CCL19-IgG Prevents Allograft Rejection by Impairment of Immune Cell Trafficking

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An adaptive immune response is initiated in the T cell area of secondary lymphoid organs, where antigen-presenting dendritic cells may induce proliferation and differentiation in co-localized T cells after T cell receptor engagement. The chemokines CCL19 and CCL21 and their receptor CCR7 are essential in establishing dendritic cell and T cell recruitment and co-localization within this unique microenvironment. It is shown that systemic application of a fusion protein that consists of CCL19 fused to the Fc part of human IgG1 induces effects similar to the phenotype of CCR7−/− animals, like disturbed accumulation of T cells and dendritic cells in secondary lymphoid organs. CCL19-IgG further inhibited their co-localization, which resulted in a marked inhibition of antigen-specific T cell proliferation. The immunosuppressive potency of CCL19-IgG was tested in vivo using murine models for T11-mediated immune responses (delayed-type hypersensitivity) and for transplantation of different solid organs. In allogeneic kidney transplantation as well as heterotopic allogeneic heart transplantation in different strain combinations, allograft rejection was reduced and organ survival was significantly prolonged by treatment with CCL19-IgG compared with controls. This shows that in contrast to only limited prolongation of graft survival in CCR7 knockout models, the therapeutic application of a CCR7 ligand in a wild-type environment provides a benefit in terms of immunosuppression.


Received July 28, 2005. Accepted June 23, 2006.

Published online ahead of print. Publication date available at www.jasn.org.

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ISSN: 1046-6673/1709-2521

Materials and Methods

Generation, Expression, and Characterization of CCL19-IgG Fusion Protein

The chimeric CCL19-IgG fusion protein was generated as described previously with minor modifications (12). For obtaining large amounts
of the fusion protein, the CCL19-human IgG1 was expressed in sus-
pension CHO cells that were adapted to grow under serum-free con-
ditions (ProCHO4-CDM; Cambrex, Verviers, Belgium). The culture
supernatant, which contained secreted CCL19-IgG1, was purified using
Protein A Sepharose (Amersham Biosciences, Freiburg, Germany) (13)
and stored at 20°C. The protein concentration was quantified by
Bio-Rad Proteinassay (Munich, Germany), and serum concentrations
in mice were measured by an ELISA capture assay as described previ-
ously (12). The biologic activity of CCL19-IgG1 was checked routinely
in a chemotaxis assay (14). For all in vitro and in vivo experiments,
equimolar ChomePure human IgG (Dianova, Hamburg, Germany)
served as control for CCL19-IgG.

Cells and Cell Lines
The CHO cell line was obtained from ATCC (Manassas, VA). Cells
were maintained in RPMI 1640 medium supplemented with 10% FCS,
2 mM l-glutamine, streptomycin (100 μg/ml), and penicillin (100
U/ml). For large-scale protein production and purification, the adher-
ent transfected CHO cells were adapted to serum-free conditions and
maintained in ProCHO4 media.

Murine T lymphocytes were purified from spleen and lymph node
(LN) suspensions of untreated mice. Purification was done using a
MACS system after magnetic labeling of non-T cells (Pan-T cell anti-
body cocktail; Miltenyi, Bergisch Gladbach, Germany). Purity of >90% of
T cells was confirmed by CD3 staining in FACS analysis.

Mice
Male inbred C57BL/6 (H2b), female Balb/c (H2b), and ovalbumin
TCR-transgenic DO11.10 mice were supplied by Charles River (Sulz-
feld, Germany) and were housed at the animal facilities of Phenos
GmbH, Hannover, University of Duisburg-Essen, or the University of
Kiel under conventional conditions. Mice that weighed between 20 and
30 g and were 8 to 12 wk of age were used for all experiments. Mice
were cared for in accordance with the institution’s guidelines for ex-
perimental animals. The animal protection committee of the local au-
thorities approved all experiments.

