b)　アザチオプリン[3)]

アザチオプリン（azathioprine）は肝で6-メルカプトプリンに代謝され，これがプリンヌクレオチド生合成を阻害することにより活性化リンパ球の増殖抑制をきたし，免疫抑制作用を発揮する．アザチオプリンは肝臓で6-メルカプトプリンに代謝された後，肝臓・腸管壁でキサンチンオキシダーゼ（xanthineoxydase）により6-チオ尿酸に代謝され，主に尿中に排泄される．したがって，キサンチンオキシダーゼ阻害薬である**アロプリノール（allopurinol）**との併用により，メルカプトプリンは蓄積して白血球減少症・血小板減少症などの重篤な副作用が出現する．したがって，アロプリノールとの併用時にはアザチオプリンの投与量をおよそ1/3～1/4に減量しなくてはならない．

c)　ミゾリビン[3)]

ミゾリビン（mizoribine）はイノシン一リン酸デヒドロゲナーゼ（IMP dehydrogenase）の阻害によりプリン合成を抑制して活性化リンパ球の細胞周期回転阻止効果を有する．また，Bリンパ球に対しては，サイクリンAのmRNAの発現を抑制するというユニークな効果のあることが証明されている．臓器移植時の拒否反応や，ステロイド無効のループス腎炎やネフローゼ症候群，抗リウマチ薬無効のRAなどに適応がある．作用は弱いが，副作用も少ない．良く見られる副作用は高尿酸血症である．

d)　シクロフォスファミド

シクロフォスファミド（cyclophosphamide）はナイトロジェンマスタード（nitrogen mustard）の誘導体で，主として肝臓において活性体である4-ヒドロキシシクロフォスファミド（4-hydroxy cyclophosphamide）に変換されて免疫抑制作用を発揮する．その薬理作用は，ほかのアルキル化薬と同様に核酸やタンパク質分子中のアミノ基，カルボキシル基，イミダゾール基，SH基部分などの求核群をアルキル化することにより発揮される．また，DNA環のグアニン基の7位もアルキル化を受けやすい．アルキル化したグアニンはイミダゾール環の開裂，DNA鎖からの脱プリン，ほかのDNA鎖のグアニンとの架橋などを生じ，DNA合成，mRNAの転写は阻害され，細胞の増殖が抑制される．Tリンパ球機能よりBリンパ球機能の抑制効果が強く，特にBリンパ球の抗体産生能を強く抑制し，長期投与例では血中の免疫グロブリンの低下が著明になることが多い．

シクロホスファミドによる**出血性膀胱炎**は頻度が高く，最も重要な副作用の一つであるが，高度の腎機能障害を呈するループ腎炎に対してより効果的な治療として導入された間欠的大量経静脈投与（intravenous cyclophosphamide pulse therapy：IVCY）が結果としての従来の経口投与より出血性膀胱炎などの副作用の発現を低く抑えることがわかり，我が国でも広く試みられるようになっている．

e)　シクロスポリン[4)]

シクロスポリン（cyclosporin）は**カルシニューリン（calcineurin）**の脱リン酸化作用を阻害することによりNFATの核内移行を阻害し，Tリンパ球のIL-2産生を抑制することにより作用すると考えられている．腎移植・肝移植・心移植・骨髄移植

における拒否反応の抑制，ベーチェット病の眼病変，ネフローゼ症候群（頻回再発型あるいはステロイド抵抗型）に対して用いられる．副作用としては腎障害に注意する．

腎障害の出現はシクロスポリンの血中濃度とよく相関することから，投与に際してはトラフレベル（服薬直前の最低値）を適宜測定し，これが適正濃度の範囲に保たれるように投与量を調節する．一般的にはトラフレベル 100 ～ 200 ng/ml の範囲を維持することが望ましい．

シクロスポリンは主としてチトクローム P-450 Ⅲ A（CYP3A4）により代謝される．したがって，CYP3A を阻害する薬剤や同じく CYP3A で代謝される薬剤はシクロスポリンの濃度を上昇させて，その作用を増強する．逆に，リファピシン（rifampicin）などは，CYP3A を含むいくつかの酵素を誘導することが知られている．このためこのような薬剤の併用によりシクロスポリンの血中濃度が低下するものと考えられる．

f) タクロリムス水和物（tacrolimus hydrate）[4]

タクロリムスもシクロスポリンと同様に，T リンパ球による IL-2 などのサイトカインの産生を抑制することにより，免疫抑制作用を発揮すると考えられている．腎移植，肝移植，心移植，骨髄移植時の拒否反応の抑制や重症筋無力症および RA に適応がある．

タクロリムスもチトクローム P-450 Ⅲ A（CYP3A4）で代謝されるので，シクロスポリンと同様に併用薬剤によりその血中濃度が影響を受ける．

g) メトトレキサート[3]

メトトレキサート（methotrexate）はグルタミン化されて活性体となり，葉酸の還元酵素であるジヒドロ葉酸リダクターゼ（dihydrofolate reductase）を阻害することにより細胞増殖を抑制する．メトトレキサートはその他，プリン代謝系に関係するチミジル酸合成酵素（thymidylate synthase）も阻害するといわれている．メトトレキサートがほかの抗リウマチ薬と比較して効果の発現が速いのは，抗炎症性オータコイドであるアデノシンの放出を促進するためではないかとする報告があるが，詳細は不明である．

メトトレキサートの副作用のうち重篤なものとしては骨髄抑制，間質性肺炎，肝障害があり，その他胃腸障害，口内炎などが報告されている．

h) ミコフェノール酸モフェチル

ミコフェノール酸モフェチル（mycophenolate mofetil）は生体内でミコフェノール酸に代謝され，これがミゾリビンと同様にイノシン一リン酸デヒドロゲナーゼを阻害することにより，プリン合成を阻害して活性化リンパ球の DNA 合成を抑制する．ミゾリビンと同様に，臓器移植時の拒否反応に適応があるが，最近ループス腎炎や重症筋無力症に対しても有効であることが明らかにされている．ミゾリビンと同様に副作用の少ない薬剤である．

i) レフルノミド

レフルノミド（leflunomide）の主たる作用機序は，吸収後生体内で生じるその活性型である A77 1726 によるジヒドロオロテートデヒドロゲナーゼ（dehydroorotate

dehydrogenase）の抑制を介して**ピリミジン合成阻害**とそれに続くDNA合成抑制に基づくと考えられている．これは，MTXがプリン合成に必要な諸酵素を抑制するのと対照的である．

レフルノミドはMTXと同様に細胞のDNA合成を抑制することから，汎血球減少や免疫抑制などの重篤な副作用を生じる可能性がある．特に，MTXやほかの免疫抑制作用を有する薬剤が併用された患者や，それらの薬剤による治療からの切換えの場合にこうした副作用が出現しやすいことが指摘されている．レフルノミドの血漿タンパク結合率は99%と高く，またその血中からの消失半減期は14日と極めて長い．したがって，投与中に骨髄抑制などの重篤な副作用が見られた場合や，レフルノミドから他剤へ切り換える場合にはウォッシュアウトが必要である．これにはコレスチラミン（cholestyramine）（12～24 g/分[3]）が用いられる．

米国で市販後に致命的な**肝障害**の発生が報告されてから，レフルノミドの肝障害についての懸念が高まった．また，本邦では2003年の発売以来，治験時には予想できなかった副作用として，**間質性肺炎**が重大な問題として認識されるに至った．これは組織型としてびまん性肺胞障害型を示すことが多い，致死率は約50％で，MTXの間質性肺炎に比しても重篤である．

〔3〕 抗体療法を中心とした生物学的製薬[5]

近年，RAやSLEなどの自己免疫疾患の治療において，種々のサイトカインや細胞表面抗原に対するモノクローナル抗体などの**生物学的製薬**を用いた治療が盛んに行われるようになってきている．

炎症性サイトカイン（**TNF-α**，**IL-6**）を標的とした生物学的製薬としては，**インフリキシマブ**（infliximab）（キメラ型抗TNF-αモノクローナル抗体），**アダリムマブ**（adalimumab）（ヒト化抗TNF-αモノクローナル抗体），**エタネルセプト**（etanercept）（TNFレセプター2IgGFc融合タンパク質），MRA（ヒト化抗IL-6Rモノクローナル抗体）があり，これらは何れもRAに対して有効である．また，インフリキシマブはクローン病に対して，MRAはキャッスルマン（Castleman）病に対しても有効であることが明らかになっている．細胞表面分子を標的とした治療では，特に悪性リンパ腫に対して用いられる**リツキシマブ**（rituximab）（キメラ型抗CD20モノクローナル抗体）がSLEやRAの治療において有用であることが示され注目されている．このほか，Tリンパ球の活性化に必要な共因子であるCD28と抗原提示細胞上のCD80/CD86の結合を阻害するCTLA-4-IgG1融合タンパク質がRAに対して有用であることも示されている．

一方，マウスIgのCDR領域をヒトIgGに組み込んだ抗IgEモノクローナル抗体である**オマリズマブ**（omalizumab）がハイリスクグループの気管支喘息の患者群で有用であることも示されている．既存の薬剤とは異なり，これらの生物学的製薬による治療は標的もはっきりしており，各種自己免疫疾患の病態解明の進歩とともに，今後も次々に新しい治療製薬が開発されていくものと考えられる．

ARTHRITIS & RHEUMATISM
Vol. 52, No. 6, June 2005, pp 1670–1675
DOI 10.1002/art.21042

Elevated Levels of Soluble Fractalkine in Active Systemic Lupus Erythematosus

Potential Involvement in Neuropsychiatric Manifestations

Nobuyuki Yajima,[1] Tsuyoshi Kasama,[1] Takeo Isozaki,[1] Tsuyoshi Odai,[1] Mizuho Matsunawa,[1] Masao Negishi,[1] Hirotsugu Ide,[1] Yosuke Kameoka,[2] Shunsei Hirohata,[3] and Mitsuru Adachi[1]

