The Journal of Immunology

Overexpression of IFN-Induced Protein 10 and Its Receptor CXCR3 in Myasthenia Gravis

Tali Feferman, Prasanta K. Maiti, Sonia Berrih-Aknin, Jacky Bismuth, Jocelyne Bidault, Sara Fuchs and Miriam C. Souroujon

J Immunol 2005; 174:5324-5331; ;
doi: 10.4049/jimmunol.174.9.5324
http://www.jimmunol.org/content/174/9/5324

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Overexpression of IFN-Induced Protein 10 and Its Receptor CXCR3 in Myasthenia Gravis[1]

Tali Feferman,* Prasanta K. Maiti,[2]* Sonia Berrih-Aknin,*† Jacky Bismuth,† Jocelyne Bidault,† Sara Fuchs,[3]* and Miriam C. Souroujon*‡

Myasthenia gravis (MG) and its animal model, experimental autoimmune MG (EAMG), are autoimmune disorders in which the acetylcholine receptor (AChR) is the major autoantigen. Microarray technology was used to identify new potential drug targets for treatment of myasthenia that would reduce the need for the currently used nonspecific immunosuppression. The chemokine IFN-γ-inducible protein 10 (IP-10; CXCL10), a CXC chemokine, and its receptor, CXCR3, were found to be overexpressed in lymph node cells of EAMG rats. Quantitative real-time PCR confirmed these findings and revealed up-regulated mRNA levels of another chemoattractant that activates CXCR3, monokine induced by IFN-γ (Mig; CXCL9). TNF-α and IL-1β, which act synergistically with IFN-γ to induce IP-10, were also up-regulated. These up-regulations were observed in immune response effector cells, namely, lymph node cells, and in the target organ of the autoimmune attack, the muscle of myasthenic rats, and were significantly reduced after suppression of EAMG by mucosal tolerance induction with an AChR fragment. The relevance of IP-10/CXCR3 signaling in myasthenia was validated by similar observations in MG patients. A significant increase in IP-10 and CXCR3 mRNA levels in both thymus and muscle was observed in myasthenic patients compared with age-matched controls. CXCR3 expression in PBMC of MG patients was markedly increased in $CD4^+$, but not in $CD8^+$, T cells or in $CD19^+$ B cells. Our results demonstrate a positive association of IP-10/CXCR3 signaling with the pathogenesis of EAMG in rats as well as in human MG patients. *The Journal of Immunology,* **2005, 174: 5324–5331.**

Myasthenia gravis (MG)[4] is a T cell-regulated, Ab-mediated autoimmune disorder in which the nicotinic acetylcholine receptor (AChR) at neuromuscular junctions is the major autoantigen. Despite the relatively vast knowledge concerning myasthenia, its etiology and the factors determining disease progression in individual patients are still unknown. Moreover, the current treatment of MG involves, as in other autoimmune diseases, nonspecific immunosuppression, which has undesirable side effects. A better understanding of the molecular and cellular mechanisms underlying the elicitation and progression of MG could contribute to a better management of the disease and to the development of novel immunotherapies for MG and other autoimmune diseases.

Experimental autoimmune MG (EAMG) can be induced in animals by immunization with Torpedo AChR; it mimics human MG in its clinical and immunopathological manifestations. EAMG induced in rats is the most reliable model for delineation of the immunopathological factors and processes involved in myasthenia and for the investigation of therapeutic strategies for MG (1, 2). DNA microarray analysis, in which lymph node cells (LNC) of myasthenic rats were compared with those of healthy controls, was used in this study to identify new, previously unknown factors that contribute to EAMG induction and progression and that could, in turn, serve as novel drug targets for the treatment of myasthenia. We focused on selected chemokines and adhesion molecules and their respective receptors that may take part in the activation, recruitment, and migration of lymphocytes into target tissues in myasthenia.

Chemokines constitute a superfamily of chemoattractant cytokines that mediate leukocyte recruitment into tissues in homeostasis and inflammation (3). IFN-γ-inducible protein 10 (IP-10) is a primary response gene that belongs to the CXC chemokine superfamily. It is a highly inducible chemoattractant for activated T cells, but has pleiotropic activities, such as stimulation of monocytes and NK cells, bone marrow progenitor maturation, modulation of adhesion molecule expression, and inhibition of angiogenesis (4). Monokine induced by IFN-γ (Mig) is an additional IFN-γ-inducible chemoattractant that, like IP-10, exerts its effect by binding and activating CXCR3, a G protein-coupled receptor. TNF-α and IL-1β work in synergy with IFN-γ to induce IP-10.

IP-10 has been reported to be expressed in many Th1-type inflammatory diseases, such as psoriasis (5, 6) and atherosclerosis (7), and in autoimmune diseases, such as multiple sclerosis (8, 9) and rheumatoid arthritis (10). In these diseases, tissue IP-10 levels correlate with the tissue infiltration of T lymphocytes, suggesting

*Department of Immunology, The Weizmann Institute of Science, Rehovot, Israel; †Centre National de la Recherche Scientifique, Unité Mixte de Recherche 8078, Unité Paris Sud, Institut Paris Sud Cytokines, Hospital Marie Lannelongue, Le Plessis Robinson, France; and ‡Open University of Israel, Raanana, Israel

Received for publication December 1, 2004. Accepted for publication February 18, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants from the Muscular Dystrophy Association of America, the Association Francaise Contre les Myopathies, the European Commission (QLG1-CT-2001-10918 and QLRT-2001-00225), The Araham and Judy Goldwasser Fund, and The Wood-Byer Foundation. S.B.A. was the recipient of a Weston Professorship from The Weizmann Institute.

[2] Current address: Department of Internal Medicine, University of Missouri, Columbia, MO 65212.

[3] Address correspondence and reprint requests to Dr. Sara Fuchs, Department of Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel. E-mail address: sara.fuchs@weizmann.ac.il

[4] Abbreviations used in this paper: MG, myasthenia gravis; AChR, acetylcholine receptor; EAMG, experimental autoimmune MG; EST, expressed sequence tag; IP-10, IFN-induced protein 10; LNC, lymph node cell; Mig, monokine induced by IFN-γ; Q-RT-PCR, quantitative real-time RT-PCR.

0022-1767/05/$02.00

that IP-10/CXCR3 signaling plays an important role in the recruitment of T cells to the site of inflammation and autoimmune attack (11). Another group of molecules involved in the mobilization of activated lymphocytes toward their tissue targets is adhesion molecules. The process of adhesion of circulating leukocytes to vascular endothelium and invasion of inflamed tissue is complex and involves a large variety of adhesion molecules as well as chemokines and their receptors (12). This process is controlled by integrins, such as VLA-4 and LFA-1, and their respective ligands, VCAM-1 and ICAM-1 (13).

In this study we used DNA microarray technology to identify genes that are dysregulated in EAMG and that may play a role in the pathogenesis of myasthenia. These lead genes, as well as genes related to them were analyzed by quantitative real-time RT-PCR (Q-RT-PCR), FACS, and immunohistochemistry. We show that the chemokine IP-10 and its receptor, CXCR3, are up-regulated in LNC and muscles of myasthenic rats and are significantly reduced after mucosal tolerance induction by an AChR fragment. A similar analysis in MG patients indicates that CXCR3 and IP-10 are also dysregulated in the human disease.

