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Restriction of De Novo Nucleotide Biosynthesis Interferes with Clonal Expansion and Differentiation into Effector and Memory CD8 T Cells1,2

Laurence Quéméneur,3 Laurent Beloeil,4,5 Marie-Cécile Michallet,5 Georgi Angelov, Martine Tomkowiak, Jean-Pierre Revillard,6 and Jacqueline Marvel6,7

Nucleotide synthesis inhibitors are currently used in neoplastic diseases or as immunosuppressive agents for the prevention of acute rejection in organ transplantation and the treatment of autoimmune disorders. We have previously described that these inhibitors interfere with proliferation and survival of primary T cells in vitro. However, the precise effects of nucleotide restriction on effector and memory functions have not been elucidated. In this study, we investigated the impact of nucleotide synthesis inhibition on CD8 T cell differentiation by using TCR transgenic mice (F5) specific for the influenza virus nucleoprotein 68 peptide presented on the H-2Dk molecule. Our results show that methotrexate and 5-fluorouracil prevent the acquisition of effector functions, such as IFN-γ, granzyme B expression, and cytotoxic function following antigenic stimulation of naive cells. Surprisingly, in the presence of mycophenolate mofetil, activated F5 cells are still able to produce granzyme B and to kill target cells but to a lesser extent compared with control. All three inhibitors interfere with the differentiation of naive cells into memory CD8 T cells. In contrast, the drugs are unable to inhibit the development of improved cytotoxic functions displayed by memory CD8 T cells. The Journal of Immunology, 2004, 173: 4945–4952.

In response to a specific Ag, naive CD8 T cells undergo a number of highly complex changes resulting in activation, proliferation, differential homing, and acquisition of effector functions (1–3). The expansion phase is critical for complete differentiation of naive cells into effector and memory T lymphocytes. Resting T cells use nucleotides from the salvage pathway for their steady state metabolic requirements. Activation of T lymphocytes is associated with an increase of purine and pyrimidine pools (4). This expansion of the nucleotide pools is a consequence of increased expression, or activity of key enzymes involved in de novo synthesis, such as inosine monophosphate dehydrogenase and thymidylate synthase (TS; Refs. 5 and 6). Recently, we have demonstrated that purine and pyrimidine nucleotides play a critical role in the cell cycle progression, proliferation, and survival of primary T lymphocytes in vitro (7, 8). As T cell differentiation is closely linked to proliferation, we investigated to what extent inhibition of nucleotide synthesis could interfere with the acquisition of effector functions. It was previously reported that pyrimidine limited-conditions induced by the immunosuppressive agent, leflunomide, altered the balance of Th1/Th2 cell differentiation (9), but the precise role of these nucleotides in the differentiation of naive CD8 T lymphocytes into effector and memory cells has not been studied.

To achieve depletion of nucleotide pools in T cells, we took advantage of well-known de novo nucleotide synthesis inhibitors, which are widely used as antineoplastic agents and/or as immunosuppressive agents in organ transplantation and the treatment of autoimmune and chronic inflammatory diseases (10). Although these drugs have been in clinical use for several decades, the precise mechanisms of action involved in suppression of the immune response are still unknown. Mycophenolate mofetil (MMF), metabolized in mycophenolic acid, is a selective inhibitor of inosine monophosphate dehydrogenase type II, which catalyzes the first step in the formation of guanine ribonucleotides. This enzyme is strongly expressed in activated T and B cells (11). The antiproliferative effect of mycophenolate acid on T cells was clearly demonstrated in vitro (7, 8, 12, 13). Methotrexate (MTX) is a folate antagonist that blocks dihydrofolate reductase and TS (14), but it can also block the first step of purine biosynthesis (15). We have previously shown that in primary T cells, MTX inhibits T cell division by specifically depleting thymidine nucleotides (8, 16). Finally, 5-fluorouracil (5-FU) is converted in 5-fluoro-2-deoxyuridine-5-monophosphate that forms a ternary complex with TS and its cofactor and renders the enzyme inactive (17).

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2 We dedicate this work to Prof. J.-P. Revillard who died on June 2, 2003.

