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The Role of the CD134-CD134 Ligand Costimulatory Pathway in Alloimmune Responses In Vivo

Xueli Yuan,* Alan D. Salama,* Victor Dong,* Isabela Schmitt,* Nader Najafian,* Anil Chandraker,* Hisaya Akiba,† Hideo Yagit,† and Mohamed H. Sayegh2*

The CD134-CD134 ligand (CD134L) costimulatory pathway has been shown to be critical for both T and B cell activation; however, its role in regulating the alloimmune response remains unexplored. Furthermore, its interactions with other costimulatory pathways and immunosuppressive agents are unclear. We investigated the effect of CD134-CD134L pathway blockade on allograft rejection in fully MHC-mismatched rat cardiac and skin transplantation models. CD134L blockade alone did not prolong graft survival compared with that of untreated recipients, and in combination with donor-specific transfusion, cyclosporine, or rapamycin, was less effective than B7 blockade in prolonging allograft survival. However, in combination with B7 blockade, long-term allograft survival was achieved in all recipients (>200 days). Moreover, this was synergistic in reducing the frequency of IFN-γ-producing alloreactive lymphocytes and inhibiting the generation of activated effector lymphocytes. Most impressively, this combination prevented rejection in a presensitized model using adoptive transfer of primed lymphocytes into athymic heart transplant recipients. In comparison to untreated recipients (mean survival time (MST): 5.3 ± 0.5 days), anti-CD134L mAb alone modestly prolonged allograft survival (MST: 14 ± 2.8 days) as did CTLA4Ig (MST: 21.5 ± 1.7 days), but all grafts were rejected within 24 days. Importantly, combined blockade further and significantly prolonged allograft survival (MST: 75.3 ± 12.7 days) and prevented the expansion and/or persistence of primed effector alloreactive T cells. Our data suggest that CD134-CD134L is a critical pathway in alloimmune responses, especially recall/primed responses, and is synergistic with CD28-B7 in mediating T cell effector responses during allograft rejection. Understanding the mechanisms of collaboration between these different pathways is important for the development of novel strategies to promote long-term allograft survival.


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3 Abbreviations used in this paper: DST, donor-specific transfusion; CD134L, CD134 ligand; MST, mean survival time; hCTLA4Ig, human CTLA4Ig; CsA, cyclosporine.
Materials and Methods

Animals

Eight- to 10-wk-old inbred male Wistar-Furth (WF, RT1<sup>a</sup>) or ACI (RT1<sup>c</sup>) rats were used as heart or skin allograft donors and Lewis (LEW, RT1<sup>b</sup>) animals as recipients (all from Harlan Sprague-Dawley, Indianapolis, IN). Athymic LEW/mol-rnu rats were purchased from M&B (Ry, Denmark) and bred in our facility. All animals were housed and cared for under National Institutes of Health guidelines.

Cardiac transplantation

Heterotopic vascularized cardiac transplants were performed by standard microvascular techniques (39, 40). Donor hearts were engrafted into the recipient’s abdomen using an end-to-side anastomosis of the donor ascending aorta and pulmonary artery with the recipient’s abdominal aorta and vena cava. Transplantation tolerance was confirmed by engrafting long-term transplant survivors with a second original donor or third-party donor heart. Graft function was assessed by daily palpation, and rejection was confirmed at autopsy.

Skin transplantation

Full thickness skin grafts were prepared from the lateral thoracic skin of the donor, cut into 2.5-cm circular pieces, and then maintained at 4°C until transplantation. Donor skin was engrafted onto the recipient’s lumbar region and checked daily for signs of rejection (39, 40).

ELISPOT

Immunospot plates (Cellular Technology, Cleveland, OH) were coated with purified anti-rat IFN-γ Ab (BD PharMingen, San Diego, CA) and stored at 4°C overnight. The next day, they were washed three times with PBS and blocked for at least 2 h with PBS containing 1% BSA. After further washing with PBS, irradiated WF spleen cells (5 × 10<sup>7</sup>/well) and splenocytes (5 × 10<sup>5</sup>/well) from naive LEW rats, or those of LEW recipients of skin transplants (7, 14, or 30 days posttransplantation), which received treatments with or without anti-CD134L Ab, CTLA4Ig, or a combination of both, were added to some wells, each in 100 μl of complete RPMI 1640 medium containing 10% FCS (Sigma-Aldrich, St. Louis, MO), 2 mM l-glutamine, 100 U/ml penicillin/streptomycin (BioWhittaker, Walkersville, MD), and 50 mM 2-ME (Sigma-Aldrich). The resulting spots were counted on a computer-assisted ELISAPort Image Analyser (Cellular Technology). The results were then calculated as cytokine-producing cells per half million splenocytes.

