technologies, Paisley, U.K.). For production in insect cells, the resulting pMT/V5-His A plasmids (9.5 μg of each plasmid) were transfected into Drosophila S2 cells together with pSi2Neo (1 μg). Stable transfectants were selected by growing the cells in SIF (Life Technologies, containing 100 ng/ml Geneticin) for 3–4 wk. The αβ heterodimer production was induced by growing the cells in the presence of 500 μM CoCl2 for 4–5 days. Subsequently, the supernatant was collected and concentrated, and the buffer was exchanged to 100 mM NaCl, 20 mM Tris (pH 8). The αβ heterodimers were purified by Co^- precipitation using 100 mM Imidazole for elution. Then αβ heterodimers were biotinylated with BirA, purified, and converted to tetramers as has been described for MHC class I tetramers (27). MHC class II tetramers were stored at 4°C in 150 mM NaClO2/30 mM Tris (pH 7)/0.5% BSA (Sigma-Aldrich, The Netherlands). MHC class II tetramers were used at a final concentration of ~0.75 μg/ml.

Cell isolation and in vitro restimulation

Spleen, lymph nodes, and lungs were isolated and homogenized using a nylon mesh filter (NBF, Emmer-Compascuum, The Netherlands). Tumors were isolated and homogenized by treatment of small tumor pieces with Collerase (1 mg/ml) and DNase (10 μg/ml) for 30 min at 37°C and were transferred through a nylon mesh filter. RBCs were removed from the cell suspensions by treatment with erythrosin buffer (155 mM NH4Cl, 10 mM KHCO3, and 0.1 mM EDTA (pH 7.4)).

For in vitro restimulation, cells were labeled with CFSE (Molecular Probes, Leiden, The Netherlands) as described (28). CFSE-labeled cells were stimulated for 6 days in IMDM supplemented with 10% FCS (Bio-Whittaker, Verviers, Belgium), 0.5 μM 2-ME (Merck, Hohebrunn, Germany), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Boehringer Mannheim) (culture medium) in either the presence or absence of H19-Ev peptide.

Flow cytometry

MHC class I tetramer staining was performed in PBS containing 0.5% BSA and 0.02% NaN3 at room temperature for 15 min. MHC class II tetramer staining was performed in culture medium at 37°C for 3–5 h. Staining with Abs was performed during the last 20 min of tetramer staining. Samples were also stained with F4/80 Ab to be able to reduce background staining of MHC class II tetramers by gating out macrophages. Subsequently, cells were washed and resuspended in PBS containing 0.2% BSA and 0.02% NaN3. Before analysis, propidium iodide was added to select for propidium iodide-negative (living) lymphocytes. Analysis was performed on a FACS Calibur using CellQuest software (BD Biosciences).

Intracelluar staining

Intracellular staining was performed as described (29). In brief, cells were incubated with peptide (10 μg/ml) for 4–5 h at 37°C in the presence of recombinant human IL-2 (10 U/ml) and brefeldin A (1 μM). After incubation, cells were stained with anti-CD8α-allophycocyanin or anti-CD4-peridinin chlorophyll protein Ab, incubated in Cytofix/Cytoperm solution (BD Biosciences) for 30 min on ice and subsequently washed with PBS containing 0.2% BSA and 0.1% NaN3. Intracellular staining was performed in culture medium at 37°C for 30–45 min. For in vitro restimulation, cells were labeled with CFSE (Molecular Probes, Leiden, The Netherlands) as described (28). CFSE-labeled cells were stimulated for 6 days in IMDM supplemented with 10% FCS (Bio-Whittaker, Verviers, Belgium), 0.5 μM 2-ME (Merck, Hohebrunn, Germany), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Boehringer Mannheim) (culture medium) in either the presence or absence of H19-Ev peptide.

Statistical analysis

Percentages and absolute numbers of tetramer-positive cells were logarithmically transformed and subsequently a repeated measurement ANOVA was used. The model was fitted using restricted maximum likelihood, assuming constant SDs over cell type as well as time. SES and p values were calculated using the sandwich estimator for the covariance matrix of the means. The p values were calculated from approximate type III F-tests and used for the construction of confidence intervals from approximate t-distributions. First, an overall test was done to determine whether the two curves differ in shape (cell*time interaction). If this was the case (p < 0.05), relative changes between adjacent days were estimated and compared between the two cell types. PROC MIXED of the SAS statistical was used for the analyses.

