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Agonistic Monoclonal Antibody Against CD40 Receptor Decreases Lymphocyte Apoptosis and Improves Survival in Sepsis

Steven J. Schwulst,* Mitchell H. Grayson,† Peter J. DiPasco,‡ Christopher G. Davis,§ Tejal S. Brahmbhatt,‡ Thomas A. Ferguson,§ and Richard S. Hotchkiss2‡

Sepsis causes a marked apoptosis-induced depletion of lymphocytes. The degree of lymphocyte apoptosis during sepsis strongly correlates with survival. CD40, a member of the TNFR family, is expressed on APCs and has potent antiapoptotic activity. In this study we determined whether an agonistic Ab against CD40 could protect lymphocytes from sepsis-induced apoptosis. Secondly, we examined potential antiapoptotic mechanisms of the putative protection. Lastly, we aimed to determine whether anti-CD40 treatment could improve survival in sepsis. CD1 mice were made septic by the cecal ligation and puncture method and treated postoperatively with anti-CD40 Ab. Treatment with anti-CD40 completely abrogated sepsis-induced splenic B cell death and, surprisingly, decreased splenic and thymic T cell death as well (p < 0.001). To investigate the mechanism of protection of anti-CD40 therapy on T cells, CD40 receptor expression was examined. As anticipated, the CD40 receptor was constitutively expressed on B cells, but, unexpectedly, splenic and thymic T cells were found to express CD40 receptor during sepsis. Furthermore, CD4+CD8− T cells were the predominant subtype of T cells expressing CD40 receptor during sepsis. Additionally, the antiapoptotic protein Bcl-xL was found to be markedly increased in splenic B and T cells as well as in thymic T cells after treatment with anti-CD40 Ab (p < 0.0025). Lastly, mice that were made septic in a double injury model of sepsis had improved survival after treatment with anti-CD40 as compared with controls (p = 0.05). In conclusion, anti-CD40 treatment increases Bcl-xL, provides nearly complete protection against sepsis-induced lymphocyte apoptosis, and improves survival in sepsis. The Journal of Immunology, 2006, 177: 557–565.

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In the present study, we used a widely used, clinically relevant murine model of septic peritonitis as well as an in vitro model of sepsis with human lymphocytes to examine the ability of an agonistic mAb against the CD40 receptor to protect lymphocytes from sepsis-induced apoptosis. We also examined the effect of the CD40 receptor agonist on Bcl-xL, an antiapoptotic member of the Bcl-2 family. Lastly, we aimed to determine whether this therapeutic effort translated into improved outcome in septic animals.

Materials and Methods

**Animal models**

**Apoptosis.** Mice used in apoptosis studies were C57BL/6 male mice 7 wk old weighing 20–25 g purchased from The Jackson Laboratory. Mice were made septic using a “double injury” model of sepsis as developed in our laboratory (J. T. Muenzer, C. G. Davis, B. S. Dunne, J. Unsinger, W. M. Dunne, and R. S. Hotchkiss, submitted for publication). In brief, the CLP technique with a single puncture from a 30-gauge needle was used to initiate sepsis. Mice received 1 ml of 0.9% saline s.c. immediately after the operation. Three hours after the operation, the mice received a single s.c. dose of imipenem at 25 mg/kg. They were then allowed free access to food and water. Animal studies were approved by the Animal Studies Committee of Washington University, St. Louis, MO.

**Survival.** Mice used in survival studies were C57BL/6 male mice 7 wk old weighing 20–25 g purchased from The Charles River Laboratories. The cecal ligation and puncture (CLP) technique as developed by Chaudry et al. (35) and modified by our laboratory was used to induce septic peritonitis. Briefly, mice were anesthetized using 2% halothane with supplemental oxygen. A 1-cm left paramedian incision was made, and the cecum was ligated below the ileocecal valve. The cecum was punctured twice with a 25-gauge needle. Sham mice had cecal manipulation only. Mice were given 1 ml of 0.9% saline s.c. immediately after the operation. At 3 h after the operation, the mice received a single s.c. dose of imipenem at 25 mg/kg. They were then allowed free access to food and water. Animal studies were approved by the Animal Studies Committee of Washington University Animal Studies Committee.

