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Expression of CD44 and L-Selectin in the Innate Immune System Is Required for Severe Joint Inflammation in the Proteoglycan-Induced Murine Model of Rheumatoid Arthritis

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Proteoglycan (PG)-induced arthritis, a murine model of rheumatoid arthritis, is characterized by autoimmunity against mouse cartilage PG and chronic joint inflammation. L-selectin (CD62L) and CD44 are major adhesion molecules on leukocytes that regulate their homing to lymph nodes and entry into inflamed tissues. In the present study, we studied the requirement for CD44 and CD62L expression for mediating lymphocyte homing, thus permitting the development of autoimmunity vs mediating the entry of leukocytes into the joints, thus allowing inflammation in PG-induced arthritis. We immunized wild-type, CD44 knockout (KO), CD62L KO, and double (CD44/CD62L) KO BALB/c mice with PG and monitored the effects of gene deficiencies on PG-specific immunity, arthritis severity, leukocyte trafficking, and the ability of lymphocytes to adoptively transfer disease to syngeneic SCID mice. Single and double KO mice demonstrated reduced PG-specific spleen cell proliferation, but the production of Th cytokines and autoantibodies was comparable in KO and wild-type mice. KO leukocytes had reduced ability to adhere tightly to the synovial endothelium in arthritic joints. This diminished leukocyte adhesion correlated with the magnitude of granulocyte (neutrophil) influx and the severity of inflammation, which were both reduced in the joints of KO mice. However, transfer of spleen cells from mildly arthritic KO donors to SCID hosts resulted in development of severe arthritis. Our results indicate that CD44 and CD62L expression in the cells of the innate immune system (granulocytes) is important for their efficient influx into the joints and also suggest that granulocytes play a crucial role in arthritis progression. The Journal of Immunology, 2006, 177: 1932–1940.

Rheumatoid arthritis (RA) is a human autoimmune disease characterized by chronic inflammation of multiple joints, ultimately leading to cartilage and bone erosion and loss of joint function (1, 2). Proteoglycan (PG)-induced arthritis (PGIA) in BALB/c mice bears strong resemblance to RA in clinical and histopathological features, immunopathology, as well as genetic susceptibility and inheritance (3–5). PGIA develops in genetically susceptible strains (BALB/c or C3H) of mice upon i.p. immunization with human cartilage proteoglycan (hPG) aggrecan in adjuvant (3, 6). A Th1-polarized response to hPG in the immunized animals is followed by recognition of self-mouse PG (mPG), and the development of polyarthritis is preceded by, or coincides with, the appearance of mPG-specific autoantibodies in the circulation (4, 5, 7, 8). Locally, PGIA is characterized by influx of leukocytes into the synovial tissue and joint cavity, synovial lining cell proliferation, and invasion of cartilage and bone by synovial pannus (3, 5).

Sustained migration of leukocytes from the blood into the synovial tissue and cavity of multiple joints is a hallmark of the chronic inflammatory process in both PGIA and RA. Cells of both the adaptive and innate immune system are recruited to the joints; T and B cells are more frequent in the synovial tissue than in the joint cavity, whereas polymorphonuclear granulocytes (neutrophils) constitute the major cell population in the synovial fluid (2, 3, 5, 9–11). To gain entry into a target tissue, leukocytes implement a sequence of adhesive interactions with the vascular endothelium, collectively known as the “multistep paradigm” of extravasation (12). Leukocytes first transiently tether to, then roll on, the endothelium under blood flow. Next, chemokine-triggered activation of leukocyte integrins leads to firm adhesion of these cells to the endothelium so that they resist detachment from endothelial cells (13, 14). Finally, leukocytes migrate across the vessel wall into the tissue (12).

L-selectin (CD62L), the lymphocyte homing receptor (15), and CD44, the hyaluronan receptor (16), are two of the major adhesion molecules expressed by leukocytes of both lymphoid and myeloid origin (17). In addition to its established role in the homing of lymphocytes to lymph nodes (LNs), CD62L has been shown to mediate leukocyte rolling on inflamed endothelium (18–20). CD44 has been shown to function primarily by supporting leukocyte rolling at inflammatory sites (21, 22), but recent observations in CD44 gene knockout (KO) mice suggest that loss of CD44 might facilitate lymphocyte homing to peripheral LNs during inflammation (23, 24).

In a dermal delayed-type hypersensitivity model, it was shown that mice deficient in CD62L could not mount a response to epicutaneous immunization due to the inability of naïve lymphocytes to home to the skin-draining LNs where they could acquire memory and effector functions (18, 25). Expression of CD62L was also required for the prompt initiation of leukocyte migration into the
knee joint in Ag-induced arthritis, a nonautoimmune, monoarticular form of joint inflammation (26). In experimental allergic encephalomyelitis, transfer of wild-type (WT) macrophages to CD62L-deficient mice was necessary to cause myelin damage in the CNS (27). Previous work from our laboratory demonstrated that DBA/1 mice deficient in CD44 developed collagen-induced arthritis at reduced incidence and severity despite normal autoimmune responses to type II collagen (28). In contrast to CD62L KO lymphocytes that migrate poorly to LNs (18, 25), lymphocytes from CD44-deficient mice showed an increased propensity to home to LNs following immunization with type II collagen, bacterial superantigen (23), or injection with conalbumin (24). The role of CD62L in autoimmune arthritis has not been investigated. Because of the intricate regulatory pathways involved in the generation of autoimmunity to a joint (cartilage) component, it is expected that induction of a chronic autoimmune form of arthritis, such as PGIA, would be difficult in CD62L KO mice. We hypothesized that the LN homing defect (18) could be corrected, at least in part, by introducing CD44 deficiency into CD62L-null mice (i.e., creation of a double KO). “Restoration” of the adaptive immune system could then allow for induction of an autoimmune response to PG in CD44/CD62L double KO mice. Dual adhesion receptor deficiency is, on the other hand, expected to manifest in impaired leukocyte recruitment at the periphery (19, 21, 26), thus resulting in reduced inflammation and joint damage.