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8 Abbreviations used in this paper: TS, thymidylate synthase; MMF, mycophenolate mofetil; MTX, methotrexate; 5-FU, 5-fluorouracil; NP, nucleoprotein; BMDC, bone marrow-derived dendritic cell; GVHD, graft-vs-host disease; GVL, graft-vs-leukemia.
To investigate the impact of nucleotide restriction induced by the inhibitors on effector and memory T cell differentiation, we used F5 TCR transgenic mice that express a TCR derived from the cytototoxic clone F5, recognizing the nucleoprotein (NP) 68 peptide. Nearly all (~95%) of the CD8⁺ cells in the F5 mice are clonotype-positive (Vα4⁺ Vβ11⁺) and have a naive CD44low phenotype. Upon exposure to a specific Ag (NP68 peptide), F5 CD8 T cells proliferate, express a high level of activation markers, and acquire effector functions such as IFN-γ expression and cytotoxic activity (18, 19). They then differentiate into memory phenotype CD8 T cells that display improved effector functions such as IFN-γ and RANTES expression, as well as hyperproliferation (20–22). To determine the impact of nucleotide inhibitors on the proliferation of Ag-stimulated T lymphocytes in vivo, we have used the fluorescent dye, CFSE, to label Ag-specific F5 CD8⁺ T cells before adoptive transfer into syngeneic mice. In the present study, we show that nucleotide restriction induces a strong inhibition of the clonal expansion of F5-specific CD8 T cells, associated with an alteration in the acquisition of effector functions, such as IFN-γ expression and cytolytic activity. Furthermore, nucleotide restriction during the primary response prevents the generation of memory phenotype CD8 T cells. Surprisingly, purine and pyrimidine inhibitors displayed a different capacity to interfere with the acquisition of effector functions.

Materials and Methods

Mice and reagents

F5 mice were a gift from D. Kioussis (Division of Molecular Immunology, Medical Research Council National Institute of Medical Research, London, U.K.). F5 mice are transgenic for a TCR recognizing the (366–374) peptide (NP68) derived from the influenza virus NP in the context of the MHC class I molecule, H2-Dª (23). C57BL/10 mice were purchased from Charles River Laboratories (Les Oncins, France). C57BL/10 and F5 mice were bred in the institute’s animal facility, Plateau de Biologie Expérimentale de la Souris-Ecole Normale Superieure de Lyon (Lyon, France). For all experiments, mice were aged between 6 and 10 wk. The mouse experiments have been approved by our institutional review board, the Comité Régional d’Ethique pour l’Expérimentation Animale.

MMF was purchased from Roche (Palo Alto, CA). MTX was obtained from Teva Pharma (Courbevoie, France). MMF (100 mg/kg per 12 h), MTX (7 mg/kg per 24 h), and 5-FU (40 mg/kg per 24 h) were diluted in PBS and injected i.p. in the i.p. cavity. The volume of the drug given to each animal was adjusted according to body weight. The dose of MMF given to mice was similar to the one used by our group (24) and others (25). The dose of MTX and 5-FU was determined by preliminary experiments (Ref. 16, and data not shown) showing that a daily administration of the selected dose over a period of 1 wk did not lead to a bone marrow immunosuppression or to increased lethality.

CFSE staining and adoptive transfer

Cell suspension of the spleens and lymph nodes from F5 mice were incubated with 7.5 μM CFSE (Molecular Probes, Pootgebouw, The Netherlands) in 2% FCS medium (3–5 × 10⁷ cells/ml) at 37°C for 13 min. Cells were washed once with DMEM, 6% FCS, and twice with PBS. Age- and sex-matched C57BL/10 syngeneic mice were adoptively transferred by i.v. injection with 3 × 10⁶ CFSE-labeled F5 CD8⁺ cells in a final volume of 200 μl of PBS into the retro-orbital sinus.

Immunization

Immunizations were performed 24 h after adoptive transfer. Mice were immunized i.p. once with bone marrow-derived dendritic cells (BMDC; 1 × 10⁷ CI6-CD11c⁺ CMH-II⁺ cells in 200 μl of PBS) previously pulsed with 1 μM NP68 peptide from the influenza virus NP: Ala-Ser-Asn-Glu-Asn-Met-Asp-Ala-Met Np⁶⁶-Np⁶⁷ (Neosystem, Strasbourg, France).