Objective. **To determine levels of the soluble form of the chemokine fractalkine (sFkn) and its receptor, CX_3CR1, in patients with systemic lupus erythematosus (SLE) with neuropsychiatric involvement (NPSLE) and in SLE patients without neuropsychiatric involvement, and to assess their relationship with disease activity and organ damage.**

Methods. **Levels of sFkn in serum and cerebrospinal fluid (CSF) were measured by enzyme-linked immunosorbent assay. Expression of Fkn and CX_3CR1 was quantified using real-time polymerase chain reaction. Surface expression of CX_3CR1 on peripheral blood mononuclear cells (PBMCs) was determined by flow cytometry. Disease activity and organ damage were assessed using the SLE Disease Activity Index (SLEDAI) and the Systemic Lupus International Collaborating Clinics/American College of Rheumatology (SLICC/ACR) Damage Index, respectively.**

Results. **Serum sFkn levels were significantly higher in patients with SLE than in patients with rheumatoid arthritis (RA) or healthy controls. In addition, significant correlations between serum sFkn levels and the SLEDAI, the SLICC/ACR Damage Index, anti–double-stranded DNA and anti-Sm antibody titers, immune complex levels (C1q), and serum complement levels (CH50) were observed. Expression of CX_3CR1 was significantly greater in PBMCs from patients with active SLE than in those from RA patients or healthy controls. Levels of sFkn were also significantly higher in CSF from untreated patients with newly diagnosed NPSLE than in SLE patients without neuropsychiatric involvement; treatment reduced both serum and CSF levels of sFkn in patients with SLE.**

Conclusion. **Soluble Fkn and CX_3CR1 may play key roles in the pathogenesis of SLE, including the neuropsychiatric involvement. Soluble Fkn is also a serologic marker of disease activity and organ damage in patients with SLE, and its measurement in CSF may be useful for the diagnosis of NPSLE and followup of patients with NPSLE.**

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by multiorgan damage with infiltration and sequestration of various leukocyte subpopulations, and by the presence of autoantibodies (1). Its etiology is known to involve dysregulation of the immune system, leading to a functional imbalance of T cell subsets, production of a wide range of autoantibodies, and polyclonal B cell activation. In addition, the importance of dysregulation of cytokine expression has been noted (2).

A variety of diffuse and focal neuropsychiatric symptoms often occur in patients with SLE. The features of this condition may include seizures, stroke, depression, psychosis, and cognitive disorders (3). Although the pathogenesis of neuropsychiatric SLE (NPSLE) has not been completely elucidated, a variety of clinical,

Presented in part at the 67th Annual Scientific Meeting of the American College of Rheumatology, Orlando, FL, November 2003.

[1]Nobuyuki Yajima, MD, Tsuyoshi Kasama, MD, PhD, Takeo Isozaki, MD, Tsuyoshi Odai, MD, Mizuho Matsunawa, MD, Masao Negishi, MD, PhD, Hirotsugu Ide, MD, PhD, Mitsuru Adachi, MD, PhD: Showa University School of Medicine, Tokyo, Japan; [2]Yosuke Kameoka, PhD: National Institute of Infectious Diseases, Tokyo, Japan; [3]Shunsei Hirohata, MD, PhD: Teikyo University School of Medicine, Tokyo, Japan.

Address correspondence and reprint requests to Tsuyoshi Kasama, MD, PhD, Division of Rheumatology and Clinical Immunology, First Department of Internal Medicine, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8666, Japan. E-mail: tkasama@med.showa-u.ac.jp.

Submitted for publication October 6, 2004; accepted in revised form February 24, 2005.

laboratory, and radiographic findings are reportedly abnormal in some, but not all, SLE patients with central nervous system (CNS) complications, and the direct and indirect effects of several inflammatory mediators have been emphasized as possible contributors (4).

The chemokine fractalkine (Fkn; CX_3CL1) is synthesized as a type 1 transmembrane protein by endothelial cells (5). The soluble form of Fkn (sFkn) reportedly exerts a chemotactic effect on monocytes, natural killer (NK) cells, and T lymphocytes and acts via its receptor, CX_3CR1, as an adhesion molecule that is able to promote the firm adhesion of a subset of leukocytes to endothelial cells under conditions of physiologic flow (6). Notably, prominent expression of both Fkn and CX_3CR1 has been observed in the CNS (7). Thus, Fkn appears to possess immunoregulatory properties that affect inflammatory/immune cell–endothelial cell interactions and inflammatory responses.

The aim of the present study was to determine serum and CSF levels of sFkn and CX_3CR1 in SLE patients (those with and those without neuropsychiatric involvement) and to assess the relationship of these levels with disease activity and organ damage.

PATIENTS AND METHODS

Patients and samples of serum and CSF. A total of 67 serum samples were obtained from 53 patients with SLE (50 women and 3 men; mean ± SEM age 35.8 ± 1.8 years). In 14 patients, serum samples were collected during both the active and inactive phases of disease. All patients previously or currently fulfilled the American College of Rheumatology (ACR) revised criteria for the classification of SLE (8). Serum samples were also obtained from 91 patients with rheumatoid arthritis (RA) (71 women and 20 men; mean ± SEM age 65.3 ± 1.3 years) who fulfilled the 1987 revised ACR (formerly, the American Rheumatism Association) criteria for a diagnosis of RA (9), and from 28 healthy volunteers (16 women and 12 men; mean ± SEM age 34.4 ± 2.7 years). CSF from the lumbar spine was collected for the purpose of diagnosing NPSLE. For ethical reasons, CSF samples were not collected from SLE patients without any neuropsychiatric involvement or from healthy volunteers.

The SLE Disease Activity Index (SLEDAI) (10) was used to estimate general disease activity, and the Systemic Lupus International Collaborating Clinics (SLICC)/ACR Damage Index (11) was used to estimate organ damage.

Because of the difficulty in confirming neurologic diagnoses and of assigning cause to SLE, we defined NPSLE as the presence of at least 1 clinical feature of neuropsychiatric syndromes (3) and at least 1 of the following: pathologic findings on brain magnetic resonance imaging, diffusely abnormal results of brain single-photon–emission computerized tomography, severely abnormal results on a neuropsychiatric test, an elevated CSF IgG index, or increased interleukin-6 (IL-6) activity in the CSF (12).

Serum levels of specific autoantibodies, complement hemolysis activity (CH50), and immune complex (C1q) as well as albumin and IgG levels in both serum and CSF were determined in the clinical laboratory at our hospital. All human experiments were carried out in accordance with protocols approved by the Human Subjects Research Committee at our institution, and informed consent was obtained from all patients and volunteers.

Soluble Fkn levels. Soluble Fkn was quantified using a double ligand enzyme-linked immunosorbent assay (ELISA) that was a modification of an assay described previously (13). Monoclonal murine anti-human Fkn (4 μg/ml; Genzyme/Techne, Cambridge, MA) and biotinylated polyclonal goat anti-Fkn (0.25 μg/ml; Genzyme/Techne) served as the primary and the secondary antibodies, respectively. This ELISA detects the chemokine domain of human Fkn, and the sensitivity limit is ~150 pg/ml.

Flow cytometry. Flow cytometric analyses of CX_3CR1 expression on peripheral blood mononuclear cells (PBMCs) were carried out as previously described (14). PBMCs were obtained from heparinized venous blood from patients with SLE, patients with RA, and healthy volunteers and then labeled with the indicated primary antibody (anti-CD3–fluorescein isothiocyanate [FITC], anti-CD4–phycoerythrin [PE], anti-CD8–PE, and anti-CD14 [monocyte]–FITC; BD PharMingen, San Diego, CA), or rabbit anti-CX_3CR1 antibody (ProSci, Poway, CA), and then with a secondary antibody (biotin-conjugated anti-rabbit IgG) and a tertiary reagent (CyChrome-conjugated streptavidin; BD PharMingen). The fluorescence intensity was measured on a 3-color FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Isolation of total RNA, and real-time polymerase chain reaction (PCR). Total RNA extracted from PBMCs was reverse transcribed, and then real-time PCR was carried out in a LightCycler (Roche Diagnostics, Mannheim, Germany). To compare quantitative results between different samples, a dilution series of complementary DNA from unstimulated human umbilical vein endothelial cells and normal human PBMCs, which served as internal standards for Fkn and CX_3CR1, respectively, were loaded every time and assigned a value of 100 units. The primers used in the real-time PCR were as follows: for human CX_3CR1, 5′-AGCAGGCATGGAAGTGTTCT (sense) and 5′-GTTGTTTTGTGTGCATTGGG (antisense); for human Fkn, 5′-GCTGAGGAACCCATCCAT (sense) and 5′-GAGGCTCTGGTAGGTGAACA (antisense); for β-actin, which served as an internal control, 5′-CCCAAGGCCAACCGCGAGAAGAT (sense) and 5′-GTCCCGGCCAGCCAGGTCCAG (antisense).

Statistical analysis. Data are expressed as the mean ± SEM. Differences between groups were analyzed using the Mann-Whitney U test. Followup data were analyzed using Wilcoxon's test. The relationship between sFkn levels and the indicated parameters was evaluated using Spearman's rank correlation. P values less than 0.05 were considered significant.

