Functional and in Situ Evidence for Nitric Oxide Production Driven by CD40-CD40L Interactions in Graft-versus-Leukemia Reactivity

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ABSTRACT

In a murine tumor model, complete tumor remission is achievable at even advanced metastasized stages by transfer of immune T cells from donor B10.D2 (H-2d, Mlsa) into tumor-bearing DBA/2 (H-2d, Mlsa) mice. We showed previously that this graft-versus-leukemia (GvL) effect is dependent on synergistic interactions of transferred CD4+ and CD8+ T cells with host sialoadhesin (SER)-positive macrophages. We now show that the CD40-CD40L (CD154) interaction is involved in the induction of inducible nitric oxide synthase (iNOS) expression during adoptive immunotherapy (ADI). We demonstrate that during ADI, the level of CD40 expression in the liver becomes significantly augmented in comparison to livers of tumor-bearing, untreated animals. CD40 expression is found mostly on SER+ macrophages and to a lesser extent on dendritic cells (DCs). In GvL animals, more SER+ macrophages express iNOS than untreated animals. iNOS expressing cells are found in close proximity to apoptotic cells, at early time points of the therapy in areas of metastasis, and at late stages around portal veins, where CD4+ and CD8+ T lymphocytes form clusters with SER+ macrophages. Blocking of CD40 in vivo at days 5 and 20, when all iNOS+ cells express CD40, leads to significantly reduced CD40 and iNOS expression as well as to a marked inhibition of the therapeutic effect. These data provide functional and in situ evidence that the increased CD40 and iNOS expression observed during ADI contribute to the eradication of liver metastases and to the clearance of donor lymphocytes from the liver.

INTRODUCTION

We described previously a highly effective ADI protocol using the well-defined ESb-MP T lymphoma model in the DBA/2 (H-2d, Mlsa) mouse, which works even in late-stage cancer disease (1, 2). For adoptive transfer, in vivo activated tumor immune spleen lymphocytes from the ESb tumor-resistant, MHC-identical but superantigen-different mouse strain B10.D2 (H-2d, Mlsa) are injected into 5 Gy-irradiated, tumor-bearing DBA/2 mice. This transfer of donor immune cells into tumor-bearing hosts leads to a complete rejection of primary tumors (1.5 diameter in size) from the skin and to the eradication of late-stage metastases in liver, spleen, and kidney. This therapy effect requires the transfer of CD4+ as well as CD8+ T cells and synergistic interactions of these cells with host SER+ macrophages (1, 2). SER is a lectin-like receptor that mediates divergent cation-independent binding but not phagocytosis of sheep erythrocytes (3). It is expressed on a number of tissue macrophage subsets in bone marrow, spleen, liver, and lymph nodes (3, 4), and it has been shown to function as an adhesion molecule for lymphocytes (5). Moreover, SER+ macrophages in this ADI model were shown to: (a) play an essential role in the GvL effect (6); and (b) form clusters with CD8 and CD4 T lymphocytes, which could be sites for T-cell activation and differentiation (7).

