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Cutting Edge: The Silent Chemokine Receptor D6 Is Required for Generating T Cell Responses That Mediate Experimental Autoimmune Encephalomyelitis

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D6, a promiscuous nonsignaling chemokine binding molecule expressed on the lymphatic endothelium, internalizes and degrades CC chemokines, and D6+/− mice demonstrated increased cutaneous inflammation following topical phorbol ester or CFA injection. We report that D6+ mice were unexpectedly resistant to the induction of experimental autoimmune encephalomyelitis due to impaired encephalitogenic responses. Following induction with myelin oligodendroglial glycoprotein (MOG) peptide 35–55 in CFA, D6−/− mice showed reduced spinal cord inflammation and demyelination with lower incidence and severity of experimental autoimmune encephalomyelitis attacks as compared with D6+/+ littermates. In adoptive transfer studies, MOG-primed D6+/− T cells equally mediated disease in D6+/+ or D6−/− mice, whereas cells from D6−/− mice transferred disease poorly to D6+/+ recipients. Lymph node cells from MOG-primed D6−/− mice showed weak proliferative responses and made reduced IFN-γ but normal IL-5. CD11c+ dendritic cells accumulated abnormally in cutaneous immunization sites of D6−/− mice. Surprisingly, D6, a “silent” chemokine receptor, supports immune response generation.

C onventional chemokine receptors are seven-transmembrane single polypeptide chains whose intracellular domains couple to heterotrimeric G proteins to mediate agonist responses and to β-arrestins for internalization. These receptors are required for the chemotactic and activating responses of leukocytes to the chemokine ligands. In addition to classical receptors, there are at least three chemokine-binding molecules that have moderate to high homology to classical receptors but lack G protein coupling motifs and are incapable of eliciting chemotactic or activating responses to a ligand (1). Two of these “silent” receptors, D6 and the Duffy Ag receptor for chemokines (DARC),3 have been characterized in some detail (1). The biological functions of DARC, which is expressed on erythrocytes and postcapillary venules, seem to be 2-fold: to transfer chemokines from parenchymal sites of synthesis across endothelial barriers and to provide a binding “sink” for chemokines in the circulation. Based on in vitro observations, D6 was proposed as a chemokine-scavenging receptor, functioning to aid in the resolution of inflammatory reactions. D6 is selectively expressed on the lymphatic endothelium. Upon transfection into lymphatic endothelial cells or human embryonic kidney cells (2, 3), D6 shuttles rapidly between plasma membrane and early endosomes independently of ligand engagement; consistent with these findings, D6 associated constitutively with β-arrestins (4). After binding its ligands, which include >12 inflammatory CC chemokines, D6 delivers these components to sites of intracellular degradation. As predicted by the scavenger receptor paradigm for D6, mice that lacked D6 exhibited markedly increased cutaneous inflammatory reactions in two distinct models (5, 6). Following phorbol ester painting, D6−/− mice showed remarkably sustained persistence of its inflammatory chemokine ligands along with psoriasiform skin changes that included skin thickening, hyperkeratosis, marked inflammatory infiltrates, and angiogenesis (5). The pathology was reversed by blocking chemokine availability with neutralizing Abs or by depleting T cells or abrogating TNF signaling, indicating its inflammatory and chemokine-dependent nature. Upon s.c. challenge with CFA, the D6−/− mice showed many of these features, including increased inflammatory infiltrates, but without hyperkeratosis (6). D6−/− mice that received CFA showed increased amounts of CC chemokines and increased cellularity in the draining lymph nodes, suggesting an enhanced flux of chemokines across the lymphatic vessels. In this regard, chemokines are transported from local inflammatory sites to draining lymph nodes and mediate the recruitment of circulating monocytes across high endothelial

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3 Abbreviations used in this paper: DARC, Duffy Ag receptor for chemokines; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; MOG, myelin oligodendroglial glycoprotein; p.i., post immunization.

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venules (7). Although chemokines clearly orchestrate this “remote control” process, its physiological importance in establishing adaptive immune responses remains completely defined.

In the current study we addressed two questions. 1) Would enhanced inflammation in the skin of D6+/− mice affect the induction of experimental autoimmune encephalomyelitis (EAE) by s.c. immunization? 2) Would reduced potential clearance of chemokines from the inflamed CNS worsen the severity of EAE? Surprisingly, we found that D6+/− mice were relatively resistant to the induction of EAE. These results widen the functional spectrum of nonsignaling chemokine receptors and suggest that the orchestrated delivery of chemokines to draining lymph nodes or their clearance from immunization sites may play an important role in the generation of immune responses.