For statistical analysis of CD4-depleted and control mice, percentages of H-2Dd tetramer containing the GagL* peptide variant Abu-Abu-Leu-Leu-Leu-Thr-Val-Phe-Leu (GagL*) peptide (D-b-GagL* tetramer)-positive cells were determined using a Student’s t test.
In situ immunohistochemical and immunofluorescence analysis

Cryostat fragments of retrovirus-induced sarcoma tissues were cut in 4-μm sections, air-dried overnight, and fixed in acetone for 10 min at room temperature. Sections were preincubated in 5% (v/v) normal goat serum (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands), or 5% (v/v) normal mouse serum in case of staining with hamster Abs.

For immunohistochemical analysis, sections were stained using a standard alkaline phosphatase protocol as described previously, with slight modifications (30). Briefly, sections were stained with primary Ab diluted in PBS containing 1% BSA (plus 10% normal mouse serum in case of staining with hamster Abs). Sections stained with FITC-conjugated Ab were subsequently incubated with alkaline phosphatase-labeled sheep anti-FITC Ab (Boehringer Mannheim). Sections stained with biotin-conjugated Ab were subsequently incubated with streptavidin/biotin-conjugated alkaline phosphatase complex (ABC-protocol; DAKO, Glostrup, Denmark). Color was developed using naphth AS-MX phosphate (0.3 mg/ml) plus New Fuchsin (0.1 mg/ml) in 0.2 M Tris-HCl buffer (pH 8.0; ABC-protocol; DAKO), and sections were counterstained with hematoxylin. Between incubation steps, sections were extensively rinsed in PBS. Within each staining procedure, isotype-matched control Abs were included and found negative.

Immunofluorescence double labeling was performed as described previously (31), with slight modifications. Briefly, sections were incubated with optimal dilutions of FITC-conjugated F4/80 Ab and biotin-conjugated mouse anti-mouse I-A\(^b\) (MHC class II) mAbs (in PBS containing 1% (v/v) BSA) for 1 h at room temperature, followed by incubation with Cy5-conjugated streptavidin (Jackson ImmunoResearch Laboratories, Palo Alto, CA). Confocal fluorescence images were obtained on a Leica TCS SP confocal system (Leica Microsystems, Heidelberg, Germany), equipped with an Ar/HeNe laser combination. Images were taken using a 40×1.25 NA objective. Color photomicrographs were taken from electronic overlays.

Results

Role of CD4\(^+\) and CD8\(^+\) T cells in the regression of MoMSV-induced sarcomas

After i.m. injection of mice with MoMSV virus, lesions develop within 2 wk, and they subsequently regress spontaneously. We determined the role of CD4\(^+\) and CD8\(^+\) T cells in immune control of MoMSV infection by analyzing lesion development in mice depleted for either CD4\(^+\) cells or CD8\(^+\) T cells. Consistent with earlier data (22, 23), CD4 depletion resulted in progressive outgrowth of MoMSV-induced lesions and subsequent death (Fig. 1), demonstrating that CD4\(^+\) T cells are required for the regression of MoMSV-induced sarcomas. Mice depleted of CD8\(^+\) cells show a delay in the progression of MoMSV-induced tumors but eventually clear the virus (Fig. 1) (22). Likewise, sarcoma regression is delayed but not abolished in perforin-deficient mice (32). Previously, it has been demonstrated that regression of MoMSV-induced lesions in immunodeficient mice can be achieved by infusion of Ag-specific CD8\(^+\) T cell clones (33). Collectively, these data indicate that, whereas CD4\(^+\) T cell immunity is crucial for the control of MoMSV infection, CD8\(^+\) T cell immunity contributes to viral clearance in conjunction with CD8-independent mechanisms.

Detection of MoMSV-specific CD4\(^+\) T cell immunity using MHC class II tetramers

Previously, Iwashiro et al. (34) defined the immunodominant I-A\(^b\) restricted epitope (H19-Env, EPLTSLPCTNTAWNLKL) of the envelope (gp70) protein of the Friend murine leukemia virus. This epitope is conserved in the Friend/Moloney/Rauscher (FMR) family of retroviruses that includes MoMSV. With the aim to visualize the Ag-specific CD4\(^+\) T cell response during MoMSV infection, we generated I-A\(^b\)-tetramers containing the H19-Env epitope of MoMSV (I-A\(^b\)-Env). To this purpose, heterodimers of the extracellular domains of the I-A\(^b\) α-chain and β-chain were produced in eukaryotic cells with the H19-Env epitope covalently attached to the I-A\(^b\) β-chain. Velcro leucine zippers were included to promote heterodimerization (35), and a His-tag and biotinylation signal (bio-tag) were attached to the I-A\(^b\) α- and β-chain for subsequent purification and tetramer formation.

