Synergistic Action of fms-Like Tyrosine Kinase 3 Ligand and CD40 Ligand in the Induction of Dendritic Cells and Generation of Antitumor Immunity In Vivo

Luis Borges, Robert E. Miller, Jon Jones, Kiley Ariail, James Whitmore, William Fanslow and David H. Lynch

*J Immunol* 1999; 163:1289-1297; http://www.jimmunol.org/content/163/3/1289

---

**References** This article cites 37 articles, 28 of which you can access for free at: http://www.jimmunol.org/content/163/3/1289.full#ref-list-1

**Subscription** Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions** Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Synergistic Action of fms-Like Tyrosine Kinase 3 Ligand and CD40 Ligand in the Induction of Dendritic Cells and Generation of Antitumor Immunity In Vivo

Luis Borges, Robert E. Miller, Jon Jones, Kiley Ariail, James Whitmore, William Fanslow, and David H. Lynch

Daily treatment of mice with fms-like tyrosine kinase 3 ligand (Flt3L) leads to a significant increase in the number of dendritic cells and induces antitumor immunity. Here, we show that Flt3L and CD40 ligand (CD40L) synergize in the generation of immune responses against two poorly immunogenic tumors, leading to complete tumor rejection in a high proportion of mice. Rechallenge of the Flt3L + CD40L-treated mice with the immunizing tumor resulted in complete inhibition of tumor growth, indicating that these animals had developed long-lasting antitumor immunity. In addition, we demonstrate that endogenous CD40L plays a critical role in antitumor immunity, since blockade of CD40-CD40L interactions in vivo prevents the generation of antitumor immunity in therapeutic and vaccination protocols. Dendritic cells generated in mice treated with Flt3L alone or in combination with CD40L were equally potent in stimulating allogeneic T cells and expressed similar levels of MHC class II, CD80, and CD86. However, mice treated with Flt3L + CD40L had significantly more dendritic cells than mice treated with either of the cytokines alone, suggesting that CD40L promotes the proliferation and/or survival of dendritic cells generated by Flt3L treatment. Dendritic cells generated in this manner are likely to be involved in the priming of antitumor immune responses.

Tumor-specific CTLs isolated from cancer patients are capable of recognizing and killing autologous tumor cells in vitro (1–4). Despite the presence of tumor-reactive T cells, tumors frequently grow and metastasize to secondary sites. In these cases, it is clear that the immune response does not effectively eliminate the cancer cells. Several mechanisms have been postulated to account for these limited antitumor immune responses: 1) effector cells are not generated in sufficient numbers or are not fully activated to mediate a strong antitumor immune response; 2) the tumor blocks or limits immune responses through the synthesis of immunosuppressive molecules; or 3) the tumor cells down-regulate expression of MHC class I molecules to prevent the presentation of tumor Ags to CTLs. Therapies aimed at boosting antitumor immune responses could potentially help the immune system overcome activation thresholds and suppressive mechanisms.

Tumor-specific CTLs are activated and expanded on interaction with APCs that have internalized and processed tumor Ags. Dendritic cells (DCs) are among the most efficient APCs that are capable of inducing both antitumor and antiviral immune responses (for a review, see Ref. 5). Although the use of DCs as vaccine vectors for tumor and infectious diseases has been proposed in the past, the low abundance of these cells in peripheral blood and lymphoid tissues has impaired their use for immunotherapeutic purposes. Recently, new protocols have been developed to generate large numbers of DCs in vitro and in vivo. For example, DCs can be generated in vitro from bone marrow precursors or PBMC grown in the presence of cytokines such as GM-CSF (6–8), IL-4 (9, 10), TNF-α (11, 12), and c-kit ligand (12, 13). Alternatively, large numbers of DCs can be isolated from the peripheral blood or lymphoid organs of mice treated with fms-like tyrosine kinase 3 ligand (Flt3L) (14, 15). Several groups have now shown that DCs stimulated in vitro with tumor Ags can be used to induce protective antitumor immunity in recipient mice. Successful vaccination of mice with DCs has been achieved after culturing these cells with tumor Ags in the form of peptides (16, 17), full length protein (18, 19), DNA (20), or RNA (21). Other less conventional approaches have also been used to promote antitumor immunity; Gong et al. (22) demonstrated that DCs could be fused with carcinoma cells and used to induce tumor-specific CTL in vivo. Zitvogel et al. (23) showed that DCs are capable of secreting vesicles called exosomes, which can be loaded with tumor peptides and used to immunize mice. These vesicles are derived from endosomes and express costimulatory receptors and functional MHC class I and II molecules.