Materials and Methods

Mice

The generation and genotyping of D6-deficient mice has been described previously (5). D6-deficient mice had been backcrossed to C57BL/6J (B6) mice for 12 generations, and D6+/− mice were intercrossed to obtain D6−/+ , D6+/− , and D6−− mice. In all experiments, age- and sex-matched littermate cohorts at 8–10 wk of age were used. All mice were housed under pathogen-free conditions in the animal facility at the Cleveland Clinic Foundation, Cleveland, OH. All protocols for animal research met the requirements of the Animal Research Committee of the Cleveland Clinic Foundation and were in compliance with the Public Health Service policy on humane care and use of laboratory animals.

Induction and analysis of EAE

Active immunization. Induction of EAE was performed as previously described (8). Briefly, mice were s.c. injected at two sites with 100 μg of rat myelin oligodendroglial glycoprotein (MOG) peptide 35–55 (MEVGYWRSPFSRVHYLIRNGK; >95% purity) (Bio-Synthesis) emulsified in CFA containing 400 IU mycobacterial lipoprotein (DncCo laboratories). On the same day (day 0) and on day 2 postimmunization (p.i.), mice were i.v. injected with 200 ng of pertussis toxin (Sigma-Aldrich). All mice were weighed, examined, and graded daily for neurological signs in a blinded manner as follows: 0, no disease; 1, decreased tail tone or slightly clumsy gait; 2, tail atony and moderately clumsy gait and/or poor righting ability; 3, limb weakness; 4, limb paralysis; and 5, moribund state. The day of EAE onset was calculated by adding the first day of clinical signs for individual mice and dividing by the number of mice in the group. The day of peak EAE was calculated by determining the first day of maximum EAE score for individual mice and dividing by the number of mice in the group. Mean maximum score was calculated by adding peak scores of individual mice and dividing by the number of mice. Cumulative EAE score was calculated by adding total EAE scores from onset until day 22 p.i. for individual mice and dividing by the number of mice. Active immunization with MOG peptide induced spontaneous EAE in B6 mice and was followed for 22 days. Animals were euthanized if scores were worse than grade 4.

Adoptive transfer. To prepare encephalogetic cells for adoptive transfer of EAE, mice were immunized with MOG/CFA in the same fashion as for active EAE. Splenies and lymph nodes were collected 8 days p.i., single cell suspensions were prepared, and RBCs were lysed. Cells (6 × 10^6 cells/ml) were cultured in RPMI 1640 medium (supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin/streptomycin, and 2 × 10−5 M 2-ME (Invitrogen Life Technologies) with MOG35−55 (20 μg/ml) and IL-12 (30 ng/ml) (R&D Systems). Three days after initiation of culture, the cells were harvested, washed in PBS, and injected into recipient mice that were irradiated sublethally (500 rad) within 16 h before cells injection. All mice were weighed, examined, and graded daily after cell transfer (9).

T cell recall responses: proliferation and cytokine production

T cell proliferation assays and quantification of cytokine production were performed as previously described (8). For T cell proliferation, draining lymph node cells from MOG35−55-immunized mice were harvested 8 days p.i., and incubated in 96-well plates (1 × 10^5 per well) with MOG35−55 or Con A (Sigma-Aldrich) at the indicated concentration or in medium alone. During the final 16 h of a total 72 h culture, cells were pulsed with 1 μCi/well [3H]thymidine (Amersham Biosciences); plates were harvested using a Tomtec harvester and analyzed with a 1450 Wallac Microbeta TriLux liquid scintillation and luminescence counter.

For quantification of cytokine production, draining lymph node cells isolated from MOG35−55-immunized D6−/+ or D6+/− mice were incubated (5 × 10^5 cells/ml) in 24-well plates with MOG35−55 (20 μg/ml). After 48 h, supernatants were collected for cytokine detection by ELISA kits for IFN-γ and IL-5 were obtained from R&D Systems. A standard curve was generated with each assay. All samples were measured in duplicate and diluted if necessary.