In vitro production of BMDC

Bone marrow cells, obtained from C57BL/10 mouse femora, were resuspended in RPMI 1640 medium (Invitrogen Life Technologies, Cergy Pontoise, France) supplemented with 10% FCS (Invitrogen Life Technologies), 10 mM HEPES pH 7 (Sigma-Aldrich), 2 mM L-glutamine (Invitrogen Life Technologies), 50 μg/ml gentamicin (Invitrogen Life Technologies), and 50 μM 2-ME (Sigma-Aldrich). Cells (2 × 10⁶/ml) were cultured for 8 days in the presence of 0.2 μg/ml recombinant human Flt3-ligand (TEBU, Le Perray en Yvelines, France) at 37°C. Cells were further matured with LPS (250 ng/ml; Sigma-Aldrich), and pulsed or non-pulsed with 1 μM NP68 peptide during 16 h. Following the maturation step, ~95% of cells were CD11c⁺ and >50% expressed very high levels of the MHC class-II molecule IAβ.

Intracellular staining

For intracellular IFN-γ detection assay, staining was preceded by a 5-h in vitro stimulation. Briefly, cells were stimulated in the presence of 0.67 μg/ml Golgi-stop (BD Pharmingen) and 1 μM NP68 peptide. After surface staining with CyChrome-conjugated anti-CD8 (clone 53-6.7) mAb (BD Pharmingen), cells were washed in PBS/ FCS/NaN₃ and incubated with Cytoxys/Cytoperm (BD Pharmingen) for 15 min. Cells were washed withperm/wash buffer (BD Pharmingen) and incubated overnight with PE-conjugated anti-IFN-γ mAb (clone XMG1.2; BD Pharmingen), or PE-conjugated anti-granzyme B mAb (clone GB12; Caltag Laboratories, Burlingame, CA). At the end of incubation, cells were washed with perm/wash buffer, and were analyzed on a FACScalibur (BD Biosciences, Mountain View, CA). A total number of 4 × 10⁶ CD8 T cells were acquired for each sample. The number of F5 CD8 T cells was calculated using the following equation: (number of viable cells × percentage of total CD8 T cells) × percentage of CFSE-Cells within the CD8 population.

In vivo cytotoxic assay

This assay was performed as described previously (26). Targets were prepared from C57BL/10 spleen and lymph node cells. Suspension was divided into two populations, pulsed or unpulsed with 1 μM NP68 for 1 h and 30 min at 37°C, washed extensively, and labeled with a high concentration of CFSE (7.5 μM) or with a low concentration of CFSE (0.75 μM), respectively. Peptide-pulsed CFSEnob cells and unpulsed CFSEsw cells were mixed together. A total of 2 × 10⁶ CFSE-labeled cells were injected (i.p.) into C57BL/10 mice previously transferred with F5 cells and immunized with NP-pulsed BMDC. After 16 h, mice were killed and splenocytes were analyzed by flow cytometry to detect the CFSE-labeled target cells. Two parameters were used to evaluate the in vivo cytotoxic activity of CD8 T cells. The ratio between peptide-pulsed targets and control targets injected in a given mouse (ratio = percentage of CFSEsw/percentage of CFSEnob) and the percentage-specific lysis that normalized cytolytic activity between primed and control mice, determined by the following formula: percentage-specific lysis = [1 – (ratio primed/ratio unprimed)] × 100.