**Ab preparations**

**Mouse.** Purified mAb to mouse CD40 was obtained from a hybridoma clone generously provided by S. Schoenberger (La Jolla Institute of Allergy and Immunology, San Diego, CA) (36). Ab was generated and purified by Harlan Bioproducts for Science. Purity was >93% by PAGE. Endotoxin concentration was <0.1 endotoxin units (EU) as determined by the quantitative kinetic chromogenic Limulus amoebocyte lysate (LAL) method. Ab was reconstituted in sterile PBS to desired concentrations just before injection into mice.

**Human.** Purified mAb to human CD40 was purchased from R&D Systems. Endotoxin concentration was <0.1 EU as determined by the LAL method. Ab was reconstituted in sterile PBS to desired concentrations just before addition to culture medium.

**Controls.** Appropriate human and mouse IgG2B isotype control Ab was purchased from R&D Systems. Endotoxin concentration was <0.1 EU as determined by the LAL method.

**In vivo murine experiments**

**Apoptosis.** Sham mice were treated with a single dose of 200 μl of 0.9% saline given i.p. 3 h after the operation. CLP mice were treated with a single dose of either 150 or 300 μg of monoclonal anti-CD40 or 300 μg of an IgG2B isotype control Ab given in 200 μl of saline injected i.p. 3 h after the operation. Mice were sacrificed between 20 and 22 h after the operation. Splenocytes and thymi were removed for histologic and flow cytometric analysis.

**Survival.** Control mice were treated with a single daily dose of 100 μl of 0.9% saline given i.p. from post-CLP day 1 through day 7. The experimental mice were treated with a single daily dose of 150 μg of monoclonal anti-CD40 in 100 μl of 0.9% saline given i.p. from post-CLP day 1 through day 7. Survival was recorded for 14 days.

**In vitro human experiments**

Fresh whole blood was obtained from healthy human volunteers. Mononuclear cells were isolated via differential migration over Ficoll-Paque Plus. These cells were then treated with monoclonal anti-CD40 in varying doses (0.1, 1, and 10 μg) or with 10 μg of an IgG2B control Ab. Cells were then incubated for 14 h with Escherichia coli (4 × 10^6 CFU) contained within 0.02 μM Anopore microwells. After incubation, mononuclear cells were harvested for flow cytometric analysis. Human studies were approved by the Washington University Institutional Review Board.

Detection and quantification of apoptosis

**Flow cytometry and apoptosis.** Splenocytes and thymocytes were obtained at the time of sacrifice in our in vivo experiments. Human PBMCs were harvested after 14 h of incubation in our in vitro experiments. Apoptosis was quantified using a commercially available PE-labeled TUNEL kit (Phoenix Flow Systems) or by staining for active caspase-3. CD40 and anti-CD40 in 100 μl of an IgG2B isotype control Ab given in 200 μl of 0.9% saline i.p. from post-CLP day 1 through day 7. The experiments were analyzed using fluorescently labeled anti-CD4 and anti-CD8 Abs. Mice were treated with a single daily dose of 150 μg of monoclonal anti-CD40 in 100 μl of 0.9% saline given i.p. from post-CLP day 1 through day 7. Survival was recorded for 14 days.

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**Survival.** Control mice were treated with a single daily dose of 100 μl of 0.9% saline given i.p. from post-CLP day 1 through day 7. The experimental mice were treated with a single daily dose of 150 μg of monoclonal anti-CD40 in 100 μl of 0.9% saline given i.p. from post-CLP day 1 through day 7. Survival was recorded for 14 days.

**5 Abbreviations used in this paper CLP, cecal ligation and puncture; EU, endotoxin unit(s); LAL, Limulus amoebocyte lysate.**
The characteristic forward and side scatter properties of lymphocytes were used to identify the lymphocyte gate as previously described (5). Back-gating of surface-marked lymphocytes (CD3 and b220 on mouse cells and CD3 and CD20 for human cells) was used to confirm the appropriate T or B lymphocyte gate. The percentage of apoptotic cells within the lymphocyte gate was quantitated by both TUNEL and active caspase-3 labeling. The investigator familiar with the histologic appearance of lymphocytes undergoing apoptosis, and the presence or absence of apoptotic cells in each specimen was determined. Stained specimens failed to show a reduction in lymphocyte apoptosis after treatment with the highest dose of monoclonal anti-CD40 Ab or saline. There was no reduction in the degree of sepsis-induced lymphocyte apoptosis after anti-CD40 treatment in CD40 knockout mice (data not shown).