Generation of Murine Bone Marrow DC and Recirculation
of DC
Preparation and culture of bone marrow (BM) cells to generate DC
has been described (15). For complete DC maturation, 1 μg/ml LPS and
1 μg/ml PGE2 (Sigma-Aldrich, Taufkirchen, Germany) were added on
day 10 for 24 h. For antigen-specific T cell priming experiments, BM-DC
were pulsed with ovalbumin by incubation with 200 μg/ml ovalbumin,
Grade VI (Sigma-Aldrich) before subcutaneous injection into footpads.
Those ex vivo generated BM-DC (>90% CD11b+/CD11c+) were flu-
orescence-labeled by PKH26 or CFSE (Sigma-Aldrich) according to the
manufacturer’s instructions. Thereafter, 1 × 106 cells were injected
subcutaneously into the left footpad. DC recirculation was assessed
after 48 h by dissection of lymphoid tissue of killed mice and by FACS
analysis and immunohistochemistry. Furthermore, a model of contact
sensitivity was used for recirculation studies. In brief, the abdominal
skin of mice was shaved and painted with 100 μl of 0.5% FITC dis-
solved in dibutylphthalate/acetone. Labeled and matured cutaneous
DC were counted after migration into secondary lymphoid tissues by
FACS analysis of dissected regional LN (inguinal and axillary) 48 h
later. Treatment consisted of daily intraperitoneal injections of 150 μg
of CCL19-IgG or the same amount of control IgG throughout the
experiments starting at least 6 h before application of cells or FITC
painting. Each group consisted of at least five mice. The experiments
were repeated at least twice.

Recirculation of Lymphocytes
For recirculation studies, purified T cells (>95% CD3+) were fluo-
rescence-labeled with PKH26 or CFSE according to the manufacturer’s
instructions and injected into the mouse tail vein (2 × 107 T cells per
mouse). Viability of >95% of cells was confirmed routinely by a trypan
blue exclusion assay. After 6 h, mice were killed and the distribution of
T cells was analyzed by determination of the fraction of labeled cells in
blood, spleen, mesenteric LN, and inguinal LN using FACS analysis
and cell count. Groups of five mice were either left untreated or treated
by intraperitoneal injections of 150 μg of CCL19-IgG or the same
amount of control IgG 3 to 6 h before cell transfer.

For redistribution analyses, Balb/c mice were treated daily with 150
μg of CCL19-IgG or the same amount of control IgG. After 5 d, mice
were killed and the cell numbers as well as the composition of the
different lymphocyte subtypes within the spleens and LN were ana-
lyzed using FACS.

Monitoring of Antigen-Induced T Cell Proliferation In Vivo
Ovalbumin TCR-transgenic T cells from DO11.10 mice were isolated
by magnetic labeling of non-T cells. A total of 107 transgenic T cells/ml
suspended in RPMI 1640 medium were labeled by incubating with 5
μM CFSE (Sigma-Aldrich) and subsequent washing. Transfer experi-
ments were performed by injecting 2 × 107 CFSE-labeled cells into the
tail vein of normal Balb/c mice.

Subsequently, 106 ovalbumin- or PBS-pulsed BM-DC were injected
in a volume of 30 μl subcutaneously into either footpad of the mice.
Concomitantly, treatment with CCL19-IgG or control IgG (150 μg
intraperitoneally daily) was started. Mice were killed 72 h later, and
draining popliteal LN were analyzed by FACS for proliferation of
ovalbumin-specific T cells, which is indicated by the dilution of CFSE-
fluorescence intensity.

Immunohistochemistry
Spleens, peripheral (axillary, inguinal, and popliteal), and mesenteric
LN were isolated from mice immediately after they were killed. These
lymphoid tissues were embedded in Tissue-Tek (Miles Inc., Elkhart, IN),
sectioned, mounted on glass slides, or frozen in liquid nitrogen and
stored at −80°C until used. Sections (5 to 7 μm) were fixed in acetone;
air-dried; rehydrated with PBS; and blocked with rat serum and stained
with Cy3-, PE-, or FITC-conjugated antibodies against the indicated
markers at 1:50 to 1:200 in blocking solution. After washing, the slides
were mounted with Citifluor (Citifluor Products, The University, Can-
terbury, UK) and examined by immunofluorescence microscopy (Leitz
DMRB). The relative distribution of transferred cells to red pulp, mar-
ginal zone, and white pulp was enumerated by image analysis of
assembled micrographs.

DTH Response In Vivo
Female Balb/c mice were sensitized on day 1 by intravenous injec-
tion of 2 × 108 sheep red blood cells (SRBC). Mice were challenged 4 d
after immunization by injection of 2 × 108 SRBC in 50 μl of PBS into the
left hind footpad (specific swelling). For determination of unspecified
swelling, an equal volume of PBS was injected into the right hind
footpad. Results were the difference of swelling of both footpads,
measured 24 and 48 h after challenge with a dial gauge caliper. The
extent of immune response was quantified further by dissection and
cell count of regional ipsilateral and contralateral LN.

