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Control of Transplant Tolerance and Intragraft Regulatory T Cell Localization by Myeloid-Derived Suppressor Cells and CCL5

Nahzli Dilek,*‡§ Nicolas Poirier,*‡§ Claire Usal,*‡§ Bernard Martinet,*‡§ Gilles Blancho,*‡§ and Bernard Vanhove*‡§

Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of immature cells that are believed to inhibit immune responses in the contexts of cancer and organ transplantation, in association with regulatory T cells (Treg). However, the way in which MDSC cooperate with Treg remains elusive. In this study, we used DNA microarrays to analyze gene expression in blood-derived MDSC from rat recipients of kidney allografts. We found CCL5 (Rantes), a chemotactic C-C motif 5 chemokine, to be strongly downregulated after treatment with a tolerizing regimen. The amount of CCL5 protein was also lower in the plasma of tolerant recipients, whereas intragraft CCL5 was unchanged. Because CCL5 is chemotactic for Treg, we hypothesized that a gradient of CCL5 between the graft and peripheral blood might contribute to the intragraft localization of Treg in tolerant animals. To test this hypothesis, we treated tolerant rat recipients of kidney allografts with recombinant rat CCL5 to restore normal plasma concentrations. This led to a strong reduction in intragraft Treg monitored by immunohistofluorescence and by quantitative real-time PCR measurement of Foxp3 mRNA. Ultimately, this treatment led to an increase in serum creatinine concentrations and to kidney graft rejection after about a month. The kidney function of syngeneic grafts was not affected by a similar administration of CCL5. These data highlight the contribution of MDSC to the establishment of a graft-to-periphery CCL5 gradient in tolerant kidney allograft recipients, which controls recruitment of Treg to the graft where they likely contribute to maintaining tolerance. The Journal of Immunology, 2012, 188: 4209–4216.

Myeloid-derived suppressor cells (MDSC), a heterogeneous population of progenitor and immature myeloid cells (1), were reported to contribute to immunosuppression in the tumor microenvironment (2, 3). These cells have also been associated with long-term survival and tolerance in transplantation (4, 5). Because of their heterogeneous nature, MDSC have the ability to use several suppressive mechanisms that disrupt both innate and adaptive immunity. These include increased arginase activity (6, 7) and upregulation of NO (8, 9), reactive oxygen species, and peroxynitrite production (10, 11). Furthermore, several recent studies demonstrated that MDSC can block T cell activation by depriving the environment of cysteine (12) and by downregulating L-selectin (CD62L) (13).

Despite their suppressive capacity, MDSC are probably not the major players in transplant tolerance. Indeed, transplant tolerance is mostly associated with the action of regulatory T cells (Treg) (14) working in synergy with MDSC (15, 16) and other suppressive cell types, such as monocytes (17), NK cells (18), plasmacytoid dendritic cells (19–22), or immature dendritic cells (23). However, the mechanisms involved in this cooperation are not well understood.

CCL5, also called RANTES, plays an essential role in inflammation and immune responses. CCL5 is a chemotactic cytokine playing an active role in recruiting leukocytes into inflammatory sites. This chemokine is produced by tumor cells in the tumor microenvironment (24, 25) and participates in the recruitment of Treg that preferentially express CCR5, thereby blocking immune responses. Disruption of CCR5/CCL5 by administration of a CCR5 inhibitor was shown to induce a reduction in migration of Treg into tumors, as well as rejection by immune cells (25). In addition, after transplantation, systemic treatment with the RANTES antagonist Met-RANTES dramatically suppressed the acute tissue damage underlying rejection and limited endothelial cell–leukocyte interactions and infiltration in allografts (26–30).

In a previous study, we reported on the role of MDSC in sustaining tolerance in rat recipients of kidney allografts treated with a CD28-targeted tolerance-inducing regimen (9). In this study, we compared gene expression in blood-derived MDSC from tolerant recipients of allogeneic kidney grafts in the same model with syngeneic grafts. In addition to modifications in genes regulating cell activation (LAT) and apoptosis (Bcl2like12 and Dapk2), we observed the strong downregulation of CCL5 in blood MDSC, as well as a reduction in CCL5 plasma levels in tolerant recipients. Knowing that CCL5 is expressed by the kidney and participates in the recruitment of leukocytes and particularly Treg, we hypothesized that a gradient of CCL5 between the graft and the periphery...
could promote intragraft localization of Treg and contribute to transplant tolerance. This hypothesis was confirmed by the observation that restoration of physiological plasma levels of CCL5 led to a reduction in intragraft localization of Treg and to kidney allograft rejection. Our data depict a situation in which peripheral MDSC repress CCL5 gene expression in tolerance and suggest a role for CCL5 in intragraft localization of Treg.