The specificity of the I-A\(^b\)-Env tetramers was first tested on H19-Env peptide-stimulated CFSE-labeled spleen cells of B6 mice that had previously been infected (40 days) with MoMSV. I-A\(^b\) Env tetramers stained a sizeable percentage of CD4\(^+\) T cells that had proliferated (i.e., were CFSE low ) on day 6 after stimulation with H19-Env peptide (Fig. 2A). Staining was found to be specific, as these CD4\(^+\) CFSElow cells did not stain with control I-A\(^b\) tetramers that contained an influenza A/NT/60/68 virus-derived peptide (NP\(_{413-435}\), SVQRNLFPDKPTMAAFTGNT) (36). Furthermore, after restimulation in the absence of the MoMSV Env-derived CD4\(^+\) T cell epitope, little to no I-A\(^b\)-Env tetramer+ T cells were detected within the CD4\(^+\) CFSElow population.

We subsequently tested the ability of the I-A\(^b\)-Env tetramer to detect Ag-specific T cells directly ex vivo. To this purpose, organs of MoMSV-infected mice were isolated on day 14 postinfection, and cell suspensions were stained with I-A\(^b\)-Env tetramers, anti-CD4, and phenotypic markers (Fig. 2B, shown for the 11B1 activation marker; see below). Low but detectable frequencies of I-A\(^b\)-Env tetramer+ CD4\(^+\) T cells are observed in the DLNs, spleen, and lesions of MoMSV-infected mice (average: 0.29, 0.50, and 1.09%, respectively) (Fig. 2B). Much lower levels of staining are observed in organs of both noninfected mice (average, 0.02 ± 0.01) and mice infected with the influenza A/NT/60/68 virus (average, 0.03 ± 0.01% (Fig. 2B)). Vice versa, CD4\(^+\) T cells of MoMSV-infected mice did not stain with control I-A\(^b\) tetramers containing the influenza A NP-derived peptide (data not shown).

Detection of MoMSV-specific CD8\(^+\) T cell immunity using MHC class I tetramers containing an altered peptide

Previously, Chen et al. (37) described the immunodominant CD8\(^+\) T cell epitope of the FMR family of retroviruses (the H-2D\(^b\)-restricted MoMSV Gag\(_{319-333}\) epitope, CCLCLTVFIL). This H-2D\(^b\)-restricted epitope contains three cysteine residues that preclude the formation of MHC tetramers by their strong propensity to form disulfide-bonded dimers. To circumvent the problems associated with the inherent reactivity of this cysteine-rich epitope, we generated a set of variant peptides in which individual amino acids were replaced by the isosteric amino acid α-aminoacrylic acid. Screening of variant peptides for their ability to induce IFN-γ secretion in CD8\(^+\) T cells of MoMSV-infected mice by intracellular IFN-γ staining reveals that an H-2D\(^b\)-restricted MoMSV Gag\(_{319-333}\) epitope variant in which all three cysteine residues were replaced by the isosteric amino acid α-aminoacrylic acid is recognized efficiently (data not shown).
not shown). D\(^\text{\text{p}}\)-tetramers can readily be produced with this triple-substituted epitope (GagL\(^*\), Abu-Abu-Leu-Abu-Leu-Thr-Val-Phe-H\(^{11001}\)).

**FIGURE 2.** Detection of MoMSV-specific CD4\(^+\) T cells. A, Purified spleen cells of mice previously infected (40 days) with MoMSV were labeled with CFSE and stimulated with or without H19-Env peptide. On day 6 after stimulation, cells were stained with anti-CD4 Ab and COS-7-expressed I-A\(^\text{\text{d}}\)-Env tetramer or the I-A\(^\text{\text{d}}\)-tetramer containing the A/NT/60/68 NP\(_{113-145}\) epitope and were analyzed by flow cytometry. Cells are gated on live CD4\(^+\) lymphocytes. B, Representative FACS profiles of cells from inguinal draining lymph node (DLN), spleen, and lesion (tumor upon MoMSV infection, lung upon influenza A/NT/60/68 infection), either 14 days postinfection with MoMSV or 8 days postinfection with influenza A/NT/60/68 virus (25 HAU), stained with anti-CD8 Ab, Db\(^-\)GagL\(^*\) tetramer, and 1B11 Ab. Cells are gated on live CD8\(^+\) lymphocytes.

