Nonimmunosuppressive chemotherapy: EM011-treated mice mount normal T-cell responses to an acute lymphocytic choriomeningitis virus infection

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Abstract

Myelosuppression and associated immunosuppression are major problems in cancer chemotherapy. Thus, infection remains a significant source of morbidity and mortality during chemotherapy of cancer patients. Viral infections, particularly herpes simplex virus, varicella zoster virus, and cytomegalovirus, result either due to reactivation of latent viruses or new infections as sequelae of chemotherapy and debilitated cell-mediated immunity. Ultimately, the resolution of these infections can only be achieved after the control of malignancy and regaining the patient’s ability to mount adequate immune responses. We show here that EM011, a tubulin-binding, nontoxic, orally available anticancer agent, does not alter absolute CD4+, CD8+, B220+, and NK1.1+ cell counts in immunocompetent mice. More importantly, EM011 treatment at tumor-suppressive dosages (300 mg/kg) does not suppress cell-mediated immune responses in mice experimentally challenged with acute lymphocytic choriomeningitis virus infection, in that mice mount robust virus-specific CD8+ and CD4+ T-cell immune responses while maintained on daily drug treatment. Thus, CD8+ and CD4+ T-cell expansion and acquisition of effector functions is not perturbed by EM011 treatment. These data provide compelling evidence to support the nonimmunosuppressive nature of EM011 therapy and provide strong impetus for combining chemotherapy with immunotherapy as a novel anticancer strategy. [Mol Cancer Ther 2007; 6(11):2891–9]

Introduction

Immunocompromisation is a major cause of opportunistic infections, activation of latent viruses, and the development of certain carcinomas such as Kaposi’s sarcoma (1, 2). This is particularly evident in many diseases that weaken the immune surveillance system, including AIDS and various cancer treatment modalities (e.g., radiation therapy, chemotherapy, and, in particular, antimicrotubule therapy), primarily because of severe myelosuppression and immunosuppression (3, 4). Finally, the resolution of these infections is dependent on the treatment modality of the malignancy and the ability of the host to mount an adequate immune response to fight off the infection (2).

Although antimicrotubule drugs constitute an important major class of anticancer drugs mainly typified by agents that either overpolymerize or bundle microtubules (such as the taxanes; ref. 5) or those that depolymerize them (such as the vincas; ref. 6, recently reviewed in refs. 7, 8), these agents, due to their gross effects on microtubules and nonselective action, affect all dividing cells and cause various serious toxicities including weakening of the immune system, leucocytopenias, alopecia, and gastrointestinal and neurologic toxicities (9–12). Furthermore, frequent development of infection restricts the repertoire of the available armamentarium of tubulin-binding anticancer drugs. This also leads to a poor quality of life during classic treatment regimens before significant morbidity and mortality of the oncology patient. Thus, there is a desperate need for drugs that are selective in their action and have improved toxicity and superior pharmacologic profiles.

Our laboratory has rationally designed and synthesized an ~10- to 40-fold more potent noscapine analogue, 9-bromonoscapine (EM011), which binds tubulin without changing the total tubulin polymer mass (13). We have shown that EM011 is a potent anticancer agent that briefly arrests a wide variety of cancer cell types in the G2-M phase of the cell cycle followed by abortive mitoses, which, in turn, activate a mitochondrially driven apoptotic program leading to cancer cell death (14, 15). We have also shown earlier that noscapinoids arrest normal cells too at mitoses but they resume normal cell cycle after the drug is removed or cleared from the system (16). Furthermore, EM011 inhibits the growth of several human neoplasms including lymphomas and breast carcinomas, without causing any gross changes in the microtubule arrays of postmitotic cells (15, 17). More importantly, we have thus far failed to detect any toxicity in tissues such as hematopoietic, gut, spleen, and long nerves that are common targets of currently used antimicrotubule drugs (15, 17). Because our animal studies to investigate the inhibition and regression of tumor growth used immunodeficient (nu/nu) mice to grow...
EM011 Chemotherapy Is Nonimmunosuppressive

human tumor xenografts, we had been thus far restricted to determine the immunosuppressive nature of EM011. Although we have previously reported the absence of alterations in immune cell counts on EM011 treatment (despite the fact that these nude mice have very low T-cell counts; refs. 15, 17), those data may not have represented a true scenario of the drug effects on the immune surveillance system.