Materials and Methods

Abs and reagents

Anti-CD80 (3H5) and anti-CD86 (24F) mouse Abs were prepared in our laboratory from a hybridoma provided by Dr. H. Yagita (Juntendo University School of Medicine, Tokyo, Japan). Biotinylated anti-Foxp3 (FJK-16s) was from eBioscience (San Diego, CA). Purified anti-MHC class II (OX6), anti-CD3 (G4.18), and anti–TCR-β (R7/3) were prepared in our laboratory from the corresponding hybridomas obtained from the European Cell Culture Collection (Salisbury, U.K.). FITC-conjugated anti-mouse and PE-conjugated streptavidin were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Alexa Fluor 568-conjugated streptavidin was from Life Technologies (Carlsbad, CA). Rat CCL5 in its recombinant form, also known as Rantes, was purchased from Creative BioMart (Shirley, NY).

Rats and transplantsations

Seven- to nine-week-old male Lewis.1W (RT1u) and Lewis.1A (RT1a) congenic rats were obtained from Janvier (Savigny/Orges, France) and maintained in our animal facility under specific pathogen-free conditions, according to institutional guidelines. Kidney allografts were performed, as previously described (31). One native Lewis.1W kidney (right side) was replaced by the LEW.1A donor allograft, and a contralateral nephrectomy was performed 7 d later, after which time the recipient’s survival depended on the proper functioning of the allograft. LEW.1W heart grafts were implanted heterotopically onto LEW.1A recipients using the Ono and Lindsey technique (32), and function was monitored daily by palpation through the abdominal wall. Rejection was defined as complete cessation of heartbeat.

Tolerance induction

The J319 (IgG1 anti-rat CD28) mouse hybridoma was a gift from Dr. Thomas Hunig (University of Würzburg, Würzburg, Germany). The J319 mAb was purified from hybridoma supernatant and administered to LEW.1A allograft recipients by i.p. injection at 0.3 mg/d for 7 d, starting on the day of transplantation. This Ab induces a transient downregulation of CD28 expression in vivo, without depleting target cells (33). Without treatment, the grafts were rejected 11 d after transplantation. Syngeneic transplants (LEW.1A to LEW.1A) served as controls. Cardiac transplant tolerance was obtained by treating rats with donor-specific blood transfusions (DST). One milliliter of heparinized LEW.1A donor blood was administered i.v. to naive LEW.1A recipients on pretransplant days −14 and −7 (34).

Administration of rCCL5

ALZET osmotic mini-pumps (model 2001) were purchased from Charles River (Wilmington, MA). Filling osmotic pumps with recombinant rat CCL5 (Creative BioMart) was done according to the instructions of the manufacturer. Pumps were surgically implanted i.p. under isoflurane anesthesia and sterile conditions. Pumps were set to deliver 14 μg/kg/d of rCCL5 to transplanted rats over a period of 14 or 21 d.

Cells and cell sorting

PBL were isolated from heparinized blood by removing erythrocytes with hypotonic lysis solution. Depletion of MHC class II+ and CD3+ cells was performed with specific mAbs, followed by anti-mouse IgG-coated Dynabeads (Life Technologies). MDSC were identified by staining for 30 min at 4˚C with purified anti-rat CD80/86 (3H5/24F) mAbs and FITC-conjugated anti-mouse IgG secondary Ab (Jackson ImmunoResearch Laboratories), as previously described (9). MDSC were isolated using automated magnetic cell sorting (AutoMACS; Miltenyi Biotec, Bergisch Gladbach, Germany) based on CD80/86 expression.

Microarray and data analysis

mRNA was extracted from sorted cells using TRIzol reagent (Life Technologies). The quality and quantity of extracted total RNA were checked by gel filtration was assessed by double staining with anti-rat TCR-β (R7/3) and Alexa Fluor 568-conjugated streptavidin (Life Technologies). Slides were analyzed using standard fluorescence confocal-like microscopy.

Statistics

The results obtained are presented as fold induction versus the control. Percent Present. Gene-expression data are presented as Fold Change (Tolerant MDSC/Syngeneic MDSC). Full microarray data are available at the Gene Expression Omnibus Web-based data repository under accession number GSE28545 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi? acc=GSE28545).