Materials and Methods

Animals and Ag preparation

Female Lewis rats, ages 6–7 wk, were obtained from the Animal Breeding Center of The Weizmann Institute of Science and were maintained in the Institute's animal facility. All experiments in this study were performed according to the institutional guidelines for animal care. Torpedo AChR was purified from the electric organ of *Torpedo californica* by affinity chromatography as previously described (14). The recombinant rat AChR fragment ($R\alpha_{1-205}$), corresponding to the extracellular domain of the rat AChR α subunit (residues 1–205) was cloned in a pET8C-derived vector, expressed in *Escherichia coli*, and purified as described previously (15). The purified protein appeared as a single band (~20 kDa) on SDS-PAGE stained with Coomassie blue and was estimated to be >95% pure (16).

Induction and clinical evaluation of EAMG

To induce EAMG, rats were anesthetized and immunized once in both hind footpads by s.c. injection of *Torpedo* AChR (40 μg/rat in 200 μl) emulsified in CFA supplemented with additional nonviable *Mycobacterium tuberculosis* H37RA (0.5 mg/rat; Difco). Control rats were immunized with CFA alone. Clinical signs of EAMG were monitored by blinded observers on alternate days for 8–10 wk after disease induction, and clinical scores were graded 0–4, where grade 0 represents healthy normal rats with no symptoms of EAMG; grade 1 indicates mildly decreased activity and weak grip with fatigability; grade 2 indicates weakness, hunched posture at rest, decreased body weight, and tremor; grade 3 indicates severe generalized weakness, significant decrease in body weight, and moribund; and grade 4, when the animal is dead (17).

Oral tolerance induction

Rats (eight per group) were fed using feeding tubes (Unoplast) on alternate days with 1 mg/ml/dose $R\alpha_{1-205}$ or with OVA as control, starting on day 7 (acute phase protocol) or on day 28 (chronic phase protocol) after induction of disease. Feeding was continued until the end of the experiment (8–10 wk after disease induction by *Torpedo* AChR).

Sample preparation for microarrays

Popliteal lymph nodes and quadriceps muscles were harvested from myasthenic and control rats. LNC were cultured (5×10^5 cells/well) in RPMI 1640 supplemented with HEPES (25 mM), sodium pyruvate (1 mM), glutamine (2 mM), 2-ME (50 mM), penicillin (20 U/ml), streptomycin (20 mg/ml), amphotericin B (0.05 mg/ml), nonessential amino acids (Ala, 8.9 mg/L; Asn, 15 mg/L; Asp, 13.3 mg/L; Glu, 14.7 mg/L; Gly, 7.5 mg/L; Pro, 11.5 mg/L; Ser, 10.5 mg/L), and 0.5% normal rat serum, either alone or in the presence of *Torpedo* AChR (5 μg/ml) for 40 h at 37°C in humidified 5% CO. Cells were washed twice in PBS and were frozen at −70°C until RNA was isolated. Total RNA from LNC was isolated using the HiPure RNA isolation kit (Roche) according to the manufacturer's protocol. Muscle total RNA was extracted from thigh muscles using the RNeasy kit (Qiagen). Two RNA samples were used for each group; each sample consisted of a pool from three individual rats. Probing and analysis of these samples were performed at The Weizmann Institute microarray unit.

GeneChip probing and analysis

The GeneChip RG-U34A arrays (Affymetrix) containing probes for 8000 rat genes and 1000 expressed sequence tags (ESTs) were used to screen and quantify the mRNA transcript level in rat LNC and muscle samples. The preparation of cRNA, hybridization, washing, and labeling were performed according to the manufacturer's instructions. After scanning with the HP GeneArray scanner (Hewlett-Packard), the fluorescence intensity of each probe was quantified using MicroArray Suite 4.0 (Affymetrix). The level of each mRNA sample was determined as the average fluorescence intensity obtained by 16 paired (perfectly matched and single nucleotide-mismatched) primers consisting of 25-base oligonucleotides and presented as average differences. Genes showing a fold change of >2 were selected for further evaluation.

Tissues from MG patients

Thymuses were obtained from five MG patients (4 women and 1 man, ages 18–25 years) undergoing thymectomy at Marie Lannelongue Hospital. Sternocleidomastoidian muscles were harvested during thymectomy from 17 MG patients (15 women and 2 men, ages 16–65 years) and four age-matched controls. Blood was drawn for routine medical tests from six patients (5 women and 1 man, ages 15–39 years). All MG patients had a generalized disease and anti-AChR Abs. Patients receiving corticosteroid treatment were excluded from the study. Normal thymi, which would otherwise be discarded, were obtained from young adults (4 women and 2 men, ages 15–24 years) undergoing cardiac surgery. All procedures were approved by the local ethics committee, Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale.

Q-RT-PCR

Q-RT-PCR of cytokines and other factors in rat samples was performed using a LightCycler (Roche). LNC were prepared and cultured as described above, and cells were collected for additional RNA analysis. RNA from LNC and muscles of individual rats was isolated as described above. RNA was reverse transcribed to cDNA from 1 μg of total RNA, which was then subjected to Q-RT-PCR performed essentially according to the manufacturer's instructions. Specific primer pairs were designed using LightCycler probe design software (Roche) and were used to amplify specific genes in the presence of 3 mM $MgCl_2$. PCR was performed in triplicate in a total volume of 20 μl of LightCycler HotStart DNA SYBR Green I mix (Roche) containing primer and 5 μl of cDNA. PCR amplification was preceded by incubation of the mixture for 10 min at 95°C, and the amplification step consisted of 35–50 cycles of denaturation, annealing, and extension. Denaturation was performed for 15 s at 95°C, annealing was performed in a transitional temperature range of 60°C (primary target temperature) to 58°C (secondary target temperature), with a step size of 0.5°C/cycle, and the extension was performed at 72°C for 20 s, with fluorescence detection at 72°C after each cycle. After the final cycle, melting point analyses of all samples were performed within the range of 62–95°C with continuous fluorescence detection. A standard curve was generated from one sample in each run. Expression levels of β-actin were used for sample normalization. The expression levels of another housekeeping gene, ribosomal protein L32, were also monitored in all samples and were found to be similar to those of β-actin. Results for each gene are presented as the relative expression level compared with β-actin. The primers were as follows: *ICAM-1* (235 bp): sense primer, 5′-AGCAGCAAGCATTTCC-3′; antisense primer, 5′-CGT TTGAAGAAGCCAACC-3′; *LFA-1* (176 bp): sense primer, 5′-ATGCCTTTGTGGGAGT-3′; antisense primer, 5′-AGCCTG GAGTGTGTTAG-3′; *CXCR3* (211 bp): sense primer, 5′-ATGCCTTTGT GGGAGT-3′; antisense primer, 5′-AGCCTGGAGTGTGTTAG-3′; *Mig* (181 bp): sense primer, 5′-ACTGTGGAGTTCGAGG-3′; antisense primer, 5′-GGGTCTAGGCAGGTTT-3′; *TNF-α* (186 bp): sense primer, 5′-CACGTCGTAGCAAACC-3′; antisense primer, 5′-GGTGAGGAGC ACATAGT-3′; *IFN-γ* (200 bp): sense primer, 5′-AGGACGGTAACAC GAAA-3′; antisense primer, 5′-CTGTGGGTTGTTCACCTC-3′; *IP-10* (202 bp): sense primer, 5′-CAAGGCTTCCCAATTCTC-3′; antisense primer, 5′-ACCTGGACTGCATTTGA-3′; *AChR α subunit* (186 bp): sense primer, 5′-ATGAAGTCAGACCAGGAG-3′; antisense primer, 5′-CAGAGGGAG GCTTAGTT-3′; *TGF-β* (104 bp): sense primer, 5′-CAAGGGCTACCAT GCCAACT-3′; antisense primer, 5′-CCGGGTTGTGTTGGTTGTAGA-3; *IL-1β* (235 bp): sense primer, 5′-ATGGTCGGGACATAGT-3′; antisense primer, 5′-GTGGTTGCCTGTCAGA-3′; *β-actin* (101 bp): sense primer, 5′-TACTGCCCTGGCTCCTAGCA-3′; antisense primer, 5′-TGGACA GTGAGGCCAGGATAG-3′; and *L32* (152 bp): sense primer, 5′-AGTT

TCTGGTCCACAATG-3′; antisense primer, 5′-GTTGGGATTGGTGA CTCT-3′.