Several recent studies indicate that the CD40-CD40L interaction plays a critical role in the humoral immune response (e.g., isotype switching, development of B-cell memory and germinal centers; Ref. 8) as well as in T-cell mediated immunity (e.g., viral target lysis, parasite clearance, and antitumor responses; Refs. 9–12). CD40 is a member of the TNF receptor family, is expressed constitutively in low levels on B cells, monocytes/macrophages, and DCs, and can be further up-regulated by IFN-γ (9, 11–13). CD40L (CD154) belongs to the TNF family and is expressed on activated T cells, predominantly on activated CD4 cells (8, 9). Triggering of CD40 on APCs enhances the levels of adhesion (intercellular adhesion molecule-1) and costimulatory molecules (CD80 and CD86; Refs. 9, 14–17), leading to enhanced antigen presentation, thereby facilitating activation of T cell-mediated immune responses. Recent publications showed that the CD40-CD40L interaction is also involved in the secretion of NO by macrophages and DCs in vitro (9, 18, 19). NO is a highly effective molecule that can exert direct cytotoxic effect toward microorganisms and tumor cells (8, 20, 21) or that can act indirectly via stimulating the DNA damage response, as well as other functions (22). CD40L (CD154) belongs to the TNF superfamily and is expressed on activated T cells, predominantly on activated CD4 cells (8, 9, 23). NO is a highly effective molecule that can exert direct cytotoxic effect toward microorganisms and tumor cells (8, 20, 21) or that can act indirectly via stimulating the DNA damage response, as well as other functions (22).
production of inflammatory mediators like TNF-α and IL-1α (20). Murine macrophages have been shown to express iNOS, which catalyzes the production of NO from L-arginine in vitro and in vivo (20, 22, 23). NO is able to induce apoptosis in several cell types including tumor cells (24). In the present study, we investigated a possible role of CD40-CD40L interactions in our murine GvL model system. The results provide in situ and in vivo evidence for the involvement of the CD40-CD40L interaction in the therapy effect. This interaction stimulates the up-regulation of iNOS expression predominantly by SER+ macrophages. The increased NO synthesis in livers of ADI-treated mice is associated with apoptosis in tumor cells early after cell transfer as well as in liver-infiltrating T cells at later stages. We suggest that the CD40-CD40L interaction contributes to the effectiveness of the antitumor response as well as to the clearance of T cells from the liver after completion of the antitumor therapy.

MATERIALS AND METHODS

Animals

DBA/2 mice were obtained from IFFA Credo (Lyon, France), and B10.D2 mice were obtained from Olac (Bicester, United Kingdom). Animals were used at 6–12 weeks of age.

Tumor Cell Lines

ESb cells represent a spontaneous highly metastatic variant of the chemically induced lymphoma L5178Y (Eb) of DBA/2 mice. The subline ESb-MP is a plastic-adherent variant of the ESb lymphoma cells. In vivo, ESb-MP cells grow progressively but show a less aggressive phenotype, metastasizing more slowly than ESb and involving multiple organs including the liver (2).

Adaptive Cellular Immunotherapy (ADI)

DBA/2 mice were anesthetized with Rompun (0.1%; Parke, Davis & Company, Berlin, Germany); Ketanest (0.25; 1% Bayer, Leverkusen, Germany); PBS at 1:1:3 (vol) to inject $2 \times 10^7$ ESb-MP tumor cells intradermally into the dermis of the shaved flank. To generate allogeneic tumor immune effector cells, B10.D2 mice were inoculated i.v. with $1 \times 10^7$ ESb-MP cells (1). Seven days later, spleen cells were isolated from the donor and transferred i.v. (2 $\times 10^7$ cells/200 μl PBS; Life Technologies, Inc., Eggenstein, Germany) into 5 Gy ($^{60}$Co source Gammatron F 80 S; Siemens, Munich, Germany) sublethally irradiated DBA/2 mice that carried tumors (>1 cm in diameter). The transfer of the antitumor immune spleen cells was made 3 weeks after intradermal tumor cell inoculation. As a control group, sublethally irradiated tumor-bearing DBA/2 mice remained untreated. To analyze GVH responses, non-tumor-bearing DBA/2 mice were irradiated and inoculated with the tumor-immune donor cells.

Antibodies and Other Reagents

The following rat mAbs were used as culture supernatants: antimouse SER (clone SER4; Ref. 5), antimouse B220 (clone GB2; Ref. 25), and antimouse CD40 (clone 3/23; Instruchemie, Hilversum, the Netherlands). The rat antimouse N418 (clone N418), which was kindly provided by Dr. B. Kyewski (German Cancer Research Center, Heidelberg, Germany), and the hamster antimouse CD40L mAbs (clone MR1; Ref. 26) were biotinylated. In vivo injected anti-CD40L mAb was unconjugated and immunohistologically detected with a goat antihamster IgG (Dianova, Hamburg, Germany). iNOS was detected by a polyclonal rabbit antimouse iNOS serum (Calbiochem, Bad Soden, Germany). SER and B220 were visualized by using polyclonal donkey antirat IgG (H+L) second antibodies linked either to PO or to AP (Dianova). Anti-N418 and anti-CD40L were detected by ExtrAvidin linked to AP (Sigma, Deisenhofen, Germany). Anti-CD40 was visualized by consecutive incubation with a rabbit antirat IgG linked to PO and a swine antirabbit IgG linked to PO (both DAKO, Hamburg, Germany). The rabbit antimouse iNOS antibody was detected via a biotinylated donkey antirabbit second antibody (Amersham, Braunschweig, Germany), and avidin was linked to PO (Sigma). Endogenous biotin was blocked by the biotin blocking system from DAKO.