**Mouse Thymus.** CLP caused a profound increase in thymocyte apoptosis, whereas postinjury treatment with monoclonal anti-CD40 resulted in a dose-dependent decrease in sepsis-induced apoptosis as demonstrated by both active caspase-3 labeling (p < 0.05) and TUNEL (p < 0.001; data not shown). Similarly, treatment with the highest dose of monoclonal anti-CD40 Ab resulted in a return to near-baseline levels of apoptosis in the anti-CD40-treated groups.

**Tissue histology of mouse spleen and thymus**

A qualitative survey of H&E-stained slides from spleens and thymi of mice subjected to either CLP or a sham operation at 20–22 h post injury was conducted in a blinded fashion by an experienced investigator familiar with the histologic appearance of lymphocytes undergoing apoptosis, and the presence or absence of apoptotic cells in each specimen was determined. Stained specimens of spleens from CLP mice revealed large numbers of apoptotic lymphocytes with compacted or fragmented nuclei. Sham mice resulted in a return to baseline levels of apoptosis in splenic B cells (Fig. 1A). There was a slightly significant difference between CLP and treatment with the IgG2B isotype control when quantitated with TUNEL, but no statistical difference when quantitated with active caspase-3. This difference highlights the importance of using both a highly sensitive and highly specific method when quantitating apoptosis with flow cytometry. In a similar manner, CLP caused a striking increase in splenic T cell apoptosis, which was ameliorated by postinjury treatment with monoclonal anti-CD40.

**Results**

**Agonistic monoclonal anti-CD40 blocks sepsis-induced lymphocyte apoptosis**

Monoclonal anti-CD40 protects thymocytes from sepsis-induced apoptosis. Isolated splenocytes from sham- or CLP-operated mice (n = 29 for B cell studies and n = 20 for T cell studies) were labeled with fluorescent cell-specific markers for B and T cells (b220 and CD3+, respectively) and for apoptosis (active caspase-3). A, Quantification by active caspase-3 reveals a return to baseline levels of B cell apoptosis in the anti-CD40-treated groups. IgG Cont., IgG2B isotype control Ab. B, Quantification by active caspase-3 also reveals a return to baseline levels of T cell apoptosis in anti-CD40-treated groups.

Quantification by active caspase-3 reveals a return to near-baseline levels of apoptosis in the anti-CD40-treated groups. IgG Cont., IgG2B isotype control Ab. A, Quantification by active caspase-3 reveals a return to baseline levels of B cell apoptosis in the anti-CD40-treated groups. IgG Cont., IgG2B isotype control Ab. B, Quantification by active caspase-3 also reveals a return to baseline levels of T cell apoptosis in anti-CD40-treated groups.

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Quantification by active caspase-3 reveals a return to near-baseline levels of apoptosis in the anti-CD40-treated groups. IgG Cont., IgG2B isotype control Ab. A, Quantification by active caspase-3 reveals a return to baseline levels of B cell apoptosis in the anti-CD40-treated groups. IgG Cont., IgG2B isotype control Ab. B, Quantification by active caspase-3 also reveals a return to baseline levels of T cell apoptosis in anti-CD40-treated groups.
had no change from baseline. Groups treated with anti-CD40 had a reduction in the number of apoptotic lymphocytes to near baseline levels (Fig. 3, A–D). As reported previously, the cortical regions of the thymus are affected more than the medulla (38). In some areas of cortical thymus the normal tissue architecture was completely obliterated, with >50% of thymocytes showing apoptotic changes in the CLP group. Groups treated with anti-CD40 had a clear reduction in the number of apoptotic lymphocytes as compared with specimens from the CLP-only group (Fig. 4, A–D). Groups treated with the IgG2B isotype control Ab showed no visual difference from CLP alone in either the spleen or the thymus (data not shown).