Flt3L is a growth factor that stimulates the proliferation of hemopoietic stem cells (24). From 9 to 11 daily injections of Flt3L into mice induce a large increase in the numbers of DCs in both the peripheral blood and lymphoid tissues. In normal human volunteers, the injection of Flt3L for 14 consecutive days results in a transient 30- to 50-fold increase in the number of DCs in the peripheral blood (C. Maliszewski, unpublished observations). Recently, we showed that Flt3L treatment of mice induced tumor regression in ~40% of tumor-bearing mice and decreased tumor growth rate in the remaining animals (25). Here, we report that the combination treatment of Flt3L and CD40 ligand (CD40L), a cytokine that promotes DC survival and activation in vitro (26), significantly increases antitumor immune responses compared with mice treated with either of the cytokines alone. In addition, we show that the combination treatment results in the development
of a memory immune response capable of blocking tumor rechallenges.

Materials and Methods

Mice

Female C57BL/10J and C3H/HeN mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and Taconic (Germantown, NY). CD40L-knockout (KO) mice were generated at Immunex (Seattle, WA) by homologous gene disruption (27), and the colony was maintained as random hybrids of C57BL/6 × 129J. All mice were age matched (8–10 wk old) at the beginning of each experiment and kept under specific pathogen-free conditions. For each experiment, the size of each treatment group was 10 mice, unless stated otherwise.

Tumors

The B10.2 sarcoma is a methylcholanthrene-induced tumor; the B10.5 and 87 sarcomas are UVB-induced tumors. The B10.2 and B10.5 tumors are of B10 origin, and the 87 tumor is of C3H/HeN origin. The B10.2 and 87 tumors are of the progressor phenotype (i.e., grow progressively in normal mice); the B10.5 tumor is of the regressor phenotype (i.e., a challenge of 5 × 10^5 tumor cells is immunologically rejected subsequent to implantation into normal mice but grows progressively in 500 R-irradiated mice). Tumor inoculations were performed by injecting mice intradermally in the abdomen with 5 × 10^5 tumor cells in a total volume of 50 μl. Tumors were measured weekly with calipers. The tumor size represents the product of two perpendicular diameters and is expressed as the average of only those mice bearing tumors within each treatment group. Immunization against the B10.2 and 87 tumors can be achieved by the inoculation of 5 × 10^5 cells, followed by excision of the tumor 10 days later.

Statistical analysis

The Wilcoxon rank sum test was used to compare tumor growth, and Fisher’s exact test was used to compare the tumor rejection frequencies.

Tumor rechallenge

C57BL/10 mice that had been treated with Flt3L + CD40L and rejected the B10.2 tumor were later rechallenged with the B10.2 and B10.5 tumors. Mice were left tumor free for 3 months and then irradiated with 500 R. After irradiation, each mouse was inoculated with 5 × 10^5 B10.2 and 5 × 10^5 B10.5 tumor cells on the left and right sides of the abdomen, respectively. Tumors were measured weekly.

Cytokines

Recombinant human Flt3L was expressed in Chinese hamster ovary cells and had a biological activity of 1.8 × 10^3 U/mg of protein as determined by its ability to promote proliferative responses of WWF7 cells in vitro. Recombinant murine leucine-zipper CD40L was expressed in Chinese hamster ovary cells and had a biological activity of 16–45 × 10^3 U/mg of protein as determined by its ability to induce the proliferation of normal mouse B cells in vitro. Flt3L and CD40L were diluted in buffered saline solution containing 1 μg/ml mouse serum albumin (MSA) and injected i.c. (intracardially) at the nape of the neck in a total volume of 100 μl. Control mice were injected in the same manner with saline buffer containing 1 μg/ml MSA.

Antibodies

The anti-CD40L (M158) Ab was produced and purified at Immunex. Purified rat IgG was purchased from Sigma (St. Louis, MO). The Abs against CD11b, CD11c, CD80, CD86, and MHC class II (I-A^d) used for FACS analysis were purchased from Pharmingen (San Diego, CA).