Immunohistochemistry

Tissue Preparation. Livers from DBA/2 mice were removed at different time points after therapy and snap-frozen in liquid nitrogen. Six-μm-thick consecutive cryostat sections were mounted on uncovered glass slides, air-dried overnight at room temperature, fixed in acetone (Merck, Darmstadt, Germany) for 10 min at room temperature, and air-dried again for 1 h.

Single Staining. After drying, the slides were washed in PBS three times for 5 min. To avoid nonspecific binding, sections were treated with 1% normal mouse serum for 15 min, followed by incubation with the first antibody for 45 min. After washing three times in PBS, the sections were treated with the second antibody, and in the case of CD40, afterward with the third antibody. Before the substrate reaction for PO or AP was performed, the slides were washed three times in PBS for 5 min. When using a biotinylated antibody, the slides were incubated with ExtrAvidin for 30 min. After staining, the sections were washed with water and mounted with glycerol-gelatin (Merck). The same protocol was performed for negative controls in which either the first or the second antibody was omitted. All steps were performed in a humid chamber and at room temperature, except for incubations with the first antibody for CD40, CD40L, and iNOS, which were done overnight at 4°C.

Double Staining. The staining procedure represents a combination of consecutive stainings for each antigen. Every single staining was completely finished with the substrate reaction before starting with the second staining. For double staining of CD40 and CD40L, sections were incubated with the two first antibodies in parallel.

Development of Enzyme Reactions. PO reactivity was revealed by incubating the sections in a solution containing 6 mg of 3-amino-9-ethylcarbazole (Merck), which was dissolved in 1.5 ml of N,N-dimethylformamide (Merck), 15 μl of 30% hydrogen peroxide, and 28.5 ml of 0.1 M acetate buffer (pH 5.0). The substrate for the development of AP consisted of 6.3 μl of 5% Neufuchsin (Sigma) or 2 mg of Fast Blue (Sigma) in 16 μl of 4% sodium nitrite (Fluka, Buchs, Switzerland), 2 mg of naphthol-As-Bi-phosphate (Sigma) in 20 μl of N,N-dimethylformamide (Merck), and 3 ml of 0.05 M Tris-HCl buffer (pH 8.7) containing 1 mM levamisole (Sigma). The freshly prepared
solutions were filtered through a 0.22 μm filter (Millipore, Eschborn, Germany) and added to the sections. Development lasted about 3–10 min, with regular checking of the staining intensity by microscope. Immunohistochemical results were evaluated by counting positively stained cells per liver lobule from three to six lobuli/mouse. The means and SDs of the data obtained from two to three mice/time point and from different experiments were calculated and presented in graphs.

**TUNEL Assay**

To detect apoptotic cells, the TUNEL assay was performed with the *in situ* cell death detection kit (Boehringer Mannheim, Mannheim, Germany) according to the instructions of the manufacturer. Briefly, frozen tissue sections were fixed in acetone at room temperature, air dried, and permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate (both Merck) for 2 min. After washing in PBS, sections were incubated with the TUNEL reaction mixture for 90 min at 37°C. After washing in PBS, the POD converter was added for 30 min at 37°C. The sections were washed with PBS, and the substrate reaction for PO was performed. Afterward, sections were washed in water, counterstained in 50% Hemalaun (Merck), and mounted with glycerol-gelatin. When the TUNEL assay was combined with immunohistology, the immunohistological staining was performed first as described above, followed by the TUNEL assay. No counterstaining with Hemalaun was performed.