Flow cytometry

Splenocytes were isolated 14 or 30 days following skin transplantation from animals treated with human CTLA4Ig (hCTLA4Ig) alone, anti-CD134L alone, or combined therapy, or from untreated allograft controls (three animals in each group), and were stained with anti-CD3-PE and anti-CD44-FITC mAbs (BD PharMingen). Cells were analyzed on a FACS-Calibur (BD Biosciences, San Jose, CA) using CellQuest software (BD Biosciences). The percentages of splenocytes expressing a CD3<sup>+</sup>CD44<sup>high</sup> phenotype were then calculated. The expansion and survival of T cells in the peripheral blood and spleen of the athymic heart recipients, adoptively transferred with sensitized splenocytes and treated with the different regimens, was examined by staining for T cells using an anti-CD3-PE mAb 19 days after transplantation and adoptive transfer.

Sensitization and adoptive transfer of sensitized spleen cells

Fourteen days following skin transplantation of a WF graft onto a LEW animal, the recipients were sacrificed. Spleens were removed, and single-cell suspensions were obtained. RBC were removed by incubating with ACK lysing buffer (BioWhittaker) for 10 min at room temperature. After several washes, cells were counted and resuspended in PBS. Immediately following transplantation of a WF heart into an athymic nude rat of LEW background, 4 × 10<sup>7</sup> of the sensitized splenocytes were adoptively transferred by i.v. injection into the nude rats.

Abs and immunosuppressive agents

Hybridoma cell lines producing mAbs directed against rat CD134L (ATM-2) (38), rat B7-1 (3H5), and B7-2 (2A4) (41) were generous gifts from Dr. H. Yagita (Juntendo University, Tokyo, Japan) and were produced by Bioexpress (West Lebanon, NH). hCTLA4Ig was a kind gift from Dr. R. Peach (Bristol-Myers Squibb, Princeton, NJ). Anti-CD134L Ab (1 mg) was given by injection (i.p.) on the day of transplantation and on days 2, 4, 6, 8, 10, and 12 following transplantation. Anti-rat B7-1 or B7-1 mAb (0.5 mg), or hCTLA4Ig (0.5 mg) were administered as a single i.p dose on day 2, in cardiac transplant recipients, or as multiple doses (0.5 mg each) on days 0, 2, 4, and 6 in the skin transplant recipients and in athymic nude rats. Cyclosporine (CSA; 10 mg/kg/day; Bedford Laboratories, Bedford, OH) and rapamycin (0.3 mg/kg/day; Wyeth-Ayerst, Madison, NJ) were given for 4 consecutive days following transplantation.

Statistics

The Kaplan-Meier test was used to calculate the graft survival and the log-rank (Mantel-Cox) test was applied to compare differences in survival between groups. A value of p < 0.05 was considered significant.

Results

The effect of CD134-CD134L and CD28-B7 blockade on cardiac allograft rejection

First, we compared the effects of B7 vs CD134L blockade on vascularized cardiac allograft rejection. In the WF into LEW rat transplant model, untreated control cardiac allografts were all rejected promptly, with a mean survival time (MST) of 8.6 ± 1.2 days (n = 8). Following treatment with a single injection of hCTLA4Ig on posttransplant day 2, allograft survival was significantly prolonged (MST: 35.7 ± 13.1 days; n = 6, p < 0.001) (Fig. 1), while the administration of anti-CD134L mAb alone had no significant effect on allograft survival (MST: 9.3 ± 3.5 days; n = 6, p = NS compared with allograft controls) (Fig. 1).

As we have previously described (39), administration of DST on the day of transplant followed by a single injection of hCTLA4Ig on day 2 resulted in all of the allografts achieving long-term survival (MST > 200, n = 6, p < 0.001 compared with untreated controls and hCTLA4Ig alone). However, DST showed no significant additional effect on graft survival when combined with anti-CD134L Ab (MST: 15.5 ± 1.732 days; n = 4, p = NS) (Fig. 1).