The 1B11 Ab has been described to recognize an activation-associated isoform of CD43 (38), which was recently found to be upregulated on effector CD8\(^+\) T cells but absent on memory CD8\(^+\) T cells, defining it as a marker for effector phase CD8\(^+\) T cells (39). The finding that MoMSV-specific CD4\(^+\) T cells, isolated from MoMSV-infected mice, also express 1B11 suggests that this marker may not only be used to detect effector CD8\(^+\) T cells, but also to define effector CD8\(^+\) T cells. The observation that ~50% (range, 28–93%) of the CD4\(^+\) T cells present within the lesion expresses 1B11 at the peak of infection is consistent with this notion (Fig. 2B).

**Function of MoMSV-specific CD8\(^+\) and CD4\(^+\) T cells**

To determine whether the T cell responses detected by I-A\(^\text{\text{d}}\)-Env and D\(^\text{\text{p}}\)-GagL\(^*\) tetramer staining correlated with Ag-induced cytokine responses, tissue samples of MoMSV-infected mice were stained separately with tetramers or with anti-IFN-\(\gamma\) Ab upon specific in vitro stimulation (Fig. 4) (combined MHC tetramer staining/intracellular cytokine staining is made impossible by Ag-induced TCR down-regulation). In the majority of organs containing a significant percentage of Ag-specific cells as determined by tetramer staining, Ag-specific IFN-\(\gamma\) staining was likewise detected.

**Table I. Phenotype of MoMSV-specific CD4\(^+\) and CD8\(^+\) T cells**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Site</th>
<th>CD8(^+) D(^\text{\text{p}})-GagL(^*)</th>
<th>CD4(^+) I-A(^\text{\text{d}})-Env</th>
</tr>
</thead>
<tbody>
<tr>
<td>1B11</td>
<td>DLN</td>
<td>85 ± 10</td>
<td>82 ± 6</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>95 ± 3 (\dagger)</td>
<td>76 ± 16 (\dagger)</td>
</tr>
<tr>
<td></td>
<td>Lesion</td>
<td>84 ± 16</td>
<td>95 ± 6</td>
</tr>
<tr>
<td>CD62L</td>
<td>DLN</td>
<td>26 ± 11</td>
<td>11 ± 6</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>5 ± 7</td>
<td>7 ± 1</td>
</tr>
<tr>
<td></td>
<td>Lesion</td>
<td>5 ± 7</td>
<td>7 ± 4</td>
</tr>
<tr>
<td>CD44</td>
<td>DLN</td>
<td>90 ± 8</td>
<td>96 ± 3</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>99 ± 1</td>
<td>94 ± 1</td>
</tr>
<tr>
<td></td>
<td>Lesion</td>
<td>94 ± 5</td>
<td>92 ± 13</td>
</tr>
</tbody>
</table>

\(\dagger\) Isolated inguinal DLN, spleen, and lesion cells of mice 14 days postinfection with MoMSV were stained with either anti-CD8 Ab and D\(^\text{\text{p}}\)-GagL\(^*\) tetramers or anti-CD4 Ab and Drosophila-expressed I-A\(^\text{\text{d}}\)-Env tetramers, together with FITC-labeled 1B11 Ab, anti-CD62L Ab, or anti-CD4 Ab. Percentages (average ± SD) of marker-positive cells within the I-A\(^\text{\text{d}}\)-Env tetramer-CD4\(^+\) T cell and D\(^\text{\text{p}}\)-GagL\(^*\) tetramer-CD8\(^+\) T cell population are shown. Data are representative for two independent experiments (\(n = 5, \sigma = 3\)). On average, 46, 22, and 12 CD4\(^+\) I-A\(^\text{\text{d}}\)-Env cells and 120, 358, and 626 CD8\(^+\) D\(^\text{\text{p}}\)-GagL\(^*\) cells were counted for DLN, spleen, and lesion, respectively.
In both the CD4⁺ and CD8⁺ T cell compartment, the frequency of tetramer⁺ cells was generally higher than the frequency of Ag-specific cells as revealed by IFN-γ production (on average two-fold). Interestingly, Ag-specific CD8⁺ T cells in the DLN appear less capable of producing IFN-γ (Fig. 4B), possibly indicating that these cells may not have fully differentiated into IFN-γ-producing cells. Further analysis of the cytokine profile of the MoMSV Env-specific CD4⁺ T cell response by intracellular cytokine staining revealed a Th1 phenotype, as manifested by the production of TNF-α, IL-2, and GM-CSF, but not IL-10 and IL-4 (data not shown).