**In Vivo Blocking of the CD40-CD40L Interaction with mAb against CD40L**

To block the function of CD40L+ T cells and thereby the interaction with CD40 molecules in the host liver after ADI, anti-CD40L mAb (clone MR1) was injected i.p. twice at days 3 and 5 or at days 18 and 20 after ADI into tumor-bearing mice (250 μg/injection; 6–10 animals/experimental group). Control tumor-bearing ADI-treated mice were injected i.p. with normal hamster immunoglobulin (Dianova) at the same concentrations and at the same days as anti-CD40L mAb. Statistical analysis was performed with the log-rank test.

**RESULTS**

**Kinetics of CD40 and CD40L Expression in the Liver during ADI.** For the investigation of CD40 and CD40L expression in the liver of GvL (tumor-bearing animals treated with ADI) and GvH animals (non-tumor-bearing mice treated with ADI) after immune cell transfer, cryostat liver sections were stained with the respective mAbs (Fig. 1). In normal livers of DBA/2 mice, neither CD40 nor CD40L could be detected (data not shown). Fig. 1A shows that in the liver of tumor-bearing DBA/2 mice, which were only lethally irradiated with 5 Gy but not treated with immune cells (control), some CD40+ cells (maximum, four cells/LL) could be detected. All animals from this group died until day 32 after tumor inoculation because of the tumor burden and metastasis. Tumor-bearing mice that were treated with tumor immune donor cells showed a steady increase of CD40+ cells with a significant peak at day 20 after cell transfer (35 cells/LL) and a subsequent decrease at day 28 (18 cells/LL). The number of CD40L+ cells was much lower than that of CD40+ cells (Fig. 1B). Maximal levels were observed at day 20 (three cells/LL), whereafter their number decreased to one cell/LL at day 28. In non-tumor-bearing mice that received the same immune cells (GvH mice), the number of CD40+ cells increased similar to the GvL animals but only until day 12, reaching a peak level of 12/LL (Fig. 1A). CD40L+ expression in the liver of either control mice (●), GvL (□), or GvH (▲) animals. Three to six lobules per liver were analyzed by light microscopy. Data represent the means from two independent experiments with two to three animals/time point; bars, SD. Data points without SD represent mean values for which the SD was <1. †, no surviving animals.

**Characterization of CD40-expressing Cell Populations.** To identify the CD40-expressing cells in the liver during ADI, we performed double stainings with different surface markers for potential APCs (macrophages, B cells, and DCs) on frozen liver sections of GvL and GvH animals after ADI. We stained CD40+ cells for F4/80 (a general macrophage marker) and for SER, a marker expressed on a liver macrophage subset that was found previously to be crucial for the effectiveness of ADI (6).

As shown in Fig. 2A, ADI caused a significant increase in the numbers of F4/80+ and SER+ single-positive cells in tumor-bearing mice. The number of CD40+ F4/80+ double-positive cells also increased in these animals. At day 20, when the highest number of CD40+ cells was detected, there was also a peak of CD40+ F4/80+ cells (22 cells/LL, which represent
23% of the total number of F4/80<sup>+</sup> cells), which represented 70% of the total CD40<sup>+</sup> cells. The highest number of CD40<sup>+</sup> SER<sup>+</sup> macrophages (72%) was also seen at day 20 (16 cells/LL, 22% of the SER<sup>+</sup> cells). Thus, at this time point, 72% of CD40<sup>+</sup> macrophages in the liver were SER<sup>+</sup>.

In GvH animals, the numbers of F4/80<sup>+</sup> and SER<sup>+</sup> cells also increased after the transfer of tumor-immune cells (Fig. 2B). Although in GvH considerably fewer CD40<sup>+</sup> cells were found, the maximal number of CD40<sup>+</sup> F4/80<sup>+</sup> cells and CD40<sup>+</sup> SER<sup>+</sup> cells were also detected at day 12, like in GvL (six and six cells/LL, respectively). As in GvL, the majority of CD40-expressing macrophages (F4/80<sup>+</sup>) were SER<sup>+</sup> in GvH as well.

Fig. 2C shows that the number of B220-positive cells (B cells) in liver sections is very low, both in GvL and GvH animals. Therefore, practically no CD40<sup>+</sup> B220<sup>+</sup> double-positive cells were found in these mice. This suggests that B cells do not play any role in ADI and that they do not contribute to the CD40-CD40L interactions in the liver.