Therefore, to rigorously investigate the effects of EM011 therapy on the immune system, we chose to use the well-established model of murine infection with lymphocytic choriomeningitis virus (LCMV). This model system reproducibly induces acute or persistent infection in vivo, and the mechanism by which it is cleared during an acute infection is well understood (18, 19). Clearance of the pathogen, although associated with several antiviral immune effector mechanisms, is primarily dependent on the activity of virus-specific CTLs restricted by H-2 molecules of the mouse MHC. If these cells fail to generate or are depleted, progression from acute to persistent infection occurs. Because this murine model is a well-defined system for studying protective CTL responses in vivo, we thought it was appropriate for addressing the immunomodulatory properties of EM011. Toward this goal, we asked if EM011 pretreatment at tumor-suppressive doses for 28 days and on continued further dosing compromised the ability of CD8 T cells to undergo adequate expansion following an acute LCMV infection. In addition, we aimed to explore if EM011 treatment altered the acquisition of normal effector functions by antigen-specific CD8 T cells. Our results show that tumor-suppressive EM011 dosages in mice do not affect the overall numbers of T cells, both the CD4 (T-helper) and CD8 (CTL) subsets. In addition, EM011 treatment did not alter the numbers of B cells (measured by counting B220+, a marker present on cells committed to the B lineage) and natural killer (NK) cells (by quantifying cells bearing the NK-specific marker NK1.1+). More importantly, at day 8 postinfection (peak of CD8 T-cell responses), mice undergoing EM011 treatment mounted robust antigen-specific CD8 T-cell responses, as evaluated by the standard tetramer staining for three different LCMV-specific nucleoprotein-derived (NP-) and glycoprotein-derived (GP-) epitope specificities of NP396+, GP33+, and GP276+ T-cell populations. The level of clonal CD8 T-cell expansion was comparable, and the immunodominance hierarchy of viral antigen-specific T cells was well maintained and similar among the vehicle- and EM011-treated groups. In addition, the ability of CD8 T cells to produce effector molecules, such as granzyme B, and functional cytokines, such as IFN-γ, tumor necrosis factor α (TNF-α), and interleukin 2 (IL-2), was unaltered. The migratory response of effector cells to the peripheral tissues and their relative homing ability in lymphoid and nonlymphoid tissues were similar for vehicle- and EM011-treated mice. These data thus strongly suggest that EM011 therapy is nonimmunosuppressive and does not compromise the ability of the host to successfully resolve an acute LCMV infection. We believe that these results provide the necessary framework for evaluating EM011 in combinatorial immunotherapeutic regimens to maximize anti-tumor outcomes with an improved quality of life during treatment.

Materials and Methods

Mice and Virus

Four- to six-week-old female C57BL/6J mice were purchased from The Jackson Laboratory. Animals were housed in the Emory University Animal Facility and experiments were done in accordance with institutional guidelines. For acute infections, mice were infected by i.p. injection of 2 × 10^5 plaque-forming units of LCMV Armstrong. Titers of virus were determined by plaque assay on Vero cells as previously described (19).

Drug Treatment

Age- and weight-matched mice were randomly divided into two groups of six animals each. One group of mice received the vehicle solution (water, pH 4) p.o. by daily gavage, whereas the other group of mice was fed with 300 mg/kg EM011 daily for 28 days before they were subjected to an acute infection of LCMV. Treatment with EM011 was continued even after the LCMV infection until sacrifice at day 8 postinfection.

Flow Cytometry and Tetramer Staining

MHC class I tetramers of H-2Db or H-2Kb complexed with LCMV NP396-404, GP33-41, or GP276-286 were produced as previously described (20). Biotinylated complexes were tetramerized with allopurinol-conjugated streptavidin (Molecular Probes). All antibodies were purchased from BD/PharMingen. Lymphocytes isolated from freshly explanted spleens, livers, and lymph nodes (10^6) were stained with PBS/2% (wt/vol) bovine serum albumin/0.2% (wt/vol) NaN3 using fluorochrome-conjugated antibodies and MHC class I tetramers and were fixed in PBS/2%/0.2% (wt/vol) parafomaldehyde and events were acquired using a FACS Calibur flow cytometer (Becton Dickinson). Dead cells were excluded on the basis of forward and side light scatter. Data were analyzed using Flo Jo 8.1.1.

Intracellular Cytokine Staining

For intracellular cytokine analysis, 10^6 splenocytes were cultured in the absence or presence of the indicated peptide (0.2 μg/mL) and brefeldin A for 5 to 6 h at 37°C. Cells were stained for intracellular cytokines using the Cytofix/Cytoperm kit according to the manufacturer’s instructions (BD PharMingen). Antibodies used for intracellular cytokine detection, anti–IFN-γ, anti–IL-2, and anti–TNF-α, and an isotype control were purchased from BD PharMingen.