**Sepsis increases CD40 expression on mouse T cells**

Although the prevention of sepsis-induced B cell apoptosis by monoclonal anti-CD40 was expected due to the presence of the CD40 receptor on B cells, the finding of protection of both splenic T cells and thymic T cells by anti-CD40 Ab was serendipitous. Although there are numerous possible mechanisms for the anti-apoptotic effect of CD40 on T cells, one potential mechanism is expression of the CD40 receptor on T cells. In fact, Bourgeois et al. (48) reported that activated human CD8+ T cells transiently express the CD40 receptor after experimental TCR stimulation. To investigate this issue, we evaluated CD40 cell surface expression

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**FIGURE 3.** Qualitative light microscopic evidence of monoclonal anti-CD40-mediated splenocyte protection against sepsis-induced apoptosis (×200 magnification). A, Tissue sections from sham-operated mice show normal follicles with no apoptotic cells. B, Tissue from CLP mice demonstrates apoptotic splenocytes with pyknotic and karyorrhectic nuclei along with prominent lipid vacuolization. C and D, Tissue sections from septic mice treated with monoclonal anti-CD40 Ab demonstrate a dose-dependent decrease in apoptotic splenocytes.

**FIGURE 4.** Qualitative light microscopic evidence of monoclonal anti-CD40-mediated thymocyte protection against sepsis-induced apoptosis. A, Tissue sections from sham-operated mice show normal thymic architecture with minimal apoptotic cells (×200 magnification). B, Tissue from CLP mice demonstrates substantial thymocyte apoptosis as well as obliteration of normal thymic architecture (×400 magnification). C and D, Tissue sections from septic mice treated with monoclonal anti-CD40 Ab demonstrate a dose-dependent decrease in apoptosis as well as a return of normal thymic architecture (×200 magnification).
in splenic and thymic T cells in both sham and septic mice. The characteristic forward and side scatter properties of lymphocytes were once again used to identify the lymphocyte gate as previously described (5). An initial screen of splenocytes and thymocytes expressing the CD40 receptor was performed using labeled anti-CD3 and anti-CD40 Abs. CD3<sup>+</sup> splenocytes from sham mice demonstrated 6.5 ± 2.7% CD40 expression vs 13.0 ± 3.4% CD40 expression in septic CLP animals (n = 10; p < 0.015). Further examination of this population revealed that the majority of CD3<sup>+</sup>CD40<sup>+</sup> splenocytes, 59.0 ± 9.0%, were CD4<sup>+</sup> T cells, whereas only 15.2 ± 1.3% were CD8<sup>+</sup> T cells (Fig. 5).

In thymocytes we found two distinct populations of CD3-expressing cells: one population with low CD3 expression and one with high CD3 expression (Fig. 6). Furthermore, only the CD3-low population was found to express CD40 after the induction of sepsis: 3.7 ± 0.5% in sham animals vs 12.9 ± 2.0% in septic CLP animals (n = 19; p < 0.006). To further evaluate the exact subtype composition of these two populations, we gated each CD3 expressing population into CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>+</sup>CD8<sup>-</sup>, CD4<sup>-</sup>CD8<sup>+</sup>, and CD4<sup>-</sup>CD8<sup>-</sup> subpopulations using four-color flow cytometry (Fig. 6). Interestingly, we found that the population of CD3-low-expressing T cells was composed primarily of CD4<sup>+</sup>CD8<sup>-</sup> and CD4<sup>-</sup>CD8<sup>+</sup> subpopulations (80.8 ± 15.1%), whereas the CD3-high-expressing population was composed primarily of mature CD4<sup>-</sup>CD8<sup>-</sup> and CD4<sup>+</sup>CD8<sup>-</sup> subpopulations (76.9 ± 2.7%). To more fully evaluate the thymocytes expressing the CD40 receptor during sepsis, we gated specifically on the CD40<sup>+</sup>CD3-low-expressing population of T cells from septic animals. As we found in splenic T cells, the predominant subpopulation of thymic T cells from septic mice expressing CD40 receptor were CD4<sup>+</sup>CD8<sup>-</sup> (50.0 ± 7.9%; Fig. 5C).