In Vivo Cell Depletion Assay
BALB/c mice received an intravenous injection of 6 × 106 PKH26-
labeled CD4+ T cells. One day later, PBS, 150 μg of CD4+ T cell—
depleting mAb GK1.5 (BD Biosciences, Heidelberg, Germany), 150 μg of control IgG, or 150 μg of CCL19-IgG was administered each in a total volume of 500 μl (n = 4 per group). Control IgG and CCL19-IgG injections were repeated (two doses of each 150 μg/d), and the efficiency of depletion was assessed after 7 d by isolation of spleen and LN cells and analyzing fluorescence-labeled cells by using flow cytometry.

Allogeneic Kidney Transplantation

Vascularized kidney transplantation from C57BL/6 to Balb/c mice was described as previously performed (16). Briefly, mice were anesthetized with isoflurane, and the left donor kidney was attached to a cuff of the aorta and the renal vein with a small caval cuff, and the ureter was removed en block. After left nephrectomy of the recipient, the vascular cuffs were anastomosed to the recipient abdominal aorta and vena cava, respectively, below the level of the native renal vessels. The ureter was anastomosed directly into the bladder (17). Cold ischemia time was 90 min, and warm ischemia time was 30 min. The right native kidney was removed through flank incision 4 d later.

Two experimental groups were evaluated. Mice were treated by intraperitoneal injection of two doses of 150 μg of CCL19-IgG versus 150 μg of control IgG per day. Treatment of the recipient was started the day before surgery and stopped after 3 wk. No other immunosuppressive treatment was administered. Mice that received a transplant were studied for renal function and survival. Serum creatinine levels were measured by an automated method (Beckman Analyzer, Krefeld, Germany). Kidney grafts were harvested 5 d after transplantation, and one half of each allograft was fixed immediately in buffered formalin and embedded in paraffin. Three-micrometer sections were stained with hematoxylin-eosin and periodic acid-Schiff stain and evaluated according to the updated Banff classification by a nephropathologist, who was masked to the experimental groups (18). For adoptive transfer experiments, 2 × 10⁷ spleen cells from CCL19-IgG-treated kidney transplant recipients were isolated and purified 9 wk after surgery and transferred by intravenous injection into naive Balb/c mice (n = 6). Kidney transplantation was performed 3 d after the adoptive transfer, using the same donor strain (C57BL/6). No additional immunosuppressive treatment was administered. Recipient mice were monitored for renal function and survival.

Allogeneic Heart Transplantation

Vascularized heterotopic heart transplantation from C57BL/6 to Balb/c mice or vice versa was performed as described previously (19). General anesthesia was provided by injection of Ketanest (Ketamine; Pfizer Pharma, Karlsruhe, Germany) and Rompun (Xylazine; Bayer AG, Leverkusen, Germany). Two experimental groups were evaluated. Mice were treated by intraperitoneal injection of two doses of 150 μg of CCL19-IgG versus 150 μg of control IgG per day. Treatment of the recipients was started the day before surgery and stopped after 2 wk. No other immunosuppressive treatment was administered. Graft function was assessed daily by palpation. Rejection was defined as the lack of palpable cardiac contraction (20). Heart grafts were harvested 6 d after transplantation, embedded in paraffin, stained with hematoxylin-eosin, and evaluated by a pathologist, who was masked to the experimental groups.

Level of alloantibodies was determined by flow cytometry as described previously (21) using sera that were obtained on days 6 and 20 after transplantation. The levels of donor-specific antibodies were expressed as mean fluorescence intensity.

Statistical Analyses

Data are shown as mean ± SEM or SD. Normal distribution was analyzed by the Kolmogorov-Smirnov test, and statistical significance was calculated by the t test for independent groups. SPSS 12.01 software was used (SPSS, Inc., Chicago, IL).

Results

For disruption of immune cell trafficking of CCR7-positive cells in vivo, prolonged high concentrations of CCR7 ligands were needed. By fusing CCL19 to the Fc part of IgG1, an extended systemic half-life of the chemokine was achieved, leading to trough level concentrations of 105 ± 19 μg of CCL19-IgG/ml after injection of two doses of 150 μg of CCL19-IgG per day per mouse, as determined by ELISA on day 3 after initiation of treatment. Accumulation of the fusion protein could not be observed, because concentration was in a similar range after 14 d of treatment. The chemotactic activity and binding avidity were identical to recombinant CCL19 as demonstrated previously (12).