RESULTS

Serum sFkn levels. We initially used ELISAs to assay the levels of sFkn in serum samples obtained from SLE patients with and those without neuropsychiatric

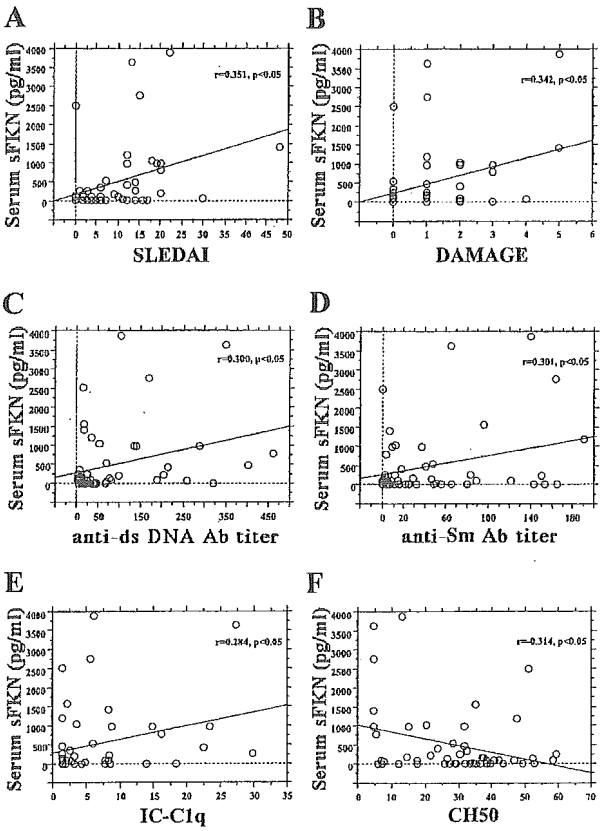

Figure 1. Correlation between serum levels of soluble fractalkine (sFkn) and various clinical parameters. The correlation between serum levels of sFkn (n = 67 samples) and the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) (**A**), organ damage (Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index) (**B**), serum anti–double-stranded DNA (anti-dsDNA) antibody (Ab) titers (**C**), serum anti-Sm antibody titers (**D**), immune complex (IC-C1q) levels (**E**), and serum complement hemolysis activity (CH50) (**F**) in patients with SLE was examined. Serum levels of sFkn were assessed by enzyme-linked immunosorbent assay. Each point represents an individual SLE patient.

involvement (n = 67 samples), patients with RA (n = 91), and healthy controls (n = 28). Serum levels of sFkn were significantly higher in patients with SLE (mean ± SEM 452.7 ± 118.0 pg/ml) than in either patients with RA (mean ± SEM 225.2 ± 53.2 pg/ml; $P < 0.05$) or healthy controls (mean ± SEM 3.2 ± 3.2 pg/ml; $P < 0.01$). We then examined the relationship between serum levels of sFkn and disease activity, organ damage, and the indicated serologic parameters (Figure 1). We observed that serum levels of sFkn were correlated with both disease activity as measured by the SLEDAI (r = 0.351, $P < 0.05$) (Figure 1A) and organ damage as measured by the SLICC/ACR Damage Index (r = 0.342, $P < 0.05$) (Figure 1B) and were also positively correlated with anti–double-stranded DNA (anti-dsDNA) antibody titers (r = 0.300, $P < 0.05$), anti-Sm antibody titers (r = 0.301, $P < 0.05$), and immune complex C1q levels (r = 0.284, $P < 0.05$) (Figures 1C–E) and were negatively correlated with CH50 (r = −0.314, $P < 0.05$) (Figure 1F).

Expression of Fkn and CX_3CR1 messenger RNA (mRNA) and cell-surface expression of CX_3CR1. To better understand the dysregulation of Fkn/CX_3CR1 expression that occurs in SLE, we examined their expression profiles. CX_3CR1 mRNA was more strongly expressed in PBMCs from SLE patients than in those from patients with RA or healthy controls (Figure 2A). In contrast, Fkn expression in PBMCs from all 3 groups was markedly weak, and no significant difference between the groups was observed (results not shown). To examine in more detail the phenotype of cells expressing CX_3CR1, we used flow cytometry to analyze the protein expression of CX_3CR1 in peripheral blood–specific cell populations from SLE patients with active or inactive disease, patients with RA, and healthy controls (Figure 2B). Although both the intensity of CX_3CR1 expression on macrophages (results not shown) and the relative number of affected cells were slightly higher in patients with active SLE than in patients with inactive SLE or healthy controls, the expression of CX_3CR1 protein was most pronounced on CD4+,CD3+ T cells and CD8+,CD3+ T cells from a patient with untreated active SLE.

Neuropsychiatric manifestations and CSF levels of sFkn. Because Fkn has been detected in the nervous system (7), we hypothesized that it may also be involved in the pathogenesis of NPSLE. To test this hypothesis, we first assayed the sFkn levels in CSF from untreated patients with newly diagnosed active SLE, with or without neuropsychiatric involvement. As shown in Figure 3, levels of sFkn in CSF samples from all but 1 SLE patient without neuropsychiatric involvement (non-NPSLE) were relatively low (n = 6, mean ± SEM 186.3 ± 177.1 pg/ml) compared with those in patients with NPSLE (n = 6, mean ± SEM 842.7 ± 190.0 pg/ml). Notably, in contrast with the results observed in CSF, no significant difference in serum sFkn levels was observed between untreated patients with newly diagnosed NPSLE (n = 6, mean ± SEM 467.4 ± 24.0 pg/ml) and SLE patients without overt neuropsychiatric involvement (n = 6, mean ± SEM 400.3 ± 182.0 pg/ml). In addition, there were no significant differences in any serologic para-

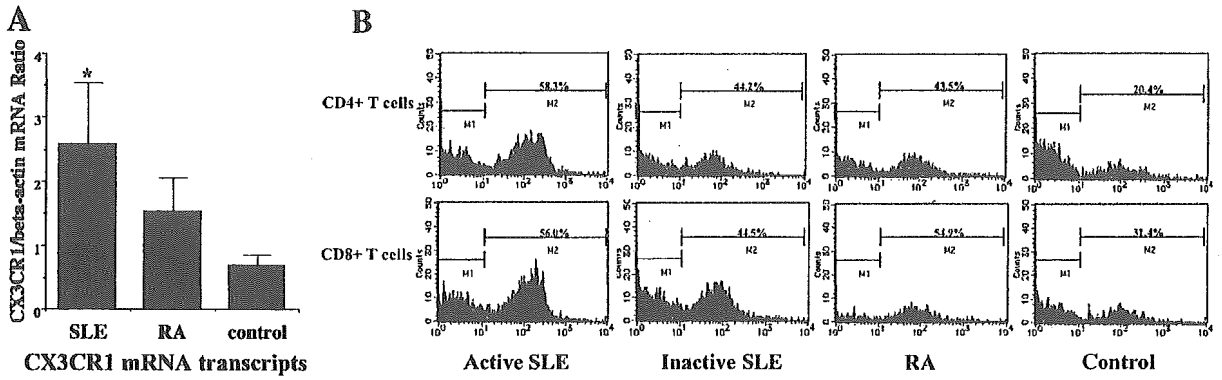

Figure 2. CX_3CR1 expression in peripheral blood mononuclear cells (PBMCs). **A,** Total RNA was isolated from PBMCs obtained from 21 patients with systemic lupus erythematosus (SLE), 30 patients with rheumatoid arthritis (RA), and 10 healthy controls, after which the cDNA was reverse transcribed, and real-time polymerase chain reaction was carried out. Levels of CX_3CR1 mRNA are expressed as the mean and SEM units. * = $P <$ 0.05 versus RA and control. **B,** PBMCs obtained from untreated patients with newly diagnosed SLE (active), treated patients with inactive SLE, patients with RA, and healthy controls were labeled with anti-CD3+, anti-CD4+, anti-CD8+, or anti-CX_3CR1 antibody. CX_3CR1 expression on gated cells (CD4+,CD3+ T cells; CD8+,CD3+ T cells) was assayed by 3-color flow cytometry. Samples obtained from patients with SLE were followed up. M1 = background intensity of isotype-matched control staining. M2 = percent of CX_3CR1-positive cells. Histograms are representative of 3 independent experiments.

meters between patients with NPSLE and SLE patients without neuropsychiatric involvement. Moreover, the IL-6 concentration was shown to be elevated in the CSF of some patients with NPSLE (12), but we found no significant correlation between CSF levels of sFkn and IL-6 activity in the CSF (P = 0.32).

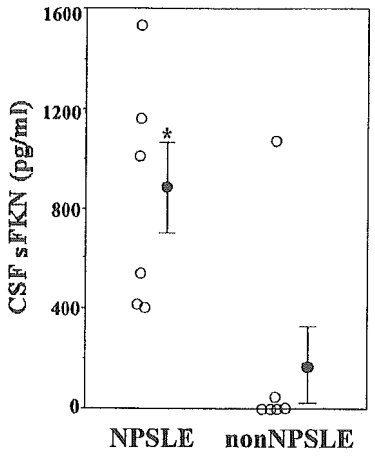

Figure 3. Levels of soluble fractalkine (sFkn) in cerebrospinal fluid (CSF). Samples of CSF were obtained from 6 untreated patients with newly diagnosed neuropsychiatric systemic lupus erythematosus (NPSLE) and 6 SLE patients without neuropsychiatric involvement (non-NPSLE; of these 6 patients who did not fulfill our criteria for NPSLE, 4 described having mild headache, and 2 had mild mood disorder). Soluble Fkn levels were determined by enzyme-linked immunosorbent assay. Each point represents an individual patient. Bars show the mean ± SEM. * = $P < 0.05$ versus non-NPSLE.

Because of the small number of samples examined, we were unable to determine the statistical significance of differences in CSF sFkn levels among patients with any particular neuropsychiatric manifestation. However, when neuropsychiatric manifestations were classified as either diffuse CNS disease (n = 2), which included psychosis, mood disorder, cognitive dysfunction, and acute states of confusion, or as focal CNS disease (n = 4), which included cerebrovascular disease, demyelinating syndrome, headache, aseptic meningitis, seizures, or myelopathy (3), sFkn levels tended to be higher in CSF from patients with focal disease (mean ± SEM 1,029.0 ± 234.1 pg/ml versus 470.0 ± 69.0 pg/ml in patients with diffuse disease).