**FIGURE 5.** CD40 receptor expression is up-regulated on splenic T cells during sepsis. Isolated splenocytes from sham- or CLP-operated mice (n = 10) were labeled with fluorescent anti-CD3, anti-CD4, anti-CD8, and anti-CD40 Ab. A, T cells from nonseptic sham-operated mice did not express the CD40 receptor. B, The CD40 receptor was expressed on splenic T cells from septic CLP-operated mice (p < 0.015). C, CD3<sup>+</sup>CD40<sup>+</sup> T cells from septic animals were gated into CD4<sup>+</sup> and CD8<sup>+</sup> subpopulations. Analysis revealed that the predominant subpopulation of splenic T cells expressing the CD40 receptor after the induction of sepsis was CD4<sup>+</sup> (59.0 ± 9.0%). APC, allophycocyanin.

**FIGURE 6.** CD40 receptor expression on CD4<sup>+</sup> thymic T cells increases during sepsis. Isolated thymocytes from sham- or CLP-operated mice (n = 19) were labeled with fluorescent anti-CD3, anti-CD4, anti-CD8, and anti-CD40 Abs. Analysis revealed the CD3<sup>+</sup>-high population to be predominately CD4<sup>+</sup> and CD8<sup>-</sup>. The CD3<sup>+</sup>-low population was predominately immature CD4<sup>-</sup>CD8<sup>-</sup> and CD4<sup>+</sup>CD8<sup>-</sup>. A, Nonseptic sham-operated mice did not express the CD40 receptor on thymic T cells. B, The CD40 receptor was expressed on T cells from CLP mice (p < 0.006). C, CD3<sup>+</sup>CD40<sup>+</sup> T cells from septic animals were gated into CD4<sup>+</sup>, CD8<sup>-</sup>, CD4<sup>-</sup>CD8<sup>-</sup>, and CD4<sup>-</sup>CD8<sup>+</sup> subpopulations. Analysis revealed that the predominant subpopulation of T cells expressing the CD40 receptor after the induction of sepsis was CD4<sup>+</sup> (50.0 ± 7.9%). APC, allophycocyanin.

Agonistic monoclonal anti-CD40 increases Bcl-x<sub>L</sub> expression in mouse lymphocytes

There have been many mechanisms proposed for the CD40-mediated reduction in lymphocyte apoptosis. One of the most thoroughly investigated mechanisms is the CD40-mediated up-regulation of genes from the antiapoptotic Bcl-2 family (25). However, this has only been investigated in vitro. To examine this question further, we evaluated the relative expression of Bcl-x<sub>L</sub> in naive mice treated with saline vs mice treated with a monoclonal anti-CD40 Ab (n = 10). Flow cytometric analysis revealed that the relative expression of Bcl-x<sub>L</sub> was significantly increased in splenic B cells (23.4 ± 0.9% vs 40.6 ± 3.3%, p = 0.0025) and T cells (13.7 ± 1.8% vs 27.2 ± 1.4%, p = 0.0003). Thymic T cells also revealed a marked increase in Bcl-x<sub>L</sub> expression (7.3 ± 3.2% vs 42.2 ± 4.0%, p = 0.0005) 20 h after injection with monoclonal anti-CD40 (Fig. 7).

**FIGURE 7.** Bclx<sub>L</sub> expression increases after CD40 induction.

**FIGURE 8.** Apoptosis improves survival in sepsis.

Agonistic monoclonal anti-CD40 protects against sepsis-induced apoptosis in human PBLs

After confirming that monoclonal anti-CD40 Ab protects mouse lymphocytes from sepsis-induced apoptosis, we investigated the effects of a CD40 agonist on human lymphocytes. To examine whether human monoclonal anti-CD40 Ab protects against sepsis-induced apoptosis, we used an in vitro system in which human PBMCs were examined for apoptosis using TUNEL labeling. TUNEL labeling for *E. coli*-induced apoptosis in human peripheral blood demonstrated near-complete protection in both peripheral B-cells (p < 0.01) and T cells (p < 0.05) (Fig. 8).