Decreased Homing of T Lymphocytes into SLO

For assessment of the recirculation and homing of T cells, mice received 10⁷ purified and fluorescence-labeled T cells intravenously 6 h before removal of LN. The cells were analyzed by FACS and microscopically. Figure 1A shows that the number of homed T cells was significantly reduced in mice that were treated with CCL19-IgG, compared with control IgG-treated mice. The findings were manifested in inguinal and mesenteric LN as well as in spleens with a homed T cell proportion of 43.2 ± 3.2, 44.1 ± 6.4, and 45 ± 8.2%, respectively (P < 0.05). In contrast, the proportion of labeled T cells in peripheral blood was significantly higher in CCL19-IgG-treated mice (161.2 ± 25.5% compared with controls; P < 0.05). No difference was noted between untreated and control IgG-treated mice. Moreover, migration of injected T cells into the T cell zones was significantly reduced in CCL19-IgG-treated mice. Figure 1B demonstrates the presence of labeled T cells restricted to the T cell areas only in control mice, but a random distribution throughout the spleen was noted in CCL19-IgG-treated mice. In summary, the systemic administration of CCL19-IgG effectively impairs homing of T cells into SLO quantitatively and qualitatively.

Impairment of Migration of DC into Draining LN

Next, we examined the effect of CCL19-IgG on the migration of matured DC into draining LN using two different models. First, we applied FITC into the abdominal skin to induce contact hypersensitivity. After 36 h, the number of migrated DC, labeled by FITC-antigen uptake, was determined by FACS and microscopically. Figure 2A shows that after CCL19-IgG treatment, significantly fewer DC had undergone migration into draining LN (control IgG 3583 ± 264 DC versus CCL19-IgG 1778 ± 130 DC; P < 0.05). The total cell count in draining LN also was reduced in CCL19-IgG–treated mice, as illustrated in Figure 2B (untreated 14.7 ± 0.17 × 10⁶ cells versus control IgG 15.2 ± 0.28 × 10⁶ cells versus CCL19-IgG 5.2 ± 0.16 × 10⁶ cells). In a second approach, ex vivo generated and matured BM-DC were injected subcutaneously into the footpads. The numbers of migrated cells were determined after 48 h, because preceding pilot experiments had shown that the...
that were treated with 150 labeled purified T cells were injected intravenously into mice. The count of migrated DC was reduced in CCL19-IgG–treated DC count in the draining LN was maximal at this time point. The major prerequisites for an adaptive immune response, recirculation and co-localization of T cells and DC, indeed are disturbed effectively by CCL19-IgG.

CCL19-IgG Inhibits DTH

We hypothesized that the potency of CCL19-IgG to impair the recirculation of immune cells results in immunosuppression. Therefore, we next analyzed the effects of the fusion protein in a model of T helper–mediated DTH.

C57BL/6 mice were immunized and challenged with SRBC. Starting 2 h before immunization, mice were treated either with daily injections of 150 μg of CCL19-IgG or 150 μg of control IgG. As shown in Figure 2A, in mice that were treated with CCL19-IgG, footpad swelling was significantly diminished by 48% 2 d after SRBC challenge (control IgG 0.90 ± 0.07 mm versus CCL19-IgG 0.47 ± 0.06 mm; P < 0.05). Untreated and control IgG-treated mice did not differ significantly. Again, the cell counts of regional LN were significantly reduced in CCL19-IgG–treated mice. Figure 3B shows that the hypercellularity of the draining popliteal LN in the challenged foot was significantly less pronounced (control IgG 82 ± 10 versus CCL19-IgG 33 ± 6 × 10^5 cells; P < 0.01). These results confirm the notion that systematically administered CCL19-IgG is capable of suppressing immune responses in T cell–dependent models.

CCL19-IgG Suppresses Antigen-Induced T Cell Proliferation in Draining LN

To test whether the observed reduction of draining LN size is not only a result of redistribution but also a consequence of impaired priming of T cells, we analyzed the effect of CCL19-IgG on antigen-induced proliferation in vitro. We injected ovalbumin-pulsed DC subcutaneously into footpads concomitantly with initiation of intraperitoneal application of CCL19-IgG or control IgG for 3 d. The mice received an adoptive transfer of CFSE-labeled ovalbumin-specific T cells before DC injection. The success of antigen-specific priming was controlled using CFSE dilution in FACS analysis. Figure 4 demonstrates that in the draining LN of the control IgG–treated mice, a robust proliferation of ovalbumin-specific T cells occurred, whereas CCL19-IgG treatment resulted in a markedly reduced proliferation of ovalbumin-specific T cells in the draining LN. LN of the contralateral footpads served as controls. Here, DC “pulsed” with PBS instead of ovalbumin antigen were injected.