Quantitative real-time PCR

RNA was extracted from sorted cells or snap-frozen renal biopsies using TRIzol reagent (Life Technologies). A total of 2 μg RNA was reverse transcribed using the Omniscript RT-PCR kit (Qiagen, Düsseldorf, Germany). The quality and quantity of RNA were controlled by infrared spectrometry (Nanodrop) and Agilent RNA 6000 Nano Assay Kit (Genomics). Quantitative real-time PCR (qRT-PCR) was performed in an Applied Biosystems GeneAmp 7900 Sequence Detection System. TaqMan gene-expression assays for rat transcripts (Applied Biosystems, Life Technologies) were used to quantify the mRNA expression of the respective genes: HPRT (Rn01527838_g1), CCL5 (Rn00579590_m1), Foxp3 (Rn01525805_m1), CCR5 (Rn00583676_g1), B2m (Rn00168052_g1), and Pdk2 (Rn01417725_m1). HPRT was used as an endogenous control gene to normalize varying starting amounts of RNA. Relative expression between a given sample and a control sample, used for all experiments, was calculated with the 2−ΔΔCt method. Expression of genes of interest was compared between tolerant animals and syngeneic controls. The results obtained are presented as fold induction versus the baseline levels in control syngeneic-transplanted rats.

ELISA and Luminex

Freshly isolated MDSC and “non-MDSC” were incubated for 16 h at 37˚C, 5% CO2, in complete medium (RPMI 1640, 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2-ME). Supernatants were collected and frozen at −20˚C before use. Plasma was collected from heparinized blood and frozen at −20˚C before use. Kidneys were harvested on day 100 posttransplantation, and their volume was evaluated. Kidneys were homogenized at 4˚C in RIPA lysis solution with an ULTRA-TURRAX (IKA, Lille, France). After agitation, protein lysate was centrifuged, and supernatant was collected and frozen at −20˚C before use. CCL5 was measured using a commercially available ELISA kit (Leinco Technologies, St. Louis, MO), according to the manufacturer’s instructions. Eotaxin, GM-CSF, G-CSF, growth-related onconecine (CXCL1), IFN-γ, IL-10, IL-12 (p70), IL-18, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IP-10, leptin, MCP-1, MIP-1α, RANTES, TNF-α, and vascular endothelial growth factor were measured using a commercially available rat MILLI-PLEX kit (Millipore, Billerica, MA).

Immunohistochemical staining

Frozen sections (10 μm) were prepared from renal biopsies. Slides were air dried at room temperature for 1 h before acetone fixation for 10 min at room temperature. Sections were saturated and permeabilized with 0.5% saponin (Sigma-Aldrich, St. Louis, MO) in the saturated solution (PBS containing 5% rat serum, 2% normal goat serum, and 4% BSA). Sections were incubated overnight with primary Abs at 4˚C, followed by fluorescent secondary Abs and nuclear staining (DAPI, Life Technologies). Treg infiltration was assessed by double staining with anti-rat TCR-β (R7/3) and anti-rat Foxp3 (followed by Alexa Fluor 568-conjugated streptavidin; Life Technologies). Slides were analyzed using standard fluorescence confocal-like microscopy.
(Aptome; Carl Zeiss, Oberkochen, Germany) and AxioVision imaging software (Carl Zeiss, Le Pecq, France). The percentage of TCR-β+ and Foxp3+ T cells was quantified manually (blind to the experimental conditions) on five pictures from two different sections of the graft separated by ≥100 μm.

### Statistical analysis

Graft survival was calculated using the Kaplan–Meier method. The log-rank test was used to compare survival times between different groups. Continuous variables were compared using the Mann–Whitney nonparametric test or analyzed by ANOVA, followed by the Kruskal–Wallis correction. The p values < 0.05 were considered statistically significant. All statistical analyses were performed on a personal computer with the statistical package GraphPad InStat (version 5.1; GraphPad Software, San Diego, CA).

### Results

#### Microarray analysis of MDSC

We previously reported that kidney transplant tolerance could be induced in the LEW.1W to LEW.1A rat model by administration of functionally antagonist anti-CD28 Abs and that the tolerant state was characterized by the presence of Treg, as well as blood MDSC (9). Because MDSC can also be found in naive and in syngeneic-transplant recipients, we aimed at identifying possible differences in MDSC from tolerant animals. Therefore, we performed a transcriptome analysis of blood MDSC from recipient rats tolerating an allograft for >3 mo or from rat recipients of a syngeneic graft. A total of 10,735 transcripts was detected in MDSC by microarray analysis, and the expression level of 525 of them was significantly different between the two groups (full data are available on the Gene Expression Omnibus Web-based data repository under GEO accession number GSE28545). Bcl2112, a gene with an assigned antiapoptotic function, was upregulated >3-fold, whereas Dapk2, an apoptosis-inducing gene, was downregulated 19-fold in MDSC from tolerant recipients compared with MDSC from syngeneic recipients (Table I). Lat, a gene known to positively regulate cell activation in T lymphocytes, was downregulated >40-fold (Table I). Additional analyses of these genes by qRT-PCR, using independent samples, confirmed these gene-expression variations for Bcl2112, Dapk2, and LAT (Fig. 1A–C).