Results

Nucleotide synthesis inhibitors affect peptide-induced proliferation of adoptively transferred CD8 T cells

We have previously described that nucleotide restriction inhibits T cell proliferation and could induce apoptosis of cycling T cells in vitro (7, 8, 27). Therefore, we investigated the effects of nucleotide synthesis inhibitors on T cell proliferation in vivo. To this end, CFSE-labeled TCR transgenic F5 CD8 T cells were adoptively transferred into naive syngeneic C57BL/10 mice. BMDC matured with LPS and pulsed with NP68 peptide were used as APC to elicit F5 CD8⁺ T cell activation and proliferation (Fig. 1). Inhibitors of nucleotide synthesis were administered 4 h before priming, and for 2 days after immunization as described in Materials and Methods. Three days after immunization, F5 CD8 T cell proliferation was analyzed on spleen (Fig. 1A) and lymph node cells (Fig. 1B) by visualizing CFSE dilution. Injection of nonpulsed BMDC did not induce cell division. Immunization with NP68-pulsed BMDC led to a strong proliferative response with cells undergoing up to seven divisions. The highest frequency of cells having divided was found in the sixth division peak, both in the spleen and lymph nodes. Treatment with nucleotide inhibitors did not obviously increase the frequency of nondividing cells in immunized mice suggesting that restriction in nucleotide synthesis did not interfere with the initial activation signals. Administration of MMF or MTX led to a decrease in the number of cell divisions with a stronger effect in the
spleen compared with lymph nodes (Fig. 1, A and B). Cells underwent a maximum of five divisions and the highest frequency of cells having divided was found under the third division peak. Administration of 5-FU drastically prevented cell proliferation in the spleen and lymph nodes, as CD8 T cells performed a maximum of three divisions. Furthermore, in the presence of MTX and 5-FU, the number of cells within each division peak dropped with an increasing number of divisions, suggesting that nucleotide restric-

FIGURE 1. Effect of nucleotide synthesis inhibitors on CD8 T cell proliferation. CFSE-labeled TCR transgenic F5 CD8 T cells from the spleen and lymph nodes were adoptively transferred to naive syngeneic C57BL/10 mice. Twenty-four hours later, recipient mice were immunized i.p. with NP68-pulsed BMDC. MMF (100 mg/kg per 12 h), MTX (7 mg/kg per 24 h), and 5-FU (40 mg/kg per 24 h) were administered to recipient mice 4 h before immunization and once (MTX and 5-FU) or twice (MMF) a day for the next 2 days. Control mice were treated with PBS. Three days after immunization, the spleen (A) and lymph nodes (B) were removed. Cells were stained with anti-CD8 and cell divisions were detected by measuring the decrease in CFSE fluorescence emission. For each sample, an equal number of host CD8 T cells was acquired. The division profile of transferred CFSE-labeled F5 cells among total CD8 T cells is shown. CFSE-negative cells on the histograms represent the unlabeled host CD8 T cells. A and B, Histograms show the CFSE profile of F5 CD8 \(^+\) T cells from naive mice (dotted line), mice immunized with NP68-pulsed BMDC (shaded histogram), or mice immunized with NP68-pulsed BMDC and treated with nucleotide synthesis inhibitors (bold line). One representative experiment of four independent experiments is shown. C, The total number of F5 CD8 T cells (CFSE \(^+\)) present in lymph nodes 2 (open) or 3 days (gray) after immunization with NP68-pulsed BMDC was calculated as described in Materials and Methods. Data presented are the mean values ± SEM of three independent experiments. Significances of differences were determined by a two-tailed Student’s t test. **, \(p < 0.05\).

Effect of nucleotide synthesis inhibitors on IFN-\(\gamma\) expression

One hallmark of the CD8 T cell response is the expression of cytokines such as IFN-\(\gamma\) (2, 3). To determine to what extent nucleotide synthesis inhibitors could affect acquisition of effector functions, we analyzed the expression of IFN-\(\gamma\) by F5 CD8 \(^+\) T cells. As reported previously (19, 21), IFN-\(\gamma\)-positive CD8 \(^+\) T cells could not be detected in naive mice after ex vivo restimulation with the peptide (Fig. 2A). Injection of LPS-matured BMDC pulsed with the NP68 peptide induced the expression of IFN-\(\gamma\) by a large fraction (41.6%) of activated F5 CD8 T cells following in vitro restimulation with the NP68 peptide (Fig. 2A). The frequency of IFN-\(\gamma\)-positive cells increased progressively as a function of cell cycle division and was highest among cells having performed more than three divisions (Fig. 2B). In the presence of MMF, MTX, or 5-FU, the frequency of IFN-\(\gamma\)-positive cells was strongly reduced to 10.4, 7.7, and 4.1%, respectively (Fig. 2A). This decrease in the frequency of IFN-\(\gamma\)-producing cells correlated with the reduced number of cell divisions observed in the presence of drugs. Indeed, the percentage of IFN-\(\gamma\)-positive cells in the different division peaks was similar in all BMDC-primed mice (Fig. 2B). As 5-FU administration led to a strong reduction in the number of cycling F5 CD8 cells, the number of IFN-\(\gamma\)-producing cells in each division could not be reliably analyzed and data are not shown. Together, these results show that nucleotide synthesis inhibition has a negative impact on the induction of CD8 T cells expressing IFN-\(\gamma\) that is correlated to the inhibition of proliferation.