Cell depletion that was induced by CCL19-IgG could not be detected by in vitro analysis of either complement-dependent cytotoxicity or antibody-dependent cell-mediated cytotoxicity using murine effector cells (data not shown). In addition, we analyzed the cell counts of labeled T cells in mice with or without treatment of CCL19-IgG after transfer of CCR7^+ ovalbumin-TCR transgenic T cells from DO11.10 mice into wild-type background. There was no significant difference in T cell recovery after 1 wk of treatment when compared with nontreated mice, clearly ruling out substantial depletion of

DC count in the draining LN was maximal at this time point. The count of migrated DC was reduced in CCL19-IgG–treated mice, as seen in Figure 2C (control IgG 5550 ± 1410 versus CCL19-IgG 1268 ± 310 labeled DC in draining LN; P < 0.05). This was associated with a significantly reduced popliteal LN cell count, as demonstrated in Figure 2D (untreated 5.1 ± 1.0 × 10^5 cells versus control IgG 5.5 ± 1.4 × 10^5 cells versus CCL19-IgG 1.2 ± 0.3 × 10^5 cells; P < 0.05). In addition, Figure 2E shows that the spleen histology exhibited altered positioning of CD11c^+ DC and T cells to each other. Thus, the major prerequisites for an adaptive immune response, recirculation and co-localization of T cells and DC, indeed are disturbed effectively by CCL19-IgG.

**Figure 1.** CCL19-IgG impairs T lymphocyte homing into secondary lymphoid organs (SLO) and induces complex redistribution within lymphoid organs. A total of 1 × 10⁷ fluorescence-labeled purified T cells were injected intravenously into mice that were treated with 150 μg of either control IgG or CCL19-IgG 2 h earlier. After 6 h, mice were dissected and the distribution of labeled cells was analyzed. (A) In CCL19-IgG–treated mice, T cells remain in blood and exhibit reduced entering into SLO. Relative distribution ± SD of labeled cells in CCL19-IgG–treated mice compared with control IgG group (set to 100%). Representative results from one of three experiments, n = 5 per group. *P < 0.05. (B) CCL19-IgG treatment disturbs specific homing into T cell areas in spleen. Histology of spleen sections: (Top) T cell areas are depicted by labeled T cells (green) in control mice, whereas random distribution is observed in CCL19-IgG spleens. (Bottom) Higher magnification displays the strict confinement of injected T cells (red) to T cell areas (green counterstaining with α-CD3-FITC) abrogated by CCL19-IgG treatment. Magnifications: ×50 in B, top; ×400 in B, bottom.
Figure 2. Influence of CCL19-IgG on migration of dendritic cells (DC). (A and B) DC recirculation after FITC painting is impaired by CCL19-IgG. Mice that were untreated or treated with either control IgG or CCL19-IgG were painted with 0.5% FITC in dibutylphthalate/acetone on the abdominal skin. Thirty-six hours later, draining lymph nodes (LN) were dissected and FITC\(^+\)/CD11c\(^+\) DC were counted using FACS analysis. Number of FITC\(^+\)/CD11c\(^+\) DC in draining LN (A) and total cell count of draining LN (B). (C and D) Recirculation of mature DC into draining LN after subcutaneous application is reduced by CCL19-IgG. A total of \(1 \times 10^6\) ex vivo matured bone marrow DC (BM-DC) were fluorescence-labeled and injected subcutaneously into the left footpad. At the time of maximal DC count (after 48 h), popliteal LN were removed and analyzed for total cell count and labeled DC. Shown is the count of labeled DC in draining LN (C) and the total cell count of ipsilateral and contralateral popliteal LN (D). (E) CCL19-IgG interferes with distribution of CD11c\(^+\) DC around T cell areas in spleen. Sections of spleen immunohistochemically stained with α-CD11c-PE (red) and α-CD3-FITC (green). Magnification, ×100 in E.
Kidney Allograft Survival Is Significantly Improved by CCL19-IgG Treatment