#### Lower expression of CCL5 in MDSC and plasma of tolerant animals

The most striking differential expression was for CCL5, which presented a 166-fold downregulation in MDSC from tolerant rats (Table I). qRT-PCR analyses on RNA extracts from independent experiments confirmed that MDSC from tolerant rats contained significantly less CCL5 transcripts than MDSC from rats grafted with syngeneic kidneys (Fig. 1D). At the protein level as well, MDSC from tolerant animals secreted less CCL5 than MDSC from controls (Fig. 2A). A reduction in CCL5 was also evident in the plasma at the protein level. CCL5 measurement, by ELISA, in the plasma of syngeneic and tolerant rats showed a 2-fold decrease (0.60 ± 0.17 versus 1.17 ± 0.48 ng/ml; p < 0.01) in tolerant rats, whereas no differences were observed between naive and syngeneic animals (Fig. 2B). To understand whether the decrease in plasma CCL5 protein could be attributed to a lower synthesis by blood MDSC, we examined the relative mRNA expression of CCL5 in total blood cells depleted of MDSC by qRT-PCR. These MDSC-depleted blood cells expressed similar levels of CCL5 transcripts in tolerant recipients and in syngeneic recipients (Fig. 2C). MDSC-depleted blood cells from tolerant animals also secreted a similar amount of CCL5 proteins compared with control animals (Fig. 2D). These data suggested at least a participation of MDSC in the reduced CCL5 protein level in the plasma of tolerant rats. Interestingly, serum levels of CCL2 and other chemokines were not reduced between tolerant and control animals transplanted with a syngeneic graft (Supplemental Fig. 1).

To understand whether the reduced levels of CCL5 is a general feature associated with transplant tolerance in the rat or is specific for the kidney-transplant model used in this study, we assessed levels of CCL5 in plasma in a heart graft model in the same LEW.1W to LEW.1A rat strain combination; tolerance was induced by pretransplant administration of DST (35). However, in these samples, no difference could be detected between plasma from tolerant heart graft recipients and age-matched recipients of syngeneic heart grafts (data not shown).

#### CCL5 gradient and Treg localization

In solid tumors, overexpression of CCL5 by tumor cells leads to the preferential recruitment of Treg and the establishment of local immunosuppression (24, 25). To assess whether an analogous situation might exist in transplantation and participate in the localization of Treg in tolerated allografts, we performed qRT-PCR on RNA samples from kidney grafts. We found an increase in CCL5 mRNA expression in tolerated allografts relative to syngeneic grafts (Fig. 3A). However, at the protein level, the slightly greater CCL5 protein found in tolerated allogeneic kidney grafts (measured by extracting total protein and measuring concentrations reported to volume) did not reach statistical significance compared with syngeneic grafts (Fig. 3B). Comparing CCL5 local concentration within the graft and concentration in the plasma (shown in Fig. 2B) revealed that ~20-fold more CCL5 was present in normal kidneys than in plasma. For syngeneic kidney grafts, the ratio was ~30:1. For tolerated allografts, it was as high as 65:1 (Fig. 3C). In naive untreated animals, the ratio was even lower (18:1), and this was mainly due to less CCL5 in naive kidneys than in kidney grafts (Fig. 3B).

mRNA levels of CCR5 (CCL5 receptor expressed on T cells) and Foxp3 were also higher in tolerated kidney allografts (Fig. 4). Consistent with these data, using immunofluorescence we observed a strong infiltration of tolerated kidney allografts by TCR-β"Foxp3+ Treg that was greater than that seen at rejection (Fig. 5).

### Table I. Microarray data analysis of MDSC from tolerant recipients of kidney allografts compared with MDSC from recipients of syngeneic grafts

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Gene Symbol</th>
<th>GenBank Function</th>
<th>Fold Change (MDSC tolerant/MDSC syngeneic)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>22372767</td>
<td>Bcl2112</td>
<td>Inhibition of apoptosis</td>
<td>3.507</td>
<td>0.001</td>
</tr>
<tr>
<td>21908913</td>
<td>Dapk2</td>
<td>Induction of apoptosis</td>
<td>0.053</td>
<td>0.01</td>
</tr>
<tr>
<td>20872514</td>
<td>Lat</td>
<td>Positive regulation of T cell activation/differentiation</td>
<td>0.022</td>
<td>0.006</td>
</tr>
<tr>
<td>20896278</td>
<td>Ccl5</td>
<td>Signaling molecule/chemoattractant chemokine</td>
<td>0.006</td>
<td>0.001</td>
</tr>
</tbody>
</table>

The top four upregulated and downregulated genes (MDSC tolerant/MDSC syngeneic) are shown. Full microarray data are available at the Gene Expression Omnibus Web-based data repository under GEO accession number GSE28545.
FIGURE 1. MDSC gene expression assessment by qRT-PCR. MDSC from rat recipients of kidney grafts were purified from blood for RNA extraction. qRT-PCR was performed to assess expression of Bcl2112 (A, syngeneic, n = 9; tolerant, n = 12), Dapk2 (B, syngeneic, n = 7; tolerant, n = 7), LAT (C, syngeneic, n = 9; tolerant, n = 9), and CCL5 (D, syngeneic, n = 4; tolerant, n = 8). *p < 0.05, **p < 0.01.