Kinetics of the MoMSV-specific CD8⁺ and CD4⁺ T cell response

We subsequently compared the kinetics and distribution of the MoMSV-specific CD4⁺ and CD8⁺ T cell response during primary MoMSV infection. Early in the response (day 10 postinfection), comparable percentages of MoMSV-specific CD4⁺ T cells (1.12%) and MoMSV-specific CD8⁺ T cells (0.71%) are detected at site of infection (Fig. 5A). Subsequently, the MoMSV-specific CD8⁺ T cell response increases dramatically, whereas the MoMSV-specific CD4⁺ T cell response is either constant or decreases slightly (22.25 and 0.72%, respectively, on day 17). To quantify this shift in T cell immunity, the absolute numbers of MoMSV-specific CD4⁺ and CD8⁺ T cells were determined at different time points after infection (Fig. 5B). At day 10 after infection, the lesion contains comparable numbers of MoMSV-specific CD4⁺ T cells (5.9 × 10⁵) and CD8⁺ T cells (8.4 × 10⁵). Subsequently, the expansion of MoMSV-specific CD8⁺ T cells outpaces that of MoMSV-specific CD4⁺ T cells, and at day 14 GagL-specific CD8⁺ T cells outnumber Env-specific CD4⁺ T cells by >75-fold.

A comparable shift in both frequency and absolute numbers of MoMSV-specific CD4⁺ and CD8⁺ T cells appears to take place in DLN and spleen (Fig. 5). Although the frequencies/absolute numbers of Ag-specific CD4⁺ and CD8⁺ T cells at day 10 after infection are too close to background levels in these organs to accurately determine the relative contribution of the different subsets at this early time point, similar numbers of Ag-specific CD4⁺ and CD8⁺ T cells appear present at both sites. In contrast, at day 14 postinfection, GagL-specific CD8⁺ T cells outnumber Env-specific CD4⁺ T cells by ~23- and 34-fold in DLN and spleen, respectively. Collectively, these data show a shift from combined CD4⁺ and CD8⁺ T cell immunity early in the MoMSV-specific T cell response, toward a T cell response that is dominated by CD8⁺ T cells at the peak of infection (Fig. 5C).

**A dual role for CD4⁺ T cells in the regression of MoMSV-induced sarcomas**

Prior studies have shown that CD4⁺ T cells provide stimulatory signals in the generation of Ag-specific CD8⁺ T cell responses (3, 40–44). To test whether the generation of Ag-specific cytotoxic T cell responses after MoMSV infection is also dependent on CD4⁺ T cells, we examined the MoMSV-specific CD8⁺ T cell response in mice lacking CD4⁺ T cells. CD4 depletion leads to a reduction in the number of GagL⁺-tetramer⁺ CD8⁺ T cells in the DLN and spleen at both day 10 and day 14 after MoMSV infection (day 14, 63 and 53%, p = 0.005 and 0.08, respectively; Fig. 6). Likewise, reduced numbers of GagL⁺-tetramer⁺ CD8⁺ T cells are detected in DLN and spleen of MHCII⁻/⁻ mice (day 14, 81 and 72%, p = 0.04 and 0.01, respectively; data not shown). In addition, CD4 depletion leads to a reduction in the number of GagL⁺-tetramer⁺ CD8⁺ T cells in lesions of CD4-depleted mice at day 10 after MoMSV infection (84%, p = 0.01, data not shown). Collectively, these data indicate that CD4⁺ T cells contribute significantly to the generation of the MoMSV-specific CD8⁺ T cell response upon viral infection. In contrast to the reduction in GagL⁺-tetramer⁺ CD8⁺ T cells early in the response, the number of Ag-specific CD8⁺ T cells in the muscle of CD4-depleted mice is increased at day 14 after MoMSV infection (3-fold, p = 0.01; Fig. 6), possibly as a consequence of increased viral load in lesions of CD4-depleted mice. Immunohistochemical analysis suggests that these CD8⁺ T cells may not have infiltrated the infected tissue in CD4-depleted mice (see below).