To dissect the requirement for CD44 and CD62L for the autoimmune vs the inflammatory response in PGIA, we generated double CD44/CD62L-deficient mice in the PGIA-susceptible BALB/c background and compared their Ag-specific T cell responses, autotaxinotides titers, and arthritis development to those in WT, CD44 KO, and CD62L KO mice upon immunization with hPG. Using intravital video microscopy (IVM), we also compared the recruitment of leukocytes to the ankle joints in WT and single and double KO mice and examined the relative involvement of leukocyte subpopulations in joint inflammation. In addition, we tested the ability of single and double KO lymphocytes to migrate to the secondary lymphoid organs of SCID mice and to adoptively transfer PGIA. We found that inflammatory, but not autoimmune, responses were compromised by the lack of CD44 and CD62L and that expression of these molecules in granulocytes was required for the development of severe arthritis.

Materials and Methods

**Mice**

WT BALB/c mice were purchased from the National Cancer Institute (NCI). Mice deficient in CD44 (in the DBA/1Jacf background) were generated by targeted gene disruption as described previously (28). CD62L-null mice (in the C57BL/6J background) (29) were purchased from The Jackson Laboratory. BALB/c mice lacking CD44 or CD62L were obtained by backcrossing into the BALB/c background, using a genomic marker-assisted method (5, 30), which generated BALB/c mice with the most complete BALB/c genetic makeup were mated with WT BALB/c females. Using this selection step before each backcross, full BALB/c background was attained faster (7–9 backcrosses) than with random cross-breeding (which usually requires >12 backcrosses). Full BALB/c background was confirmed by whole genome screening with 244 simple sequence-length polymorphic markers (30). Finally, CD44−/−, CD62L−/−, and CD44−/−CD62L−/− heterozygous males and females of pure BALB/c background were intercrossed, and offspring, homozygous for single and double gene mutations, were identified by genomic PCR (26). The phenotype of adult mice (cell surface CD44 and CD62L expression or lack thereof) was also confirmed by flow cytometry. Female SCID mice of BALB/c background (NCI/Cic.C.B-17/scid/scid), used for cell transfer experiments, were obtained from the NCI and were maintained under germfree conditions. All animal procedures were conducted under a protocol approved by the Institutional Animal Care and Use Committee of Rush University Medical Center.

**Induction of primary PGIA and adoptive transfer to SCID mice**

hPG was isolated from osteoarthritic cartilage, which was obtained from patients undergoing joint replacement surgery, and was provided through the Orthopedic Tissue, Transplant, and Implant Repository of Rush University Medical Center, with the approval of the Institutional Review Board. hPG was extracted from pooled cartilage samples described in our previous publications (3–6). WT, single KO, and double KO female BALB/c mice at 10–16 wk of age were immunized i.p. with 100 μg of hPG (measured as protein) in dimethyliodoacetadyl ammonium bromide adjoint four times at 3-wk intervals, according to an established protocol (5, 6). Adoptive transfer of PGIA to SCID mice was performed as described previously (31). In brief, each SCID mouse was injected i.p. with 5 × 107 spleen cells from donors with primary PGIA, along with 200 μg of hPG. Two weeks later, each mouse received 2.5 × 107 donor spleen cells and 100 μg of hPG. Animals were inspected for symptoms of arthritis after the third immunization and the first cell transfer for primary and adoptive PGIA, respectively. According to the presence of redness (erythema) and degree of swelling, the paws were visually scored on a scale of 0–4 per paw (0–16 per mouse). The severity of arthritis in each paw was determined as follows: 0, no swelling or redness; 1, mild swelling and erythema; 2, moderate swelling; 3, severe swelling; and 4, severe swelling with hardening of the periarthritic tissue (5, 6). The same investigator inspected and scored the paws twice a week in a blinded manner.

**Cell isolation, flow cytometry, and histology**

Blood was collected in heparin-containing tubes from anesthetized mice through retro-oral puncture. At sacrifice (wk 0, 8, or 11 of immunization), spleen and joint-draining axillary, brachial, inguinal, and popliteal) LNs were harvested, and single-cell suspensions were prepared. RBC in the spleen and blood samples were eliminated by hypotonic lysis. Cell counts and viability were determined by trypan blue exclusion. Ankle synovial fluid cells were collected after repeated flushing of the open joint cavity with PBS, followed by gentle scraping of the synovial intima with a pipet tip. Cells in LN, spleen, blood, or joint fluid samples were immunostained with fluorescently labeled mAbs against adhesion molecules, including CD44, CD62L, and P-selectin glycoprotein ligand 1 (PSGL-1), as well as to leukocyte subpopulation markers such as CD3, CD45RB220, Gr-1 (granulocytes) (BD Biosciences), and F4/80 (monocytes/macrophages) (Serotec). Nonspecific binding of the fluorescence-conjugated mAbs to FCRs was inhibited by preincubation of the cells with unlabeled mAbs to CD16 and CD32 (Fc block; BD Biosciences). Flow cytometry was performed using a FACSCalibur instrument and CellQuestPro software (BD Biosciences). The hind limbs of naïve or arthritic mice were dissected, fixed in formalin, decalcified, and embedded in paraffin. The tissue sections were stained with H&E.