Total RNA from muscles and thymi of MG patients and normal controls was extracted using the TRIzol reagent (Invitrogen Life Technologies). cDNA was synthesized from 1 μg of total RNA with specific primers designed using LC Probe software (Roche). Quantitative cDNA amplification was performed according to the manufacturer's instructions, as described above. Each sample was run in duplicate, and mean values were used for calculation. The CXCR3 and IP-10 mRNA levels were normalized to the 28S rRNA expression in each sample. The primers were as follows: *CXCR3* (190 bp): sense primer, 5′-AGCTTTGACCGCTACCTGAA-3′; antisense primer, 5′-TGTGGGAAGTTGTATTGGCA-3′; *IP-10* (279 bp): sense primer, 5′-AAGGATGGACCACACAGAGG-3′; antisense primer, 5′-TGGAAGATGGGAAAGGTGAG-3′; and *28S* (180 bp): sense primer, 5′-CGGGTAAACGGCGGGAGTAA-3′; antisense primer, 5′-GGTAGG GACAGTGGGAATCT-3′. A standard curve was obtained with serial dilutions of a reference cDNA sample amplified concomitantly with the tested samples. CXCR3 and IP-10 mRNA levels were determined by comparing experimental levels to the standard curves and are expressed as arbitrary units.

Immunofluorescence of thymus and muscle sections

Acetone-fixed, 6-μm-thick frozen sections from thymi or muscles of MG patients or healthy controls were incubated for 30 min at room temperature with 20 μg/ml mAb anti-CXCR3 (R&D Systems) or mAb anti-IP-10 Abs (R&D Systems). Control sections were incubated with mouse IgG1 (DakoCytomation). After three washes in PBS, the sections were incubated with a secondary goat anti-mouse Ab (DakoCytomation) coupled to FITC for 30 min. After three additional washings in PBS, the sections were mounted in fluorescence mounting medium (DakoCytomation) and viewed with a Leica DMB3 fluorescence microscope. Images were acquired on a Sony DXC 930P color-cooled, charge-coupled device camera connected to a Leica MSP 930 image analysis system. Quantification of fluorescence intensity was analyzed on whole sections using Image J software. The results were expressed as the mean fluorescence intensity obtained on the whole section after deduction of the background fluorescence intensity determined in the control section.

Immunofluorescence flow cytometry

Splenocytes from myasthenic and healthy rats were washed once with PBS, resuspended in FACS wash buffer (PBS, 5% BSA, and 0.05% sodium azide), and incubated with primary Abs to the tested molecule (10 μg/ml) for 30 min at 4°C. Cells were washed and analyzed immediately on a FACScan flow cytometer. The following primary Abs were used for flow cytometry: PE-conjugated mouse anti-rat CD8 β (MCA938 PE), PE-conjugated mouse IgG anti-rat CD4 (MCA55 PE), FITC-conjugated mouse IgG2a anti-rat LFA-1 (MCA774F), FITC-conjugated mouse IgG1 anti-rat VLA-4 (MCA1383F; all from Serotec), and FITC-conjugated mouse IgG1κ anti-rat ICAM-1 (554969; BD Biosciences). Negative controls consisted of FITC-conjugated mouse IgG1 (MCA1209F), PE-conjugated mouse IgG1 (MCA1209PE), and FITC-conjugated mouse IgG2a (MCA1210F; all from Serotec).

Human PBMC were purified on 15-ml Leucosep tubes (Greiner; VWR) according to the manufacturer's instructions. Incubation steps were performed at 4°C in HBSS with 5% horse serum, 3% HEPES (1 M), and 0.1% sodium azide. Cells (0.5×10^6) were incubated first with anti-CXCR3 mAbs (MAB160; R&D Systems) for 30 min. After washing, FITC-conjugated goat anti-mouse Ig (F0479; DakoCytomation) was added for 30 min. After two washings, the cells were incubated with mouse IgG1 (X0931; DakoCytomation) for 45 min to saturate the free sites of the FITC-goat anti-mouse Ig. After two washings, the cells were incubated for 15 min with one of the following mAbs: anti-CD19-R-PE (R0808; DakoCytomation) anti-CD4-R-PE-Cy5 (R7069; DakoCytomation), or anti-CD8-R-PE (R0806; DakoCytomation). The cells were then fixed with 0.02% paraformaldehyde in PBS and analyzed on a FACSCalibur using CellQuest software (BD Biosciences).

Statistical tests

The significance of the results was analyzed by Student's *t* test or Mann-Whitney *U* test using GraphPad software.

Results

Selection of lead genes associated with EAMG by DNA microarray analysis

DNA microarray analysis was performed on LNC from myasthenic rats in which the disease was induced by immunization with *Torpedo* AChR and from control healthy rats immunized with CFA. A large number of genes were differentially expressed in sick vs healthy rats: 170 of 8000 genes and seven of 1000 ESTs were up-regulated, and 35 genes and two ESTs were down-regulated in myasthenic rats. We chose to further analyze genes known to code for factors involved in lymphocyte activation and migration as well as genes related to them. These included genes coding for chemokines and their receptors as well as genes coding for adhesion molecules.

CXC chemokine gene expression in LNC and muscles of EAMG rats

IP-10, belonging to the CXC chemokine subfamily that acts via the CXCR3 and attracts T lymphocytes, was one of the up-regulated lead genes in EAMG identified by the DNA microarray and was chosen by us for additional analysis by Q-RT-PCR. As shown in Fig. 1*A*, IP-10 mRNA expression in LNC was increased in the EAMG group compared with controls, as expected from the microarray data. In addition, the mRNA level of Mig, a monokine that, like IP-10, is induced by IFN-γ, was also increased significantly in LNC of EAMG rats as was the mRNA level of CXCR3,

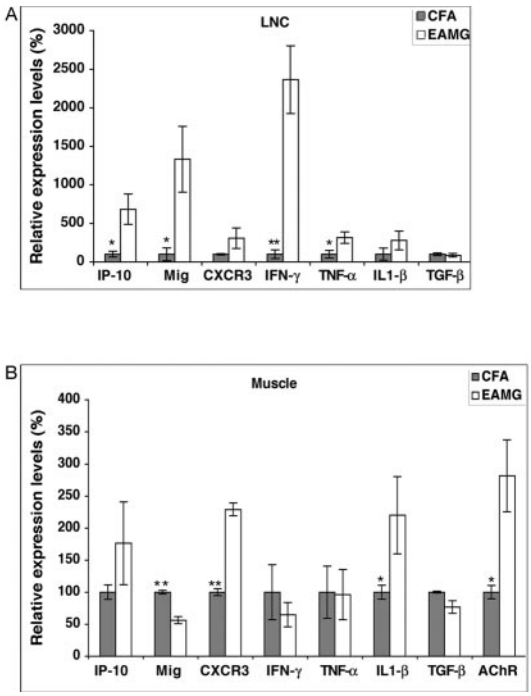


FIGURE 1. mRNA expression of chemokines and cytokines in LNC and muscles of EAMG and healthy rats. LNC and muscle samples from at least four individual rats from each experimental group were harvested in each experiment. Myasthenic rats were killed when they reached a clinical score of 2; for each myasthenic rat, a control rat was killed at the same time point. mRNA expression levels of cytokines and chemokines in LNC (*A*) and muscles (*B*) from EAMG and control CFA healthy rats were determined by Q-RT-PCR, and data are presented as the relative expression value for every EAMG group compared with its control, which was assigned a value of 100. β-Actin was used as an inner control for normalization for each cytokine/chemokine. *, $p < 0.05$; **, $p < 0.005$. The data shown are representative of five independent experiments

the receptor for IP-10 and Mig, although to a lesser extent. IFN-γ levels were markedly elevated in LNC of myasthenic rats, and TNF-α and IL-1-β, which act synergistically with IFN-γ to induce IP-10, were increased. TGF-β mRNA levels were low and similar in EAMG and healthy control rats.