As shown in Fig. 2D, the number of DCs as defined by expression of N418 increased in the GvL significantly from 0 to 28 cells/LL at day 20. At this time point, the maximal number of CD40-expressing DCs was detected (13 cells/LL). This corresponded to 46% of the DCs and to 40% of the total number of CD40<sup>+</sup> cells at this time point. In GvH, the number of DCs also increased and reached a peak at day 12 after the transfer of immune cells (13 cells/LL). At this time point, 38% of DCs (five cells/LL) also expressed CD40.

In conclusion, macrophages and DCs are the two APC populations in the liver expressing CD40 during ADI, whereas B cells do not.

**iNOS Expression by SER<sup>+</sup> Macrophages during ADI.**

It has been shown that the CD40-CD40L interaction is involved in the production of NO by macrophages and DCs in vitro (9, 19). Therefore, we analyzed iNOS expression in situ in the liver of ADI-treated mice, in particular by SER<sup>+</sup> macrophages. In normal, non-treated livers, no iNOS expression could be found (data not shown). Fig. 3 shows that the maximal number of iNOS-expressing cells in the liver of GvL (N418<sup>+</sup>) was detected at day 20 (25 cells/LL). A lower peak was already seen at day 8 (14 cells/LL). At these time points, most of the iNOS-expressing cells (>80%) were SER<sup>+</sup>. Double staining for iNOS and N418 revealed that DCs also expressed iNOS, but the cell numbers were much lower than those for SER<sup>+</sup> macrophages. The maximum number of iNOS<sup>+</sup> DCs was seen at day 20 with six cells/LL (data not shown). Thus, it can be concluded that SER<sup>+</sup> macrophages represent the major population and DCs only a minor population of iNOS-expressing cells during ADI.
CD40/iNOS Coexpression in the Liver during ADI.

To investigate whether CD40-CD40L interactions are involved in the induction of iNOS expression, we evaluated coexpression of CD40 and iNOS during ADI.

In the liver of GvL animals (Fig. 4A), the maximal number of iNOS-expressing cells (25 cells/LL) was detected at day 20 after donor cell transfer, coinciding with the highest level of CD40-expressing cells (33 cells/LL; Fig. 1). At this time point, all iNOS-expressing cells were CD40+. In GvH mice, maximal iNOS expression also correlated with the presence of CD40 (similar to the GvL situation). The number of double-positive cells was much lower than in GvL animals and reached a maximum at day 8 (eight cells/LL). iNOS expression, therefore, seems to be induced in CD40+ cells during ADI.

Colocalization of iNOS-Expressing Cells and Apoptotic Cells. To assess whether NO is involved in the induction of apoptosis during ADI, we analyzed the colocalization of iNOS-expressing cells and apoptotic cells by using the TUNEL assay in combination with immunohistological staining for iNOS. At day 8 after donor cell transfer, the highest number of apoptotic cells was detected within metastases, suggesting that these had been tumor cells. Fig. 4B shows iNOS-positive cells and apoptotic cells in the metastatic area at day 5 after transfer of donor cells. At day 20, apoptotic cells were detected around portal veins, where clusters containing CD4+, CD8+ T cells and SER+ macrophages were demonstrated previously (6). These apoptotic cells might represent liver-infiltrating T cells that had to be removed after successful elimination of the metastases. Combination of the TUNEL assay and immunohistological staining for the donor cell marker β2-microglobulin revealed that at day 20, 45% of the apoptotic cells were donor cells, whereas at day 5, only 6% were of donor origin (data not shown). At both time points, iNOS-expressing cells were clearly found in close proximity to cells undergoing apoptosis (three and three cells/LL at days 5 and 20, respectively), indicating that NO might induce apoptosis in tumor cells at the early time points and in normal T cells at later stages of ADI. In GvH animals, 3–5 apoptotic cells/LL were also found at day 20 after cell transfer, of which some were in close proximity to iNOS-expressing cells (data not shown).