Followup studies of the effect of treatment on CSF and serum sFkn levels. Figure 4 summarizes the results of followup studies of serum levels of sFkn in 14 patients with SLE (with or without neuropsychiatric manifestations) before and 2–3 months after treatment with glucocorticoids and other immunosuppressive drugs (12 patients received glucocorticoids alone, and 2 patients received glucocorticoids plus cyclophosphamide or cyclosporin A). Notably, serum sFkn levels in patients with active SLE were significantly diminished following successful treatment and clinical improvement (mean 559.4 pg/ml in patients with active disease versus 102.1 pg/ml in patients inactive disease). Although the mean reduction in the CSF

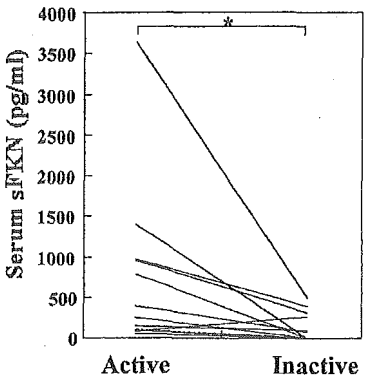

Figure 4. Followup measurements of soluble fractalkine (sFkn) levels in serum from patients with systemic lupus erythematosus (SLE), with or without neuropsychiatric involvement. Paired samples of serum were obtained from 14 patients with SLE (with or without neuropsychiatric involvement) at the time of active disease (newly diagnosed, untreated) and after treatment (inactive disease). Each line represents an individual patient. * = $P < 0.05$.

of 4 patients with NPSLE was quite pronounced (from 877.3 pg/ml to 155.3 pg/ml), it did not reach statistical significance.

DISCUSSION

In the present study, we showed that serum sFkn levels were significantly higher in patients with SLE than in patients with RA or healthy controls and were positively correlated with disease activity, organ damage, anti-dsDNA and anti-Sm antibody titers, and immune complex levels and were negatively correlated with CH50 activity. In addition to the increased expression of sFkn itself, increased expression of its receptor, CX_3CR1, was also detected, especially on CD4+ and CD8+ T cells from patients with active SLE. Finally, levels of sFkn in the CSF were elevated in patients with NPSLE, and both serum and CSF levels of sFkn were reduced by successful treatment with glucocorticoids and other immunosuppressive drugs.

This study is the first to demonstrate increases in sFkn levels in the peripheral blood and CNS of patients with active SLE and patients with NPSLE, respectively. Recent evidence indicates that receptor expression determines the spectrum of action of chemokines in Th1 and Th2 cells. Indeed, Fraticelli et al recently reported that CX_3CR1 was preferentially expressed in Th1 cells, and that Th1 cells, but not Th2 cells, respond to Fkn (15). Furthermore, Fkn also acts via CX_3CR1 as an adhesion molecule and as a chemoattractant, recruiting monocytes, NK cells, and T lymphocytes to endothelial cells. Thus, Fkn likely plays multiple roles in the development of SLE, via Th1 cell–endothelial cell interactions.

Intracranial increases in a variety of cytokines, including IL-6, have been observed in patients with NPSLE (12). This suggests that these various proinflammatory and antiinflammatory cytokines all play specific roles during the progression of NPSLE. In the present study, however, we observed no significant correlation between the levels of sFkn and IL-6 in the CSF of patients with NPSLE, which may indicate that the expression of Fkn and IL-6 is differentially regulated by these 2 mediators during the evolution of the neuropsychiatric manifestations in patients with SLE. Furthermore, we observed that patients with focal neuropsychiatric manifestations had higher CSF levels of sFkn than did those with diffuse disease. These findings are not consistent with the results reported by Erichsen et al (16), who found that sFkn levels in the CSF of human immunodeficiency virus type 1 (HIV-1)–infected patients with cognitive impairment (diffuse disease) were significantly higher than those in HIV-1–infected patients without cognitive impairment. It would be interesting to know whether this difference reflects a difference in the underlying mechanism of the pathogenesis of NPSLE and HIV-induced encephalopathy, and the extent to which Fkn participates in those processes.

In healthy individuals, surface expression of CX_3CR1 has been demonstrated in NK cells, monocytes, and effector T cells (17). CX_3CR1 is also expressed on CD4+ and CD8+ T cells in patients with RA (18). Consistent with those findings, we observed increased expression of CX_3CR1 mainly on CD4+ and CD8+ T cells in patients with active SLE. Moreover, T cell expression of CX_3CR1 was significantly reduced by treatment that diminished disease activity. Although there have been few studies of the expression and regulation of CX_3CR1 under pathologic conditions, it is noteworthy that CX_3CR1 expression on immune cells parallels the sFkn levels, suggesting that CX_3CR1 mediates activation of recruited inflammatory cells, especially CD4+ and CD8+ T cells, during active SLE.

In conclusion, sFkn and CX_3CR1 may play important roles in the pathogenesis of SLE, including the neuropsychiatric involvement. Soluble Fkn is also a serologic marker of disease activity and organ damage in patients with SLE, and its measurement in CSF may be useful for the diagnosis of NPSLE and the followup of patients with NPSLE.

REFERENCES

1. Ruiz-Irastorza G, Khamashta MA, Castellino G, Hughes GR. Systemic lupus erythematosus. Lancet 2001;357:1027–32.
2. Dean GS, Tyrrell-Price J, Crawley E, Isenberg DA. Cytokines and systemic lupus erythematosus. Ann Rheum Dis 2000;59:243–51.
3. ACR Ad Hoc Committee on Neuropsychiatric Lupus Nomenclature. The American College of Rheumatology nomenclature and case definitions for neuropsychiatric lupus syndromes. Arthritis Rheum 1999;42:599–608.
4. Jennekens FG, Kater L. The central nervous system in systemic lupus erythematosus. II. Pathogenetic mechanisms of clinical syndromes: a literature investigation. Rheumatology (Oxford) 2002;41:619–30.
5. Bazan JF, Bacon KB, Hardiman G, Wang W, Soo K, Rossi D, et al. A new class of membrane-bound chemokine with a CX3C motif. Nature 1997;385:640–4.
6. Imai T, Hieshima K, Haskell C, Baba M, Nagira M, Nishimura M, et al. Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion. Cell 1997;91:521–30.
7. Pan Y, Lloyd C, Zhou H, Dolich S, Deeds J, Gonzalo JA, et al. Neurotactin, a membrane-anchored chemokine upregulated in brain inflammation. Nature 1997;387:611–7.
8. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982;25:1271–7.
9. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315–24.
10. Bombardier C, Gladman DD, Urowitz MB, Caron D, Chang CH, and the Committee on Prognosis Studies in SLE. Derivation of the SLEDAI: a disease activity index for lupus patients. Arthritis Rheum 1992;35:630–40.
11. Gladman DD, Urowitz MB. The SLICC/ACR damage index: progress report and experience in the field. Lupus 1999;8:632–7.
12. Hirohata S, Miyamoto T. Elevated levels of interleukin-6 in cerebrospinal fluid from patients with systemic lupus erythematosus and central nervous system involvement. Arthritis Rheum 1990;33:644–9.
13. Kasama T, Shiozawa F, Kobayashi K, Yajima N, Hanyuda M, Takeuchi HT, et al. Vascular endothelial growth factor expression by activated synovial leukocytes in rheumatoid arthritis: critical involvement of the interaction with synovial fibroblasts. Arthritis Rheum 2001;44:2512–24.
14. Shiozawa F, Kasama T, Yajima N, Odai T, Isozaki T, Matsunawa M, et al. Enhanced expression of interferon-inducible protein 10 associated with Th1 profiles of chemokine receptor in autoimmune pulmonary inflammation of MRL/lpr mice. Arthritis Res Ther 2004;6:R78–86.
15. Fraticelli P, Sironi M, Bianchi G, D'Ambrosio D, Albanesi C, Stoppacciaro A, et al. Fractalkine (CX3CL1) as an amplification circuit of polarized Th1 responses. J Clin Invest 2001;107:1173–81.
16. Erichsen D, Lopez AL, Peng H, Niemann D, Williams C, Bauer M, et al. Neuronal injury regulates fractalkine: relevance for HIV-1 associated dementia. J Neuroimmunol 2003;138:144–55.
17. Foussat A, Coulomb-L'Hermine A, Gosling J, Krzysiek R, Durand-Gasselin I, Schall T, et al. Fractalkine receptor expression by T lymphocyte subpopulations and in vivo production of fractalkine in human. Eur J Immunol 2000;30:87–97.
18. Ruth JH, Volin MV, Haines GK III, Woodruff DC, Katschke KJ Jr, Woods JM, et al. Fractalkine, a novel chemokine in rheumatoid arthritis and in rat adjuvant-induced arthritis. Arthritis Rheum 2001;44:1568–81.

Differential effects of IFN-α on the expression of various T_H2 cytokines in human $CD4^+$ T cells

Hideki Shibuya, MD, PhD, and Shunsei Hirohata, MD *Tokyo, Japan*

Background: In both human subjects and mice, T helper cells are classified into 2 subsets, T_H1 and T_H2 cells, on the basis of the cytokines they produce. Although IFN-α has been shown to enhance human T_H1 responses, its influences on human T_H2 responses have not yet been fully characterized. In addition, the mechanism for induction of T_H1 responses by IFN-α has not been fully delineated.

Objective: The present study was undertaken to explore the direct effects of IFN-α on the expression of various cytokines in human $CD4^+$ T cells with a system using immobilized anti-CD3, which permits activation of $CD4^+$ T cells in the complete absence of accessory cells.

Methods: Highly purified $CD4^+$ T cells obtained from healthy donors were stimulated with immobilized anti-CD3 with or without IFN-α and IL-12 in the complete absence of accessory cells. The production of cytokines was estimated by means of ELISA. The expression of mRNA for various cytokines, as well as transcription factors, was evaluated by using quantitative PCR.