A short course of CsA (10 mg/kg i.p. on days 0, 1, 2, and 3) resulted in some prolongation of allograft survival but all grafts were ultimately rejected (MST: 20.5 ± 6.5 days; n = 6, p = 0.001 compared with CsA alone) (Fig. 1).
Synergy between CD134-CD134L and CD28-B7 blockade

Although CD134-CD134L blockade alone had no effect on allograft survival, the combination of anti-CD134L mAb and hCTLA4Ig demonstrated significant synergy (Fig. 3A) with indefinite survival being achieved in all animals (MST > 200 days; n = 8, p < 0.001 when compared with hCTLA4Ig alone or anti-CD134L alone). Moreover, donor-specific tolerance was demonstrated by the acceptance of a second WF heart graft (>100 days) and rejection of a third-party ACI strain heart within 10 days (Table I). To further elucidate the mechanism of this synergy between hCTLA4Ig and anti-CD134L, blocking anti-B7-1 and -B7-2 mAbs were used. Anti-B7-1 and anti-B7-2 mAb alone or in combination (0.5 mg for each, i.p. on posttransplantation day 2) modestly prolonged allograft survival (MST: 13.5 ± 1.3 days, n = 4; 15.4 ± 1.8, n = 5; and 18 ± 1.8, n = 4, respectively). However, while the addition of anti-CD134L to either anti-B7-1 (MST: 13.3 ± 3.6 days, n = 7) or anti-B7-2 (23.1 ± 13.6 days, n = 7) produced no further prolongation of allograft survival (p = NS when compared with isolated anti-B7-1 or -B7-2 mAb treatment, respectively), co-administration of anti-CD134L with both anti-B7-1 and -B7-2 mAbs resulted in long-term allograft survival in all recipients (MST > 200 days; n = 6, p < 0.001 compared with the combination of CD134L mAb with either anti-B7-1 or anti-B7-2 alone or with anti-B7-1 and anti-B7-2 combined) (Fig. 3B). Therefore, blockade of both B7-1 and B7-2 is necessary to achieve long-term allograft survival with anti-CD134L mAb.

In vivo blockade of CD134-CD134L and CD28-B7 diminishes the frequency of alloreactive IFN-γ-producing lymphocytes and prevents the development of activated cells

Using ELISPOT analysis, the frequency of alloreactive IFN-γ-producing lymphocytes from skin transplant recipients, treated with or without anti-CD134L mAb, hCTLA4Ig, or their combination, was measured 7, 14, and 30 days after transplantation. On day 7, the allograft control group had an elevated frequency of alloreactive IFN-γ-producing lymphocytes compared with that of naive control animals (Fig. 4). Treatment with anti-CD134L mAb alone diminished the IFN-γ frequency by 21%, but a greater reduction was achieved with hCTLA4Ig and combined hCTLA4Ig and anti-CD134L mAb (74.3 and 64.2% reduction, respectively). However, by day 14, when animals treated with hCTLA4Ig were rejecting their grafts, the frequency of alloreactive IFN-γ-producing cells in this group increased significantly, while the combined treatment group of hCTLA4Ig and anti-CD134L mAb maintained low alloreactive frequencies consistent with the frequencies on day 7. By day 30, a time when most grafts treated with combined blockade are in the process of rejection, the alloreactive T cell frequency in untreated recipients was unchanged, while the frequency in the combined treatment group had increased, compared with day 14 (Fig. 4). Furthermore, 14 days posttransplantation, the combined treatment group demonstrated the greatest reduction of activated effector T cells, as judged by a CD3+CD44high phenotype (43% reduction) (Fig. 5), while single therapy with hCTLA4Ig (22%
with hCTLA4Ig, 55.6/H11006 6; hCTLA4Ig, 13.5/H11006 controls, 8.2 graft rejection, while either agent alone had minimal effect (MST: allograft fi
plus B7-2 mAb, 23.1/H11005 3.6 days, n = 7; anti-CD134L mAb plus B7-2 mAb, 23.1 ± 13.6 days, n = 7; and anti-CD134L mAb plus B7-1 mAb plus B7-2 mAb, >200 days, n = 6). C. Skin model. Only the combi-
was not sufficient to achieve long-term allograft survival, combined anti-B7-1 and -B7-2 mAb with anti-CD134L did (MST: allograft controls, 8.6 ± 1.2 days, n = 8; hCTLA4Ig, 35.7 ± 13.1 days, n = 6; anti-CD134L mAb, 9.3 ± 3.5 days, n = 6; hCTLA4Ig plus anti-CD134L mAb, >200 days, n = 8; B7-1 mAb, 13.5 ± 1.3 days, n = 4; B7-2 mAb, 15.4 ± 1.8 days, n = 5; B7-1 mAb plus B7-2 mAb, 18 ± 1.8 days, n = 4; anti-
were obtained from LEW rats presensitized with WF skin
reduction) or anti-CD134L (26% reduction) resulted in more mod-
est decreases in the percentage of effector CD44high cells. More-
reduction) or anti-CD134L (26% reduction) resulted in more mod-
erance.