Isolation of DCs from cytokine-treated and control mice

Single-cell suspensions of splenocytes from Flt3L- or Flt3L + CD40L-treated mice were prepared by gently mashing the spleens between two frosted glass slides. Splenocytes were first resuspended in NH_4Cl to eliminate erythrocytes, passed through a nylon mesh to exclude splenic debris, and then diluted into DMEM medium. Isolation of splenocytes from MSA- and CD40L-treated animals required digestion with collagenase and DNase, as described in Ref. 14. DCs were enriched from splenocytes by negative depletion with mAbs to Thy-1, B220, NK1.1, and Ter119 for 30 min at 4°C. After incubation, the cells were washed, mixed with anti-IgG-coated magnetic beads (Dynabeads, Dynal, Oslo, Norway), and kept on ice for 30 min. Cells coated with Ab were removed using a magnet. Unbound cells were centrifuged and resuspended in fresh medium.

Mixed lymphocyte reaction

Mixed lymphocyte reaction assays were performed in 96-well round-bottom tissue culture plates. DC-enriched splenocytes from C57BL/10J mice treated with MSA, CD40L, Flt3L, or Flt3L + CD40L were added to triplicate wells and diluted serially at 1:3 ratio starting at 10,000 cells/well and down to 1.5 cells/well. The stimulator DCs were irradiated with 3000R. Allergenic T cells isolated from the lymph nodes of C3H/HeN mice were then added to the DCs at 100,000 T cells/well. The mixed cultures were incubated for 5 days and then pulsed overnight with 0.5 μCi [3H]thymidine. On the next day, the cells were harvested and the incorporated [3H]thymidine was counted on a gas-phase beta counter.

RNase protection assays

Total RNA was isolated from the spleens or tumors of C57BL/10 mice treated for 20 days with MSA, CD40L, Flt3L, or Flt3L + CD40L. After surgical removal, the spleens and tumors were immediately placed in liquid nitrogen. The frozen tissues were reduced to powder using a stainless steel mortar that had been cooled in liquid nitrogen and then lysed in Trizol reagent (Life Technologies, Grand Island, NY). Total RNA was isolated according to the recommendations of the supplier. For the RNase protection assay, we used the RiboQuant multiprobe mcK-2b system (Pharmingen). This system contains a series of mouse cytokine templates that allow for the synthesis of multiple probes in the same reaction vial. The RNA probes were labeled with [α-32P]UTP and hybridized to the splenic or tumor RNA. After RNase treatment and purification, the protected probes were run on a polyacrylamide denaturing gel and analyzed with Phosphorimager (Storm 860, Molecular Dynamics, Sunnyvale, CA).

Results

Combining Flt3L with CD40L augments antitumor immunity against the B10.2 and 87 sarcomas

Since Flt3L promotes the generation of DCs (14, 28) and CD40L induces DC maturation (26), we sought to determine whether these two cytokines would synergize in the treatment of tumor-bearing mice. Fig. 1A shows the combined results of two independent experiments in which C57BL/10 mice were treated for 19 days with MSA, CD40L, Flt3L, or a combination of Flt3L and CD40L. The tumors grew progressively and at the same rate in all of the MSA- and CD40L-treated animals. In the Flt3L and Flt3L + CD40L treatment groups, the tumors grew considerably more slowly than in the MSA or CD40L treatment groups. No significant differences in tumor growth rate were detected when the Flt3L- and Flt3L + CD40L-treated mice were compared. However, at the end of the experiments (6 wk post-tumor inoculation), 56% of the Flt3L + CD40L-treated animals had rejected the tumors, compared to only 24% of the mice treated with Flt3L alone. Even though these differences were not found to be statistically significant (p = 0.08), we observed the same biological trend in both experiments, i.e., a larger proportion of mice rejected the tumors in Flt3L + CD40L combination treatment. In the first experiment, 62.5% of the Flt3L + CD40L-treated mice rejected the B10.2 tumor, whereas only 25% of the Flt3L-treated mice rejected the tumors. None of the mice treated with CD40L alone was able to eliminate the tumor. In the second experiment, 50% of the Flt3L + CD40L-treated mice rejected the B10.2 tumor, whereas only 25% of the Flt3L-treated and none of the CD40L-treated mice rejected the tumors.