Thus, the increased differential between intragraft and plasma levels of CCL5 correlated with the localization of Treg in tolerated allografts. To explore whether the enhanced graft-to-periphery gradient of CCL5 might influence intragraft localization of Treg in our experimental model, we aimed at restoring the physiological plasma concentration of CCL5 in tolerant recipients of kidney allografts by i.p. implantation of osmotic mini-pumps filled with recombinant rat CCL5 (rCCL5) delivering 14 μg/kg/d over ≥14 d. Assessment of plasma CCL5 concentration showed that osmotic pump implantation increased plasma levels from 670 to ~1800 pg/ml within 2 wk. In recipients of syngeneic kidney grafts, pump implantation also increased the titer from 1500 to 3000 ng/ml (Supplemental Fig. 2). After 1 mo, kidney graft function started to decline. Animals were euthanized for transplant analyses. No difference was observed in CCL5 mRNA expression between tolerant grafts treated or not with rCCL5 (Fig. 3A). Interestingly, mRNA levels of CCR5 and Foxp3 were reduced in tolerated grafts after systemic rCCL5 administration (Fig. 4). By quantification of cell infiltration by immunofluorescence, we also observed a decrease in intragraft TCR-β"Foxp3" Treg infiltration after systemic rCCL5 administration (Fig. 5). In another grafted animal, instead of using an osmotic pump, we gave CCL5 by i.p. administration of 1.5 μg/kg soluble protein twice daily for 21 d. This also induced similar findings in kidney biopsies at 1 mo and also resulted in graft rejection (data not shown).

CCL5 administration controls kidney transplant tolerance

It was demonstrated that Treg localization in the graft is required for induction and maintenance of transplant tolerance in the rat (36, 37). To further understand the impact of rCCL5 administration and reduction of intragraft infiltration by Treg on transplant tolerance, we monitored graft function. First, we investigated the role of rCCL5 in the tolerance-induction phase. Implantation of osmotic pumps delivering rCCL5, in addition to the anti-CD28 Ab-based tolerance-induction regimen, prevented tolerance induction of kidney allografts, whereas monotherapy with anti-CD28 Ab induced long-term survival over ≥4 mo (Fig. 6A). Kidney graft function (assessed by measurement of creatininemia) also decreased in the bitherapy group (Fig. 6B), indicating a rejection of the transplanted kidney in these animals. Measurement of plasma urea concentration confirmed these data (Supplemental Fig. 3A). Administration of rCCL5 to rats transplanted with syngeneic kidneys had no effect on survival, creatinine (Fig. 6A, 6B), or urea (Supplemental Fig. 3A). Second, we studied the effect of rCCL5 administration in established tolerance. Implantation of osmotic pumps delivering rCCL5 starting from 120 d after transplantation led to kidney graft rejection arising after ~50 d (Fig. 6C). This rejection was confirmed by measurement of plasma creatininemia (Fig. 6D) and urea (Supplemental Fig. 3B). Thus, rCCL5 administration abrogated kidney transplant tolerance during the induction and maintenance phases. Together, these data suggest that increasing peripheral levels of CCL5 impacted intragraft localization of Treg and inhibited transplant tolerance.

In contrast, in the heart allotransplant model in the same rat strain combination, in which tolerance is induced by pretransplant administration of DST (35), implantation of CCL5-delivering osmotic pumps for >1 mo did not brake established tolerance (Supplemental Fig. 4).