**Assays of Ag (PG)-specific immune responses**

These assays were performed as described before (5–7, 31). In brief, spleen or LN cells were cultured in DMEM (Sigma-Aldrich) supplemented with 10% FBS (HyClone) in round-bottom 96-well plates (200 μl/well, in triplicate wells) with or without hPG (25 μg protein/ml). To determine hPG-specific cell proliferation, the cells were pulsed with [3H]thymidine (1 μCi/well) on the fourth day of culture and harvested on day 5. Radioisotope incorporation was measured by scintillation counting and expressed in counts per minute. PG-specific IL-2 production by spleen cells was determined using a CTLL-2 bioassay, where IL-2 bioactivity was measured in the supernatants after 24 h of culture. To determine the PG-specific production of IFN-γ and IL-4, spleen cells were cultured for 5 days using the same cell density and Ag dose as indicated for the proliferation assays. The concentration of IFN-γ and IL-4 in the culture supernatants was measured using ELISA (BD Biosciences).

PG-specific Abs in serum were measured using ELISA (6, 31). Briefly, mice were bled at defined time points, and serum samples were stored at −20°C. Maxisorp microplates (Nunc) were coated with hPG or mPG (Nunc) were coated with hPG or mPG (Nunc) at 10 μg/ml (in sodium carbonate coating buffer (pH 9.5)) overnight at 4°C. Fat-free milk was used to block nonspecific protein binding sites. Serially diluted serum samples and internal standard samples (pooled sera from arthritic mice with known anti-PG Ab titers) were incubated with the immobilized PGs, and plate-bound hPG- or mPG-specific Abs were detected using peroxidase-conjugated rabbit IgG against mouse IgG1 and IgG2a isotypes (Zymed Laboratories, respectively).

**Intravital video microscopy**

IVM on the mouse ankle synovium was performed as described previously (11). In brief, the mouse was anesthetized i.m. injection of a xylazine-ketamine mixture. The ankle was immobilized on a customized stage, and...
the Achilles tendon was cut off the calcaneus to expose the ankle synovial tissue to the intravital fluorescence microscope (model Eclipse E600FN; Nikon). The synovium of the open ankle was continuously superfused with warm PBS through a fine silicon tube attached to the objective lens. To visualize leukocytes in the circulation, the mouse was injected i.v. with 10 μg of rhodamine 6G (Molecular Probes), a cell-permeable fluorescent DNA-binding dye (11, 26). Leukocyte trafficking was recorded by streamline acquisition of images with a digital video camera (CoolSnap CF; RS Photometrics) fitted to the microscope. Blood vessel diameters were measured, and leukocyte adhesion behavior was analyzed offline using MetaVue software (Universal Imaging). Leukocytes present in postcapillary venules with diameters ranging from 20 to 50 μm were selected for analysis. The parameters measured were the number of rolling leukocytes per 10 μm (1 mm vessel perimeter per minute, rolling velocity (micrometer per second)), and the number of adherent leukocytes per 100 μm² of endothelial surface per minute (11, 26). For in vivo detection of CD44 and CD62L expression or the presence of granulocytes in the synovial vessels, mice were injected with fluorescence-conjugated mAbs against CD44, CD62L expression or the presence of granulocytes in the synovial vessels, and leukocyte adhesion behavior was analyzed offline using MetaVue software (Universal Imaging). Leukocytes present in postcapillary venules with diameters ranging from 20 to 50 μm were selected for analysis. The parameters measured were the number of rolling leukocytes per 10 μm (1 mm vessel perimeter per minute, rolling velocity (micrometer per second)), and the number of adherent leukocytes per 100 μm² of endothelial surface per minute (11, 26). For in vivo detection of CD44 and CD62L expression or the presence of granulocytes in the synovial vessels, mice were injected with fluorescence-conjugated mAbs against CD44, CD62L, or Gr-1 (4 μg mAb/mouse) instead of rhodamine 6G.

Lymphocyte trafficking assays

To compare the ability of WT and KO spleen cells to migrate to lymphoid or extra-lymphoid organs, we performed short-term mixed-cell homing assays (23, 28). First, lymphoid tissue (and homoeostasis) was reconstituted in the immunodeficient mice by i.p. injection of splenocytes (5 × 10⁷ cells) from immunocompetent WT or KO BALB/c mice with primary PGIA, as described for the adoptive transfer experiments. Two weeks later, the reconstituted SCID mice received a mixture of WT and KO spleen cells from arthritic donor mice after labeling the donor cells with stable cell-tracing fluorophores of different colors. WT spleen cells were labeled with either CellTracker Green (catalog no. C7025; 3 μM) or CellTracker Orange (catalog no. C2927; 5 μM) and KO cells with CellTracker Orange, according to the manufacturer’s instructions (Molecular Probes). The green fluorescent (WT) and “orange” (red) fluorescent (WT or KO) spleen cells were mixed at a 1:1 ratio, and a total of 2 × 10⁷ viable cells (with 100 μg of hPG) in a final volume of 150 μl was injected i.v. into each recipient. SCID mice previously reconstituted with WT spleenocytes received a mixture of red and green fluorescence-labeled WT donor cells, and those injected with KO splenocytes received a mixture of red KO and green WT donor cells (23). The hosts were killed 24 h later, and single-cell suspensions were prepared from peripheral blood, spleen, joint draining LNs, and ankle synovial fluid. Labeled donor cells in these suspensions, along with those in the original samples of mixed donor cells (before injection), were detected using two-color flow cytometry. The net ratios of red:green fluorescent donor cells that had migrated into SCID tissues were calculated after normalizing the actual ratios of red:green-labeled cells retrieved from the host’s LNs and spleen (R LN and R spleen, respectively) to the ratios of red:green donor cells in the mixtures before injection (R mix) (23). SCID mice injected with a 1:1 mixture of red and green fluorescent WT donor cells served as internal controls, where the migration efficiency of differentially labeled WT cells was expected to be identical.