To determine whether the Flt3L + CD40L combination therapy would also be beneficial in other tumor settings, we tested this combination on C3H/HeN mice inoculated with the 87 sarcoma. This is a more aggressive tumor than the B10.2 sarcoma; it develops faster and its growth rate is not significantly affected by treatment with Flt3L alone. For this experiment, C3H/HeN mice were challenged with 0.5 × 10^6 cells intradermally and treated for 20 days with MSA, CD40L, Flt3L, or Flt3L + CD40L (Fig. 1B). The CD40L treatment was started 7 days after the initiation of the MSA and Flt3L treatments: in the combination treatment, the animals were initially treated with Flt3L alone from day 1 to day 6, followed by Flt3L + CD40L from day 7 to day 20. In mice treated...
with MSA, CD40L, or Flt3L alone, the tumors grew progressively and at about the same rate. In mice treated with Flt3L + CD40L, the tumors grew at a slower rate, and the tumor rejection frequency was substantially higher (50%) than in the mice treated with MSA (0%), CD40L (0%), or Flt3L (10%) alone.

Due to the numbers of mice per group (8–10 animals) in the experiments described above, the differences in rejection frequencies between Flt3L- and Flt3L + CD40L-treated mice did not achieve statistical significance on any individual experiment. However, the same biological trend was observed in the experiments with the B10.2 and 87 sarcomas; i.e., a larger proportion of mice rejected tumors in the combination treatment than in the treatment groups receiving Flt3L or CD40L alone. If the data from both tumor systems are combined, the sample size is markedly increased and the difference in rejection frequency between Flt3L-treated (18.5%) and Flt3L + CD40L-treated mice (54%) is statistically significant (p = 0.01).

The possibility that Flt3L or CD40L had any direct cytotoxic or cytostatic effects on the B10.2 or 87 tumor cells was evaluated by culturing these cells in the presence of various concentrations of Flt3L and CD40L. No effects on cell growth or cell viability were found when these cultures were compared with B10.2 or 87 cells grown in standard medium alone or medium + MSA (Fig. 1C).

**Figure 1.** B10.2 and 87 tumor growth in mice treated with Flt3L and CD40L. A, Combined results of two independent experiments in which C57BL/10 mice were challenged with $5 \times 10^5$ B10.2 tumor cells and treated for 19 days with MSA (0.1 μg/day), CD40L (10–30 μg/day), Flt3L (10 μg/day), or a combination of Flt3L (10 μg/day) and CD40L (10–30 μg/day). Treatments were started at the same day as the tumor inoculations. B, C57BL/10 mice were challenged with $5 \times 10^5$ B10.2 tumor cells and treated with MSA (0.1 μg/day), CD40L (10 μg/day), Flt3L (10 μg/day), or a combination of Flt3L and CD40L (each at 10 μg/day). Treatments were started the day after tumor inoculation and extended for 20 days, except for CD40L either alone or in combination, which was started only 7 days after the initiation of the MSA and Flt3L treatments. The tumor size represents the average of only those mice bearing tumors within each treatment group. The number of tumor-bearing mice over the total number of mice challenged is shown for the last time point. C, Growth of B10.2 and 87 tumor cells in vitro in the absence or presence of MSA (0.1 μg/ml), CD40L (0.1 μg/ml), Flt3L (0.1 μg/ml), or a combination of Flt3L and CD40L (each at 0.1 μg/ml).
Mice were sublethally irradiated (500 R) to abolish primary immune responses without compromising memory T cell responses, and then challenged bilaterally with B10.2 and B10.5 tumor cells. The second tumor challenge was performed 3 months after the initial tumor rejection. In control mice, both the B10.2 and the B10.5 tumors grew progressively in most of the animals (Fig. 2). In the rechallenged group, the B10.2 tumor failed to grow, whereas the B10.5 tumor grew in all of the mice, thereby demonstrating the immunological specificity of a long-lasting antitumor immune response. The difference in the B10.2 tumor rejection frequencies between the rechallenged group and the control mice was highly significant ($p < 0.0001$). Similar rechallenge experiments performed in C3H/HeN mice that had rejected the 87 sarcoma subsequent to treatment with Flt3L + CD40L demonstrated that these animals also developed a long-lasting memory response (data not shown).