FIGURE 2. CCL5 synthesis assessment by MDSC and in plasma. (A) Blood MDSC from recipients of syngeneic kidney grafts (n = 4) or from tolerated kidney allografts (n = 4) were maintained in vitro for 16 h, and CCL5 was evaluated by ELISA in the supernatant. (B) Quantification of CCL5 protein levels by ELISA in plasma of nongrafted rats (n = 3), recipients of syngeneic kidney grafts (n = 6), and recipients of kidney allografts tolerated by injection of anti-CD28 Abs (n = 7). Measurements were performed 3 mo posttransplantation. (C) Quantification by qRT-PCR of CCL5 mRNA in WBCs from which MDSC were removed ("non-MDSC" cells), prepared from syngeneic (n = 3) and tolerant (n = 4) recipients of kidney allografts. (D) Similar to (A), quantification by ELISA of CCL5 in the supernatant of cultured WBCs, from which MDSC were removed ("non-MDSC" cells), prepared from syngeneic (n = 4) and tolerant (n = 4) recipients of kidney allografts. *p < 0.05, **p < 0.01.
FIGURE 3. Intragraft CCL5 expression. (A) qRT-PCR measurement of CCL5 mRNA in kidney grafts 3 mo posttransplantation, from either syngeneic recipients (Syngeneic; n = 3), allogeneic recipients with the tolerizing protocol (Tolerant; n = 11), or allogeneic recipients with the tolerizing protocol plus implantation of osmotic pumps delivering rCCL5 (analyses 30 d postimplantation; Tolerant + rCCL5; n = 3). (B) Kidneys from naive LEW.1A rats (n = 4) or kidney grafts from either syngeneic recipients (Syngeneic; n = 4) or allogeneic recipients with the tolerizing protocol (Tolerant; n = 3) were collected 3 mo posttransplantation and homogenized for the evaluation of local CCL5 protein concentration. Concentration is expressed in ng/volume of kidney. (C) Ratios were calculated between the expression level of CCL5 protein in the kidney (Naive) or kidney grafts from syngeneic recipients (Syngeneic) or allogeneic recipients with the tolerizing protocol (Tolerant) and levels in the corresponding plasma. Ratios were calculated from data shown in Fig. 2B and (B). *p < 0.05.

Discussion

We previously reported on the role of peripheral MDSC in the maintenance of kidney-transplant tolerance in the rat mainly acting through the inducible NO enzyme (9). Although we noticed a synergy between MDSC and Treg to suppress effector T cells, it was not clear how these two cell types cooperated in vivo. Also, because MDSC from tolerant animals actually presented similar suppressive activities ex vivo compared with control MDSC, the extent to which MDSC from both origins differed from each other was unclear. In this study, we showed that the expression of several genes is significantly different between MDSC from tolerant kidney graft recipients compared with recipients of syngeneic grafts. Microarray analyses revealed two interesting features that could shed light on our previous results. First, the expression of LAT, a gene coding for a transmembrane adaptor protein (38), was significantly downregulated in MDSC from tolerant recipients. LAT is known to be expressed in thymocytes, mature T cells, NK cells, mast cells, megakaryocytes, and pre-B cells (39–41) and is an essential regulator of cell activation, terminal differentiation (42), and maturation (43). To our knowledge, the expression of LAT by MDSC had not been reported previously. Because MDSC are a heterogeneous population of immature cells that lose their suppressive function after maturation, the underexpression of a maturation factor, such as LAT, is not a surprising observation. It is possible that LAT plays a role in the control of MDSC maturation in tolerant animals, but further experiments will be needed to confirm this hypothesis.

The second feature was the modification in the expression of genes involved in apoptosis. mRNA for Bcl2i12 was upregulated 3-fold in MDSC from tolerant animals, according to the microarray data, and the upregulation was confirmed by qRT-PCR from independent samples. Bcl2i12 is an antiapoptotic gene member of the Bcl2 family of apoptosis-related genes (44). In addition, mRNA for Dapk2 was downregulated in MDSC from tolerant animals, and this was confirmed by qRT-PCR. The Dapk2 gene belongs to the serine/threonine protein kinase family that functions as a positive regulator of apoptosis (45). Again, the upregulation of an antiapoptotic gene and downregulation of a proapoptotic gene in MDSC from tolerant animals is compatible with their previously reported accumulation in tolerant animals (9), but a link between these observations would need further investigation.

In parallel, we observed that CCL5 was strongly repressed in MDSC from tolerant recipients, and this was confirmed by qRT-PCR and by ELISA. In addition, we found reduced levels of the CCL5 protein in the tolerant animals. CCL5 is a chemokine expressed commonly in inflammatory environments. It has a described immunomodulatory role in cancer, is also expressed and secreted in kidney glomeruli (46), and has been associated with renal transplant rejection or, alternatively, with long-term function (47, 48). In this study, CCL5 mRNA was found to be upregulated long-term posttransplantation (our experiments were performed 3–4 mo posttransplantation) in tolerant kidney allografts compared with syngeneic kidney grafts (Fig. 3A). However, statistical significance observed for CCL5 mRNA was not reached at the protein level, which could be due to differential regulation of CCL5 transcription and translation or to experimental variation. The intragraft overexpression of CCL5 mRNA might reflect a sustained inflammatory environment in tolerated kidney allografts, despite normal kidney function and indefinite allograft acceptance. In these allografts, it is possible that expression of CCL5 resulted in the attraction of Treg from peripheral blood, and this might be amplified by the 2-fold decrease in plasmatic CCL5 concentration. Indeed, when measuring CCL5 protein ratio between the graft and the plasma, we observed a solid and significant increase in this ratio in tolerant animals. The decrease in plasma CCL5 might be caused by a reduced production by MDSC, because CCL5 expression by other blood cell types than MDSC was not reduced (Fig. 2B). However, we cannot exclude the possibility that endothelial cells (49) and platelets (50), which also produce CCL5 in inflammatory conditions, significantly contribute to the regulation of plasma concentrations of CCL5. Another question was whether the plasma decrease in CCL5 is a general feature of transplant tolerance in the rat model. Our observation was that tolerance induction to a heart allograft in the same rat strain combination, induced by donor-specific transfusions (35), did not result in modifications of the plasma levels of CCL5. Interestingly, in this heart allograft model, no implication of MDSC was reported. Therefore, it is possible that the peripheral repression of CCL5 synthesis in the rat is a feature associated with kidney transplantation and with a role for MDSC in transplant tolerance.