Isolation of Lymphocytes from Lymphoid and Non-lymphoid Tissues

Lymphocytes were isolated from the spleen, lymph nodes, and peripheral blood (20–22). For isolation of lymphocytes from nonlymphoid tissues (liver), mice were euthanized, the abdomen was opened, the hepatic vein was cut, and 5-mL ice-cold PBS was injected directly into the hepatic artery to perfuse the liver. The gall bladder was...
EM011 Treatment Does Not Interfere with the Clonal Expansion of T Cells in Nonlymphoid and Lymphoid Tissues in Response to an Acute LCMV Infection

It has recently been shown that the complex differentiation process that occurs during a CD8 T-cell response to viral infection involves dramatic cellular changes as T cells transition through the three characteristic phases of an antiviral response: initial activation and expansion, death phase, and formation of memory T cells (23). Each of these three phases of the T-cell response is accompanied by extensive transcriptional and functional changes that result in naive T cells expanding and gaining effector functions, survival of 5% to 10% of the effectors through the death phase, and the gradual acquisition of memory properties by the surviving virus-specific T cells (24). Because the dynamics of a CD8 T-cell in response to an acute LCMV infection results in a rapid CD8 T-cell expansion that is closely associated with acquisition of effector functions and viral clearance, we next evaluated if EM011 treatment affected T-cell expansion at day 8 after LCMV infection. To this end, we assessed the level of CD8 T-cell expansion in both nonlymphoid and lymphoid tissues (Fig. 2). Concurrently, we also investigated the effect of EM011 on NK1.1+ and B220+ cells in all of these tissues. Figure 2A(i–iv) shows dot plots of percentages of CD4+, CD8+, NK1.1+, and B220+ cells in the blood from vehicle- and EM011-treated mice. Figure 2A(v) bar-graphically shows percentages of these immune cells in blood from vehicle- and EM011-treated mice that are comparable at day 8 postinfection. Figure 2B(i–iv) shows dot plots depicting percentages of immune cell counts in hepatic lymphocytes, and Fig. 2B(v) is a bar-graphical representation of absolute cell counts of CD4+, CD8+, NK1.1+, and B220+ cells in the liver from control- and EM011-treated mice. We observed a sharp increase in the CD8+ T-cell responses at day 8 postinfection compared with CD8+ T cells in EM011-treated naive mice. We also investigated perturbations, if any, caused by EM011 treatment in the percentage of immune cells in lymphoid tissues such as the spleen and lymph node. Our results showed similar profiles for mice from both groups, and we did not observe any differences in the percentages of CD4+, CD8+, NK1.1+, and B220+ cells in the spleen [Fig. 2C(i–iv)] and lymph node [Fig. 2 D(i–iv)] among the vehicle- and EM011-treated groups. Figure 2C(v) and D(v) shows bar graphs depicting absolute cell counts of CD4+, CD8+, NK1.1+, and B220+ cells in splenic and lymph node lymphocytes, respectively, from vehicle- and EM011-treated groups.

Having identified that EM011 treatment does not perturb the expansion of CD8+ T cells on an acute LCMV infection, we next asked if there were any differences in the number of virus-specific CD8+ T cells among the vehicle- and EM011-treated groups.

EM011 Treatment Does Not Alter Primary Virus-Specific CD8+ T-Cell Responses during an Acute LCMV Infection

Infection of immunocompetent mice with the Armstrong strain of LCMV results in an acute infection, which is...
resolved in 8 to 10 days (19, 22). Early in the infection, the LCMV-Arm virus replicates predominantly in the tissue macrophages found in spleen, lymph nodes, liver, lung, etc. (25). In LCMV-Arm–infected mice, after attaining peak viral titers on day 3 postinfection, viral load in the tissues drops precipitously after day 5 and most of the infection is resolved by day 8 postinfection (26). It has been estimated that, in the naïve CD8+ T-cell pool in the mouse, T cells specific for a given peptide/MHC complex exist at a frequency of 1/10^5 T cells (27), with similar estimates for humans (28). During the initial phase of a CD8+ T-cell response to infection, this population, which corresponds to 10^2 cells per spleen in the mouse, can expand to >10^7 cells per spleen (27). Robust CD8+ T-cell responses have also been observed for humans during the acute phase of viral infections (29). This 10^3- to 10^5-fold expansion indicates that, at the peak of proliferation, antigen-specific CD8+ T cells can divide approximately every 6 to 8 h (20).