Reduced ADI Effect Caused by in Vivo Treatment with Anti-CD40L mAb. To determine whether the CD40-CD40L interaction is crucial for the therapeutic effect of ADI, we injected a blocking anti-CD40L mAb (MR1) into tumor-bearing animals either at days 3 and 5 or at days 18 and 20 after ADI. One hundred % of ADI-treated, tumor-bearing animals that were injected with normal hamster immunoglobulin were alive at the end of the experiment (at day 60 after tumor cell inoculation; Fig. 5A, ADI + control Ig). In contrast, both groups that were injected with anti-CD40L mAb showed reduced survival. Thus, only 49% of mice injected at days 3 and 5 after ADI and 37% of mice injected at days 18 and 20 (Fig. 5A) were alive at the end of the experiment, which is statistically significant in comparison to the control (P < 0.0001). Animals that received neither ADI nor antibody treatment were all dead at day 53 after tumor inoculation. The injected anti-CD40L mAb could be detected with a goat antimouse second antibody in the liver of the treated animals, indicating that the injected antibodies were able to target CD40L+ cells. The observed effect is most probably attributable to blocking of the receptor and not to depletion of these cells. We further looked whether the elimination of the effector T cells at late time points is abrogated by the blocking of CD40-CD40L interaction. Interestingly, immunohistological staining for CD4+ and CD8+ revealed that in livers of mice injected with control immunoglobulin, the number of CD4+ and CD8+ T cells decreased from day 7 to day 22 (from 36 to 29 CD4+ and from 32 to 24 CD8+ T cells; Fig. 5B), whereas in mice treated with the anti-CD40L mAb, the number of both T-cell subsets increased (from 41 to 50 CD4+ and from 37 to 50 CD8+ T cells; P < 0.05; Fig. 5B). At day 22, ~2-fold more CD4+ and CD8+ T cells were found in the anti-CD40L mAb-treated group than in the group injected with control immunoglobulin. The CD40-CD40L interaction thus appears to be of importance at early as well as at late time points of ADI.

Down-Regulation of CD40 and iNOS Expression after Treatment with Anti-CD40L mAb. To determine whether iNOS expression during ADI is mediated by CD40-CD40L interaction, livers from mice treated with control immunoglobulin or the anti-CD40L mAb were stained for CD40 and iNOS. Livers were removed either at day 7 after ADI in the case of early mAb injections or at day 22 in the case of the late antibody treatment. In livers of ADI-treated animals that received the control immunoglobulin, 13 CD40+ cells/LL were detected at day 7 and 25 cells/LL at day 22 (Fig. 5C). In contrast, the expression of CD40 was significantly lower in livers of mice injected with anti-CD40L mAb than in ADI treated animals without antibody injection (four cells/LL at day 7 and 11 cells/LL at day 22; P < 0.05; Fig. 5C). Treatment with anti-CD40L mAb also caused a reduction in the number of iNOS+ cells (from 9 cells/LL in the control group to 3 cells/LL at day 7, and from 20 cells/LL to 6 cells/LL at day 22; P < 0.05; Fig. 5C).

DISCUSSION

The data presented in this study demonstrate a significant increase in CD40 expression in the liver of tumor-bearing DBA/2 mice that received a cellular therapy with tumor immune
cells from B10.D2 mice. We found CD40 expression on DCs and macrophages, in particular SER1 macrophages but not on B cells. Both the in situ data and the in vivo functional experiments suggest the involvement of the CD40-CD40L interaction in the therapy effect. This interaction seems to up-regulate iNOS expression, predominantly by SER1 macrophages. The increased NO synthesis in livers of ADI-treated mice is associated with apoptosis of tumor cells early after transfer and apoptosis of normal lymphocytes at a later time point. iNOS up-regulation through the CD40-CD40L interaction thus seems to be biphasic and bifunctional, leading to the eradication of liver metastases at early stages (antitumor effect) and of T cells from the liver at later stages of ADI. The overall effective protection achieved through allogeneic T-cell transfer in this GvL model thus involves cytotoxic reactivity against tumor cells as well as against normal T cells. These cytotoxic effects could be exerted through: (a) T cells (which can be cytotoxic but do not produce NO); (b) NO-producing cells (macrophages, DCs, and endothelial cells); or (c) other mechanisms. NO has been shown to induce apoptosis in various cell types including tumor cells (21, 22, 24). This study identified a possible contribution of CD40-CD40L interaction to this mechanism.