Endogenous CD40L is necessary for the generation of antitumor immunity in mice treated with Flt3L alone

Because exogenous CD40L synergizes with Flt3L during the generation of antitumor immune responses, we set out to determine whether CD40-CD40L interactions were necessary for the generation of antitumor immunity in mice treated with Flt3L alone.

In one series of experiments, groups of 10 mice were treated with MSA, Flt3L + anti-CD40L mAb, Flt3L + control rat IgG, or Flt3L + CD40L. Whereas a high proportion (80%) of mice treated with Flt3L + control rat IgG completely rejected the tumors, only a small proportion (30%) of mice treated with Flt3L + anti-CD40L mAb was able to eliminate the tumors (Fig. 3A). A critical role for CD40L in the generation of antitumor immunity is further supported by a series of experiments performed in CD40L-KO mice. In one such experiment, we challenged CD40L-KO mice with the B10.2 sarcoma and then treated the animals with either MSA or Flt3L for 19 days (Fig. 3B). Even though the tumors grew significantly more slowly in the Flt3L-treated than in the MSA-treated CD40L-KO mice ($p = 0.02$), no complete tumor rejections were observed in any of these animals. In contrast, 70% of the normal C57BL/10 mice treated with Flt3L rejected the tumor.

We also sought to determine whether the blockade of CD40-CD40L interactions would prevent tumor vaccination in animals that were inoculated with live tumor cells (Fig. 3C). For this experiment, mice were challenged intradermally with B10.2 tumor cells and then subdivided into two groups. One group received a neutralizing anti-CD40L mAb and the second group was injected with control rat IgG. The tumors were excised 10 days after immunization, and 40 days later the animals were rechallenged with B10.2 tumor cells. Sixty percent of the mice that had been injected with control rat IgG rejected the tumor, whereas none of the mice treated with the neutralizing anti-CD40L mAb was able to eliminate the tumor ($p = 0.013$). In those mice that did not eliminate the tumors, the tumor growth rate was still significantly lower in the mice that had been vaccinated in the presence of control rat IgG than in the mice vaccinated in the presence of neutralizing anti-CD40L mAb ($p = 0.014$). These results indicate that CD40L is required in vivo for effective vaccination against tumor cells.

Flt3L + CD40L-treated animals have more splenic DCs than animals treated with either of the cytokines alone

During the course of this study, we noticed that the spleens of mice treated with Flt3L + CD40L were considerably larger than those of animals treated with Flt3L or CD40L alone. To determine whether more DCs were being generated in the combination treatment, C57BL/10 mice were treated with MSA, Flt3L, CD40L, or Flt3L + CD40L. After 13 days of treatment, the spleens were harvested, and the splenocytes were counted and stained with Abs against CD11b and CD11c. When the absolute numbers of CD11cbright DCs were compared, we found that there were significantly more DCs in the spleens of mice treated with Flt3L or Flt3L + CD40L than in the spleens of mice treated with MSA or CD40L alone (Table 1). There was a significantly greater number of DCs in mice treated with Flt3L + CD40L than in mice treated with only Flt3L. The fold increase in the number of splenic DCs in the Flt3L + CD40L-treated animals is slightly higher in the CD11chighCD11bdull DC subpopulation than in the CD11clowCD11bhigh DC subpopulation. Similar increases in the number of DCs have also been detected in C3H/HeN and C57BL/10 mice treated with Flt3L + CD40L for 16 or 20 days (data not shown).

Splenic DCs from Flt3L- or Flt3L + CD40L-treated mice express similar levels of MHC class II and costimulatory molecules

To analyze the phenotype of the DCs generated under Flt3L and CD40L treatment, splenocytes were isolated from mice treated with Flt3L or Flt3L + CD40L, stained with Abs against CD11b, CD11c, CD80, CD86, and MHC class II; and subjected to three-color flow cytometric analysis. Even though there were more DCs in the spleens of animals treated with Flt3L + CD40L, no differences in the expression level of CD80, CD86, and MHC class II were detected between DCs isolated from mice treated with Flt3L.
alone or in combination with CD40L (Fig. 4). Also, no differences were detected in the level of expression of CD25, CD40, CD44, and CD54 (data not shown).