Because CTLs play an important role in defense against several viral infections including LCMV, our next question was if EM011 treatment (4 weeks before the LCMV infection and 1 week after the infection before sacrifice) affected the expansion of CD8+ T cells specific to the two D^b-restricted immunodominant viral epitopes (NP396 and GP33) and the subdominant epitope GP276. It is recognizable that the recent introduction of MHC tetramer technology has greatly facilitated the analysis of CD8+ T-cell responses by allowing accurate enumeration and phenotypic characterization of antigen-specific T cells using flow cytometric techniques (22, 30). Moreover, the use of MHC class I tetramers to identify antigen-specific T cells obviates the need to use TCR transgenic cells. We thus used MHC-I tetramers to investigate the effect of acute infection with LCMV-Arm on the expansion of CD8+ T cells specific to epitopes NP396, GP33, and GP276 in vehicle- and EM011-treated mice. Figure 3 details the profiles of antigen-specific T cells visualized in the blood [3A(i–iii)], liver [3B(i–iii)], spleen [3C(i–iii)], and lymph nodes [3D(i–iii)] using MHC class I tetramers complexed with defined viral epitopes. As shown in Fig. 3, high numbers

Figure 1. EM011 treatment at tumor-suppressive doses (300 mg/kg fed by oral gavage, once daily) for 28 d does not alter the immune composition of blood. A, i to iv, dot plots indicating percentages of CD4+, CD8+, NK1.1+, and B220+ cells in blood from control vehicle-treated and EM011-treated mice before an LCMV infection. B, bar-graphical comparison of the percentages of immune cells from vehicle- and EM011-treated mice (P > 0.05).
of LCMV-specific CD8+ T cells were detected in the blood and spleens of LCMV-Arm–infected mice. Interestingly, the percentages of NP396+, GP33+, and GP276+ CD8+ T cells in LCMV-Arm–infected mice were comparable between the vehicle- and EM011-treated mice. Furthermore, the immunodominance hierarchy of three investigated viral epitopes (NP396 > GP33 ≥ GP276) in the primary CD8+ T-cell response in LCMV-Arm–infected mice was well maintained and similar results were observed in both the vehicle- and EM011-treated mice. Figure 3A(v) is a bar-graphical comparison of the percentages of antigen-specific T cells in the blood from vehicle- and EM011-treated mice. Similarly, Fig. 3B(v), C(v), and D(v) depicts bar-graphical comparisons of the absolute cell counts of the antigen-specific T cells in the liver, spleen, and lymph node, respectively, from the vehicle- and EM011-treated mice.

As naive T cells differentiate into effector cells, their migration patterns are altered. Effector T cells have a reduced potential for homing to lymph nodes, owing to decreased expression of lymph node homing receptors such as the CC-chemokine receptor 7 and L-selectin (CD62L), but a greater capacity to migrate to inflamed tissues, owing to increased expression of chemokine receptors such as CC-chemokine receptor 5 and CC-chemokine receptor 2 (31). To examine if EM011 treatment interferes with the migration patterns of epitope-specific CD8+ T cells, we evaluated the homing potential of the NP396+, GP33+, and GP276+ cells to the liver, spleen, and the lymph nodes. Figure 3E to G shows that the relative homing potential of all antigen-specific T cells (NP396+, GP33+, and GP276+) to the liver, spleen, and lymph node was similar for vehicle- and EM011-treated mice, with maximum localization in the liver followed by spleen and lymph nodes.

Having visualized directly the physical presence of antigen-specific CD8+ T cells by MHC tetramer staining and their relative homing potential on EM011 treatment, we were curious to know if EM011 altered the functional activity of virus-specific CD8+ T cells. To this end, we measured antiviral T-cell effector functions by cytokine production assays.

**EM011 Treatment Does Not Alter Functional Properties of CD8+ Effector T Cells**

CD8+ T cells recognize peptidic antigens derived from intracellular proteins, complex with cell-surface MHC class
I molecules, and embark on a rapid proliferation phase. Along with the dramatic proliferation, CD8+ T cells also undergo activation and differentiation to acquire antiviral effector functions including the ability to rapidly produce cytokines such as IFN-γ and TNF-α. On antigenic stimulation, CD8+ T cells also up-regulate the expression of cytotoxic granule proteins such as granzymes and perforin, become cytolytic, and gain the ability to enter nonlymphoid tissues (23, 31–34).