Statistical analysis

Data were analyzed using one-way ANOVA (SPSS). The post hoc Dunnett’s t test was used to determine significant differences (p < 0.05) between the WT and any of the three KO groups of mice. For time course experiments conducted on the same sets of mice, repeated measures ANOVA was also performed to determine significant differences (p < 0.05) between the groups in the overall responses.

Results

The onset of PGIA is delayed in CD62L KO and CD44/CD62L double KO mice, and disease severity is reduced in CD44 KO, CD62L KO, and double KO mice

To determine whether expression of CD44 and CD62L was required for the development of PGIA, we immunized four genotypes (WT, CD44 KO, CD62L KO, and double KO) of BALB/c mice with hPG. To achieve maximum PGIA incidence and severity in all groups, mice were given four i.p. injections of hPG in adjuvant at wk 0, 3, 6, and 9. They were monitored for the onset of PGIA and the severity of arthritic symptoms until wk 11. As shown in Fig. 1A, the incidence of PGIA was reduced in all KO mice as compared with age-matched WT animals. CD62L KO and double KO mice also showed a delay in arthritis onset, but the majority (70–100%) of the mice developed arthritis by wk 11 regardless of genotype (Fig. 1A). The severity of PGIA was reduced in both single and double KO mice relative to WT (Fig. 1B). This difference was significant for the CD62L KO and double KO groups until the end of the observation period (wk 11) and for the CD44 KO group on wk 7, 9, and 10 (Fig. 1B).

It was very unlikely that genetic “contamination” (microchimerism remaining from the original backgrounds) accounted for the reduced incidence and severity of PGIA in KO BALB/c mice, as genome screening with 244 polymorphic markers (including those covering quantitative trait loci that control arthritis susceptibility, severity, and immune responses in PGIA (30)) detected no difference between the WT and any of the KO groups of mice (data not shown).

Ag-specific cell proliferation is reduced, but cytokine and Ab responses are normal in hPG-immunized CD44KO, CD62LKO, and double KO mice

CD62L has been shown to play a crucial role in LN homing and subsequent activation of lymphocytes upon contact with Ag (18–20). Therefore, the delay in arthritis development and reduced disease severity, particularly in mice lacking CD62L (Fig. 1B), could be the consequence of inadequate response to PG immunization due to impaired lymphocyte homing. We compared the magnitude and kinetics of immune responses in the four genotypes of mice by

**FIGURE 1.** Incidence and severity of primary PGIA in WT, CD44 KO, CD62L KO, and CD44/CD62L double KO BALB/c mice. Mice (n = 20/group) were immunized with hPG four times at 3-wk intervals and monitored for the incidence and severity of PGIA. Arrows indicate the third and fourth hPG injections given on wk 6 and 9, respectively. A, Incidence is expressed as the percentage of mice that developed arthritis. B, Disease severity (cumulative score) is the sum of paw inflammation scores divided by the number of arthritic mice. The results shown are the means ± SEM. The symbols denote significant differences (p < 0.05; Dunnett’s t test) between the WT and CD44 KO (*), WT and CD62L KO (**), and WT and double KO (†) groups at the defined time points. Repeated measures ANOVA indicated that the differences between the WT and KO groups were significant (p < 0.05) during the entire observation period.
measuring PG-specific LN and spleen cell proliferation, production of IL-2, IL-4, and IFN-γ by splenocytes in vitro, as well as serum levels of mPG-specific autoantibodies (both Th1- and Th2-polarized isotypes) at wk 0 (before immunization), wk 8 (2 wk after the third PG injection), and wk 11 (2 wk after the fourth PG immunization). The hPG-specific proliferation of spleen cells from all KO mice was significantly weaker than the proliferation of WT immunization). The hPG-specific proliferation of spleen cells from both wk 8 and 11 of immunization (Fig. 2A). LN cells from the immunized mice (including WT) generally gave very poor proliferative responses to in vitro stimulation with hPG; proliferation was essentially undetectable in all KO LN cultures at wk 8 but was detectable by wk 11 in CD44 KO LN cultures (Fig. 2A). IL-2 was produced in approximately equal amounts by hPG-stimulated splenocytes, with the highest levels in double KO cultures, but IL-2 could not be detected in the LN cell cultures (data not shown). PG-specific IFN-γ production by spleen cell cultures was comparable in all genotypes, and less IL-4 was found in the CD44KO and double KO than in the WT cultures at wk 8 but not at wk 11 (Fig. 2B). Serum levels of mPG-specific IgG2a and IgG1 autoantibodies were not reduced in KO mice; in fact, we found significantly elevated levels of the IgG2a isotype of these autoantibodies in serum samples of CD44 KO mice at both wk 8 and 11 and of the IgG1 isotype in the sera of CD62L KO mice at wk 11 of immunization (Fig. 2C). Taken together, except for a reduced proliferative response of spleen cells from all three groups of KO mice to in vitro Ag stimulation (which correlated well with the reduced severity of PGIA in these groups), adaptive immunity did not seem to be compromised in PG-immunized CD44 KO, CD62L KO, and double KO mice.