Histological and immunohistochemistry

Histological and immunohistochemical analysis of spinal cords at different stages of EAE was done as previously described (8) using rat anti-mouse CD45 mAbs at 1/2000 dilution (clone MCA 1388; Serotec) and rabbit anti-human myelin basic protein (MBP) at 1/4000 dilution (DakoCytomation). Fresh skin tissues from CFA/MOG peptide-injected mice at day 3 or day 8 p.i., both ipsilateral and contralateral to the injection site, were dissected and embedded in OCT on dry ice. Eight-micron sections were prepared and dried overnight at room temperature and stored at −80°C before using. For H&E and immunohistochemical staining, skin tissues sections were air dried for 30 min, fixed in acetone at room temperature for 10 min, rinsed in PBS, incubated with 3% hydrogen peroxide in PBS, blocked with avidin/biotin blocking kits (SP-2001; Vector Laboratories) and by incubation with 10% goat or rabbit serum at room temperature for 30 min, and then incubated at 37°C for 1 h with the following primary Abs at the dilutions indicated: hamster anti-mouse CD3 at 1/100 dilution (145−2c11; R&D Systems); CD11c at 1/100 dilution (HL3; BD Pharmingen); and rat anti-mouse Gr1 at 1/500 dilution (Ly-6G; BD Pharmingen). Tissues were then incubated with the appropriate biotinylated secondary Ab (goat anti-hamster (Jackson Immunoresearch Laboratories) or rabbit anti-rat (Vector Laboratories), at 1/500 dilution for 30 min at room temperature and then with an avidin/biotin complex kit at 1/1000 dilution (PK-6100; Vector Laboratories). Sections were washed thrice with PBST buffer (PBS with 0.2% Triton X-100) after each incubation step (except for goat serum). All Abs, as well as the avidin/biotin complex, were diluted in 1% BSA in PBST. Sections were developed with 3, 3-diaminobenzidine tetrahydrochloride (SK-4100, Vector Laboratories) with hydrogen peroxide for 5 min at room temperature. Following development with 3, 3-diaminobenzidine tetrahydrochloride, tissues were rinsed in distilled-deionized H₂O, counterstained with 50% hematoxylin for 5 s, and then dehydrated and mounted. All slides were visualized by light microscopy (Leica), digitized under a ×2.5, ×5, or ×10 objective, and captured with a 3-CCD color video camera interfaced with a MagnaFire analysis system (Optronics).

RT-PCR analysis

RNA was extracted using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer’s instructions. Reverse transcription was performed using SuperScript first strand synthesis system for RT-PCR (12571-019; Invitrogen Life Technologies) according to the manufacturer’s instructions. To measure the expression of D6 using RT-PCR, the following three different pairs of primers were used (from 5’ to 3’; antisense to sense): D6-1, CACTGCTCTCTCACCCGTC and GGAAGAGGATGGCGCAAGGTCG (expected PCR product size 590 bp): D6-2, AGCTTCTACGTCGAACTCCTCGG and AAGAGGAAGACTATGCGCCAGA (expected PCR product size 441 bp) (5): and D6-3, GGAAGAGGACATAGTGAAAGGC and GTGACAGAGCTTGGCCTTC (374 bp) (10). The housekeeping gene GAPDH primers GGTTGAGGTCGGAAATCAGG and CAAGATTGTCTGATGGACC were also used as a positive control.

Statistical analyses

The Student t test was used for the comparisons of disease severity, day of onset, peak, and cumulative score, percentage of EAE, levels of T cell proliferation, and cytokine expression in comparisons between D6−/+ and D6+/− mice. A χ² test was used for the comparisons of disease incidence or mortality between D6−/+ and D6−/− mice; p < 0.05 was considered significant.

Results and Discussion

D6−/−, D6+/+, and D6−/+ mice were generated for these experiments and studied in gender-matched littermate cohorts to minimize effects of background genes. We conducted three separate immunizations to induce EAE, which exhibited typical onset and course in D6+/+ mice, with most mice developing hind limb paralysis. We observed greatly reduced and significantly reduced EAE severity in D6−/+ mice by analysis of the pooled data (Fig. 1, A and B). The incidence of disease was also somewhat reduced in D6−/+ mice; those failing to exhibit signs of disease in the 22-day period of observation were removed from analysis of disease score. D6−/− mice consistently showed reduced peak severity of disease along with improved resolution
of the EAE attack, as compared with their wild-type littermates. The day of EAE onset and the day of peak severity were equivalent in D6−/− and D6+/+ mice (Fig. 1B), arguing against altered kinetics of disease as an explanation for these findings. To evaluate the pathological basis for these neurological signs, we examined spinal cord histology by immunohistochemistry using anti-CD45 Abs to monitor inflammation and anti-MBP Abs to reveal demyelination (Fig. 1C) (11). We found that inflammation and demyelination mirrored neurobehavioral severity, being uniformly more extensive in wild-type mice with EAE than in D6−/− animals.