Effect of CD134-CD134L and CD28-B7 blockade on primed alloreactive lymphocytes

LEW background athymic nude rats, lacking T cells, failed to re-
ject MHC-mismatched WF cardiac grafts (MST > 100 days, n =
5). Upon adoptive transfer of $4 \times 10^7$ lymphocytes from naive LEW rats, allografts were rejected in 12–23 days after transplantation (MST: 17 ± 4.6 days; n = 4). If the adoptively transferred cells were obtained from LEW rats presensitized with WF skin grafts, rejection was significantly accelerated (MST: 5.3 ± 0.5 days; n = 4, p < 0.0058 compared with naive lymphocyte trans-
fused recipients) (Fig. 6). We used this model to examine the role of B7 and CD134L on transplant rejection mediated by primed/
effector T lymphocytes. Both anti-CD134L mAb and hCTLA4Ig when administered alone delayed the allograft rejection (MST: 14 ± 2.8 days, n = 4, p = 0.009; and 21.5 ± 1.7 days, n = 4, p < 0.001, for anti-CD134L mAb and hCTLA4Ig, respectively, com-
pared with the untreated control group) mediated by sensitized

Discussion

CD134 and CD134L are members of the TNF and TNFR super-
families, respectively, and have been recently recognized as ef-
cient T cell costimulatory molecules (25, 42, 43). CD134 is ex-
pressed on activated T cells, primarily on CD4+ cells, although
CD8+ T cell expression is also found but to a lesser degree (18, 44). CD134L has also been found on activated B cells (22, 23),
dendritic cells (22), and vascular endothelial cells (24), and has
been shown to be up-regulated following the engagement of the
TCRs with MHC-peptide complexes. The expression peaks 24–48 h
after T cell engagement with Ag and is down-regulated by 72–96 h (25). CD134L-transfected APC have little ability to induce either
IL-2 secretion or the proliferation of naive T cells; however, en-
hanced proliferation is observed when B7 is coexpressed on the
APC, and thereafter proliferation persists for several days (25). Thus, signaling through this pathway promotes T cell survival and
helps determine the development of CD4+ T cell memory by reg-
ulating primary clonal expansion (26). Furthermore, signaling
through the CD134-CD134L pathway has been shown to reverse
peripheral tolerance (45). CD134+ T cells have also been associ-
ated with various autoimmune diseases (30, 32–34), and targeting
of the CD134-CD134L pathway is believed to hold therapeutic
potential in the treatment of autoimmunity and possibly in enhanc-
vaccine efficacy (46).

Although numerous in vitro studies have shown that CD134-
CD134L interaction plays an important role in T cell activation to
alloantigens (25, 47, 48), there is no clear evidence of its effect on
in vivo alloimmune responses. Using our allogenic transplantation

Table I. Survival of second allografts from original or unrelated third-party donors in long-term survived recipients