Leukocytes show altered adhesion behavior in the inflamed synovial vessels of KO mice

As both CD44 and CD62L have been shown to support leukocyte rolling (18, 20, 32–34), the reduced severity of arthritis, observed in mice lacking one or both of these receptors (Fig. 1B), could also be attributed to impaired leukocyte rolling and subsequent recruitment in the target organs. We performed IVM on mouse ankle joints (11) to determine whether the adhesion behavior of leukocytes in the synovial postcapillary vessels of ankle synovium were different in the four genotypes of mice. Both nonarthritic and arthritic animals were used for IVM experiments. In the absence of CD44 or CD62L or both, we expected the number of leukocytes that rolled on synovial endothelium to decrease, and rolling velocity to rise, as a result of loss of one or two major receptors that can mediate rolling. However, the number of rolling cells was not reduced in the synovial vessels of arthritic CD44 KO and CD62L KO ankles and was even significantly elevated in double KO, compared with WT mice (Fig. 3A). The velocity of rolling cells was significantly higher in double KO than WT mice (Fig. 3B). A compensatory increase in the circulating pool of leukocytes expressing PSGL-1, another adhesion molecule that mediates rolling via endothelial or platelet selectin binding (35), could explain the increased propensity of double KO cells to roll, but we found little evidence for such compensation (the average proportion of PSGL-1+ cells was 31% in WT and 39% in double KO blood). Although the percentage of leukocytes expressing CD62L was lower in CD44 KO than in WT mice (11 and 22%, respectively), the absence of CD44, combined with low CD62L expression in CD44 KO cells, did not result in increased rolling interactions (Fig. 3A) or elevated rolling velocity (Fig. 3B). Importantly, the number of firm adherent cells was reduced in the vessels of all KO mice and was significantly lower in CD62L and double KO than in WT mice (Fig. 3C). The difference between WT and KO leukocytes, with respect to firm adherence to endothelium, remained consistent when WT and KO ankle joints with similar arthritis scores were compared (data not shown).

CD44- and CD62L-expressing cells of the innate immune system contribute to the development of severe PGIA

PGIA can be adoptively transferred to syngeneic SCID mice using spleen cells isolated from BALB/c donors with primary PGIA (31). SCID mice lack functional T and B cells, but they possess an intact innate immune system. Therefore, lymphocyte transfer to SCID mice could provide help in determining whether expression of CD44 and CD62L in the innate or adaptive arm of the immune system is more important for development of a severe form of adoptive PGIA. If expression of CD44 and/or CD62L in the adaptive immune system is required for severe disease, then SCID mice reconstituted with splenocytes from CD44 KO, CD62L KO, or double KO donors with low-severity PGIA are expected to develop mild arthritis. However, SCID mice that received spleen cells from arthritic KO donors all developed PGIA promptly (Fig. 4A) and with similar or even higher severity (Fig. 4B) than their

**FIGURE 2.** PG-specific immune responses in WT, CD44 KO, CD62L KO, and double KO BALB/c mice. A, hPG-specific proliferation of spleen cells (left graph) and joint-draining LN cells (right graph) were assayed before (wk 0) and after immunization with hPG (wk 8 and 11) using [3H]thymidine incorporation as described in Materials and Methods (n = 3–9 mice/group/time point). B, The concentrations of IFN-γ (left graph) and IL-4 (right graph) were measured in hPG-stimulated spleen cell culture supernatants by ELISA (n = 6 mice/group/time point). C, The amounts of mPG-specific IgG2a (left graph) and IgG1 (right graph) autoantibodies in serum samples (collected at wk 0, 8, and 11) were determined using ELISA (n = 10–15 mice/group/time point). The error bars represent SEM. The symbols are as described in the legend of Fig. 1 and denote significant differences between the WT and the KO groups.
counterparts reconstituted with cells from arthritic WT donors. This observation suggested that the development of adoptive disease was not hampered by the reduced ability of KO donor spleen cells to proliferate in the presence of PG (Fig. 2A) and that host cells to proliferate in the presence of PG (Fig. 2A), as described in Materials and Methods. Similar numbers of WT and KO ankles were compared at each level of inflammation (score 1 through score 4). IVM experiments were scheduled according to the onset and progression of ankle inflammation in the four genotypes of mice and were completed in all groups by wk 16 of immunization. Results are expressed as the means ± SEM (n = 20–23 mice/group). Asterisks indicate significant differences (p < 0.05; Dunnett’s t test) between the WT and KO mice.

“Arthritogenic” spleen cells home to the LNs in a CD62L-dependent manner and to the spleen in a CD62L-independent manner but are undetectable in the joint fluid of SCID mice

As spleen (not LN) cells were used for the transfer of PGIA from arthritic donors to SCID mice, we could not rule out the possibility that CD62L-independent occupancy of the SCID LNs by the splenocytes accounted for the induction of severe disease, particularly in animals injected with cells from CD62L-deficient or double KO donors. Furthermore, spleens from arthritic donors could contain an “effector” population of either lymphoid or nonlymphoid cells, capable of migrating to the joints directly. To investigate these possibilities, we conducted in vivo trafficking assays by transferring fluorescence-labeled spleen cells from arthritic WT and KO donors into SCID mice. As described in Materials and Methods, WT donor splenocytes were labeled with a green fluorophore and mixed at 1:1 ratio with red fluorescence-labeled cells from each genotype of the KO donors, which allowed for simultaneous detection of WT and KO cells upon their retrieval from the SCID organs 24 h after transfer. As shown in Fig. 5, A and B, splenocytes from CD44 KO donors migrated significantly better than WT cells to the LNs of SCID hosts, whereas LN homing of CD62L KO cells was impaired severely. Because CD44 KO splenocytes demonstrated enhanced LN occupancy, it was expected that CD44 deficiency could compensate for this homing defect of CD62L-null cells. This was not the case, however, as splenocytes lacking both CD62L and CD44 showed very modest improvement in LN homing compared with spleen cells from
in the SCID LNs and spleen were detected by two-color flow cytometry 24 h later. The net ratio of WT and KO cells in the LNs or spleen was differentially labeled cells were mixed and injected into the SCID hosts as described in Materials and Methods. Green and orange (red) fluorescent donor cells in the SCID LNs and spleen were detected by two-color flow cytometry 24 h later. The net ratio of WT and KO cells in the LNs or spleen was calculated from the ratio of red:green cells in the retrieved (Rln or Rsp) and injected (Rinj) samples. Data shown are means ± SEM (n = 6–7 SCID mice/group). The asterisks indicate significant differences (p < 0.05; Dunnett’s t test) between the net ratios of retrieved WT and KO donor cells. B and D, Representative samples of the flow cytometry profiles of labeled donor cells in the cell suspensions of SCID LNs and spleens, respectively.