CD4⁺ T cell immunity is indispensable for the regression of MoMSV-induced lesions. Although CD8⁺ T cell immunity contributes to viral clearance, it is not essential. It is therefore apparent that there must be an additional role(s) for Ag-specific CD4⁺ T cell immunity in sarcoma regression, besides controlling the magnitude of the Ag-specific CD8⁺ T cell response. In particular, the

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**FIGURE 4.** Correlation of tetramer⁺ and IFN-γ⁺ T cell responses. Purified DLN (diamonds), spleen (squares), and lesion (triangles) cells of mice (n = 5) 17 days postinfection with MoMSV were stained with anti-CD4 Ab and Drosophila-expressed I-A²-Env or anti-CD8 Ab and D¹-GagL⁺ tetramers. In parallel, cell suspensions from the same tissue samples were stimulated for 4 h at 37°C in the presence of 10 U/ml IL-2 and H19-Env peptide, GagL⁺ peptide, or no peptide and were subsequently stained for CD4/CD8 and intracellular IFN-γ expression. H19-Env/GagL⁺-specific IFN-γ staining was determined by subtracting the background IFN-γ staining (IFN-γ staining after stimulation in the absence of peptide) from the IFN-γ staining after specific stimulation with peptide. Data show the percentage of tetramer-positive cells within the CD4⁺CD8⁺ T cell compartment vs peptide-specific IFN-γ-staining within the CD4⁺CD8⁺ T cell compartment.
Kinetics of MoMSV-specific CD4+ and CD8+ T cell responses. Mice were infected i.m. with MoMSV virus and sacrificed at day 10, 14, 17, or 21 postinfection. Cells from DLN, spleen, and lesion were isolated and stained with either Drosophila-expressed I-Aβ-Env tetramer/anti-CD4 Ab or Dβ-GagL+ tetramer/anti-CD8 Ab and were analyzed by flow cytometry. A, The percentage (average ± SD) of I-Aβ-Env tetramer-positive cells within the CD4+ lymphocyte population and Dβ-GagL+ tetramer-positive cells within the CD8+ lymphocyte population are depicted. Cells from mediastinal lymph nodes, spleen, and lung of mice on day 8 post-influenza A/NT/60/68 virus infection (25 HAU) showed on average 0.14 ± 0.01% I-Aβ-Env tetramer+ and 0.10 ± 0.08% Dβ-GagL+ tetramer+ cells within the CD4+ and CD8+ T lymphocyte population, respectively. n = 6–7/group; ND = not determined. A significant difference in the kinetics of the percentage of tetramer positive CD4+ and CD8+ T cells was found for all organs analyzed (p < 0.001). The median relative increase in the frequency of GagL-specific CD8+ vs Env-specific CD4+ T cells between day 10 and day 14 is 5.1, 8.8, and 18.8; 95% confidence intervals 1.14–22.6, 4.1–18.9, 8.3–42.8 for DLN, spleen, and lesion, respectively. B, Absolute numbers of CD4+ I-Aβ-Env tetramer-positive cells and CD8+ Dβ-GagL+ tetramer-positive cells were calculated from the total number of cells recovered. Averages ± SD are depicted. A significant difference in kinetics of the absolute number of tetramer-positive CD4+ and CD8+ T cells was found for all organs analyzed (p < 0.002). The median relative increase in the absolute number of GagL-specific CD8+ T vs Env-specific CD4+ T cells between day 10 and day 14 is 5.1, 11.3, and 31.6; 95% confidence intervals 1.21–28.7, and 12.8–78.1 for DLNs, spleen, and lesion respectively. C, Shift in MoMSV-specific T cell immunity during sarcoma regression. Bars represent the ratio of CD8+ Dβ-GagL+ tetramer+ cells/CD4+ I-Aβ-Env tetramer+ cells in the lesion at the indicated time points, as determined from the absolute numbers of MHC class I and MHC class II tetramer-positive cells.
CD4 depletion results in a decreased infiltration of cells positive for the macrophage marker F4/80 in the lesion (Fig. 7, C and D). Infiltration of cells that express the dendritic cell marker CD11c or the granulocyte marker Gr1 does not appear decreased significantly (data not shown). In IHC sections of CD4-depleted mice there is a significant reduction of CD8+ cells (Fig. 7, A and B), even at a time point at which CD8+ numbers as measured by flow cytometry are increased. This difference between CD8+ cell infiltration as measured by flow cytometry and IHC is consistently observed in multiple experiments and may suggest an altered distribution of the CD8+ T cells in lesions of CD4-depleted mice. Further analysis will be required to resolve this issue. Most strikingly, a dramatic reduction in the number of cells expressing MHC class II is observed in sarcomas of CD4-depleted mice (Fig. 7, E and F). To study which cells express MHC class II in the lesions and how this is affected by CD4 depletion, sections of MoMSV-induced sarcomas were simultaneously stained with F4/80 and anti-I-Aβ Ab (Fig. 7, G and H). In MoMSV-induced lesions of control mice, colocalization of MHC class II and F4/80 dominates, indicating that most of the MHC class II-expressing cells within the lesion are macrophages and that most of the macrophages within the lesion are activated. In CD4-depleted mice, the picture is strikingly different. Although single positive cells (i.e., F4/80+ MHC II- and F4/80+ MHC II+) are still present, there is a very strong reduction in the number of activated, MHC II+ macrophages. Because only few B220+ cells and significant numbers of CD11c+ cells are found in the lesion (data not shown), the remaining (F4/80+ MHC II- MHC II+ cells are most likely dendritic cells, the accumulation of which appears less dependent on CD4+ T cells. Collectively, these data indicate that CD4+ T cell immunity contributes to the infiltration/accumulation of immune cells and in particular the accumulation and activation of macrophages at the effector site during MoMSV infection.