First, we used a model of allogeneic kidney transplantation in mice that resulted in lethal renal failure as a result of graft rejection within 10 d (16). Figure 5A shows the survival of mice that received a transplant and were treated either with 150 μg/d CCL19-IgG or with 150 μg/d control IgG for 21 d. All recipients that were treated with control IgG died 5 to 9 d after surgery. In contrast, 90% of CCL19-IgG–treated recipients survived until weeks 15 to 20. Graft function as determined by serum creatinine concentration was preserved in the CCL19-IgG treatment group until week 9. The slight increase in creatinine concentration, shown in Figure 5B (creatinine on day 7: 113 ± 17 μmol/L), normalized in this group within 10 d. In contrast, graft function rapidly deteriorated in untreated or control IgG–treated mice (creatinine on day 7: 256 ± 26 μmol/L [untreated], 221 ± 31 μmol/L [control IgG]), which was accompanied by marked histomorphologic alterations. Five days after transplantation, the histologic analysis showed evidence of acute tubulointerstitial, cellular rejection with significant interstitial infiltrates and mild to moderate tubulitis (Banff borderline n = 6, grade IA n = 4) in all control IgG–treated mice as indicated in Figure 5C. In four mice, focal attachments of activated lymphocytes to swollen endothelial cells of medium-sized arteries (i.e., marginalization) were present; however, endothelitis was not detected. All control IgG–treated mice exhibited marked diffuse acute tubular necrosis accompanied by dense tubular interstitial mononuclear infiltrates, as well as pericapillary inflammation. In contrast, the CCL19-IgG–treated group showed unremarkable cortical and medullar renal tissue with very limited interstitial cell infiltrates. Mild diffuse acute tubular necrosis was observed; however, tubulitis and pericapillary inflammation were not observed (all without rejection, Banff i0t0). Furthermore, 9 wk after surgery, the CCL19-IgG–treated allografts showed almost normal renal architecture without tubulitis or endothelitis and displayed only focal perivascular mononuclear infiltrates (data not shown). However, the long-term survival without overt signs of renal insufficiency in CCL19-IgG–treated mice over 9 wk was not accompanied by induction of peripheral tolerance toward the grafted kidney. We adoptively transferred 2 ×10^7 splenocytes from transplanted, CCL19-IgG–treated mice 9 wk after transplantation into untreated Balb/c mice. Three days later, those mice underwent kidney transplantation under the same conditions without any additional treatment. The recipients died within 6 to 10 d after surgery as did control IgG–treated mice. Loss of renal function within 5 d after surgery was similar in treated and control IgG–treated mice. Thus, there was no evidence for a transferable tolerance in the CCL19-IgG–treated mice.

Prolongation of Allograft Survival by Treatment with CCL19-IgG also Is Valid for Murine Heart Transplantation

To exclude that the observed sustained allograft survival of kidneys is an issue of graft type, we tested the immunosuppressive effect in another transplant model. In a murine model of heterotopic fully vascularized heart transplantation, we could demonstrate again that treatment with CCL19-IgG induces significant longer survival of the grafted organs than in mice that were treated with control IgG or untreated (Figure 6A). The mean survival in the CCL19-IgG–treated group was 22 ± 2.9 versus 9 ± 0.75 d (SD) in the control IgG group (P < 0.05). The latter did not differ significantly from the heart survival in untreated mice (7.8 ± 0.83 d). To exclude that both the renal and the cardiac graft survival is a consequence only of a favorable mouse strain combination, we additionally performed the heart transplantation with reversed strain combination. Figure 6A (bottom) shows that CCL19-IgG also is effective under these conditions.

Consistently, grafts from mice that were treated with control IgG showed massive cellular infiltration with diffuse cardio-
Figure 4. CCL19-IgG functionally disturbs interaction between T cells and DC in vivo and does not deplete CCR7+ cells in vivo. (A) PKH-labeled ovalbumin TCR-transgenic T cells from DO11.10 mice were adoptively transferred into Balb/c mice. For monitoring antigen-specific T cell proliferation responses in vivo, ovalbumin-pulsed BM-DC were injected subcutaneously into footpads and compared with mice that received a transfer but were untreated (no DC). Concomitantly, administration of PBS, control IgG (2 × 150 μg/d intraperitoneally), or CCL19-IgG (2 × 150 μg/d intraperitoneally) was started. After 3 d, draining popliteal LN were analyzed by FACS for proliferation of ovalbumin-specific T cells (gated for lymphocytes). Graph shows analysis of pooled popliteal LN of five mice in each group, region A marks labeled nonproliferated cells, B marks proliferated cells, C marks unlabeled cells. (B) Balb/c mice received an injection of fluorescence-labeled CD4+ cells and treated with PBS, depleting α-CD4 mAb, control IgG, or CCL19-IgG. After 1 wk, depletion of labeled cells was analyzed by FACS in spleen and LN. Graph shows representative analysis of splenocytes; regions mark labeled cells.
myocyte necrosis, whereas grafts from CCL19-IgG–treated mice showed capillary congestion and a mild scattered infiltration with mononuclear cells of the cardiac muscle tissue. The integrity of cardiomyocytes was preserved (Figure 6C).