CD62L single KO mice (Fig. 5, A and B). In contrast to LNs, the spleen was occupied with similar efficiency by WT and KO donor splenocytes, although relative to WT cells, the ratio of CD44 KO cells was slightly decreased, and the ratios of CD62L and double KO cells were increased in the spleens of SCID mice (Fig. 5, C and D). We were unable to detect fluorescent donor cells in the ankle synovial fluid of SCID mice 24 h (or even 72 h) after transfer (data not shown).

The severity of arthritis is determined by the abundance of innate immune cells (granulocytes) in the inflamed joints

As described above, severe PGIA developed promptly in SCID mice after transfer of KO spleen cells (Fig. 4B), although the LNs were poorly populated in mice reconstituted with cells from CD62L KO or double KO donors (Fig. 5), and arthritogenic donor splenocytes did not seem to migrate to the joint cavity. On the other hand, following transfer of unlabeled spleen cells from double KO donors to SCID mice, leukocytes expressing CD44 and CD62L (obviously of host origin) were detected by IVM in the synovial vessels of inflamed SCID joints (Fig. 4C). These observations indicated that innate immune cells significantly contribute to the severity of joint inflammation in PGIA. The most likely candidates are the granulocytes because these cells (mainly neutrophils) are present in large numbers in the joints of mice with PGIA (3, 5, 11) and other models of immune-mediated arthritis (26, 36), as well as in the inflamed joints of RA patients (37). Therefore, we investigated the relationship between granulocyte influx and disease severity, first focusing on PGIA in WT mice. IVM following in vivo immunostaining for Gr-1 revealed the presence of very few (mostly rolling) granulocytes in the synovial venules of noninflamed ankles (score: 0), but the number of cells that interacted with the endothelium increased in correlation with the progression of arthritis (from score 1 to 3) in these joints (Fig. 6A). The relative number of granulocytes in the joint fluid followed a similar pattern: very few Gr-1+ cells were detected by flow cytomtery in the fluid of noninflamed ankles, but the proportion of granulocytes "skyrocketed" with arthritis progression (Fig. 6B). This trend was also observed on histological sections of the ankles: as inflammation advanced, more and more polymorphonuclear leukocytes accumulated in both the synovial tissue and the joint cavity of the affected ankles (Fig. 6C). When comparing the cellular composition of synovial fluid cells from WT and KO joints of matching inflammation scores, we found that the proportions (%) of Gr-1+ cells were somewhat lower in KO than WT joints at a low inflammation score but were similarly high (>90%) at an advanced degree of inflammation (Fig. 6D). T and B cells and monocytes/macrophages were minor constituents of the synovial fluid exudate in both WT and KO mice, and, in contrast to granulocytes, these mononuclear cell populations did not show much change in size during arthritis progression (data not shown).

Discussion

As in RA (1, 2, 38, 39), cells of both the adaptive and the innate immune systems are involved in the initiation and perpetuation of chronic joint inflammation in PGIA (6). Autoimmunity to mouse PG in BALB/c mice develops after an induced response to a heterologous cartilage Ag (hPG) (3, 5, 6). Recognition of hPG in the adaptive immune system and breakdown of tolerance to a self-cartilage component (mPG), which both occur in the secondary lymphoid organs (LNs and spleen), are followed by the sustained migration of effector cells into the target organs (joints). Leukocyte entry to LNs and inflammatory sites has been shown to depend on the expression of adhesion molecules, including CD62L and CD44 (18–23). In this study, we used BALB/c mice lacking CD44, CD62L, or both receptors to elucidate the requirement for these adhesion molecules for lymphocyte migration to the lymphoid organs (and subsequent generation of the immune response), as well as for leukocyte entry into the joints in PGIA.