Effect of nucleotide synthesis inhibitors on the cytotoxic function of effector CD8 T cells

Cytolytic activity displayed by effector CD8 T cells is dependent on at least two pathways, the perforin-granzyme and the FasL pathways. Expression of these molecules is acquired in naive cells following their activation and differentiation in effector cells. To evaluate the frequency of potential cytotoxic effectors among activated CD8 cells, we measured the expression of granzyme B (Fig. 3A). As expected, naive cells did not contain any detectable granzyme B, whereas ~15% of effector cells induced by injection of peptide-pulsed BMDC expressed granzyme B. Interestingly, this expression is detected in cells having performed the highest number of divisions. In the presence of MMF, ~7% of the cells were still able to produce granzyme B and this expression was restricted to cells that had accomplished the highest number of divisions (i.e., 4–5 divisions). Conversely, in the presence of pyrimidine inhibitors, MTX and 5-FU, granzyme B-positive cells could not be detected.
As multiple pathways are involved in the cytotoxic effector function of CD8 T cells, we have also measured the cytolytic activity of CD8 T cells in vivo. We used an in vivo CTL assay to determine the ability of CD8 T cells to eliminate CFSE-labeled cells pulsed with NP68 peptide (Fig. 3B). In control BMDC-immunized mice, ~94% of the NP68-pulsed targets were eliminated in a 16-h assay. Strikingly, in the presence of MMF, 70.8% of NP68-pulsed target cells were eliminated, suggesting that CD8 T cells activated in MMF-treated mice have acquired a significant cytolytic activity despite their weak proliferative response compared with immunized mice (Fig. 1). In contrast, in vivo cytotoxic activity could not be detected in mice treated with MTX or 5-FU. These in vivo results are in agreement with the frequency of the granzyme B-expressing cells we have found on day 3 after priming (Fig. 3A). Together, these results indicate that while the pyrimidine synthesis inhibitors, MTX and 5-FU, totally blocked induction of the granzyme B-expressing cells, the purine inhibitor MMF only had a minor impact on the granzyme B expression and the cytotoxic activity of CD8 T cells.

Nucleotide synthesis inhibition during primary immunization impairs the generation of memory CD8 T cells

After the proliferative burst which takes place during the primary T cell response, most effector T cells die by apoptosis, while only a minor fraction of these cells persists and differentiates in memory T cells (28). In F5 mice primed with peptide-pulsed BMDC, memory cells can be identified by the expression of higher levels of CD44 surface expression and by their capacity to produce IFN-γ following a short-term (5 h) peptide stimulation (18, 19). To assess whether nucleotide restriction interfered with memory CD8 T cell generation, Ag-specific F5 CD8 T cells were visualized 38 days after priming by using NP68 peptide-loaded MHC class-I D b tetramers. As shown in Fig. 4A, tetramer-positive CD8 T cells were detectable in naive recipient mice. In immunized mice, the number of tetramer-positive cells increased to \( 2.7 \times 10^3 \) in the lymph nodes. Following MMF or MTX treatment, the number of tetramer-positive cells recovered in the lymph nodes from primed mice was strongly reduced to \( 1.4 \times 10^3 \) and \( 1.1 \times 10^3 \), respectively, and in the presence of 5-FU, the number of tetramer-positive CD8 T cells was comparable to that found in naive mice. In
BMDC-primed mice, based on the expression of CD44, cells had a memory phenotype compared with naive mice (Fig. 4B). However, nucleotide restriction during the primary response led to a decrease in the percentage of CD44-positive cells, i.e., in the generation of CD8 T cells expressing a memory phenotype.