Recent studies of CD8+ T cells have shown that the link between the commitment to clonal expansion and effector-cell differentiation is remarkably tight; the same duration of antigenic stimulation that drove naïve CD8+ T cells to proliferate was sufficient for them to differentiate into effector cells that could secrete IFN-γ and TNF-α and produce granzyme B to kill infected cells (35–37). To determine if EM011 treatment led to suboptimal and/or altered effector T-cell functions, we examined the intracellular levels of IFN-γ, TNF-α, and IL-2 on peptidic stimulation with GP33. Figure 4A(i) shows dot plot profiles of IFN-γ- and TNF-α-producing cells on GP33 stimulation from vehicle- and EM011-treated mice. Our results show that EM011 treatment does not abate the ability of CD8+ T cells to acquire effector functions to resolve an LCMV infection in an acute setting. Figure 4A(ii) is a bar-graphical comparison of absolute counts of IFN-γ- and IFN-γ + TNF-α-producing splenic CD8+ T cells on GP33 stimulation of vehicle- and EM011-treated mice. Similarly, we found no changes in the profiles of IFN-γ and IL-2 on EM011 treatment. Figure 4B shows dot plot profiles of IFN-γ- and IL-2-producing cells from vehicle- and EM011-treated mice. Nonstimulated controls showing...
negligible levels of cytokines have been included as controls. Figure 4B(ii) is a bar-graphical comparison of absolute counts of IFN-γ– and IFN-γ + IL-2–producing splenic CD8+ T cells on GP33 stimulation of vehicle- and EM011-treated mice.

**EM011 Treatment Does Not Alter Functional Properties of CD4+ Effector T Cells**

Although a similar type of developmental program drives the formation of effector CD4+ T cells, it has been reported that the formation of effector CD4+ T cells might be influenced to a greater extent than for CD8+ T cells by extrinsic factors, such as the duration of antigen exposure and the type of cytokines that are present (38–40). We next examined the effect of EM011 treatment on CD4+ T-cell differentiation to effector cells by measuring the absolute counts of CD4+ T cells that produce inflammatory cytokines on GP61 stimulation (a MHC class II–restricted CD4 epitope derived from LCMV glycoprotein; Fig. 4). Figure 4C(i) shows dot plot profiles of IFN-γ– and TNF-α–producing CD8+ T cells on GP61 stimulation of vehicle- and EM011-treated mice. Figure 4C(ii) is a bar-graphical comparison of absolute counts of IFN-γ– and IFN-γ + TNF-α–producing splenic CD8+ T cells on GP61 stimulation of vehicle- and EM011-treated mice. Nonstimulated controls that show negligible levels of cytokines have been included as controls. Figure 4D(ii) is a bar-graphical comparison of absolute counts of IFN-γ– and IFN-γ + IL-2–producing cells from vehicle- and EM011-treated mice.

**EM011 Treatment Does Not Affect Granzyme B Production and Levels of Bcl-2 Expression during an Acute LCMV Infection**

We next evaluated if the production of effector molecules such as granzyme B was influenced by EM011 treatment. Figure 5A shows histogram overlays of granzyme B production and levels of Bcl-2 expression during an acute LCMV infection.
production by NP396+ (left) and GP33+ (right) T cells from vehicle- and EM011-treated animals. It is clearly observable that EM011 treatment does not affect the capacity of CD8+ T cells to produce effector molecules that kill the infected cells to help resolve the infection.

One of the most widely studied regulators of cell death is the Bcl-2 superfamily. This family is composed of more than 15 family members in humans and mice. Some members of the family are proapoptotic (Bad, Bax, and Bid) whereas others (Bcl-2 and Bcl-xL) exhibit antiapoptotic functions (41). At any given time, cell susceptibility to apoptosis is determined by the ratio of the proapoptotic to antiapoptotic factors. The prototypic member of this gene family is Bcl-2, whose overexpression provides protection against cell death by preventing release of cytochrome c after mitochondrial damage. Because EM011-induced mitochondrially driven apoptotic cell death in cancer cells is through the decrease of the proapoptotic/antiapoptotic ratio (15, 17), we were inquisitive to learn if EM011 altered the expression levels of Bcl-2 in CD8+ T cells. The expression of Bcl-2 in splenic CD8+ T cells from vehicle- and EM011-treated mice was determined on day 8 postinfection, a time point that precedes the onset of the contraction phase of the CD8+ T-cell response. As shown in Fig. 5B, interestingly, the levels of Bcl-2 expression in NP396+ and GP33+ T cells were similar for vehicle- and EM011-treated mice. In summary, we report that EM011 treatment does not cause any immunosuppressive effects in immunocompetent mice as seen by similar profiles of immune cells from vehicle- and EM011-treated mice. In addition, on an acute LCMV infection, both vehicle- and EM011-treated mice could mount strong nucleoprotein- and glycoprotein-specific primary CD8+ T-cell responses and successfully eradicate the infection with normal kinetics.

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References


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