DCs from mice treated with Flt3L, CD40L, or Flt3L + CD40L are as potent as control DCs at stimulating allogeneic T cells

To determine whether the cytokines used in this study altered the functional capabilities of DCs, we cocultured splenic DCs with allogeneic T cells. DCs from the spleens of mice that had been treated for 20 days with MSA, Flt3L, CD40L, or Flt3L + CD40L were isolated and irradiated before culture with allogeneic T cells. DCs isolated from the three different cytokine treatment groups showed the same potency as control DCs in stimulating the proliferation of allogeneic T cells (Fig. 5). The same results were obtained when we compared splenic DCs from animals that had been treated with the various cytokines for only 10 days (data not shown).
Treatment of mice with Flt3L + CD40L up-regulates IL-12 p40 message levels within the tumors

Mice treated with Flt3L have extensive leukocytic infiltrates within the tumors (25). In addition to lymphocytes, the infiltrate includes mononuclear cells with large, vesiculated, and folded nuclei that are morphologically consistent with DCs. We have used RNase protection assays to analyze gene expression within the tumors of animals that had been treated with MSA, Flt3L, CD40L, or Flt3L + CD40L. Although this kind of analysis does not permit the identification of cells that express a specific molecule, it does allow the comparison of gene expression among the different treatment protocols. We have observed that there is a strong up-regulation of IL-12 p40 message levels in the tumors, but not in the spleens, of mice that had been treated with Flt3L and CD40L for 20 days compared with mice treated with either cytokine alone (Fig. 6). No differences were detected among all of the treatment groups when we compared the message levels for IL-12 p35, IL-1α, IL-1β, IL-1Ra, IL-10, and IL-18.

Discussion

In this study, we have demonstrated that a strong antitumor immune response can be generated in vivo using a cytokine combination therapy that promotes the proliferation and survival of DCs. Treatment of tumor-bearing mice with Flt3L and CD40L significantly enhanced antitumor immunity against two murine tumors, the B10.2 and the 87 sarcomas. We chose these two tumors because they grow progressively in all of the challenged animals and they do not express any known tumor Ags, a situation that is reminiscent of most human tumors. Our immunotherapeutic strategy was designed to stimulate the immune system and allow the APCs and effector cells to select the relevant tumor Ags to mount an immune response. We found that Flt3L and CD40L act synergistically in vivo to promote the generation of DCs. By significantly enhancing the Ag-presenting capacity of the immune system, strong antitumor immune responses were generated in vivo.

The number of splenic DCs is higher in mice treated with Flt3L + CD40L than in mice treated with either cytokine alone. In mice injected with both cytokines, there was a 38-fold increase in the number of DCs compared with mice treated with MSA, whereas in mice treated with only Flt3L the number of DCs was increased 26-fold over control mice. Interestingly, when we compared DC profiles from Flt3L- and Flt3L + CD40L-treated mice, we observed that a CD11chighCD11bdull subpopulation accounted for a large fraction of the increase in the DC numbers. This subpopulation of DCs is the same as subpopulation D/E described by Maraskovsky et al. (14) in mice treated for 10 days with Flt3L. These cells express high levels of MHC class II and >50% are CD8α positive (data not shown). This phenotype is similar to that of the putative lymphoid-related DCs (29), suggesting that the combination treatment of Flt3L and CD40L promotes the proliferation of a lymphoid precursor that can differentiate into DCs. However, CD11chighCD11bdull CD8αnegative DCs are also generated in these mice, indicating that a DC myeloid precursor is also involved in the generation of DCs in mice treated with Flt3L + CD40L. This same DC subpopulation was also described by Maraskovsky et al. (14) and shown to have a larger phagocytic capacity and to secrete less IL-12 than the putative lymphoid-related DCs (28). In addition, the lymphoid-related DCs appear to selectively enhance Ag-specific, Th1-like responses in mice (30). We are currently investigating the role played by these two DC subpopulations during the generation of antitumor immune responses in vivo.