As expected, the incidence and severity of arthritis were reduced in the mutant animals as compared with WT mice, and resistance
Localization and abundance of granulocytes in noninflamed and inflamed ankles of mice with primary PGIA. A, Gr-1<sup>+</sup> cells were visualized in the ankle synovial vessels by IVM following in vivo labeling of blood leukocytes with fluorescence-conjugated anti-Gr-1 mAb. The IVM snapshots (column A) show granulocytes (Gr-1<sup>high</sup> cells) interacting with endothelium in a noninflamed ankle (score: 0, top panel), a mildly inflamed (score: −1, middle panel), or a severely inflamed (score: −3, bottom panel) ankle joint in WT mice. (Note that the video camera captures rolling or adherent granulocytes but not cells freely flowing in the bloodstream.) B, Granulocytes were also identified in the synovial fluid of WT ankles by in vitro immunolabeling and flow cytometry. The top, middle, and bottom graphs show the percentage of Gr-1<sup>+</sup> cells in the joint fluid of noninflamed, mildly inflamed, and severely arthritic ankles, respectively. Synovial fluid samples were harvested and pooled from at least three ankle joints at each level of inflammation. C, Histopathology of ankles from WT mice illustrates the abundance and distribution of inflammatory cells in the joint compartments at different clinical scores. Very few leukocytes are seen at score 0. Infiltration of both the synovial tissue (St) and joint cavity (Jc) by leukocytes rises with increasing joint swelling (from score −1 to −3). High-magnification insets demonstrate that the majority of infiltrating cells in both mildly and severely inflamed joints are neutrophil granulocytes (showing segmented nuclei). H&E staining, Bo: bone (calcaneus). Scale bars, 250 μm (50 μm for the insets). D, Percentage of granulocytes in the synovial fluid of ankle joints from WT, CD44 KO, CD62 KO, and double KO mice at different levels of inflammation. Synovial fluid cells were harvested (n ≥ 3 ankles-score/group) and pooled according to score and genotype. The pooled cells were labeled with anti-Gr-1 mAb and analyzed by flow cytometry. The data shown are from two experiments and indicate the mean percentage of Gr-1<sup>+</sup> cells in pooled synovial fluid samples of WT and KO ankle joints.

FIGURE 6. Localization and abundance of granulocytes in noninflamed and inflamed ankles of mice with primary PGIA. A, Gr-1<sup>+</sup> cells were visualized in the ankle synovial vessels by IVM following in vivo labeling of blood leukocytes with fluorescence-conjugated anti-Gr-1 mAb. The IVM snapshots (column A) show granulocytes (Gr-1<sup>high</sup> cells) interacting with endothelium in a noninflamed ankle (score: 0, top panel), a mildly inflamed (score: −1, middle panel), or a severely inflamed (score: −3, bottom panel) ankle joint in WT mice. (Note that the video camera captures rolling or adherent granulocytes but not cells freely flowing in the bloodstream.) B, Granulocytes were also identified in the synovial fluid of WT ankles by in vitro immunolabeling and flow cytometry. The top, middle, and bottom graphs show the percentage of Gr-1<sup>+</sup> cells in the joint fluid of noninflamed, mildly inflamed, and severely arthritic ankles, respectively. Synovial fluid samples were harvested and pooled from at least three ankle joints at each level of inflammation. C, Histopathology of ankles from WT mice illustrates the abundance and distribution of inflammatory cells in the joint compartments at different clinical scores. Very few leukocytes are seen at score 0. Infiltration of both the synovial tissue (St) and joint cavity (Jc) by leukocytes rises with increasing joint swelling (from score −1 to −3). High-magnification insets demonstrate that the majority of infiltrating cells in both mildly and severely inflamed joints are neutrophil granulocytes (showing segmented nuclei). H&E staining, Bo: bone (calcaneus). Scale bars, 250 μm (50 μm for the insets). D, Percentage of granulocytes in the synovial fluid of ankle joints from WT, CD44 KO, CD62 KO, and double KO mice at different levels of inflammation. Synovial fluid cells were harvested (n ≥ 3 ankles-score/group) and pooled according to score and genotype. The pooled cells were labeled with anti-Gr-1 mAb and analyzed by flow cytometry. The data shown are from two experiments and indicate the mean percentage of Gr-1<sup>+</sup> cells in pooled synovial fluid samples of WT and KO ankle joints.

How could CD62L KO mice mount a globally “normal” (auto)immune response in the face of a LN homing defect? The answer most likely lies in the systemic (i.p.) route of Ag administration used to induce PGIA. As confirmed by our cell transfer results in the present study, CD62L is required for LN homing, but it is not necessary for lymphocyte entry into the spleen (40). The requirement for CD62L for acquisition of full immunological memory was therefore bypassed by the i.p. route of immunization, allowing for Ag delivery to the spleen, which lymphocytes could enter in a CD62L-independent fashion. The weak LN (vs strong spleen) immune responses in the PG-injected mice suggest a greater contribution of the spleen than of the LNs to the generation of immunological memory, but the best evidence for the prominent role of the spleen in PGIA development is the ability of splenocytes from arthritic BALB/c donors to transfer disease to immunodeficient mice (8, 31). In the present study, spleen cells from WT, CD62L KO, CD44 KO, and double KO BALB/c mice were equally prompt and effective in inducing severe arthritis upon transfer to SCID mice. This suggested that in PGIA, the spleen was capable of gathering lymphocytes and APCs in a CD62L- and CD44-independent manner, as well as providing a tissue microenvironment for the assembly and function of the adaptive (auto)immune system. Furthermore, the cell transfer studies indicated that splenocytes from PG-immunized CD62L KO and double KO donors that readily populated the spleen, but migrated poorly to the LNs of SCID mice, were still potent in “setting the stage” for severe joint inflammation in the SCID host. This raised the possibility that the failure of these arthritogenic spleen cells to trigger severe disease in the primary form of PGIA could be due to inefficient recruitment of KO effector cells to the inflammatory site.
Using IVM, we found that leukocytes in the synovial venules of double KO mice with primary PGIA exhibited significantly increased rolling interactions, elevated rolling speed, and a reduced ability to adhere firmly to endothelium as compared with leukocytes in WT mice. The simplest explanation for the increased proportion of rolling cells in double KO mice is that leukocytes, which interacted with the endothelium but failed to adhere, kept rolling. In this respect, the lack of CD62L and CD44 function could have been compensated for by PSGL-1 and perhaps also by “partially activated” integrins that are able to support rolling (41). It is likely that double KO cells were more easily carried away in a fast rolling motion by the shear force of the blood flow than their better-equipped WT or single KO counterparts. Fast rolling, in turn, shortened the exposure of leukocytes to chemokines, thus lowering the rate of integrin-mediated arrest and firm adhesion (14). In the synovial microcirculation of inflamed joints, all KO leukocytes demonstrated decreases in firm adhesion compared with WT cells in the order of double KO < CD62L KO < CD44 KO < WT, which correlated quite well with the severity of primary PGIA in these groups of mice. As firm adhesion is a committed step that is usually followed by transendothelial migration (12), decreased leukocyte adherence could lead to reduced extravasation and less severe inflammation. As noted above, spleen cells from these mildly arthritic KO donors were able to trigger severe arthritis in SCID mice, suggesting that the bulk of leukocytes migrating into the joints in primary PGIA did not represent the cells of the adaptive immune system.