The target organ of the autoimmune attack in myasthenia is muscle tissue, which had been suggested to play an active role in the production of immunomodulatory molecules. We therefore checked the mRNA levels of these cytokines and of CXCR3 in muscle samples from myasthenic vs healthy rats. As shown in Fig. 1*B*, mRNA levels of IP-10 and CXCR3 were increased in muscles of EAMG rats compared with controls, whereas the expression of Mig was decreased. No change was observed for IFN-γ or TNF-α. IL-1β was elevated, and TGF-β was slightly decreased. As previously reported (18), AChR mRNA levels were up-regulated in the muscles of myasthenic rats.

CXC chemokine expression after suppression of EAMG

Our next aim was to evaluate the physiological relevance of the studied molecules in EAMG: in other words, what happens to the expression of these chemokines and their receptors in rats in which EAMG was successfully suppressed. Rats were fed a recombinant fragment corresponding to the extracellular domain of the rat AChR α subunit (Rα_{1-205}), which had been recently shown by us to suppress EAMG when treatment is initiated at the acute as well as at the chronic phase of disease (19). The mechanism of action of this mucosal tolerance induction was shown to be a shift of the anti-AChR immune response from Th1 to Th2/Th3 cell regulation. For the acute phase protocol, treatment was initiated 1 wk after disease induction; for the chronic phase protocol, treatment was initiated 4 wk after disease induction. Rats were killed 8–10 wk after disease induction, and LNC were cultured in the presence of AChR. At the time animals were killed, the mean clinical score of Rα_{1-205}-treated rats in the acute phase protocol was 1.2, as opposed to 2.5 in OVA-treated controls, and the mean clinical score of Rα_{1-205}-treated rats in the chronic phase protocol was 1.4, as opposed to 2.8 in OVA-treated controls. As shown in Fig. 2*A*, mRNA expression levels of IP-10 were markedly reduced in LNC of rats treated by the acute phase protocol or the chronic phase protocol compared with those in control OVA-treated EAMG rats. The expression of CXCR3, the receptor for IP-10, as well as that of IFN-γ and TNF-α were also reduced significantly after treatment in LNC of myasthenic rats. mRNA levels of IL-1β and TGF-β remained unchanged, as also demonstrated in our previous studies (19). Changes in the expression levels of IP-10, its receptor CXCR3, and some of its inducers and coinducers (IFN-γ and TNF-α) were more significant in rats in which treatment was initiated during the acute phase. This seems to be in agreement with our reports that treatment initiated during the acute stage of disease is more effective than treatment starting during the chronic stage of disease (19).

Fig. 2*B* shows the relative expression of these molecules in the muscles of Rα_{1-205}-treated rats compared with control OVA-treated rats. Similar to the changes observed in LNC, IP-10 is strongly affected by mucosal tolerance induced by Rα_{1-205} in both acute and chronic phase protocols. Mig, CXCR3, and IL-1β are reduced as well, but to a lesser extent. In contrast to the observed changes in LNC of treated rats, where the effect of treatment was more pronounced when treatment was initiated during the acute phase (rather than the chronic phase), in muscle the effects in the acute phase protocol were not more striking than those in the chronic phase treatment.

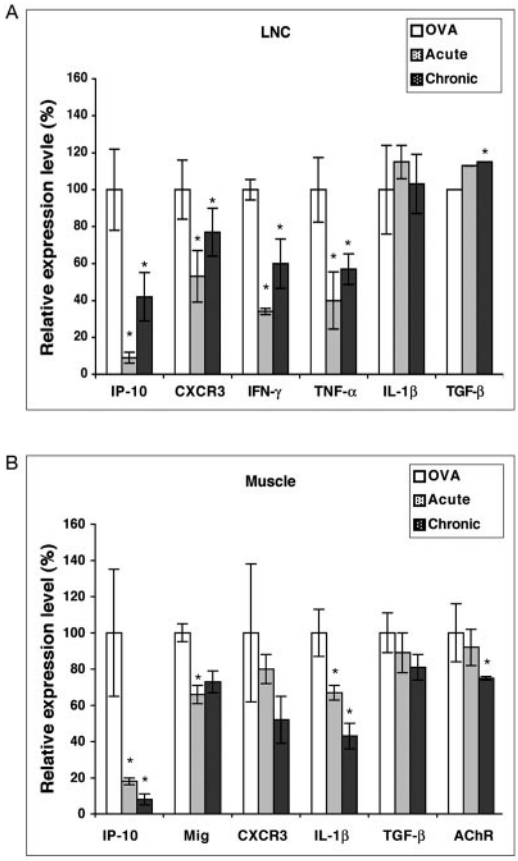


FIGURE 2. Cytokine and chemokine mRNA expression after successful treatment of EAMG by Rα_{1-205}. LNC and muscle samples from at least four individual rats from each experimental group were harvested in each experiment. mRNA expression levels of cytokines in LNC (*A*) and muscles (*B*) from rats in which treatment with Rα_{1-205} started during the acute or chronic phase of EAMG were determined by Q-RT-PCR 8 wk after disease induction, and data are presented as the relative expression value for the Rα_{1-205}-treated group compared with OVA-treated controls, which were assigned a value of 100. β-Actin was used for normalization for each cytokine/chemokine. *, $p < 0.05$. The data shown are representative of three independent experiments.

Adhesion molecule gene expression in LNC and muscles of EAMG rats

IP-10 and Mig are produced and secreted from monocytes, macrophages, and T cells in response to IFN-γ stimulation. This results in activation of naive CD4$^+$ cells into activated Th1 cells, which, in turn, express adhesion molecules that are involved in T cell recruitment and trafficking. To test whether the observed changes in IP-10/CXCR3 are accompanied by changes in molecules involved in the trafficking of reactive lymphocytes into the target of the autoimmune response, namely, the muscles, or into lymphoid organs, we analyzed the expression levels of selected adhesion molecules in EAMG and healthy rats. The mRNA expression levels of two key integrins, VLA-4 and LFA-1, as well as the LFA-1 ligand, ICAM-1, were monitored by Q-RT-PCR in LNC and muscles of myasthenic and control CFA-immunized healthy rats. The mRNA expression levels of ICAM-1 and, to a lesser extent, LFA-1 were increased in LNC of EAMG rats compared with healthy controls (6.4- and 2.1-fold, respectively; Fig. 3*A*), whereas VLA-4 mRNA levels were unchanged (data not shown).