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<tr>
<th>Second Donor</th>
<th>Allograft Survival (Days)</th>
<th>Number of Animals</th>
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<tr>
<td>WF (RT1u)</td>
<td>&gt;100</td>
<td>3</td>
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<tr>
<td>ACI (RT1u)</td>
<td>10</td>
<td>2</td>
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FIGURE 3. Synergy between CD28-B7 and CD134-CD134L blockades in prolongation of allograft survival and induction of transplantation tolerance. A and B, Heart model. All of the allografts treated with combined hCTLA4Ig and anti-CD134L achieved long-term survival. Although selective blockade of either B7-1 or B7-2 in combination with anti-CD134L was not sufficient to achieve long-term allograft survival, combined anti-B7-1 and -B7-2 mAb with anti-CD134L did (MST: allograft controls, 8.6 ± 1.2 days, n = 8; hCTLA4Ig, 35.7 ± 13.1 days, n = 6; anti-CD134L mAb, 9.3 ± 3.5 days, n = 6; hCTLA4Ig plus anti-CD134L mAb, >200 days, n = 8; B7-1 mAb, 13.5 ± 1.3 days, n = 4; B7-2 mAb, 15.4 ± 1.8 days, n = 5; B7-1 mAb plus B7-2 mAb, 18 ± 1.8 days, n = 4; anti-CD134L mAb plus B7-1 mAb, 13.3 ± 3.6 days, n = 7; anti-CD134L mAb plus B7-2 mAb, 23.1 ± 13.6 days, n = 7; and anti-CD134L mAb plus B7-1 mAb plus B7-2 mAb, >200 days, n = 6). C, Skin model. Only the combi-

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models, we found that, in both heart and skin transplants, CD134-CD134L blockade alone had no significant effect on allograft rejection, and its combination with DST, CsA, or rapamycin was not as efficacious as CD28-B7 blockade in prolonging allograft survival. However, the combination of anti-CD134L mAb and hCTLA4Ig (or both anti-B7-1 and anti-B7-2) induced donor-specific tolerance in all cardiac allograft recipients, as evidenced by long-term graft survival, acceptance of second donor WF hearts, and rejection of third-party ACI allografts. Moreover, it significantly prolonged survival in a more stringent model using fully allogenic skin grafts. Following in vivo blockade of both CD134-CD134L and CD28-B7 pathways, the frequency of allogeneic T cells was markedly reduced and was maintained at low levels. Furthermore, the percentage of T cells expressing an activated/effector phenotype was also maximally inhibited by combined CD134-CD134L and CD28-B7 blockade. Interestingly, the decrease in activated phenotype persisted, at a time when the frequency of IFN-γ-producing alloreactive lymphocytes began to recover to control values. Taken together, these data demonstrate that the effect of combined blockade is on the development and persistence of effector/memory T cells.

Furthermore, unlike other therapies, this combination was able to suppress transplant rejection mediated by primed/effector T cells from presensitized recipients. The extent of graft survival prolongation achieved has never been reported using other sensitized models. Onodera et al. (49), using a LBNF1 (LEW × BN F1 hybrid) donor heart transplanted into a LEW recipients presensitized with Brown-Norway skin grafts a week before, demonstrated that grafts were rejected within 36 h. Treatment with CTLA4Ig in the sensitization phase resulted in a cardiac graft survival of 4.4 ± 1.3 days, while treatment in both sensitization and effector phases prolonged survival to only 10.5 ± 8.5 days. Even the highly efficacious combination of anti-CD40L Ab and DST, which can induce long-term survival of allografts in fully MHC-mismatched mouse heart transplant models, failed to prevent cardiac graft rejection in a presensitized model (50). The demonstration of very low percentages of recovered CD3+ T cells from reconstituted nude recipients treated with the combination of anti-CD134 mAb and hCTLA4Ig, as compared with untreated controls and animals treated anti-CD134L mAb or hCTLA4Ig alone, suggests that only the combined therapy effectively inhibits T cell expansion and/or decreases cell survival. These findings are consistent with the known functions of CD28-B7 (51) and CD134-CD134L (25, 26) in promoting expansion and survival of Ag-specific T cells in vivo. Specifically, the CD134-CD134L pathway appears to be crucial for regulating the extent of CD4+ T cell expansion in the primary response.

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immune response, and thus the ability of T cells to persist as a population over time (26). CD134-deficient T cells secrete IL-2 and proliferate normally during the initial period of activation, but cannot sustain this during the latter phases of the primary response and exhibit decreased survival over time. Mice lacking CD134 generated lower frequencies of Ag-specific CD4+ T cells late in the primary response in vivo and generated lower frequencies of surviving memory cells as compared with WT mice (26).

This study represents the first demonstrating the role of the CD134-CD134L pathway in transplant rejection, and defines the interactions between CD134-CD134L and CD28-B7 pathways in naive and primed/effector allosensitization in vivo. Moreover, it indicates that combined blockade of CD134L and B7 is over, it indicates that combined blockade of CD134L and B7 is

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