The splenic DCs derived from Flt3L + CD40L-treated animals are as efficient as control or Flt3L-generated DCs in stimulating the proliferation of allogeneic T cells. The higher number of DCs present in mice treated with Flt3L and CD40L is likely to increase the capacity of the immune system to capture and process tumor Ags for presentation to effector T cells. In tumor-bearing animals, the increment in immune function appears to be directed against tumor, and not normal tissues. In this regard, we have followed
mice that were successfully treated with Flt3L and CD40L for up to six mo after cessation of cytokine treatment and have seen no adverse reactions. In addition, no signs of autoimmunity were detected in normal mice treated for up to 28 days with Flt3L (D. Lynch, unpublished results). Preliminary studies performed in normal human volunteers injected with Flt3L over 14-day periods have shown that Flt3L is well tolerated (C. Maliszewski, unpublished observations). These results suggest that the number of DCs can be transiently increased in vivo without marked adverse effects. This observation is important in that one can envision the use of DC-based therapies to treat not only tumors but also infectious diseases.

DCs isolated from the spleens of Flt3L-treated animals and cultured overnight in the presence of CD40L (10 ng/ml) up-regulate the expression of the costimulatory molecules CD80 and CD86 (data not shown). In tumor-bearing mice treated with Flt3L alone for 13–20 days, DCs express class II, CD80, and CD86, suggesting that the cells have been activated to a certain extent. However, addition of CD40L to the Flt3L treatment did not result in further increase in the expression levels of these molecules in splenic DCs in vivo. The differences in the ability of CD40L to up-regulate the expression of class II and costimulatory molecules in DCs stimulated in vitro vs in vivo could be related to both the kinetics of up-
and down-regulation of CD80 and CD86 and the pharmacokinetic properties of the leucine-zipper CD40L fusion protein used in this study. Pharmacokinetic analysis showed that the serum concentration of CD40L decreased by 87% within the first 22 min of injection (data not shown). Most of the CD40L cleared from circulation accumulated in the liver, but there was also significant accumulation in the spleen. During the elimination phase, the half-life of CD40L was only 366 min in the blood and 328 min in the spleen (data not shown). As a result, the DCs in mice treated with CD40L are exposed to CD40L for significantly shorter periods of time than cultured DCs. This disparity could explain, at least in part, the differences in the ability of CD40L to up-regulate and/or sustain the expression of CD80 and CD86 in DCs exposed to CD40L in vitro and in vivo. Given the synergy between Flt3L and CD40L in the generation of antitumor immunity, we cannot exclude the possibility that CD40L could specifically affect a very restricted population of DCs that is involved in the capture, processing, and presentation of tumor Ags. In fact, IL-12 p40 mRNA transcripts are more abundant in the tumors, but not in the spleens, of mice treated with the combination of Flt3L and CD40L than in those of animals treated with either of the cytokines alone. Because IL-12 is synthesized almost exclusively by DCs (31, 32) and monocytes/
macrophages (33), it is likely that the increase in IL-12 p40 message levels occurs within the APCs that invade the tumor. These tumor-infiltrating APCs are likely to be involved in capturing and processing tumor Ags for presentation to the effector arm of the immune system. Increased concentrations of IL-12 could shift the immune response toward a Th1 phenotype, where cell-mediated immunity takes precedent. Unfortunately, further characterization of the phenotype of the DCs present in the tumors has been hampered by the lack of Abs capable of specifically recognizing DC markers in histological sections.

Besides CD80 and CD86, CD40L might also trigger expression of other molecules by DCs, which can play an important role in the activation of effector cells. For instance, the expression of chemokines and their receptors in DCs has been shown to be modulated by CD40L. Human DCs cultured in the presence of CD40L down-regulate the expression of the chemokine receptors CCR1 and CCR5 and strongly up-regulate the expression of another chemokine receptor, CCR7 (34). Expression of the chemokines macrophage-inflammatory proteins-1a and 1b and RANTES is enhanced in monocyte-derived DCs stimulated with CD40L (35). In vivo, DCs capture Ags in the periphery and then migrate to lymph nodes and the spleen where they present the Ags to the effector arm of the immune system. Given the capacity of CD40L to modulate the expression of chemokines and their receptors in cultured DCs, it is plausible that CD40L treatment could play a similar role in vivo and alter the migratory properties of DCs. The increase in IL-12 message levels detected in the tumors of mice treated with Flt3L + CD40L could indicate that the tumor-infiltrating APCs are being activated further in these mice or that more APCs are being recruited to the tumor site. That could be, at least in part, mediated by changes in chemokine/chemokine receptor expression in DCs after stimulation by CD40L. The modulation of chemokine/chemokine receptor expression in DCs could affect not only the trafficking of these cells to the tumor site but also their subsequent migration to the secondary lymphoid organs.