We next checked whether the up-regulated mRNA levels of LFA-1 and ICAM-1 resulted also in respective changes in their

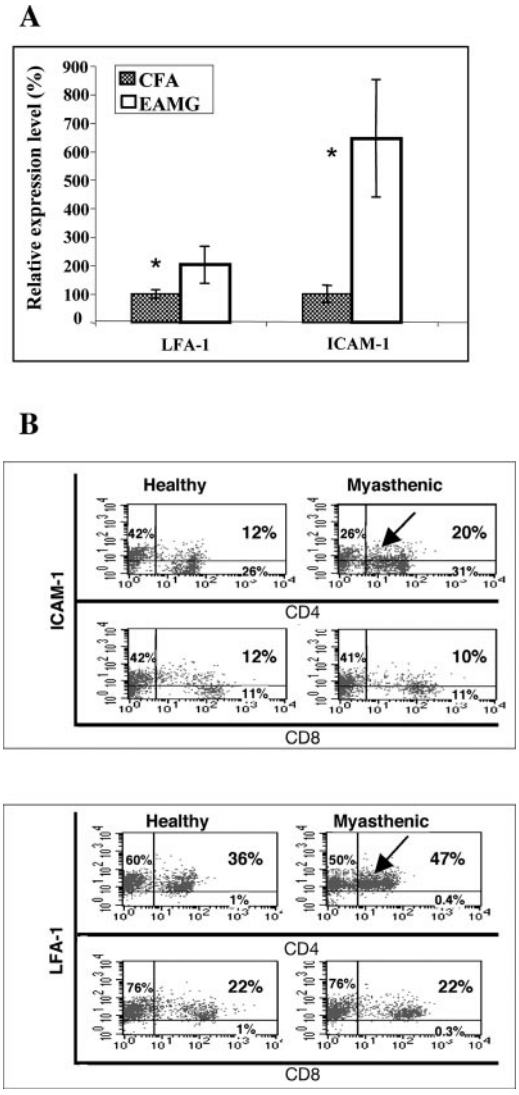


FIGURE 3. mRNA and protein expression levels of ICAM-1 and LFA-1 in LNC and splenocytes of EAMG and healthy rats. LNC and splenocytes from EAMG and healthy CFA-injected control rats were harvested when rats reached a clinical score of 2. *A*, mRNA expression levels of ICAM-1 and LFA-1 from EAMG and control CFA healthy rats were determined by Q-RT-PCR, and data are presented as the relative expression value for every EAMG group compared with its control, which was assigned a value of 100. β-Actin was used as an inner control for normalization for each gene. $*$, $p < 0.05$ ($n = 4$). The data shown are representative of five experiments. *B*, Protein levels of ICAM-1 and LFA-1 were determined in splenocytes from EAMG and healthy CFA-injected control rats (two or three rats in each group) that were subjected to FACS analysis with PE-conjugated anti-CD4 or anti-CD8 Abs combined with FITC-conjugated anti-ICAM-1 or anti-LFA-1 Abs. Shown is a representative experiment of five independent experiments performed.

protein levels. Splenocytes from EAMG and control CFA-immunized healthy rats were subjected to FACS analysis in which cells were stained with PE-conjugated anti-CD4 and anti-CD8 Abs together with FITC-conjugated anti-ICAM-1 or anti-LFA-1 Abs. As shown in Fig. 3*B*, EAMG was characterized by an increase in $CD4^+$ cells expressing LFA-1 (47 vs 36%) and its ligand ICAM-1 (20 vs 12%). There was no significant difference between myasthenic and control rats in the protein expression of LFA-1 or ICAM-1 in the $CD8^+$ splenocyte population (Fig. 3*B*). VLA-4 protein levels were similar in splenocytes of EAMG and control rats (data not shown). The mRNA expression of these molecules in LNC or muscles from rats successfully treated with $R\alpha_{1-205}$ was similar to that in control OVA-treated EAMG rats (data not shown). It should be noted that no apparent changes in mRNA expression levels of LFA-1, VLA-4, and ICAM-1 were detected in muscles of EAMG rats compared with healthy controls (data not shown). It thus seems that migration of lymphocytes controlled by the tested adhesion molecules is not directly associated with the pathogenesis of EAMG at the time point that we monitored.

IP-10 and CXCR3 are up-regulated in human MG

Because IP-10/CXCR3 signaling seems to be associated with the pathogenesis of EAMG, we checked whether similar changes in the expression of these molecules were observed in the human disease. The mRNA levels of IP-10 and CXCR3 in human MG were analyzed by Q-RT-PCR in the target organ of the autoimmune response (muscle), in PBMC, and in an effector organ (thymus). A significant up-regulation of mRNA levels was observed for both IP-10 ($p < 0.005$) and its receptor, CXCR3 ($p < 0.05$), in thymuses from MG patients vs age-matched controls (Fig. 4). To localize the CXCR3-expressing cells, we performed immunofluorescence analyses of thymuses and found that the CXCR3 protein is expressed on most thymocytes in both normal and MG thymuses (not shown), making it difficult to assess the identity of the specific cells overexpressing CXCR3 in the thymus of MG patients. The distribution of CXCR3 in normal thymus was similar to that previously described (20). IP-10 was expressed in few cells of the medulla and appeared to have a similar distribution in normal and MG thymuses (not shown). CXCR3 was also analyzed in the lymphocyte gate of PBMC by flow cytometry. As shown in Fig. 5, CXCR3 was significantly increased in $CD4^+$ cells from MG patients, but not in their $CD8^+$ or $CD19^+$ cells. The increased expression of CXCR3 in circulating $CD4^+$ cells was compatible with the role attributed to these activated cells in autoimmune pathogenic processes.

Muscle biopsies from 17 MG patients and four controls were analyzed to study the expression of IP-10 and CXCR3 proteins in the target organ of MG. As shown in Fig. 6, IP-10 as well as CXCR3 mRNA levels were significantly increased in muscles of MG patients compared with healthy age-matched controls ($p < 0.003$ and $p < 0.004$, respectively). Moreover, a correlation between the expression levels of IP-10 and CXCR3 in muscles of individual MG patients ($p < 0.0002$) was observed (Fig. 6, *inset*).

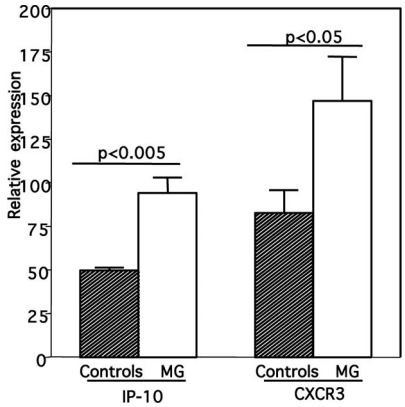


FIGURE 4. mRNA expression levels of IP-10 and CXCR3 in the thymus of MG patients and healthy controls. mRNA expression levels of IP-10 and CXCR3 were analyzed by Q-RT-PCR in thymuses of MG patients ($n = 5$) compared with controls ($n = 6$). The results are shown as the mean $\pm$ SEM. The p values were obtained using the nonparametric Mann-Whitney U test.

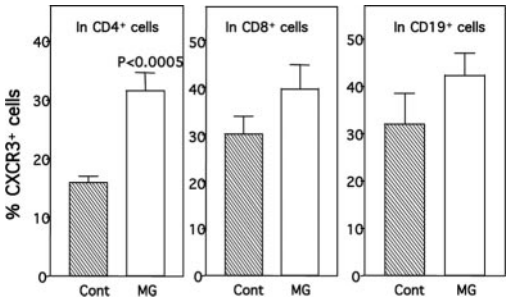


FIGURE 5. CXCR3 surface expression in PBMC of MG patients and controls. PBMC from MG patients ($n = 6$) and age-matched controls ($n = 7$) were incubated first with anti-CXCR3 Abs, followed by FITC-conjugated goat anti-mouse Ig. The cells were further incubated with mouse IgG1 to saturate the free sites of the FITC-goat anti-mouse Ig. After washings, the cells were incubated with one of the following mAbs: anti-CD19-R-PE, anti-CD4-R-PE-Cy5, or anti-CD8-R-PE. The results are shown as the mean ± SEM. The p values were obtained using the nonparametric Mann-Whitney U test.