We then investigated whether these cells produced IFN-γ after a short-term in vitro restimulation (Fig. 4C). In naive mice, a negligible number of IFN-γ-positive CD8 T cells could be detected. In immunized mice, 1.4 × 10⁵ CD8 T cells were able to produce IFN-γ. In mice immunized in the presence of MMF, the number of cells producing IFN-γ was strongly reduced. Following MTX or 5-FU treatment, IFN-γ-producing cells were not generated as the number of IFN-γ-positive cells was comparable to the one found in naive mice (Fig. 4B). The proliferative capacity of memory cells generated in these different conditions was also measured in vitro as described in Pihlgren et al. (Ref. 18; data not shown). Memory cells generated in the presence of MMF exhibited an increased proliferative capacity identical with memory cells generated in the absence of drugs, whereas administration of MTX during the primary response abrogated the generation of cells displaying an increased proliferative capacity (data not shown). Altogether, these results suggest that MTX and 5-FU completely inhibit the generation of memory phenotype CD8 T cells if administered during the primary response. In contrast, MMF treatment allows the generation of cells with an intermediate memory phenotype, i.e., cells that have acquired some memory phenotype characteristics such as IFN-γ secretion or in vitro proliferation.

Effect of nucleotide synthesis restriction on cytotoxic function following secondary immunization

We investigated the impact of nucleotide restriction on memory cytotoxic activity following the protocol described in Fig. 5A. Mice were boosted with NP68-pulsed BMDC 48 h before the in

![FIGURE 4. Impact of nucleotide synthesis inhibitor treatment during the primary response on the generation of memory phenotype CD8 T cells. Nonlabeled F5 CD8 T cells were adoptively transferred to naive syngeneic C57BL/10 mice and recipient mice were immunized with NP68-pulsed BMDC in the presence or absence of nucleotide synthesis inhibitors. Thirty-eight days later, lymph node cells were removed. A, Detection of F5 CD8 T cells. Cells were surface-stained for CD8 and NP68 peptide-loaded MHC class-I D b tetramer. The number of tetramer-positive cells among CD8 T cells is indicated for each immunization condition. B, CD44 expression by tetramer-positive F5 CD8 cells. C, Expression of IFN-γ by F5 CD8 cells. Cells were restimulated in vitro with NP68 10 nM for 5 h in the presence of monensin, cells were then stained for CD8, permeabilized, and stained for intracellular IFN-γ. The number of IFN-γ-positive cells among the CD8 T cell population was calculated as described in Materials and Methods.](http://www.jimmunol.org/Download)
vivo cytotoxic assay. As expected, in primed mice, >90% of peptide-pulsed target cells were killed by F5 cells, compared with only ~30% in naive mice (Fig. 5B). This result was consistent with the faster acquisition of cytotoxic activity by memory phenotype CD8 T cells compared with naive cells described in other systems (29, 30). We first analyzed the effects of MMF and MTX administration during the primary response on the cytotoxic function of memory CD8 T cells (Fig. 5C). The effect of 5-FU was not determined (due to its drastic effect in primary response). Memory cells generated in the presence of MMF gained the ability to lyse target cells but to a lesser extent than control memory cells. In contrast, there was a strong reduction of cytotoxic activity following treatment with MTX. In these mice, ~30% of target cells were eliminated in vivo. This is comparable to the cytotoxic activity observed in boosted naive mice, suggesting that F5 cells primed in the presence of MTX are still capable of responding to a boost but do not have the ability to acquire accelerated cytotoxic effector functions.

We next investigated the impact of nucleotide restriction on Ag-experienced T cells. To this end, MMF or MTX were administered on the day of the boost and during the next 3 days (Fig. 5D). In contrast to what was found with naive cells (Fig. 3), treatment with these drugs did not affect the cytotoxic activity of memory cells. Altogether, these results demonstrate that nucleotide synthesis inhibitors interfere with the development of effector function during the primary response and with the generation of memory cells. However, they have little effect on the expression of cytotoxic effector function by memory phenotype CD8 T cells.