FIGURE 4. Intragraft CCR5 and Foxp3 mRNA expression. qRT-PCR measurement of CCR5 (A) and Foxp3 (B) mRNA transcripts in kidney grafts collected 3–4 mo posttransplantation from recipients of syngeneic grafts (n = 3) or tolerant recipients of allografts (n = 11). Quantification was also performed 1 mo after treatment with rCCL5 in recipients of syngeneic grafts (n = 2) or tolerant recipients of allografts (n = 3). *p < 0.05.
Another question was how CCL5 potentially regulates transplant tolerance. Similar to the situation known in solid tumors (2, 3) we speculated that the chemokine CCL5 can recruit, into the graft, Treg that preferentially express CCR5 (25). However, CCR5 is also expressed in macrophages, activated and memory T cells, monocytes, and NK cells, and it is not clear why Treg should be pref-

FIGURE 5. Increased Treg infiltration in tolerated kidney allografts. Left panel, Confocal-like immunofluorescence microscopy analysis of kidney grafts from recipients of syngeneic grafts, recipients of tolerated allografts (anti-CD28 Ab treatment), or recipients of tolerated allografts after treatment with rCCL5-delivering osmotic pumps. Analyses were performed 3–4 mo posttransplantation. A rejected kidney allograft implanted in an untreated recipient is also shown. In that case, analyses were performed on day 7 posttransplantation. Photographs are representative of three to five experiments. Green color, TCR-β staining; red color, Foxp3 staining. Scale bar, 10 μm. Right panel, Quantification of TCR-β+Foxp3+ graft-infiltrating T cells was performed from the same animal samples (n = 3 animals) and in each case, five independent replicates (i.e., quantification performed from five immunohistology slides from a grafted kidney) were performed. *p < 0.05.

FIGURE 6. rCCL5 in vivo prevents induction of tolerance and brakes established tolerance after kidney transplantation. (A) Kidney graft survival in untreated rats (Untreated controls; n = 3), in rats treated with the modulating anti-CD28 JJ319 Ab (Anti-CD28; n = 5) or with the same regimen plus the implantation of an osmotic pump delivering 14 μg/kg/d rCCL5 for 14 d (Anti-CD28 + rCCL5; n = 3), or in rats grafted with a syngeneic kidney graft plus rCCL5 infusion (Syngeneic + rCCL5; n = 3). (B) Kidney function assessed once a week for 28 d by creatininemia measurement in Anti-CD28 + rCCL5 (n = 3) and Syngeneic + rCCL5 (n = 3) recipients. (C) Kidney allograft survival in recipient rats with established tolerance to a kidney allograft (n = 5) or in similar animals receiving an osmotic pump delivering rCCL5 (14 μg/kg/d) at 120 d posttransplantation (n = 3). Rat recipients of syngeneic grafts receiving the same rCCL5 administration at 120 d posttransplantation are also shown (Syngeneic + rCCL5; n = 3). (D) Kidney function assessed once a week for 28 d by creatininemia measurement in Anti-CD28 + rCCL5 (n = 3) and Syngeneic + rCCL5 (n = 3) recipients after implantation of osmotic pumps filled with rCCL5, 120 d posttransplantation. *p < 0.05, **p < 0.01.
erentially recruited. The fact that CCR5 blockade by antiagonist Abs or CCR5 deficiency in mice prolonged graft survival (51, 52) indeed indicated the importance of this receptor for the acute action of effector cells. In this study, kidney allograft biopsies showed a strong infiltration by TCR-β⁺ T cells with a high percentage of TCR-β⁺Foxp3⁺ Treg in tolerated allografts and a lower percentage during rejection (Fig. 5). In parallel, CCR5 and Foxp3 mRNA were elevated in tolerant allografts (Fig. 4). Together, our analyses indicated that, in tolerant kidney allografts, the majority of infiltrating T cells are Treg, whereas in rejected kidney allograft, Treg represent a small percentage of the overall infiltrate. Other infiltrating cells in this model are composed of macrophages, activated and memory T cells, monocytes, or NK cells that are involved in the rejection process (17). The hypothesis that Treg migrate to the tolerated allograft as a consequence of a blood-to-graft CCL5 gradient is compatible with our observations that restoring normal CCL5 plasma levels led to a strong reduction in intragraft TCR-β⁺Foxp3⁺ Treg infiltration and Foxp3 mRNA expression, an increase in creatinine and urea concentrations, and, ultimately, to kidney graft rejection. It is unlikely that administration of rCCL5 directly altered kidney graft function itself, because rats transplanted with syngeneic kidneys and receiving rCCL5 did not present kidney dysfunction. This control group indicated that a proinflammatory action of CCL5 was not directly involved in changes in creatinine and urea levels. In the literature, the impact of systemic CCL5 on graft outcomes is 2-fold: high CCL5 chemokine plasma levels were observed in chronic renal transplant dysfunction and chronic renal failure (53). In addition, CCL5 antagonists significantly attenuated the inflammatory response and delayed heart (54) and kidney (26, 27) allograft rejection in mice. However, after liver transplantation, high early systemic CCL5 levels were reported to play a protective role in immunological recognition leading to immune tolerance (55). Therefore, it is possible that CCL5 presents inflammatory, as well as tolerogenic, properties, depending on many environmental factors.