The up-regulated mRNA expression levels of IP-10 and CXCR3 in muscles of MG patients raise a question concerning the identity of the cells that express these molecules (muscle cells or infiltrating immune cells). We performed immunofluorescence analysis on muscle biopsies to address this question. Fig. 7 shows a representative immunofluorescence in which CXCR3 is expressed on myotubes in muscle biopsies from a healthy control (Fig. 7*A*) and an MG patient (Fig. 7*B*) and demonstrates that the expression of CXCR3 is higher in the myotubes of the MG patient. A quantification of the mean fluorescence of muscle biopsies from six MG patients and four controls confirmed that the CXCR3 protein expression is higher in MG patients than in controls ($p < 0.001$; Fig. 7*C*). IP-10 was undetectable by immunofluorescence in human muscle sections from controls or MG patients (not shown). The significantly increased expression of CXCR3 and IP-10 in MG patients suggests that these molecules are involved in the pathogenesis of MG in effector cells and in the target organ.

Discussion

The goal of this study was to identify genes and gene products that are still not known to be involved in the pathology of MG and could, in turn, serve as novel drug targets for improved treatment of MG. We performed DNA microarray analysis on LNC from

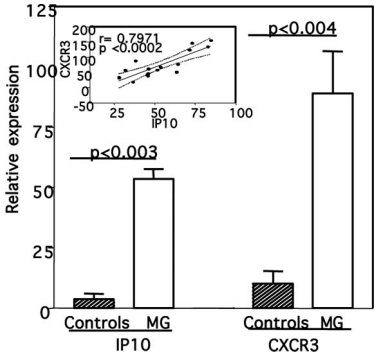
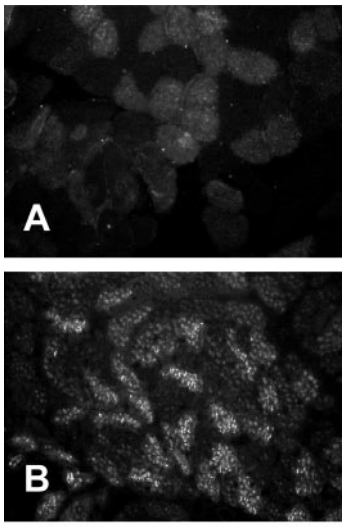


FIGURE 6. mRNA expression levels of IP-10 and CXCR3 in muscles of MG patients. mRNA expression levels of IP-10 and CXCR3 were analyzed in muscles of MG patients compared with controls. The results are shown as the mean ± SEM of 17 MG patients and four controls. The *inset* shows a positive correlation between the expression levels of IP-10 and CXCR3 in human muscles biopsies. The p values were obtained using the nonparametric Mann-Whitney U test.



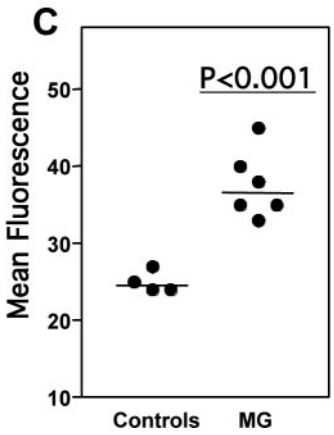


FIGURE 7. CXCR3 protein in myotubes of MG patients and controls. Frozen sections from the muscle of an MG patient (*A*) and a normal control (*B*) were stained with anti-CXCR3 Abs. The mean fluorescence was quantified on the whole section using Image J software (*C*). The results are expressed as the mean fluorescence intensity of CXCR3-stained sections of six MG patients and four controls (after deduction of the relevant background fluorescence intensity).

EAMG and control rats as major players in the autoimmune response. The expression levels of selected lead genes that presented disease-associated differential expression and genes related to them were verified by Q-RT-PCR of LNC and muscles from individual EAMG and control rats. We focused in this study on genes coding for selected chemokines and their respective receptors that may play a role in lymphocyte activation, recruitment, and migration in myasthenia. Our results clearly show that the mRNA levels of IP-10 and its receptor, CXCR3, are significantly increased in myasthenic rats. The relevance of IP-10 and CXCR3 in myasthenia is supported by two additional observations: 1) IP-10 and CXCR3 expression levels are markedly decreased after suppression of EAMG by mucosal tolerance induction; and 2) IP-10 and CXCR3 expression levels are significantly increased not only in EAMG, but also in muscle, PBMC, and thymus of human MG patients. In fact, the involvement of an IFN-γ-induced chemokine in the pathogenesis of myasthenia is not surprising in view of the key role attributed to IFN-γ in EAMG (21–24).

The increased expression of CXCR3 and IP-10 in muscle and LNC in EAMG probably results from the autoimmune disease in rats rather than from active immunization with *Torpedo* AChR,

because these changes in gene expression were also observed in the spontaneous disease in humans. CXCR3 and its chemokine ligands are thought to participate in the recruitment of inflammatory cells, as evidenced in several pathological situations. The IP-10 protein expression level has been reported to increase in some Th1-type inflammatory autoimmune diseases, including multiple sclerosis (8, 9) and rheumatoid arthritis (10), where it plays a role in recruiting activated T cells into the site of tissue inflammation. Studies with IP-10-deficient mice have demonstrated that IP-10 has a role not only in T cell trafficking, but also in T cell development and activation (11). CXCR3-activating chemokines were also shown to play a significant role in the recruitment and maintenance of T cell infiltrates in inflammatory skin diseases (5). In inclusion body myositis, IFN-γ up-regulates the production of proinflammatory chemokines, which, in turn, participate in the recruitment of activated T cells and contribute to the inflammation (25). CXCR3 was present on virtually all CD3-positive T cells in brain sections of one subtype of multiple sclerosis (8) and in thyroid glands of patients suffering from Graves' disease, CXCR3 was present on infiltrating inflammatory cells (26). Recently, Chen et al. (27) demonstrated that CXCR3 expression by inflammation-inducing Th1 cells fluctuates during cell activation and migration and is down-regulated upon re-exposure of these cells to the Ag in the target organ.

In rheumatoid arthritis, IP-10 induction within synovial joints was shown to be mediated via specific adhesion molecules, including ICAM-1 (28). However, we did not observe changes in ICAM-1 or its ligand, LFA-1, in the muscle of myasthenic rats, suggesting that in the muscle, IP-10 could be up-regulated independently of an increase in ICAM-1, although we cannot exclude that ICAM-1$^+$ cells influenced the expression of IP-10 in earlier stages of the disease. We did observe elevated mRNA levels of ICAM-1 and LFA-1 in LNC and elevated protein expression of these molecules in splenocytes of myasthenic rats compared with healthy controls. However, the expression of ICAM-1 and LFA-1 was unchanged after EAMG suppression by mucosal tolerance induction (data not shown), suggesting that they are not involved in the mechanism of suppression by this specific tolerization procedure.