We showed that leukocytes expressing CD44 and CD62L were being recruited in the synovial venules of SCID recipients after transfer of splenocytes from double KO donors, thus providing in vivo evidence for the participation of host-derived innate immune cells in the local inflammatory process. Furthermore, in primary PGIA, we demonstrated a positive relationship between the degree of inflammation and the abundance of granulocytes (neutrophils) that interacted with synovial endothelium and subsequently migrated into the synovial tissue and joint cavity. Although T and B cells, as well as macrophages, could be identified in both noninflamed and inflamed joints, these cells were present in low proportions, and their numbers did not increase markedly with arthritis progression. Collectively, these results suggest that in both the primary and adaptive forms of PGIA, the severity of inflammation largely depends on the abundance of innate immune cells (granulocytes) in the joint. We also found that the ankle joint fluid of KO mice contained fewer granulocytes than the synovial fluid of WT mice at a low degree of inflammation in primary PGIA, but KO granulocytes “caught up” with WT cells in ankle joints at an advanced stage of inflammation. These results, together with the IVM data, suggest that the lack of CD62L and CD44 expression lowers the rate of granulocyte influx into the joints, thereby delaying the onset and slowing down the progression of PGIA. However, as we have shown earlier in WT mice with PGIA (11), at an advanced stage of inflammation (score ~3), the joints become saturated with inflammatory cells, leading to a decline in the rate of leukocyte extravasation. Thus, with slowly continuing recruitment, KO granulocytes could catch up and eventually infiltrate the joints to the same extent as WT cells did. This was supported by the observation that the severity of primary PGIA, although reduced initially in CD44 KO, CD62L KO, and double KO mice, reached a degree similar to WT by wk 15 of immunization (B. Sarraj and K. Mikecz, unpublished data). Adoptively transferred PGIA developed and progressed in a WT-like fashion in SCID mice reconstituted with KO spleen cells, suggesting that CD44 and CD62L, expressed in the hosts’ innate immune system, facilitated leukocyte recruitment. Therefore, the innate immune cells that contributed mostly to disease severity must have been neutrophil granulocytes from the SCID mice. Using IVM after in vivo coadministration of fluorescent mAbs to Gr-1 and CD44, we found that the majority of leukocytes interacting with the synovial endothelium of SCID mice (which developed arthritis upon transfer of unlabeled double KO spleen cells) coexpressed Gr-1 and CD44 (B. Sarraj and K. Mikecz, unpublished results), indicating that most of the cells recruited in the inflamed joints were indeed host-derived granulocytes.

Murine neutrophils lacking CD62L (42) or CD44 (43) have been shown to exhibit impaired recruitment in vivo in response to, for example, local stimulation with proinflammatory cytokines such as TNF-α or chemokines. The requirement for CD62L (20) or CD44 (22) for T cell migration into inflammatory sites has been proposed but not firmly established. Because of the paucity of T cells that extravasate in the joint in PGIA (11), it is difficult to ascertain whether expression of these adhesion receptors is necessary for the migration of T lymphocytes to this site. Our adoptive transfer study suggests that, if CD62L and/or CD44 had been critical for the entry of activated (arthritogenic) T cells into the joint, inflammation would have been reduced in SCID mice reconstituted with lymphocytes lacking one or both of these adhesion molecules, but this was not the case.

Taken together, neutrophil granulocytes emerge as the major inflammatory cell population reliant on the expression of CD62L and, to a lesser extent, of CD44 for efficient influx into the joint in PGIA. In RA, neutrophils emigrate from postcapillary venules located in the synovial sublining area into the joint cavity in quite large numbers, causing severe damage to the cartilage matrix (9, 37, 44). Among other factors, elevated serum concentrations of TNF-α, and the presence of autoantibodies-containing complement-fixing immune complexes in the joint, have been implicated in neutrophil recruitment in RA (39). As demonstrated by in vivo studies, TNF-α-activated vascular endothelium promotes neutrophil recruitment (45), and autoantibodies-containing immune complexes (e.g., serum from arthritic K/BxN mice) preferentially localize to the joints and initiate neutrophil-dependent arthritis upon transfer to naive mice (36, 46). These finding suggest that TNF-α and autoantibodies, which are mainly (TNF-α) or exclusively (autoantibodies) produced by adaptive immune cells, can access the joint and attract granulocytes, thereby effectively connecting the adaptive and innate arms of the immune system in susceptible individuals. Therefore, the clinical efficacy of TNF-α- and B cell-targeting therapies in RA can be, in part, attributed to inhibition of neutrophil recruitment by reducing the interactions between adaptive and innate immunity (47, 48). Agents that antagonize CD62L and CD44 function, downstream of these interactions, could provide further aid in the therapeutic effort to suspend neutrophil influx into the joints in RA.

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Disclosures
The authors have no financial conflict of interest.

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