Discussion

Here we use MHC class II tetramers to characterize the distribution and kinetics of the CD4+ T cell response against the oncornavirus MoMSV and compare this response with the CD8+ T cell response. Low but detectable numbers of I-Aβ–Env tetramer-positive CD4+ T cells are present in the DLNs of MoMSV-infected mice. In addition, sizeable numbers of Ag-specific CD4+ T cells are detected within lesions of MoMSV-infected mice (Fig. 5A). In organs containing a significant percentage of I-Aβ–Env tetramer+CD4+ cells, MoMSV Env-specific IFN-γ staining was likewise detected. The enrichment of (cytokine-producing) Ag-specific CD4+ T cells at the site of infection provides indirect (numerical) support for the notion that provision of T cell help in the draining lymphoid organ is only a single aspect of Ag-specific CD4+ T cell immunity (see below).

The majority of MoMSV-specific CD4+ T cells detected by MHC tetramer staining are of an effector/memory phenotype as defined by the expression levels of CD62L and CD44. Interestingly, a large fraction of Ag-specific CD4+ T cells express the 1B11-reactive, activation-induced isoform of CD43 that was recently shown to define effector CD8+ T cell populations. Based on these data it is plausible that the 1B11 marker can also be used to define effector CD4+ T cells, but formal proof for this will require functional analysis of 1B11+ and 1B11- Ag-specific CD4+ T cells at different time points after infection. Although virtually all MHC class I and MHC class II tetramer-positive cells were 1B11+, MHC tetramer+ cells constituted only 1–2% and 9–16% of the 1B11+CD4+ and 1B11+CD8+ T cells, respectively. This is unlikely to be a result of the presence of large numbers of Ag-specific T cells that are directed against other MoMSV-derived epitopes, as the epitopes used in this study have been previously characterized as the immunodominant epitopes in the T cell response against FMR retroviruses (Ref. 37 and F. Ossendorp, unpublished data). The finding that MHC tetramer+ cells constitute only a minor fraction of activated (1B11+) cells might conceivably be due to the lack of MHC tetramers to identify all Ag-specific cells. In a number of viral infection systems, a failure of MHC class I tetramers to identify all Ag-specific CD8+ T cells has been demonstrated, in particular in situations of high viral load (49, 50). Likewise, it has previously been described for CD4+ T cell clones in a number of different systems that MHC class II tetramers containing the antigenic peptide fail to stain a subset of (low-avidity) Ag-specific hybridomas (10, 12). In line with this, we found that I-Aβ–Env tetramers do not bind to a previously characterized Env-specific CD4+ T cell clone (Ref. 24 and data not shown). However, the finding that MHC tetramers and intracellular cytokine staining identify similar numbers of Ag-specific CD4+ and CD8+ T cells argues against an underestimate of T cell responses as measured by MHC tetramer staining. The 1B11+ MHC tetramer-negative T cell populations might consist of Ag-specific T cells that have become refractory to both MHC tetramer staining and Ag-specific cytokine production. Alternatively, they may consist of bystander T (memory) cells activated by the inflammatory conditions, two possible explanations that deserve further study.