Results: IFN-α enhanced IL-4 protein and mRNA expression in immobilized anti-CD3–stimulated $CD4^+$ T cells, irrespective of the presence of IL-12, whereas IFN-α suppressed the expression of IL-5 and IL-13. Of note, IFN-α enhanced the expression of mRNA for c-Maf, T-bet, and Fox-P3, irrespective of the presence of IL-12, but not that for GATA-3, in anti-CD3–stimulated $CD4^+$ T cells.

Conclusion: These results indicate that IFN-α enhances the induction of T_H1 responses through upregulation of T-bet mRNA expression, as well as the induction of T_H2 responses through upregulation of c-Maf mRNA expression, followed by IL-4 expression. Moreover, the data also suggest that IFN-α might suppress the expression of IL-5 and IL-13 in differentiated T_H2 cells. (J Allergy Clin Immunol 2005;116:205-12.)

Key words: Human, T_H1, T_H2, IL-4, IL-5, IL-13, c-Maf, GATA-3, T-bet, Fox-P3

Abbreviations used
IL-12Rβ2: IL-12 receptor β2
NK: Natural killer

In both human subjects and mice, activated $CD4^+$ T cells can be classified into 2 subsets, T_H1 and T_H2 cells, on the basis of the cytokines they produce.[1-4] Thus T_H1 cells produce IFN-γ and IL-2, which are involved in cell-mediated immune responses, whereas T_H2 cells produce mainly IL-4, IL-5, and IL-13, which are involved in humoral immune responses.[5,6] IL-4 has a major role in B-cell activation and isotype switching, particularly in IgE production.[7] On the other hand, IL-5 activates mature eosinophils, prolongs their survival, and contributes to their accumulation at sites of inflammation.[8] In T_H2 cells the transcription factors GATA-3 and c-Maf are selectively expressed and have been shown to regulate T_H2 cytokine expression.[9,10] Thus c-Maf is required for the expression of IL-4,[11] whereas GATA-3 is involved in the expression of IL-5 and IL-13.[12,13]

IFN-α presents potent antiviral actions, as well as immunoregulatory activities, including enhancement of cytotoxic activity of T cells and natural killer (NK) cells.[14] IFN-α has been shown to enhance human T_H1 responses, which is reflected by IFN-γ production in the presence of accessory cells with stimulation by PHA.[15-17] We have recently revealed that IFN-α by itself did not enhance IFN-γ production or mRNA expression in anti-CD3–stimulated human $CD4^+$ T cells in the absence of accessory cells or exogenous IL-12.[18] Consistently, IFN-α enhanced IL-12 receptor β2 (IL-12Rβ2) mRNA expression in $CD4^+$ T cells.[18] It is therefore indicated that the induction of human T_H1 responses by IFN-α requires the presence of IL-12.[18] As for human T_H2 responses, it was previously shown that IFN-α inhibits IL-5 production and mRNA expression in $CD4^+$ T cells.[19] However, the effects of IFN-α on the production of IL-4 in human $CD4^+$ T cells have been uncertain, possibly because of contaminating accessory cells or other supplemental cell lines to cross-link CD3 molecules through soluble anti-CD3. In addition, IL-13 is one of the T_H2 cytokines that has very similar biologic actions of IL-4.[20] Although the regulation of IL-13 production is pivotal in the function of T_H2 cells, the effects of IFN-α on the production of IL-13 have not been determined. The current studies were therefore undertaken to explore the direct effects of IFN-α on the expression of the T_H2 cytokines IL-4, IL-5, and IL-13 in activated human $CD4^+$ T cells by using a system with immobilized anti-CD3, which permits stimulation of T cells in the complete absence of accessory cells or other supplemental cell lines. Special attention was paid to the effects of IFN-α on the expression of mRNA for a variety of transcription factors that regulate the polarization of

From the Department of Internal Medicine, Teikyo University School of Medicine.

Supported by 2004 grant (C2) 16590996 from the Ministry of Education, Culture, Science, and Sports of the Japanese Government and by a grant from Manabe Medical Foundation, Tokyo, Japan.

Received for publication September 24, 2004; revised February 23, 2005; accepted for publication March 14, 2005.

Available online May 2, 2005.

Reprint requests: Shunsei Hirohata, MD, Department of Internal Medicine, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-8605, Japan. E-mail: shunsei@med.teikyo-u.ac.jp.

0091-6749/$30.00

doi:10.1016/j.jaci.2005.03.016

T_H1 and T_H2 cells. The results demonstrate that IFN-α suppresses the expression of IL-5 and IL-13 and enhances the expression of IL-4 in $CD4^+$ T cells. More importantly, IFN-α enhanced the expression of mRNA for c-Maf, T-bet, and Fox-P3, but not for GATA-3, in $CD4^+$ T cells. The data indicate that IFN-α exerts a variety of effects on human T_H1 and T_H2 responses through regulation of mRNA for various transcription factors.

METHODS

mAbs and reagents

Anti-CD3 mAb 64.1 (an IgG2a mAb directed at the CD3 molecule on mature T cells) was a gift of Dr P. E. Lipsky (National Institute of Health, Bethesda, Md). Recombinant human IL-12 was purchased from PeproTech (Rocky Hill, NJ). Recombinant human IFN-α2a was a gift of Nippon Roche (Tokyo, Japan).

Culture medium

RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 100 U/mL penicillin G, 100 μg/mL streptomycin, 0.3 mg/mL L-glutamine, and 10% FBS (Life Technologies) was used for all cultures.

Cell preparation

PBMCs were obtained from healthy adult volunteers by means of centrifugation of heparinized venous blood over sodium diatrizoate-Ficoll gradients (Histopaque; Sigma Chemical Co, St Louis, Mo). PBMCs were depleted of monocytes and NK cells by means of incubation with 5 mM L-leucine methyl ester HCl (Sigma) in serum-free RPMI 1640, as described elsewhere.[21] T cells were obtained from the treated cell population by rosetting with neuraminidase-treated sheep red blood cells, as previously described.[22] Purified $CD4^+$ T cells were further prepared by means of positive selection with anti-CD4 microbeads and MACS (Miltenyi Biotec, Auburn, Calif). The $CD4^+$ T-cell population obtained in this manner contained less than 0.1% esterase-positive cells, less than 0.1% NK cells, less than 0.1% $CD19^+$ cells, and greater than 96% $CD4^+$ T cells.

Cell cultures

Anti-CD3 mAb 64.1 was diluted in RPMI 1640 (2 μg/mL), and 50 μL was placed in each well of 96-well flat-bottomed microtiter plates (no. 3596; Costar, Cambridge, Mass) and incubated at room temperature for 1 hour.[21] The wells were then washed once with culture medium to remove nonadherent mAb before the cells were added. Purified $CD4^+$ T cells (2×10^5/well) were cultured in wells with immobilized anti-CD3 with or without IFN-α (1×10^5 IU/mL) and IL-12 (10 ng/mL). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

RNA isolation and real-time quantitative PCR

Total RNA was isolated from cultured cells with Trizol reagent (Life Technologies), according to the manufacturer's application protocol, and quantified spectrophotometrically. cDNA samples were prepared from 1 μg of total RNA by using the SuperScript reverse transcriptase preamplification system (Life Technologies) with oligo (dT) primer and subjected to real-time quantitative PCR.

Real-time quantitative PCR was performed with the LightCycler rapid thermal cycler system (Roche Diagnostics Ltd, Lewes, United Kingdom), with ready-made primer sets for human IFN-γ, IL-4, IL-5, IL-12Rβ2, GATA-3, T-bet, Fox-P3, or β-actin (LightCycler-Primer Set; Roche Diagnostics GmbH, Heidelberg, Germany) and LightCycler-Fast Start DNA Master SYBR Green I (Roche Diagnostics Ltd). The primers for human c-Maf were designed as follows: forward, 5′-GGTCAGCAAGGAGGAGGT-3′; reverse, 5′-TCTCCTGCTTGAGGTGGTC-3′. PCR reaction condition was identical for all genes except for c-Maf (shown in parentheses): incubation at 95°C for 10 minutes, followed by 35 cycles (40 cycles) of 95°C for 10 seconds, 68°C (60°C for c-Maf) for 10 seconds, and 72°C for 16 seconds (6 seconds for c-Maf). Melting-curve analysis was then carried out to confirm the quality of the performance of the PCR by using 1 cycle of 95°C for 0 seconds and 58°C for 10 seconds (70°C for 15 seconds for c-Maf), with continuous increase to 95°C (rate, 0.1°C/s), followed by cooling at 40°C for 30 seconds. A standard curve was generated in each experiment by using a standard solution in each primer set, and quantitative analysis was performed with LightCycler Software version 3.5. All results were calibrated to the copy number (copies per microliter) of β-actin from each cDNA sample.

Measurement of IL-4, IL-5, and IL-13

IL-4 and IL-13 contents in the supernatants were measured with ELISA kits (Cytoscreen; BioSource International, Camarillo, Calif). The detection limits of the assays were approximately 2.0 and 12.0 pg/mL for IL-4 and IL-13, respectively. The assay is specific for natural and recombinant human IL-4 and IL-13. IL-5 contents in the supernatants were measured with a Human IL-5 ELISA development kit (PeproTech). The detection limit of the assay was approximately 2.0 pg/mL IL-5.

Statistical analysis

The results were analyzed for statistical significance by using the Wilcoxon signed-rank test.