Discussion

In this study, the effect of nucleotide restriction on the immune response and its consequence on CD8 T cell response was evaluated for the first time in vivo at the single cell level by using the fluorescent dye, CFSE. We have demonstrated that nucleotide restriction induced by specific inhibitors of purine or pyrimidine de novo synthesis leads to an inhibition of in vivo proliferation of Ag-specific CD8+ T cells. This inhibition is not due to an interference with initial activation signals as nontreated or treated cells both express a high level of CD69 1 day after immunization (data not shown). Proliferation of F5 CD8+ T cells was not completely abrogated in the presence of the three inhibitors tested. Indeed, in the presence of MMF and MTX, cells underwent a maximum of five and four divisions, respectively. The most potent inhibitor was 5-FU as cells underwent a maximum of three divisions. This is in contrast to what we have observed in vitro, using human primary T lymphocytes where we found a complete inhibition of cell cycling in the presence of these inhibitors (7, 8). However, in vitro activation only partially mimics in vivo priming and activation conditions in vivo are likely to be more optimal. As a result, cell cycling might be less sensitive to inhibition by nucleotide synthesis inhibitors than in vitro.

Additional mechanisms could contribute to the inhibition of proliferation by nucleotide inhibitors in vivo. Indeed, it has previously been shown that the differentiation of dendritic cells is impaired in the presence of MMF (25). However, in our study, as we immunized mice with Flt3-ligand-differentiated dendritic cells matured in vitro with LPS, the inhibition of DC maturation is not likely to contribute to the inhibition observed. Moreover, we have recently shown in a model of contact hypersensitivity that MMF and MTX did not interfere with the migration of mature dendritic cells (31). Hence, in the experimental conditions used in this paper, the inhibition of dendritic cell functions is not likely to contribute significantly to the suppression of CD8 T cell response observed in the presence of nucleotide synthesis inhibitors.

The inhibition of proliferation induced by nucleotide restriction was also associated with an induction of apoptosis of activated T cells in vivo. Indeed, although cells had divided a number of times by day 3, this did not lead to an increase in the number of F5 CD8+ T cells in mice treated with nucleotide synthesis inhibitors (Fig. 1C). These results are in agreement with our own in vitro study (8). Unfortunately, the relative contribution of apoptosis to the inhibition of the primary response by nucleotide inhibitors was difficult to assess directly, as apoptotic cells are eliminated too efficiently in vivo. The proapoptotic effect of these drugs was restricted to activated CD8+ T cells stimulated by the peptide without any detectable effect on bystander host cells. Indeed, at all time points, an equivalent number of host cells was recovered from lymphoid organs in nucleotide-treated and control mice (data not shown).

The acquisition of effector functions such as IFN-γ synthesis by Ag-primed CD8 T cells was also affected by nucleotide inhibitors. This effect could result from the inhibition of the cell cycle induced by the different drugs. Indeed, we show that in any given division
peak, the proportion of cells expressing IFN-γ was not modified by MMF or MTX treatment, indicating that expression of this cytokine is not inhibited by these drugs. These results indicate that the ability of nucleotide inhibitors to block IFN-γ expression is tied to their anti-proliferative effects. This is in agreement with previous studies showing that there is a strong correlation between the number of cell divisions performed by naive CD8 T cells following their activation, and the acquisition of effector function such as IFN-γ synthesis (32). Similarly, the commitment to memory cell differentiation also correlated with the number of cell divisions performed by activated CD8 T cells (33, 34). A similar correlation between the acquisition of IL-4 and IFN-γ production and the number of cell cycles performed by the cells has been shown for CD4 T cells (35–37). These functions are among the ones that will be displayed more rapidly by memory CD8 T cells following antigenic re-stimulation (38, 39). The more rapid expression of these proteins results from epigenetic modification such as DNA demethylation and chromatin remodeling. Although progression through the cell cycle can provide a window of opportunity to remodel chromatin and gene expression profiles to create new cellular phenotypes (1, 40, 41), the existence of a mechanistic link between cell division and the acquisition of cytokine synthesis capacity is still a matter of debate (42, 43).