Furthermore, the analysis of level of alloantibodies did not show significant differences between groups. At day 6, alloantibodies of IgG type were not detectable, whereas at day 20 after transplantation, all groups exhibited comparable levels of donor-specific antibodies as determined by FACS analysis (mean fluorescence intensity 12.5 ± 0.4 [untreated] versus 8.9 ± 0.3 [control-IgG] versus 9.1 ± 0.4 [CCL19-IgG]).

Discussion

Initiation of an immune response depends on recirculation of lymphocytes and DC, as well as their co-localization within SLO (22–24). Studies in gene-targeted mice that are unable to express either the chemokine receptor CCR7 or their ligands CCL19 and CCL21 have shown that these molecules play pivotal and nonredundant roles in this complex process. Those mice are unable to launch an appropriate immune response (3,23,25,26). However, therapeutic targeting of CCR7 for immunosuppressive purposes was not yet feasible because appropriate tools were lacking. We demonstrated the immunosuppressive potency of a CCR7-binding CCL19-IgG fusion protein.

The agonist fusion protein CCL19-IgG, when given for only a few days, induced similar effects on T cell and DC migration that are known from CCR7−/− mice (3). Explanations might be, first, under both conditions (CCL19-IgG treatment as well as CCR7 deficiency), T cell entry into SLO is hindered and the cells remain in circulation (3). Second, DC migration into draining LN is reduced (27,28). Third, cellular congestion in draining LN is markedly suppressed (29). Finally, compartmental migration and co-localization of CD11c+ DC and CD3+ T cells within the spleen is disturbed and accordingly the distribution of lymphocytes in different SLO is altered (3).

How can an agonistic chemokine fusion protein induce similar effects as those observed by deleting the corresponding receptor? Cells that recognize certain locally expressed chemo-
Moreover, receptor desensitization and downregulation have to be taken into account, for CCR7, in particular. For sustained concentrations of CCL19 that exceed 1 μM, an impairment of extravasation as a result of lack of integrin activation could be due to desensitization (36,37). We assume that this mechanism contributes to our findings, because serum concentrations in CCL19-IgG-treated mice exceeded this level of 1 μM.

Receptor downregulation could be even more important in light of very recent observations made by Marsland et al. (38) as well as Kursar et al. (39). Marsland et al. (38) could identify CCL19 and CCL21 as potent adjuvants for activating capacities of DC. Kursar et al. (39) could show that priming of subsets of CD8+ T cells also requires CCR7. Both pointed out the importance of the co-stimulatory function to build up an cellular immune response. This is not in contrast to our observations, because CCL19 induces rapid downregulation of CCR7 (40); therefore, an important co-stimulatory molecule would be missing in repeated stimulation. In fact, we also could demonstrate downregulation of CCR7 by CCL19-IgG (12). After 1 d of CCL19-IgG treatment, substantial downregulation was present also in vivo (data not shown). Although the co-stimulatory properties of CCR7 in general and the influence of CCL19-IgG as well still remain to be analyzed further, it is intriguing that downregulation of this receptor contributes to the observed immunosuppression by deprivation of activating signals.

Taken together with the profound alteration of cell trafficking behavior, the net effect of CCL19-IgG is a reduced clonal T cell response as demonstrated in the model for DC-mediated antigen-specific T cell priming (Figure 4A). A drastically reduced proliferation of antigen-specific T cells results from treatment with CCL19-IgG. Therefore, a central part of the adaptive immune system is critically impaired by this fusion protein.

Consistent with the mechanisms discussed above, in the T cell–dependent DTH model, the CCL19-IgG–induced decrease of the immune response was associated with a markedly reduced lymphocytic accumulation in LN. However, because a tool for therapeutic targeting of CCR7 is missing, a relevant question was whether CCL19-IgG also would be able to interfere with alloreaction in solid-organ transplantation.

First, we assessed this in a model of allogeneic kidney transplantation, which was adapted to a lethal outcome unless the mice were treated. Indeed, untreated as well as control-IgG–treated mice died within 10 d as a result of rapid, histologically proven allograft rejection that resulted in kidney graft failure and uremia (17). Three weeks of treatment with the CCL19-IgG fusion protein improved survival to 90% at 15 wk. The renal histology confirmed a nearly absent immunologic response at day 5, compared with the acute cellular rejection in control-IgG–treated mice.