RESULTS

The induction of T_H1 responses in immobilized anti-CD3–activated $CD4^+$ T cells by IFN-α totally depends on the presence of IL-12

We have previously shown that IFN-α enhanced IFN-γ production and mRNA expression only in the presence of IL-12.[18] Initial experiments were carried out to reexamine these direct effects of IFN-α on IFN-γ mRNA expression in activated human $CD4^+$ T cells. As summarized in Table I, IFN-α did not enhance IFN-γ mRNA expression of immobilized anti-CD3–activated $CD4^+$ T cells throughout the cultures, whereas IL-12 significantly enhanced this expression. Of note, IFN-α significantly enhanced IFN-γ mRNA expression in the presence of exogenous IL-12, but not in the absence of IL-12, as early as 3 hours of culture. The results therefore confirm the conclusion that upregulation of the expression of IFN-γ mRNA by IFN-α totally depends on the presence of IL-12.[18] Of note, IFN-α enhanced the expression of IL-12Rβ2 mRNA in anti-CD3–activated $CD4^+$ T cells, irrespective of the presence of IL-12 as early as 3 hours of culture (Table I), as is consistent with the results of a previous study.[18] Taken together, these data suggest that the induction of T_H1 responses by IFN-α is mediated through upregulation of the expression of functional IL-12R, although the precise

TABLE I. Effects of IFN-α and IL-12 on the expression of mRNA for IFN-γ and IL-12Rβ2 in immobilized anti-CD3–activated $CD4^+$ T cells

		× 10^{-2} to β-actin mRNA copies (mean ± SD)			
mRNA	Incubation	Nil	IFN-α	IL-12	IFN-α + IL-12
IFN-γ	3 h	9.8 ± 4.6	10.7 ± 5.1	14.6 ± 6.8*	20.9 ± 6.0*†
	24 h	6.9 ± 3.8	6.4 ± 3.1	27.8 ± 15.0*	38.4 ± 14.7*†
IL-12Rβ2	3 h	0.091 ± 0.072	0.473 ± 0.302*	0.133 ± 0.122*	0.613 ± 0.348*†
	24 h	1.017 ± 0.722	2.497 ± 1.149*	1.728 ± 1.279*	3.673 ± 2.187*†

$CD4^+$ T cells (2×10^5/well) from 6 healthy individuals were cultured in wells with immobilized anti-CD3 (mAb 64.1, 100 ng/well) with or without IFN-α (1×10^5 IU/mL) and IL-12 (10 ng/mL). After 3 or 24 hours of incubation, total RNA was isolated, and real-time quantitative PCR was performed with specific primers for IFN-γ, IL-12Rβ2, and β-actin. All results were calibrated to the copy number of β-actin (copies per microliter) from each cDNA sample.
*$P < .05$ compared with cultures without cytokines *(Nil)*.
†$P < .05$ compared with cultures with IL-12.

mechanism for the upregulation of IL-2R2 mRNA by IFN-α remains unclear.

Differential effects of IFN-α on the expression of T_H2 cytokines in anti-CD3–activated $CD4^+$ T cells

It was previously shown that IFN-α inhibits IL-5 production and mRNA expression in human $CD4^+$ T cells.[19] However, the effects of IFN-α on the production of IL-4 in human $CD4^+$ T cells have been unclear in these studies.[19] It was possible that contaminating accessory cells or supplemental cells to facilitate cross-linkage of CD3 with soluble anti-CD3 might influence the results.[19] The next experiments therefore compared the direct effects of IFN-α on the production of the T_H2 cytokines IL-4, IL-5, and IL-13 in immobilized anti-CD3–activated $CD4^+$ T cells in the complete absence of accessory cells or other supplemental cells. As shown in Fig 1, the production of IL-4 appeared to reach its peak at 24 hours of culture, whereas that of IL-5 and IL-13 markedly increased between 24 and 72 hours. More importantly, IFN-α appeared to enhance the production of IL-4 as early as 24 hours of culture in a dose-dependent manner. By contrast, IFN-α seemed to decrease the production of IL-5 and IL-13 at 72 hours of culture.

The next experiments were carried out to confirm the effects of IFN-α on the expression of IL-4, IL-5, and IL-13 in anti-CD3–stimulated $CD4^+$ T cells. In accordance with previous studies,[19] IFN-α markedly suppressed IL-5 production at 72 hours of culture and IL-5 mRNA expression at 24 hours of culture, irrespective of the presence of IL-12 (Table II). Of note, IFN-α also significantly suppressed the production of IL-13 by $CD4^+$ T cells stimulated with immobilized anti-CD3 for 24 and 72 hours, irrespective of the presence of IL-12 (Table III). The data therefore indicate that IFN-α suppresses the expression of IL-5, as well as that of IL-13.

In contrast with IL-5 and IL-13, the production of IL-4 was increased very modestly between 24 and 72 hours of culture (Fig 1). More importantly, IFN-α significantly enhanced IL-4 production and mRNA expression of anti-CD3–activated $CD4^+$ T cells, irrespective of the presence of IL-12 (Table IV). The results indicate that IFN-α by itself promotes the expression of IL-4 protein and mRNA in spite of its enhancing effects on IL-12Rβ2 mRNA expression.

Effects of IFN-α on the expression of mRNA for a variety of transcription factors in anti-CD3–activated $CD4^+$ T cells

It has been revealed that the transcription factors c-Maf and GATA-3 are expressed exclusively in T_H2 cells and regulate T_H2 cytokine expression.[9,10] Thus c-Maf is required for the expression of IL-4,[11] whereas GATA-3 is critical for the expression of IL-5.[12] It was therefore possible that the effects of IFN-α on the mRNA expression of IL-4 and IL-5 in anti-CD3–activated $CD4^+$ T cells might result from changes in the expression of these transcription factors. On the other hand, previous studies have demonstrated that T-bet plays a critical role in the induction of T_H1 responses.[23] In addition, recent studies have demonstrated that Fox-P3 is required for the development of $CD4^+CD25^+$ regulatory T cells.[24] To examine the effects of IFN-α on the expression of mRNA for these transcription factors, total RNA was isolated from cultured cells, and real-time quantitative PCR was performed with specific primers for c-Maf, GATA-3, T-bet, Fox-P3, and β-actin.

As shown in Fig 2, IFN-α enhanced the expression of mRNA for c-Maf, T-bet, and Fox-P3 in a dose-response manner, whereas it did not appear to affect the expression of GATA-3 mRNA in $CD4^+$ T cells stimulated with immobilized anti-CD3 for 3 hours. Consistently, as can be seen in Fig 3, IFN-α significantly enhanced the expression of mRNA for c-Maf, T-bet, and Fox-P3 in anti-CD3–activated $CD4^+$ T cells, irrespective of the presence of IL-12, whereas IFN-α did not significantly affect the expression of GATA-3 mRNA. Of note, IL-12 also significantly upregulated the expression of mRNA for T-bet and Fox-P3. IFN-α further enhanced their expression in the presence of IL-12. These results suggest that IFN-α might enhance the production of IL-4 in anti-CD3–activated $CD4^+$ T cells by upregulating the expression of c-Maf mRNA. Moreover, the data indicate that IFN-α induces T_H1 responses through upregulation of T-bet. Finally, it is unlikely that the suppression of the expression of IL-5 and IL-13 by IFN-α might be accounted for by the downregulation of GATA-3 mRNA expression. Because

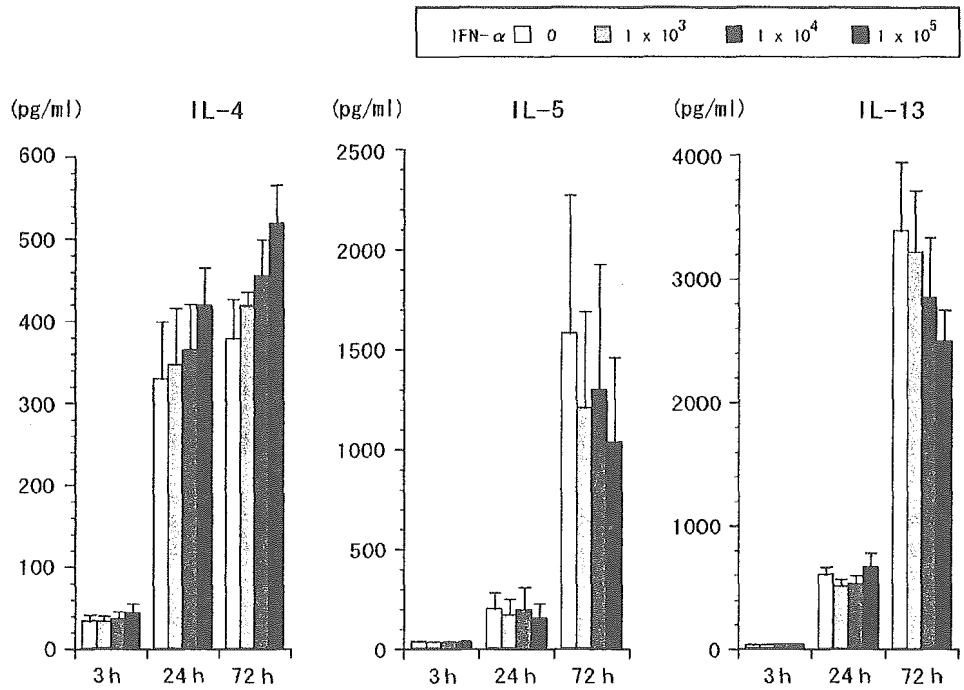

FIG 1. Effects of IFN-α on the production of IL-4, IL-5, and IL-13 by immobilized anti-CD3–activated $CD4^+$ T cells. $CD4^+$ T cells (2×10^5/well) were cultured in wells with immobilized anti-CD3 (mAb 64.1, 100 ng/well) with various concentrations of IFN-α. After 3, 24, or 72 hours of incubation, the supernatants were assayed for IL-4, IL-5, and IL-13 contents by means of ELISA. Mean values of 2 independent experiments are shown. *Error bars* represent the SD of 2 independent experiments.

TABLE II. Effects of IFN-α and IL-12 on IL-15 protein production and mRNA expression in immobilized anti-CD3–activated $CD4^+$ T cells

IL-13 production, pg/mL (mean ± SD)	IL-5 production, pg/mL (mean ± SD)	IL-5 mRNA (mean ± SD)
Nil	77.3 ± 52.3	5.183 ± 5.323
IFN-α	16.2 ± 26.5*	1.402 ± 1.671*
IL-12	87.2 ± 61.8	4.510 ± 3.995
IFN-α + IL-12	20.4 ± 11.6*†	1.643 ± 1.400*†

$CD4^+$ T cells (2×10^5/well) from 6 healthy individuals were cultured in wells with immobilized anti-CD3 (mAb 64.1, 100 ng/well) with or without IFN-α (1×10^5 IU/mL) and IL-12 (10 ng/mL). After 72 hours of incubation, the supernatants were assayed for IL-5 contents by means of ELISA. After 24 hours of incubation, total RNA was isolated, and real-time quantitative PCR was performed with specific primers for IL-5 and β-actin. All results were calibrated to the copy number of β-actin (copies per microliter) from each cDNA sample.