Agonistic monoclonal anti-CD40 improves survival in sepsis

Mice treated with agonistic monoclonal anti-CD40 had a survival advantage in a “double injury” model of sepsis compared with
mice treated with saline only (p ≤ 0.05). Survival between the two groups was not different after CLP (the “first injury”). However, when analyzed after the pneumonia challenge (the “second injury”), survival was significantly different. After 14 days, survival was 36% in the Ab-treated group vs 15% in the saline-treated group (p = 0.0005; Fig. 9). Traditional “single hit” models of CLP-induced sepsis were also performed. These studies showed no difference in survival between the Ab-treated group and the saline-treated group (data not shown).

Discussion

Sepsis causes a striking apoptosis-induced depletion of T and B lymphocytes (49, 50). The resulting lymphopenia leads to an inability of the host organism to combat the ongoing source of infection and predisposes the host to secondary opportunistic or nosocomial infections, thereby worsening mortality. The first major finding of the present study was the remarkable ability of the monoclonal anti-CD40 receptor Ab to block this sepsis-induced lymphocyte apoptosis. At the highest concentration, the anti-CD40 receptor Ab almost completely protected splenic B cells from death and provided significant protection in both splenic and thymic T cells as well. Multiple independent investigative groups have shown that the prevention of lymphocyte apoptosis improves survival in sepsis (51–53). Mice that overexpress the antiapoptotic protein Bcl-2 in their lymphocytes have a survival advantage in sepsis as compared with controls (31, 51). Similarly, there was an overall survival benefit when lymphocytes overexpressing Bcl-2 were adoptively transferred to septic animals. Additionally, knockout mice for the Fas death ligand have improved survival during sepsis as do mice treated with anti-caspase compounds (52, 53). The data contained herein are the first to describe the remarkably potent protective effects of an agonistic monoclonal anti-CD40 receptor Ab within the well established and clinically relevant CLP model of sepsis. The studies discussed above, along with data from the present study, provide persuasive evidence that compounds that block lymphocyte apoptosis, such as an agonistic monoclonal anti-CD40 Ab, may have a promising future in the treatment of sepsis-induced lymphopenia.

An unusual aspect of the antiapoptotic effect of the agonistic CD40 Ab was its ability to protect T cells. This protective effect is most dramatically seen in the thymic histologic sections that show a major sparing of cortical and medullary T cells (Fig. 4). Given the fact that T cells are not generally considered to express CD40 receptor but rather CD40L, we were surprised at the degree of T cell protection. To investigate potential mechanisms for the protective effect of the anti-CD40 receptor Ab on T cells, we examined CD40 receptor expression in splenic and thymic (Figs. 5 and 6). During this investigation we found that there was minimal CD40 receptor expression on splenic and thymic T cells from sham-operated mice (Figs. 5A and 6A). However, CD40 receptor expression increased significantly in both splenic and thymic T cells of septic CLP mice (Figs. 5B and 6B). Furthermore, it was
determined that the predominant subtype of T cells expressing CD40 after the induction of sepsis were CD4+CD8− T cells in both the spleen and the thymus (Fig. 5C and 6C). The present results showing increased expression of CD40 receptor on CD4+CD8− T cells are complimentary to the work by Bourgeois et al. (48), who demonstrated that mature peripheral blood CD4+CD8− T cells are capable of transiently expressing CD40 receptor after experimentally providing a TCR activation signal. Although we speculate that additional mechanisms such as a “by-stander” effect may also be contributing to the antiapoptotic effect of CD40 receptor agonist (15, 16, 36), it is likely that a major mechanism of T cell protection is up-regulation of the CD40 receptor on T cells.

An additional major aim of the present study was to determine potential mechanisms for the antiapoptotic action of the anti-CD40 receptor agonist. Previous work by Wang et al. (25) has shown that CD40 receptor activation in vitro induces significant expression of Bcl-xL, a potent antiapoptotic member of the Bcl-2 family. Work from our group has established that transgenic mice who overexpress Bcl-xL in T cells are resistant to sepsis-induced apoptosis and have improved survival (32). In the current study we demonstrated that administration of the CD40 receptor agonist causes a significant increase in Bcl-xL expression in both B and T lymphocytes (Fig. 7). In addition to up-regulating Bcl-xL, CD40 receptor stimulation induces multiple other signaling pathways, including NF-κB (54). In septic settings, NF-κB has potent antiapoptotic properties and, therefore, the induction of NF-κB is another potential mechanism for the protective effect of the CD40 receptor agonist (55, 56).