Maximal CD40 expression was detected at day 20 after immune cell transfer. We showed previously that at this time point, the number of SER1 macrophages also reached its maximum. These cells formed tight clusters with CD41 and CD81 donor T cells (7). Ridge et al. (27) and Schoenberger et al. (28) presented a new dynamic model of CTL activation. CD41 helper T cells expressing CD40L engage and “condition” APCs (e.g., DCs) via CD40-CD40L interaction, which leads to the up-regulation of CD40 and other costimulatory and adhesion molecules (9, 11, 17, 27, 29). It has been shown that after immunization with keyhole limpet hemocyanin, few CD40L1 cells are enough to activate antibody-producing B cells (~1 CD40L+ cell per 10–12 B cells; Ref. 30). It is thus likely that the few CD40L+ cells detected after immune cell transfer represent donor helper T cells. These might activate host APCs, which then are able to activate CD8+ T cells to become cyto-

![Fig. 4 Coexpression of CD40 and iNOS in the liver of GvL and GvH animals and colocalization of iNOS+ cells with apoptotic cells. A, iNOS-expressing cells in GvL (■) and GvH (○) and CD40+ iNOS+ double-positive cells in GvL (▲) and GvH (◆) were stained with respective Abs on frozen tissue sections. The means are shown from two representative experiments with two to three animals/time point; bars, SD. Data points without SD represent mean values for which the SD was <1. B, iNOS+ cells (blue) were found in close proximity to apoptotic cells (brown) localized in metastases at day 5 after ADI treatment. The picture at day 20 looks similar. ×200. Pp, portal vein.]
toxic. The observed clusters during ADI, thus, could be sites of T cell-macrophage/DC interactions, which lead to the activation of cytotoxic and helper T cells. Our data on the proliferation of CD4\(^+\) and CD8\(^+\) donor T cells after ADI support this model (7).

It has been shown that cytotoxic CD8\(^+\) T cells can induce apoptosis (31). We have demonstrated recently that the endogenous viral superantigen 7 (Mlsa) encoded by the mouse mammary tumor virus provirus Mtv-7 is expressed in DBA/2 mice and also in the ESb-MP tumor cells thereof derived but not in B10.D2 mice (32). In DBA/2 mice, viral superantigen 7 causes deletion of superantigen-reactive T cells with certain V\(β\) chains (\(e.g., V\β6\)) from their repertoire, whereas in B10.D2 tumor-resistant mice, V\(β6^+\) T cells are present. These particular T cells can infiltrate the liver metastases during ADI (7). We showed that V\(β6^+\) T cells are able to kill superantigen-expressing ESb-MP tumor cells by apoptosis in vitro.\(^4\) It is also likely that T cells with specificities against other tumor antigens contribute to the antitumor response. Apoptosis mediated by CTLs apparently does not involve NO because T cells do not express iNOS.

The CD40-CD40L interaction is not only involved in priming, differentiation, and activation of CD4\(^+\) and CD8\(^+\) T cells but also in the mediation of effector functions of DCs and macrophages such as the production of inflammatory cytokines (\(e.g.,\) TNF-\(α\) and IL-1) or NO (9, 11, 18). Ligation of CD40 on macrophages and DCs leads to secretion of IL-12 and other inflammatory cytokines that are critical for the differentiation of Th1 cells and therefore for the induction of antitumor immunity (29, 33, 34).

NO is known to be produced by activated macrophages, DCs, and endothelial cells in vitro by iNOS (19, 21, 24) and can induce apoptosis in tumor cells including the ESb-MP cell line (21, 22, 24). iNOS was predominantly expressed by SER+ macrophages and to a lower extent by DCs (data not shown). iNOS expression was observed in the liver close to metastases at early time points and at late time points close to portal veins, where the clusters containing CD4\(^+\), CD8\(^+\), and SER+ macrophages and DCs are located (6, 7). The question thus arises

\(^4\) Unpublished data.
whether in this model, NO may function as a cytotoxic and/or as a regulatory molecule.