In this study we show that IP-10 levels are up-regulated in the muscles of EAMG rats and MG patients. The production of IP-10 by muscle cells has been reported in inclusion body myositis (25) and the LE1 muscle cell line (29). IP-10 is also expressed by a variety of cells, including endothelial cells, keratinocytes, fibroblasts, astrocytes, monocytes, neutrophils, and T cells after Ag activation (13). Increased muscle levels of other cytokines (TNF-α, IL-1, and IL-6) were previously reported in EAMG, with a peak in expression at the acute stage (30). The source of these cytokines was infiltrating macrophages. However, muscle tissue can itself be the source of cytokines, because rat skeletal muscle exposed in vitro or in vivo to AChR-reactive Abs has been shown to produce immunologically relevant factors, such as IFN-γ, IL-1, IL-15, NO, and MCP-1, that promote the generation of disease symptoms (29, 31, 32). In addition, Reyes-Reyna et al. (29) demonstrated the production of MCP-1, RANTES, and IP-10 by the LE1 muscle cell line in response to IFN-γ stimulation in addition to immunologically relevant surface molecules, such as ICAM-1. These factors may be important at early stages of myasthenia. However, although CXCR3 and its chemokine ligands are thought to participate in the recruitment of inflammatory cells, we found that the myotubes themselves express IP-10 and CXCR3, and there was no evidence of an inflammatory reaction in the muscles of EAMG rats (data not shown) and MG patients. The latter observation is in agreement with previous reports indicating that inflammatory cells do not play a major role in the pathogenesis of muscle in MG (33).

Our finding that CXCR3 is expressed in myotubes and is elevated in muscles of myasthenic rats and MG patients is in agreement with other reports indicating that CXCR3 is expressed by various cells types. CXCR3 was found to be constitutively expressed by neurons in normal brain (34), by endothelial cells in vitro, and on small vessels in vivo, with increased expression in inflamed and neoplastic tissue (35). Moreover, Northern blot analyses revealed that normal skeletal muscle and normal heart express high levels of the CXCR3-A common isoform (36). The physiological role of CXCR3 in myotubes is still not fully understood. It was shown that the binding of IFN-inducible T cell α chemoattractant caused transient mobilization of intracellular calcium as well as chemotactic migration in activated T cells, in transfected cell lines expressing CXCR3 (37), as well as in neurons (34). All muscle fibers use Ca^{2+} as their main regulatory and signaling molecule. Contractile properties of muscle fibers are dependent on the variable expression of proteins involved in Ca^{2+} signaling and handling (38). It is thus possible that the calcium influx induced by the binding of IP-10 to its receptor on myotubes contributes to the contractile properties of the muscle. The positive correlation observed between CXCR3 and IP-10 expression in the muscle suggests that the regulation of the genes coding for them is controlled by a common factor that affects both or that they affect one another.

In MG patients we found a high number of CXCR3$^+$ cells among CD4 PBMC. However, because these cells were not found in the muscle target organ of the patients, at least at the time points tested, CXCR3$^+$ PBMC probably migrate to other sites, such as lymph nodes and/or thymus. We could not identify the cells in the thymus of MG patients that express increased protein levels of CXCR3 and IP-10, but infiltrating PBMC could be responsible for this elevated expression. The latter possibility is supported by recent studies indicating that the thymus of MG patients presents an inflammatory signature (39). However, it cannot be excluded that PBMC migrate to other immune organs, such as lymph nodes, especially in light of the observed increase of CXCR3 and IP-10 mRNA expression in lymph node cells of EAMG rats in this study.

Our results suggest that IP-10/CXCR3 signaling is associated with the pathogenesis not only of EAMG, but also of human MG. If, in the future, modulating CXCR3/IP-10 signaling is shown to affect the course of myasthenia, these molecules could serve as new drug targets to treat MG and other autoimmune diseases.

Acknowledgments

We thank the microarray facility, Department of Biological Services, The Weizmann Institute of Science, for their support and help with the microarray analysis.

Disclosures

The authors have no financial conflict of interest.

References

1. Fuchs, S. 1979. Immunology of the acetylcholine receptor. *Curr. Top. Microbiol. Immunol. 85:1.*
2. Drachman, D. B. 1996. Immunotherapy in neuromuscular disorders: current and future strategies. *Muscle Nerve 19:1239.*
3. Luster, A. D. 1998. Chemokines: chemotactic cytokines that mediate inflammation. *N. Engl. J. Med. 338:436.*
4. Neville, L. F., G. Mathiak, and O. Bagasra. 1997. The immunobiology of interferon-γ inducible protein 10 kD (IP-10): a novel, pleiotropic member of the C-X-C chemokine superfamily. *Cytokine Growth Factor Rev. 8:207.*
5. Flier, J., D. M. Boorsma, P. J. van Beek, C. Nieboer, T. J. Stoof, R. Willemze, and C. P. Tensen. 2001. Differential expression of CXCR3 targeting chemokines CXCL10, CXCL9, and CXCL11 in different types of skin inflammation. *J. Pathol. 194:398.*