In addition to protecting mouse lymphocytes from sepsis-induced apoptosis, the ability of human monoclonal anti-CD40 to protect human lymphocytes from E. coli-induced apoptosis (Fig. 8) has significant implications. It has been demonstrated by Sugimoto et al. (8) that CD40 is up-regulated on peripheral blood mononuclear cells during sepsis. Furthermore, it was found that the level of CD40 expression strongly correlated with survival. Our data demonstrate that treatment of isolated E. coli-induced lymphocytes with the monoclonal anti-CD40 Ab has profound antiapoptotic effects. Taken together, these findings provide compelling evidence for continued investigation on the potential benefits of CD40-targeted therapy in septic patients. In fact, phase I clinical trials of recombinant human CD40L in cancer patients have already been concluded and demonstrate that the compound has a safe therapeutic window, warranting continuation into phase II trials (57).

The major limitation of this study is the conflicting survival data within various models of sepsis after CD40-targeted therapy. Some studies have demonstrated a survival advantage in infected animals after treatment with recombinant CD40L; however, these studies have used atypical or indolent models of infection, i.e., S. dublin, Pneumocystis carinii, and Trypanosoma cruzi (26–28). Other investigators have reported conflicting results. Gold et al. (58) reported decreased mortality in CD40−/− mice after CLP. These results suggest that CD40 stimulation actually contributes to mortality in CLP-induced sepsis. A possible explanation for the contrasting results regarding CD40 in sepsis may be the differences in severity of infection. In the study of Gold et al. (58), the sepsis model used was overwhelmingly lethal (control mice had 100% mortality in <36 h, suggesting that the animals died of fulminate septic shock). CD40−/− mice in this study would be anticipated to have longer survival than controls, given their inability to mount the deadly “cytokine storm” seen in fulminate septic shock. Most deaths in patients with sepsis occur after 72 h at a time point when the hyperinflammatory phase has evolved into a hypoinflammatory state (59). We hypothesized that the immunostimulatory and lymphoprotective effects of CD40-targeted therapy will prevent the immunosuppression seen during the hypoinflammatory phase of sepsis and prevent secondary nosocomial infections, thus improving survival. To test this hypothesis, we developed a “double injury” model of sepsis (S. J. Schwulst, J. T. Muenzer, C. G. Davis, B. S. Dunne, J. Unsinger, W. M. Dunne, and R. S. Hotchkiss, submitted for publication) whereby mice are made septic by an initial CLP injury. The mice are then allowed 5 days to develop the hypoinflammatory phase of sepsis, at which point they are challenged with an intranasal injection of pneumonia-inducing bacteria. We found that mice treated with anti-CD40 after the initial CLP insult had significantly improved survival as compared with

![FIGURE 8. Monoclonal anti-CD40 receptor Ab protects human PBLs from E. coli-induced apoptosis. A. Quantification of T cell apoptosis by TUNEL also revealed a return to near-baseline levels of apoptosis in all treatment groups.](image-url)
saline-treated controls after the pneumonia challenge (p < 0.05; Fig. 9).

In summary, an agonistic monoclonal anti-CD40 Ab protected lymphocytes from sepsis-induced apoptosis and provided a survival advantage in a "double injury" model of sepsis. This protective effect was seen in both murine and human models of sepsis in vivo and in vitro, respectively. Additionally, anti-CD40 treatment was found to up-regulate the expression of the antiapoptotic protein Bcl-xL in both B cells and T cells. Furthermore, it was observed that sepsis induced the expression of CD40 receptor on T cells, offering insight into the unexpected finding of CD40-mediated T cell protection. These results show that CD40-targeted therapy may be an effective treatment in sepsis-induced immune dysfunction.

Disclosures
The authors have no financial conflict of interest.

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