We addressed whether mice lacking D6 could express an encephalitogenic response to cells from D6-deficient MOG-primed mice by using D6−/− or D6+/+ mice as recipients of encephalitogenic cells from D6+/+ mice. D6−/− and D6+/+ mice exhibited equivalent severity of disease (Fig. 2A), demonstrating that the presence of D6 was dispensable for expression of EAE in response to encephalitogenic cells. This result also argued that D6 was not required for the clearance of inflammatory CC chemokines from the CNSs of mice with this model disorder. Consistent with this interpretation, we used semi-quantitative RT-PCR to show that D6 mRNA was expressed at low levels in the spinal cords of healthy mice without change in expression levels at the peak of EAE (data not shown).

We therefore turned our attention to determining whether the priming of encephalitogenic T cells might be impaired in D6−/− mice. This issue was addressed in reciprocal adoptive transfers by using cells from D6−/− or D6+/+ mice to induce disease in D6−/− recipients, with findings that closely resembled those in active immunizations; cells from MOG-immunized D6−/− mice induced EAE poorly in D6−/− animals as compared with those from D6+/+ littermates (Fig. 2B). Together, these findings suggested impaired T cell priming in D6−/− mice. To extend these findings, we evaluated recall proliferation and cytokine production by draining lymph node cells from D6-deficient and wild-type mice, following immunization with MOG35-55 peptides and CFA. Cells from D6−/− mice proliferated well after incubation with mitogen (Fig. 3A) but poorly in response to Ag (Fig. 3B). Furthermore, cells from D6−/− mice produced significantly less IFN-γ than cells from wild-type mice (Fig. 3C) but equal amounts of IL-5 (Fig. 3D). These findings indicated that D6−/− mice were impaired in the generation of MOG-specific T cell responses. There was no evidence that the failure to generate robust type 1 cytokine production was caused by a shift to a type 2 response. It was not clear whether the suboptimal response to MOG immunization in D6−/− mice was attributable to a primary T cell defect or to impaired Ag presentation. The status of D6 as a nonsignaling receptor favored the interpretation that the absence of D6 affected the microenvironment required for efficient Ag presentation rather than causing a cell-intrinsic T cell defect.

We previously showed altered and enhanced inflammation with necrotic foci, leukocyte infiltrates, and angiogenesis 3 days after the injection of CFA in D6−/− mice (6). It was of interest to determine whether s.c. injection of CFA with MOG peptide elicited a similar reaction. Morphology of the skin of D6−/− and D6+/+ littermates was similar contralateral to the injection (data not shown), but D6−/− mice showed increased inflammatory infiltrates, augmented vascularity, and necrotic foci after injection of CFA with MOG peptide (Fig. 4, A and B), using...
the protocol for induction of EAE. We used immunohistochemistry to characterize the cutaneous inflammatory infiltrates, with Abs to CD11c for dendritic cells (DCs), Abs to CD3 for T cells, and Abs to Gr1 for myeloid cells. At day 3 p.i., accumulations of CD3+ lymphocytes and Gr1+ myeloid cells were equivalent in the skin of D6+/+ and D6−/− mice (Fig. 4, C and D). CD11c+ DC aggregates in cutaneous immunization sites of D6+/+ mice showed hypercellular lymph nodes 3 days after injection, with increased CCL2 concentration 7 days postinjection (6), arguing against a cardinal role for D6 in chemokine transfer to the draining lymph node (7). However, CFA-injected D6−/− mice showed hypercellular lymph nodes 3 days after injection, with increased CCL2 concentration 7 days postinjection (6), arguing against a cardinal role for D6 in chemokine transfer to the draining lymph node. Future investigations will focus on whether timing and concentration of chemokine accumulation in local lymph nodes or gradients between skin and lymph nodes are critical for efficient generation of immune responses to cutaneous immunization.

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Disclosures
The authors have no financial conflict of interest.

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