We observed a reduction in long-term CCL5 plasma levels posttransplantation after application of a tolerizing regimen based on the administration of a modulating anti-CD28 Ab. CD28 antagonist Abs have been used in several other transplant models in rodents and in primates and are thought to induce tolerance by selectively preventing CD28-mediated signals, while preserving CTLA-4–mediated regulatory signals, and, consequently, by inducing Treg (33, 56). In the heart allograft tolerance model induced by pretransplant DST, in which intragraft Treg were also reported (37), we detected no modification in blood CCL5, and administration of rCCL5 had no effect on heart graft survival. Therefore, establishment of a graft-to-periphery CCL5 gradient might be dispensable for intragraft localization of Treg after cardiac transplantation and might represent a special feature of our kidney transplant model. Indeed, Treg trafficking can also be controlled by other chemokines with redundant activities, such as CCL2, 3, 4, 7, 13, and 22 (57). For example, analysis of multiple chemokine pathways in a murine cardiac allograft model showed that recruitment of Treg and tolerance were dependent on the intragraft upregulation of CCR4 and one of its ligands, CCL22 (58).

In summary, our study shows that, in the anti-CD28 Ab tolerance-induction model, peripheral MDSC repress CCL5 gene expression in infiltrated kidney graft recipients, which contributes to the establishment of a graft-to-periphery gradient. This phenomenon contributes to the recruitment of Treg to the graft where they maintain tolerance. This novel role for MDSC in transplant tolerance might synergize with the suppressive action that these cells directly exert on effector T cells and contributes to long-term maintenance of tolerance. Our observations warrant further research to understand whether similar mechanisms can operate in other transplant models and in humans.

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Disclosures

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References

Plasma concentrations of cytokines/chemokines by Luminex assay.

Quantification of CCL2, CXCL1, IP-10, Eotaxin, IL-1a, IL-4, IL-6, IL-10, IL12p70, IL-17 and IL-18 protein levels by Luminex in plasma of recipients of syngeneic kidney grafts (n=3) or recipients of kidney allografts tolerized by injection of anti-CD28 antibodies (n=5). Measurements were performed 3 months after transplantation.
Recombinant rat CCL5 infusion restores *in vivo* CCL5 plasma level in tolerant recipients

Quantification of CCL5 protein levels by ELISA in plasma of recipients of syngeneic kidney grafts (n=2) or recipients of kidney allografts tolerized by injection of anti-CD28 antibodies (n=3). Measurements were performed once a week post-implantation of rCCL5 filled osmotic pump.
Recombinant rat CCL5 in vivo prevents induction of tolerance and brakes established tolerance.

(A) Kidney function assessed once a week for 28 days by plasma urea measurement in anti-CD28-treated recipients (n=3) and syngeneic recipients (n=3), treated with rCCL5 on day 0. 
(B) Kidney function assessed once a week for 28 days by plasma urea measurement in anti-CD28-treated recipients (n=3) and syngeneic recipients (n=3) after implantation of osmotic pumps filled with rCCL5, 120 days post transplantation. *, p≤0.05.
Recombinant rat CCL5 *in vivo* does not break established tolerance in heart allotransplanted recipients.

Heart allograft survival in untreated rats (Untreated controls; *n* = 3) or in rats treated with DST (DST; *n* = 3) or with the same regimen plus implantation on day 30 of an osmotic pump delivering 14 μg/kg/day rCCL5 for 14 days (DST + rCCL5; *n* = 3).