*$P < .05$ compared with cultures without cytokines *(Nil)*.

†$P < .05$ compared with cultures with IL-12.

IFN-α also enhanced Fox-P3 and T-bet mRNA expression, it is more likely that induction of T_H1-like regulatory cells that express Fox-P3[25] might be involved in suppression of the expression of IL-5 and IL-13.

DISCUSSION

Previous studies have reported that type 1 interferons (IFN-α/β) act directly on human, but not mouse, T cells to drive T_H1 development, bypassing the need for IL-12–induced signaling.[15] However, our previous and present studies demonstrated that IFN-α did not affect IFN-γ protein and mRNA expression in $CD4^+$ T cells unless IL-12 was present, confirming that IFN-α by itself might not be sufficient for the optimal induction of T_H1 responses.[18] It should be noted that previous studies explored IFN-α–induced T_H1 responses in the presence of accessory cells with stimulation by PHA.[15-17] Because accessory cells produce IL-12, it is likely that the induction of T_H1 responses by IFN-α in PHA-stimulated cultures might be mediated by cooperative actions of IFN-α and IL-12. In fact, no IL-12 could be detected in the culture supernatants of anti-CD3–activated $CD4^+$ T cells in our system.[18] These findings confirm that the presence of IL-12 is essential for the upregulation of IFN-γ expression by IFN-α. Consistently, we and others have demonstrated that IFN-α enhances the expression of IL-12Rβ2 mRNA in anti-CD3–activated $CD4^+$ T cells.[16,18] It was therefore most likely that IFN-α–induced T_H1 responses observed in the previous studies[15-17] might be mediated through upregulation of the responsiveness to IL-12 secreted from accessory cells. In fact, a recent study also suggests that IFN-α might enhance IFN-γ production in human T cells through IL-12–dependent mechanisms.[26]

We have shown that IFN-α enhanced the expression of T-bet mRNA in immobilized anti-CD3–activated $CD4^+$ T cells. In this regard it has recently been disclosed that IL-27 and IFN-α activate signal transducer and activator

TABLE III. Effects of IFN-α and IL-12 on IL-13 production by immobilized anti-CD3–activated $CD4^+$ T cells

	IL-13 production, pg/mL (mean ± SD)			
Incubation	Nil*	IFN-α	IL-12	IFN-α + IL-12
24 h	137.6 ± 131.5	71.5 ± 59.5*	113.1 ± 88.5	62.4 ± 45.6*†
72 h	1278.8 ± 640.8	650.9 ± 429.0*	1310.8 ± 797.6	685.0 ± 496.0*†

$CD4^+$ T cells (2×10^5/well) from 7 healthy individuals were cultured in wells with immobilized anti-CD3 (mAb 64.1, 100 ng/well) with or without IFN-α (1×10^5 IU/mL) and IL-12 (10 ng/mL). After 24 or 72 hours of incubation, the supernatants were assayed for IL-13 contents by means of ELISA.
*$P < .05$ compared with cultures without cytokines *(Nil)*.
†$P < .05$ compared with cultures with IL-12.

TABLE IV. Effects of IFN-α and IL-12 on IL-4 protein production and mRNA expression in immobilized anti-CD3–activated $CD4^+$ T cells

		Addition			
Assays	Length of incubation	Nil	IFN-α	IL-12	IFN-α ± IL-12
IL-4 protein production (pg/mL) (mean ± SD)	24 h	46.9 ± 42.3	75.1 ± 58.8*	53.5 ± 49.1	77.1 ± 57.3*†
	72 h	103.1 ± 69.4	121.2 ± 81.6*	121.4 ± 71.8*	143.4 ± 86.5*†
IL-4 mRNA expression ($\times 10^{-3}$ to β-actin mRNA copies) (mean ± SD)	3 h	2.172 ± 1.662	4.602 ± 2.718*	1.403 ± 0.835*	4.145 ± 2.445*†
	24 h	1.478 ± 1.052	1.943 ± 1.044*	1.213 ± 0.781*	1.688 ± 1.242†

$CD4^+$ T cells (2×10^5/well) from 8 healthy individuals were cultured in wells with immobilized anti-CD3 (mAb 64.1, 100 ng/well) with or without IFN-α (1×10^5 IU/mL) and IL-12 (10 ng/mL). After 24 or 72 hours of incubation, the supernatants were assayed for IL-4 contents by means of ELISA. After 3 or 24 hours of incubation, total RNA was isolated, and real-time quantitative PCR was performed with specific primers for IL-4 and β-actin. All results were calibrated to the copy number of β-actin (copies per microliter) from each cDNA sample.
*$P < .05$ compared with cultures without cytokines *(Nil)*.
†$P < .05$ compared with cultures with IL-12.

of transcription 1 and 3 to induce T-bet mRNA in naive T cells.[27] Induction of T-bet resulted in upregulation of IL-12Rβ2 on naive T cells.[27] It was thus possible that T-bet might act upstream of IL-12Rβ2 in early T_H1 differentiation. However, IFN-γ induced expression of T-bet, but not IL-12Rβ2, in naive T cells.[27] It is therefore most likely that T-bet and IL-12Rβ2 might be regulated by independent mechanisms. In addition, our data indicate that T-bet and IL-12Rβ2 are not sufficient for the induction of optimal T_H1 responses, although they are important for early T_H1 commitment.[27]

Several studies showed that the T_H2 cytokines IL-4, IL-5, and IL-13 are regulated by a coordinated mechanism.[28,29] On the other hand, a number of other studies showed evidence for differential regulation of the expression of IL-4 and IL-5 in murine and human T cells.[30-32] The results in the current studies have disclosed that IFN-α displays differential effects on the expression of these T_H2 cytokines. Thus IFN-α suppressed the expression of IL-5 and IL-13, whereas it enhanced the expression of IL-4 in anti-CD3–activated $CD4^+$ cells. Of note, in the report by Cousins et al,[29] the expression of IL-5 is well correlated with that of IL-13, but not with that of IL-4. Taken together, it is most likely that the expression of various T_H2 cytokines might be differentially regulated in $CD4^+$ T cells.

Some studies disclosed that IFN-α inhibited the differentiation of T_H2 cells producing IL-4 and IL-5 in bulk cultures of PBMCs,[33,34] whereas other studies showed that IFN-α enhanced the production of IL-4 by PBMCs from patients with chronic hepatitis C.[35] Of note, it has been also demonstrated that IFN-α by itself directly inhibited the production of IL-5 by $CD4^+$ T cells stimulated with PMA and anti-CD28.[19] Although IL-5 production was strongly inhibited in this study, IL-4 production was either upregulated or unchanged by IFN-α.[19] It was thus suggested that the effects of IFN-α on IL-4 production might depend on the system considered. Moreover, it was also possible that the contaminating non-T cells[33-35] or supplemental fibroblasts[19] might result in conflicting results as to the effects of IFN-α on IL-4 expression. In this regard the results in the current studies have clearly demonstrated that IFN-α directly upregulates IL-4 production and mRNA expression in $CD4^+$ T cells stimulated with immobilized anti-CD3 in the complete absence of other cell components, such as NK cells, accessory cells, and B cells.

The time kinetics of IL-5 and IL-13 expression were quite different from those of IL-4 expression in cultures of immobilized anti-CD3–stimulated $CD4^+$ T cells. Thus the production of IL-4 appeared to reach its peak at 24 hours of culture, whereas that of IL-5 and IL-13 markedly increased between 24 and 72 hours, during which the

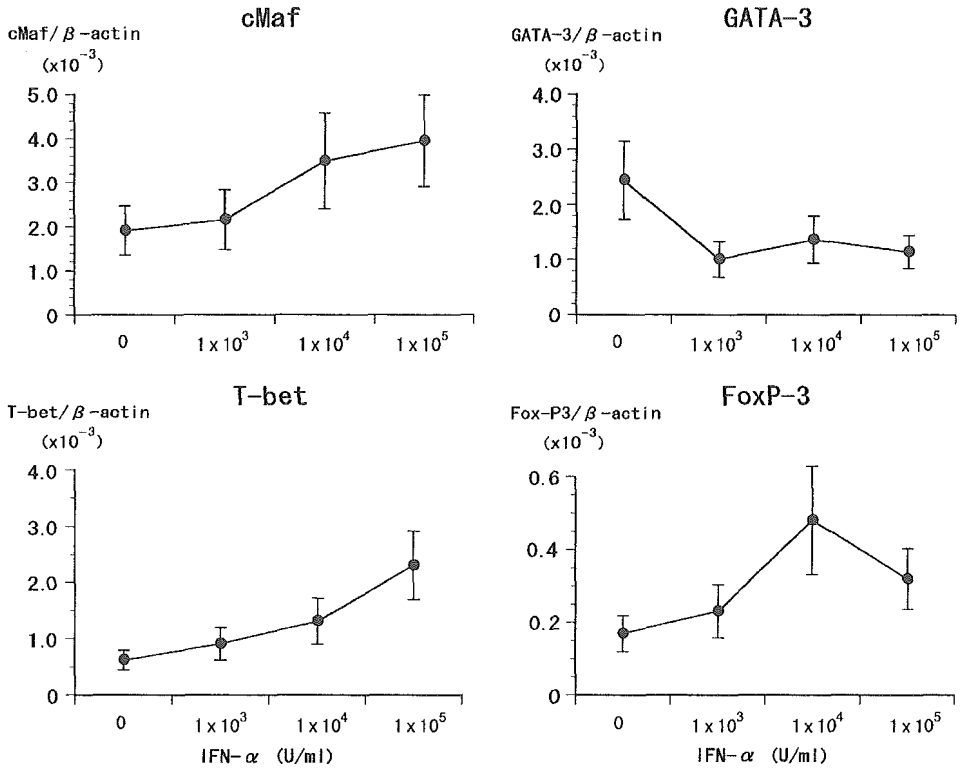

FIG 2. Effects of IFN-α on the expression of mRNA for various transcription factors in anti-CD3–activated $CD4^+$ T cells. $CD4^+$ T cells (2×10^5/well) were cultured in wells with immobilized anti-CD3 (mAb 64.1, 100 ng/well) with various concentrations of IFN-α. After 3 hours of incubation, total RNA was isolated, and real-time quantitative PCR was performed with specific primers for c-Maf, GATA-3, T-bet, Fox-P3, and β-actin. All results were calibrated to the copy number of β-actin (copies per microliter) from each cDNA sample. *Error bars* indicate the SD of duplicated determinations.