In addition to demonstrating that exogenous CD40L synergizes with Flt3L, our results have also shown that endogenous CD40L plays a critical role in the generation of antitumor immune responses. Flt3L-treated animals that were injected with a blocking anti-CD40L mAb had significantly impaired antitumor immune responses, as determined by an increase in tumor growth rate and a decrease in tumor rejections. The requirement for CD40L is also supported by the observation that CD40L-KO mice are unable to reject tumor challenges, despite treatment with Flt3L. Impaired immunity is not due to failure of Flt3L to up-regulate the numbers of DCs in these animals. Equivalent numbers of DCs are generated in CD40L-KO and C57BL/6 mice when they are treated with Flt3L for 10–14 days (data not shown). The failure of these animals to mount an effective antitumor immune response is more likely to be due to an impairment to generate a Th1-type response. In this regard, Campbell et al. (36) have shown that CD40L-KO mice fail to mount a protective Th1 response against Leishmania major. This failure appeared to be linked to an impairment of APCs to be activated to secrete IL-12, since treatment of these mice with exogenous IL-12 prevented the progression of leishmaniasis.

Some of the major advantages of immunotherapy over conventional radiation and chemotherapy are the abilities of the immune system to specifically target the tumor cells and to induce a long-lasting antitumor response. By focusing the immune response specifically on the tumor, the side effects of conventional therapies are eliminated. In addition, the generation of specific antitumor memory mechanisms should provide the patient with a preventive mechanism that could be activated to eliminate the growth of tumor metastasis. Our results have demonstrated that animals that have successfully eliminated the primary tumor upon treatment with Flt3L + CD40L are protected against later rechallenges with the same tumor. This memory response is specific for the initial tumor, since rechallenged mice did not eliminate an unrelated syngeneic tumor. CD40L plays a critical role during the generation of both primary and memory immune responses; blockade of CD40-CD40L interactions impairs the development of protective immunity in tumor-bearing mice and precludes vaccination against tumors. These findings are reinforced by the observations by Mackey et al. (37) that administration of an anti-CD40 Ab prevents the vaccination against two other tumors, the MCA-105 sarcoma and the B16 melanoma. The inability to generate protective immunity against the MCA-105 and B16 tumors correlates with a failure to induce Th1-type cytokine production following tumor vaccination. These data indicate that the interaction of CD40 with its ligand on the surface of APCs is critical for the induction of antitumor immune responses.

In summary, our data indicate that cytokines can be combined in vivo to induce potent antitumor immune responses against tumors the rejection Ags of which are not known. Because the vast majority of the human tumors carry unknown rejection Ags, our studies could have important corollaries for the development of anti-tumor immunotherapies in human patients. Most of the attempts at using cytokines to treat tumors in vivo have relied on the use of single agents. Our results show the advantage of combining different cytokines. Several treatment protocols can be envisioned using molecules that could activate not only DCs but also CD4+ and CD8+ T cells. For instance, a Flt3L + CD40L therapy could be used to generate large numbers of DCs, followed by treatment with cytokines that could activate cytolytic T cells. We are currently pursuing other therapies that combine cytokines with pro-apoptotic or antiangiogenic molecules that can directly limit tumor growth. Given the highly diverse phenotypes of tumor cells, no single therapy should be expected to efficiently eliminate most of the tumors. Future cancer therapies should focus on developing new strategies that target multiple mechanisms required for tumor growth and survival.

Acknowledgments

We thank Charles Maliszewski, David Cosman, and Doug Williams for critically reviewing and discussing the manuscript; Joann Schuh for her valuable comments on the histopathology of the tumors; Anne Aumell for editorial assistance; Gary Carlton for preparation of some of the figures; and Dana Shack and his group at the mouse facility for help with the injections and animal husbandry.

References