Previously, Ag-specific CD4+ T cell frequencies have been estimated by indirect enumeration, using limiting dilution assays,
ELISPOT and intracellular cytokine staining. These studies suggested that, as based on functional assays, the frequencies of Ag-specific CD4⁺ T cells are considerably lower than the frequencies of Ag-specific CD8⁺ T cells (8, 51, 52). Both the results by Homann et al. (15), which use tetramer technology to analyze the LCMV-specific CD4⁺ and CD8⁺ T cell response, and the results described here support the idea that the number of virus-specific CD4⁺ T cells are indeed considerably lower (~20–40) than the number of virus-specific CD8⁺ T cells.

The most striking observation from the comparison of the epitope-specific CD4⁺ and CD8⁺ T cell responses is the pronounced shift toward a CD8⁺ T cell-dominated T cell response at the site of infection. Early during MoMSV infection, the MoMSV-specific T cell response at the effector site is of comparable size in the CD4⁺ and CD8⁺ T cell compartment, whereas at later stages of infection, GagL*+-specific CD8⁺ T cells outnumber Env-specific CD4⁺ T cells by 75-fold. Because these two epitopes appear immunodominant in the T cell response against FMR retroviruses (Ref. 37 and F. Osendorp, unpublished data), this likely reflects the kinetics of the entire MoMSV-specific T cell response. In line with this, a similar albeit less dramatic, shift in the total CD4⁺ CD8⁺:CD8⁺ CD8⁺ ratio is observed within the lesion during the course of regression (CD4⁺ CD8⁺:CD8⁺ CD8⁺ ratio day 10 1:3.5; CD4⁺ CD8⁺:CD8⁺ CD8⁺ ratio day 14 1:9.4).

The shift toward a CD8⁺ T cell-dominated T cell response during the progression of MoMSV infection is unlikely to be a consequence of differences in migration properties of MoMSV-specific CD4⁺ and CD8⁺ T cells as GagL*-specific CD8⁺ T cells outnumber Env-specific CD4⁺ T cells in all three organs analyzed during the peak of the response. However, other intrinsic differences between CD4⁺ and CD8⁺ T cell responses may contribute to the observed kinetic difference. Recently, Foulds et al. (53) demonstrated that CD4⁺ T cells are programmed to divide a limited number of times upon Ag exposure. In addition, Ag-specific CD4⁺ T cells may be more susceptible to apoptosis, as previously has been suggested for memory CD4⁺ T cells (15). Furthermore, encounter of high Ag concentrations can result in T cell anergy and concomitant loss of MHC class II tetramer binding in vitro assays (54).

In addition to a possible contribution of such lineage-intrinsic differences, pathogen-specific factors are likely to affect the kinetics of Ag-specific CD4⁺ and CD8⁺ T cell immunity. Indeed, the observation that a kinetic difference between primary CD4⁺ and CD8⁺ T cell responses is not apparent during LCMV infection (15) could indicate that pathogen-specific factors may exert an overriding effect. Such pathogen-specific factors could include the kinetics of epitope generation but also the precursor frequency of the pathogen-reactive CD4⁺ and CD8⁺ T cell compartment. The ability to mount an early CD4⁺ T cell response may be essential for the development of protective immunity against certain pathogens. Prior studies have revealed that I-Aβ is a protective MHC class II allele during FMR infection, and the current data suggest that this may be a reflection of the rapid CD4⁺ T cell response against FMR Env-I-Aβ complexes. Likewise, the ability to mount an early CMV-specific CD4⁺ T cell response appears to correlate with a favorable clinical course during primary CMV infection in transplant recipients (L. Gamadia, personal communication).

As has been previously shown in other models (3, 40–44), CD4⁺ T cells promote the generation of Ag-specific CD8⁺ T cell immunity during MoMSV infection. This role of CD4⁺ T cells might be mediated either via a direct effect of CD4⁺ T cell-secreted cytokines/chemokines or through the licensing of APCs. A second role for CD4⁺ T cell immunity is suggested by the accumulation of significant numbers of Ag-specific CD4⁺ T cells within the lesion early during infection. These CD4⁺ cells contribute to the inflammatory environment in the lesion by promoting the accumulation of large numbers of macrophages and by mediating the activation of these macrophages as revealed by MHC class II expression, possibly through IFN-γ secretion (5, 55–57). Collectively, these data provide support for a central role of CD4⁺ T cell immunity both in the initiation of CD8⁺ T cell immunity and in the interplay between acquired and innate immunity. It will be challenging to dissect the various facets of CD4⁺ T cell immunity in human disease conditions associated with diminished CD4⁺ T cell counts, such as Epstein Barr virus B cell lymphomas and cytomegalovirus disease.

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