Different regulatory functions of NO have been described. Marcinkiewicz et al. (21) suggested a positive feedback mechanism in which NO enhances the synthesis of TNF-α and IL-1α. Both cytokines are known to stimulate NO generation and function as cytotoxic and inflammatory molecules. This kind of feedback mechanism could occur at the early time points of the therapy, when metastases should be eradicated from the liver. At day 5 after donor cell transfer, large metastases could still be seen in the liver. In the central areas of the metastases, apoptotic cells could be detected in high numbers (data not shown). Because metastases were completely eradicated at day 12 after ADI and iNOS+ cells were seen before this time point in close association with apoptotic cells in areas of metastases, these findings suggest a cytotoxic role for NO in the eradication of metastases during this early time period of ADI. Additionally, ESB-MP tumor cells could be killed by NO5 in vitro, which supports the latter suggestion.

NO can also act by a negative feedback mechanism, inhibiting the secretion of the Th1 cytokines IFN-γ and IL-2 (35). It is further able to limit Th1 cell activity by supporting down-regulatory IL-4 production and by inhibition of MHC II expression (35, 36). This mechanism may be operative at a later stage of ADI, when local T-cell responses have to be terminated and the liver has to be cleared from undesired immune reactive cells. At these time points, iNOS-expressing cells as well as apoptotic cells were detected around periportal veins, where the clusters containing CD8+ and CD4+ T cells are also located (7). Some of these CD8+ and CD4+ T cells were shown to be apoptotic. Moreover, at this time point, 45% of the apoptotic cells were of donor origin (positive for the donor cell marker β2-microglobulin; data not shown), indicating a role for apoptosis in elimination of donor cells from the liver. After day 20, the numbers of CD8+ and CD4+ liver-infiltrating T cells decreased markedly (7).

Double staining for iNOS and CD40 revealed that at day 20, when the maximum of NO production and CD40 expression was reached, all iNOS-expressing cells were positive for CD40. It has been described that macrophages are able to secrete NO in vitro after interaction with CD4+ T cells or plasma membranes of T cells activated with anti-CD3 antibodies and that this effect is mediated by CD40-CD40L interaction (18, 23). It was also shown for Dcs that ligation of CD40 in vitro induces a significant elevation in NO synthesis (19). Our in situ observations of CD40/iNOS coexpressing cells suggest that the CD40-CD40L interaction is also involved in the induction of iNOS in the situation of GvL reactivity. Furthermore, the in vivo blockade of CD40L during ADI led to a marked inhibition of the therapeutic effect. In the livers of anti-CD40L mAb-treated animals, iNOS and CD40 expressions were reduced significantly. Moreover, in vivo experiments in which iNOS activity was blocked by the inhibitor N-(3-aminoethylbenzyl)acetamidin (1400W) at early and at late time points after ADI treatment also showed a marked reduction of the survival,5 supporting the importance of the induced NO production for the therapeutic effect.

Interestingly, the number of CD4+ and CD8+ T cells in the liver of mice treated with anti-CD40L mAb at days 18 and 20 after ADI was not decreased 22 days after ADI, such as in animals treated with control immunoglobulin, but rather increased (Fig. 5B). The same phenomenon was observed when we suppressed iNOS activity by a specific inhibitor in vivo. Because at these later time points the metastases were already eradicated, the animals that received anti-CD40L mAb injections died not from metastases but more likely from GvH disease. GvH disease can be caused by donor T cells that were not eliminated via HvG reactivity from the liver, which is likely to be the standard physiological ADI situation. The blockage of the NO induction may have led to reduced HvG reactivity so that fewer donor cells were eliminated and that their proliferative expansion was not inhibited. Such expanding T-cell clusters may involve HvG- and GvH-reactive T cells, such as in mixed lymphocyte reactions.

This study demonstrates that at early and at late stages of the therapy, CD40-CD40L interactions are important for the GvL therapy effect of the ADI. The resultant production of NO would contribute to the destruction of liver metastases at early time points and also at a later stage to the removal of the liver-infiltrating T cells.

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5 Unpublished observations.


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