This beneficial effect was substantiated by equivocal results after heart transplantation. Although CCL19-IgG could not induce a graft survival as long as in the kidney model, it resulted in significant prolongation compared with untreated and control-IgG–treated mice.

These results in two different transplant models clearly point out that the favorable survival of the grafts indeed is a specific effect of CCL19 fusion protein as a result of the observed...
changes in T cell homeostasis. Thereby, as previously shown for this model (41), we confirm that the effects on T cell responses are sufficient to provide a prolongation of survival, because results from analysis of donor-specific antibodies show that B cell–mediated alloresponses were not impaired by CCL19-IgG.

Although it is known that the survival of different solid organs varies substantially depending on organ type (42), the longer survival of kidney over heart grafts might indicate a further specific effect mediated by CCL19-IgG. Because one important difference in the two models is the longer ischemia time in the kidney model, it well could be that CCL19-IgG additively effects ischemia-reperfusion injury. It could be demonstrated that reperfusion injury is critically dependent on T cells and their adhesion on epithelial cells (43). As discussed above, this adhesion could be reduced by missing co-stimulation as a result of CCL19-IgG–mediated downregulation of CCR7. Supporting this thesis, histology of the kidney grafts of CCL19-IgG–treated mice showed indeed less pronounced acute tubular necrosis. Whether CCL19-IgG is able to suppress ischemia-reperfusion injury is being investigated in further studies.

In previous literature, the effect of targeting CCR7 or its ligands on solid-organ transplantation was assessed only in knockout models, but these studies demonstrated only limited prolongation of organ survival in the CCR7−/− as well as plt−/− groups compared with wild-type mice (10,11,44,45). In contrast, the graft survival in CCL19-IgG–treated mice was superior to that reported for transplants in knockout mice in both models. Even in the same model (heterotopic, fully MHC-mismatched, vascularized heart transplantation), there is a marked better survival in CCL19-IgG–treated mice than if hearts were grafted into CCR7 knockout mice (10).

These different results seem to be conflicting, but, first, there are more examples published of differences between a permanent genetic knockout and a time-limited therapeutic blocking approach in an immune-competent host, and, second, the agonistic fusion protein potentially could activate pathways that result in the observed effects. A well-known example for difference between knockout and therapy are animals that are deficient for IL-2 and display an exaggerated immune response that results in autoimmune phenotype (46). In contrast, the widely used immunosuppressive calcineurin inhibitors interfere with transcription of the IL-2 gene to suppress the immune response. Similarly IL-2 receptor antagonists provide additional immunosuppression in transplant settings (47,48). Another example is the block of IFN-γ, which leads on the one hand to susceptibility to autoimmunity in the genetic knockout (49) and on the other hand to immunosuppression as a result of inhibition of the action in a, immunocompetent genetic sufficient host if blocking antibodies are used therapeutically (50). For this reason, for immunocompetent hosts, conclusions cannot be drawn easily from knockout models, and, as we could demonstrate earlier for an IL-2 fusion protein (13), there can be considerable differences between agonistic blockers of a cytokine and the phenotype of a mouse that is deficient for the same cytokine.

It is not understood in detail which pathways of signal transduction are activated by CCR7, and it is not analyzed, yet, whether those pathways are rescued by signaling of other receptors in CCR7-deficient mice. Given that CCR7 is co-stimulatory on T cell proliferation and Th1 polarization (51), however, a timely limited block of CCR7 could interfere with T cell co-stimulation. This influence could not be present in the knockout model because of escape mechanisms and might contribute to prolongation of graft survival that was observed in the therapeutic approach only.

Conclusion

Our data show that, in terms of immunosuppression, therapeutic targeting of CCR7 is effective and made possible by a CCL19-IgG fusion protein that provides a tool to achieve disruption of cell trafficking in a clinical setting. This fusion protein affects key steps of initiating an immune response, namely appropriate recirculation of DC and T cells and co-localization of both cell types within SLO. As a consequence, antigen-specific T cell proliferation is impaired. We demonstrated the immunosuppressive potency of systemic high-dosage application of CCL19-IgG by improved survival in allogeneic solid-organ transplantation, superior to survival in transplantation models with mice that are deficient for either CCR7 or its ligands, so further analyses of the potency of therapeutic interference with the CCR7 system should be warranted.

Acknowledgments

We thank Mareike Newsy, Jeun-Koon Park, and Herle Chlebusch for expert technical assistance. We also thank Jaba Gamrekelashvili and Tim Greten for performing adoptive transfer experiments.

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