Cytotoxic CD8+ T lymphocytes can destroy their targets either by the perforin and granzymes pathway, or by engaging cell surface death receptors such as CD95 (44–46). It was previously described that development of cytotoxic effector functions did not require multiple rounds of divisions. Indeed, CD8 T cells that have undergone as few as one or two divisions can become cytolytic (32, 34). In this study, we demonstrate that treatment with MMF did not lead to a significant inhibition of cytotoxic activity, whereas treatment with MTX or 5-FU completely abrogated the generation of cytotoxic effector cells. In both cases, some treated cells had undergone more than two divisions. The analysis of granzyme B expression showed that its expression is inhibited by treatment with MTX or 5-FU. In contrast, MMF treatment did not abrogate the expression of granzyme B. These results indicate that nucleotide inhibitors have the different potential to inhibit granzyme B expression by naive CD8 T cells and to block their acquisition of cytotoxic functions. The reduction in the elimination of peptide-pulsed targets induced by the MTX and 5-FU could also result from the reduced frequency of activated F5 CD8 T cells generated in these conditions.

One major unexpected result of this study was the differential effect of purine vs pyrimidine inhibitors on the differentiation of naive CD8 T cells in effector cells and memory phenotype T cells. In the presence of MMF that blocked purine synthesis, F5 cells were still able to develop cytotoxic functions in primary and secondary responses, despite their decreased proliferative response compared with control mice. In contrast, in pyrimidine-restricted conditions, cells had little effector functions and F5 CD8 T cells that survived had not developed a memory phenotype. This effect of MTX does not seem to result from a stronger inhibition of the cell cycle, as the maximal number of divisions performed by CD8 T cells following MTX or MMF treatment only differed by one division. These results suggest that pyrimidine synthesis inhibitors could regulate differentiation into effector and memory T cells by acting on cell division but also by other pathways (47).

Although nucleotide inhibitors strongly interfere with primary CD8 T cell response, and therefore, with the generation of memory T cell population, our results demonstrate that nucleotide restriction applied on memory cells had no effect on secondary cytolytic response (Fig. 5). This indicates that once cells have acquired improved effector functions that are displayed rapidly before the first division, nucleotide synthesis inhibitors are unable to interfere with the expression of these functions. However, they could, if used in chronic treatment, interfere with the maintenance of these cells. Indeed, it has recently been shown by Bellier et al. (48) that division of experienced T cells is an absolute requirement for immunological memory maintenance.

Following transplantation, recipient T cells can act against donor cells resulting in graft rejection (host vs graft). Mature donor T cells present in the graft can also react against some recipient target organs (skin, liver, and the gastrointestinal tract) leading therefore, to the development of graft-vs-host disease (GVHD; Ref. 49). To decrease GVHD incidence and severity, patients receive a prophylaxis with nucleotide inhibitors that impair T cell function (10). Our results suggest that if, after transplantation, mainly naive cells are activated, nucleotide restriction will block proliferation of Ag-specific naive CD8+ T cells and their differentiation into cytotoxic effectors. However, if Ag-specific memory CD8 T cells are predominant in the host, these drugs will be less efficient in controlling CD8-mediated damage. Moreover, it has been shown that preexisting memory cells against a graft play a key role in the rejection process (50–52). GVHD could be avoided by depleting the hematopoietic stem cell graft of T cells. However, in leukemic patients, this does not seem to be beneficial as the rate of graft rejection or the rate of tumor relapse is increased in these conditions (53). Indeed, in these patients, graft-vs-leukemia (GVL) effect, where donor T cells eliminate the malignant cells, is an important parameter in the treatment success. Recent studies have shown that donor T cells make differential use of their cytolytic pathways in mediating GVHD and GVL effects (54) and therefore, could induce GVL without GVHD (55). Several studies have demonstrated a predominant role of the perforin-granzyme B pathway in the GVL effect (56–58). Treatment with MMF that preserves the development of effector cells with granzyme-dependent cytotoxic activity might be preferable to MTX treatment that abrogates the generation of such effectors.

In conclusion, the present study reveals differential effects of purine and pyrimidine inhibitors on naive and memory cell effector functions and therefore, provides new rationale for their use in clinic.

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References