6. Gottlieb, A. B., A. D. Luster, D. N. Posnett, and D. M. Carter. 1988. Detection of a γ interferon-induced protein IP-10 in psoriatic plaques. *J. Exp. Med. 168:941*.
7. Mach, F., A. Sauty, A. S. Iarossi, G. K. Sukhova, K. Neote, P. Libby, and A. D. Luster. 1999. Differential expression of three T lymphocyte-activating CXC chemokines by human atheroma-associated cells. *J. Clin. Invest. 104:1041*.
8. Sorensen, T. L., M. Tani, J. Jensen, V. Pierce, C. Lucchinetti, V. A. Folcik, S. Qin, J. Rottman, F. Sellebjerg, R. M. Strieter, et al. 1999. Expression of specific chemokines and chemokine receptors in the central nervous system of multiple sclerosis patients. *J. Clin. Invest. 103:807*.
9. Balashov, K. E., J. B. Rottman, H. L. Weiner, and W. W. Hancock. 1999. $CCR5^+$ and $CXCR3^+$ T cells are increased in multiple sclerosis and their ligands MIP-1α and IP-10 are expressed in demyelinating brain lesions. *Proc. Natl. Acad. Sci. USA 96:6873*.
10. Patel, D. D., J. P. Zachariah, and L. P. Whichard. 2001. CXCR3 and CCR5 ligands in rheumatoid arthritis synovium. *Clin. Immunol. (Oxf.) 98:39*.
11. Dufour, J. H., M. Dziejman, M. T. Liu, J. H. Leung, T. E. Lane, and A. D. Luster. 2002. IFN-γ-inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector T cell generation and trafficking. *J. Immunol. 168:3195*.
12. Bonecchi, R., G. Bianchi, P. P. Bordignon, D. D'Ambrosio, R. Lang, A. Borsatti, S. Sozzani, P. Allavena, P. A. Gray, A. Mantovani, et al. 1998. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J. Exp. Med. 187:129*.
13. Luster, A. D., and J. V. Ravetch. 1987. Biochemical characterization of a γ interferon-inducible cytokine (IP-10). *J. Exp. Med. 166:1084*.
14. Aharonov, A., R. Tarrab-Hazdai, I. Silman, and S. Fuchs. 1977. Immunochemical studies on acetylcholine receptor fraction from *Torpedo californica. Immunochemistry 14:129*.
15. Im, S. H., D. Barchan, S. Fuchs, and M. C. Souroujon. 2000. Mechanism of nasal tolerance induced by a recombinant fragment of acetylcholine receptor for treatment of experimental myasthenia gravis. *J. Neuroimmunol. 111:161*.
16. Im, S. H., D. Barchan, M. C. Souroujon, and S. Fuchs. 2000. Role of tolerogen conformation in induction of oral tolerance in experimental autoimmune myasthenia gravis. *J. Immunol. 165:3599*.
17. Lennon, V. A., E. H. Lambert, K. R. Leiby, T. B. Okarma, and S. Talib. 1991. Recombinant human acetylcholine receptor α-subunit induces chronic experimental autoimmune myasthenia gravis. *J. Immunol. 146:2245*.
18. Asher, O., S. Fuchs, D. Zuk, D. Rapaport, and A. Buonanno. 1992. Changes in the expression of mRNAs for myogenic factors and other muscle-specific proteins in experimental autoimmune myasthenia gravis. *FEBS Lett. 299:15*.
19. Maiti, P. K., T. Feferman, S. H. Im, M. C. Souroujon, and S. Fuchs. 2004. Immunosuppression of rat myasthenia gravis by oral administration of a syngeneic acetylcholine receptor fragment. *J. Neuroimmunol. 152:112*.
20. Romagnani, P., F. Annunziato, E. Lazzeri, L. Cosmi, C. Beltrame, L. Lasagni, G. Galli, M. Francalanci, R. Manetti, F. Marra, et al. 2001. Interferon-inducible protein 10, monokine induced by interferon γ, and interferon-inducible T-cell α chemoattractant are produced by thymic epithelial cells and attract T-cell receptor (TCR) $\alpha\beta^+$ $CD8^+$ single-positive T cells, TCR$\gamma\delta^+$ T cells, and natural killer-type cells in human thymus. *Blood 97:601*.
21. Zhang, G. X., B. G. Xiao, X. F. Bai, A. Orn, P. H. van der Meide, and H. Link. 1998. IFN-γ is required to induce experimental autoimmune myasthenia gravis. *Ann. NY Acad. Sci. 841:576*.
22. Balasa, B., C. Deng, J. Lee, L. M. Bradley, D. K. Dalton, P. Christadoss, and N. Sarvetnick. 1997. Interferon γ (IFN-γ) is necessary for the genesis of acetylcholine receptor-induced clinical experimental autoimmune myasthenia gravis in mice. *J. Exp. Med. 186:385*.
23. Wang, H. B., H. Li, F. D. Shi, B. J. Chambers, H. Link, and H. G. Ljunggren. 2000. Tumor necrosis factor receptor-1 is critically involved in the development of experimental autoimmune myasthenia gravis. *Int. Immunol. 12:1381*.
24. Link, J. 1994. Interferon-γ, interleukin-4 and transforming growth factor-β mRNA expression in multiple sclerosis and myasthenia gravis. *Acta. Neurol. Scand. 158(Suppl.):1*.
25. Raju, R., O. Vasconcelos, R. Granger, and M. C. Dalakas. 2003. Expression of IFN-γ-inducible chemokines in inclusion body myositis. *J. Neuroimmunol. 141:125*.
26. Romagnani, P., M. Rotondi, E. Lazzeri, L. Lasagni, M. Francalanci, A. Buonamano, S. Milani, P. Vitti, L. Chiovato, M. Tonacchera, et al. 2002. Expression of IP-10/CXCL10 and MIG/CXCL9 in the thyroid and increased levels of IP-10/CXCL10 in the serum of patients with recent-onset Graves' disease. *Am. J. Pathol. 161:195*.
27. Chen, J., B. P. Vistica, H. Takase, D. I. Ham, R. N. Fariss, E. F. Wawrousek, C. C. Chan, J. A. DeMartino, J. M. Farber, and I. Gery. 2004. A unique pattern of up- and down-regulation of chemokine receptor CXCR3 on inflammation-inducing Th1 cells. *Eur. J. Immunol. 34:2885*.
28. Hanaoka, R., T. Kasama, M. Muramatsu, N. Yajima, F. Shiozawa, Y. Miwa, M. Negishi, H. Ide, H. Miyaoka, H. Uchida, et al. 2003. A novel mechanism for the regulation of IFN-γ inducible protein-10 expression in rheumatoid arthritis. *Arthritis Res. Ther. 5:R74*.
29. Reyes-Reyna, S., T. Stegall, and K. A. Krolick. 2002. Muscle responds to an antibody reactive with the acetylcholine receptor by up-regulating monocyte chemoattractant protein 1: a chemokine with the potential to influence the severity and course of experimental myasthenia gravis. *J. Immunol. 169:1579*.
30. Li, H., F. D. Shi, X. Bai, Y. Huang, A. Diab, B. He, and H. Link. 1998. Cytokine and chemokine mRNA expressing cells in muscle tissues of experimental autoimmune myasthenia gravis. *J. Neurol. Sci. 161:40*.
31. Stegall, T., and K. A. Krolick. 2001. Myocytes respond in vivo to an antibody reactive with the acetylcholine receptor by upregulating interleukin-15: an interferon-γ activator with the potential to influence the severity and course of experimental myasthenia gravis. *J. Neuroimmunol. 119:377*.
32. Garcia, Y. R., J. J. May, A. M. Green, and K. A. Krolick. 2001. Acetylcholine receptor-reactive antibody induces nitric oxide production by a rat skeletal muscle cell line: influence of cytokine environment. *J. Neuroimmunol. 120:103*.
33. Nakano, S., and A. G. Engel. 1993. Myasthenia gravis: quantitative immunocytochemical analysis of inflammatory cells and detection of complement membrane attack complex at the end-plate in 30 patients. *Neurology 43:1167*.
34. Xia, M. Q., B. J. Bacskai, R. B. Knowles, S. X. Qin, and B. T. Hyman. 2000. Expression of the chemokine receptor CXCR3 on neurons and the elevated expression of its ligand IP-10 in reactive astrocytes: in vitro ERK1/2 activation and role in Alzheimer's disease. *J. Neuroimmunol. 108:227*.
35. Romagnani, P., F. Annunziato, L. Lasagni, E. Lazzeri, C. Beltrame, M. Francalanci, M. Uguccioni, G. Galli, L. Cosmi, L. Maurenzig, et al. 2001. Cell cycle-dependent expression of CXC chemokine receptor 3 by endothelial cells mediates angiostatic activity. *J. Clin. Invest. 107:53*.
36. Lasagni, L., M. Francalanci, F. Annunziato, E. Lazzeri, S. Giannini, L. Cosmi, C. Sagrinati, B. Mazzinghi, C. Orlando, E. Maggi, et al. 2003. An alternatively spliced variant of CXCR3 mediates the inhibition of endothelial cell growth induced by IP-10, Mig, and I-TAC, and acts as functional receptor for platelet factor 4. *J. Exp. Med. 197:1537*.
37. Cole, K. E., C. A. Strick, T. J. Paradis, K. T. Ogborne, M. Loetscher, R. P. Gladue, W. Lin, J. G. Boyd, B. Moser, D. E. Wood, et al. 1998. Interferon-inducible T cell α chemoattractant (I-TAC): a novel non-ELR CXC chemokine with potent activity on activated T cells through selective high affinity binding to CXCR3. *J. Exp. Med. 187:2009*.
38. Berchtold, M. W., H. Brinkmeier, and M. Muntener. 2000. Calcium ion in skeletal muscle: its crucial role for muscle function, plasticity, and disease. *Physiol. Rev. 80:1215*.
39. Poea-Guyon, S., P. Christados, R. Le Panse, T. Guyon, M. De Baets, A. Wakkach J. Bidault, S. Tzartos, and S. Berrih-Aknin. Effects of cytokines on acetylcholine receptor expression: implications for myasthenia gravis. *J. Immunol. In press*.