production of IL-4 was almost unchanged. Moreover, the upregulation of IL-4 production by IFN-α was clearly observed as early as 24 hours of culture, whereas the downregulation of IL-5/IL-13 production by IFN-α became evident at 72 hours of culture, when the effect of IFN-α on IL-4 production was less marked. These results suggest that IL-4 and IL-5/IL-13 might be expressed at different stages of activation of $CD4^+$ T cells or be expressed in different subsets of $CD4^+$ T cells. In fact, previous studies showed that IL-4 is prominently produced by naive T_H0 cells in contrast to IL-5 and IL-13, which are generally limited to T_H2-like effector-memory cells.[36] Moreover, it has been revealed that the presence of IL-4 in initial priming of $CD4^+$ T cells directs the development of T_H2-like effector cells, although the source of IL-4 initiating this process is debated.[37,38] It is therefore possible that IFN-α might facilitate the development of T_H2 cells through upregulation of IL-4 expression in naive T_H0 cells. Further studies are required to clarify this point.

It has been revealed that the transcription factors c-Maf and GATA-3 are selectively expressed in T_H2 cells and have been shown to regulate T_H2 cytokine expression.[9,10] Thus c-Maf is required for the expression of IL-4,[11] whereas GATA-3 is critical for the expression of IL-5[12] and IL-13.[13] It was therefore possible that the differential effects of IFN-α on the expression of IL-4, IL-5, and IL-13 in anti-CD3–activated $CD4^+$ T cells might result from changes in the expression of these transcription factors. Of note, we demonstrate that IFN-α enhances the expression of c-Maf mRNA, whereas it does not affect the expression of GATA-3 mRNA in $CD4^+$ T cells stimulated with immobilized anti-CD3. These results therefore suggest that IFN-α might enhance the expression of IL-4 in anti-CD3–activated $CD4^+$ T cells by upregulating the expression of c-Maf mRNA. Moreover, it is also suggested that IFN-α might suppress the expression of IL-5 and IL-13 through unknown mechanisms that do not involve the expression of GATA-3 mRNA.

Recent studies have demonstrated the presence of a population of regulatory T cells that developed from naive $CD4^+CD25^-$ T cells during a T_H1 response distinct from $CD25^+$ regulatory T cells.[25] These regulatory T cells expressed Fox-P3 and T-bet and potently inhibited the development of airway hyperreactivity.[25] Of note, we have also shown in the present study that IFN-α upregulates the expression of mRNA for Fox-P3, as well as T-bet, in immobilized anti-CD3–stimulated $CD4^+$ T cells. It is

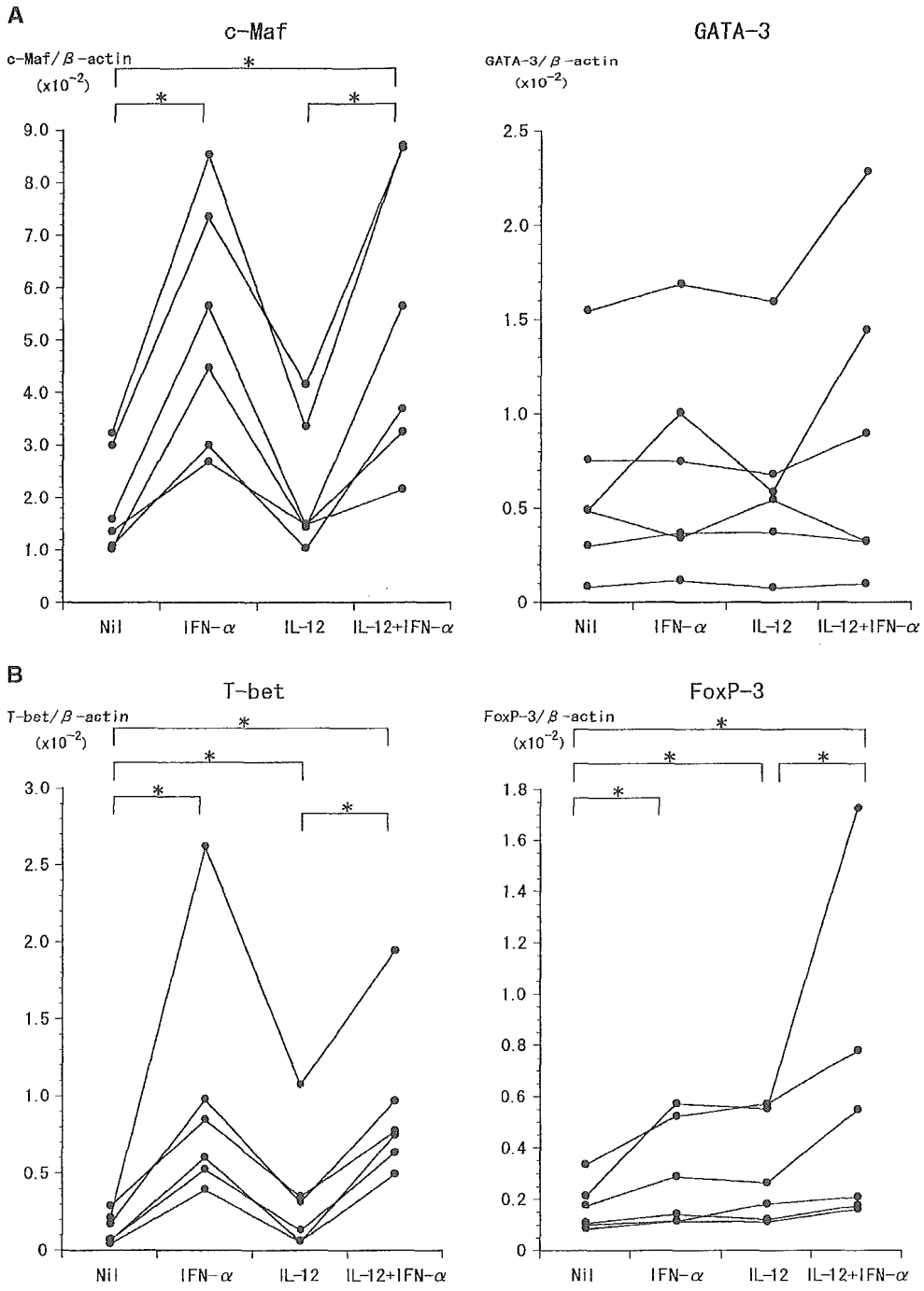

FIG 3. Effects of IFN-α and IL-12 on the expression of mRNA for various transcription factors in anti-CD3–activated $CD4^+$ T cells. $CD4^+$ T cells (2×10^5/well) were cultured in wells with immobilized anti-CD3 (mAb 64.1, 100 ng/well) with or without IFN-α (1×10^5 IU/mL) and IL-12 (10 ng/mL). After 3 hours of incubation, total RNA was isolated, and real-time quantitative PCR was performed with specific primers for c-Maf, GATA-3, T-bet, FoxP3, and β-actin. All results were calibrated to the copy number of β-actin (copies per microliter) from each cDNA sample. Each *line* on the graph is representative of the same cell preparation from the same donor. $*P < .05$.

therefore possible that IFN-α might promote the development of T_H1-like regulatory T cells, which might suppress T_H2 responses, including the expression of IL-5 and IL-13.

In summary, taken together with data from previous studies,[17,19,33] the data in the present study provide a rational basis for therapeutic use of IFN-α therapy in T cell–mediated disorders associated with IL-5 hyperproduction, such as hypereosinophilic syndrome.[39] Of note, it has been recently reported that IFN-α treatment rapidly improved the clinical condition of patients with

corticosteroid-resistant asthma.[40] The establishment of a correct T_H1/T_H2 balance and the induction of the IL-10 gene have been suggested as potential mechanisms of action.[40] It is also possible that induction of T_H1-like regulatory T cells might be involved in the IFN-α–mediated improvement of corticosteroid-resistant asthma. Further studies designed to explore the capacity of IFN-α to induce T_H1-like regulatory T cells would be important for a complete understanding of its role in the treatment of bronchial asthma and atopic diseases.

We to thank Drs A. Ishii, T. Sawada, Y. Morita, and K. Yamamoto for helpful advice and Ms C. Kawashima for preparing the manuscript.

REFERENCES

1. Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. Nature 1996;383:787-93.
2. Constant SL, Bottomly K. Induction of Th1 and Th2 CD4+ T cell responses: the alternative approaches. Annu Rev Immunol 1997;15: 297-322.
3. Lichtman AH, Abbas AK. T-cell subsets: recruiting the right kind of help. Curr Biol 1997;7:R242-4.
4. Romagnani S. The Th1/Th2 paradigm. Immunol Today 1997;18:263-6.
5. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 1989;7:145-73.
6. Kamogawa Y, Minasi LA, Carding SR, Bottomly K, Flavell RA. The relationship of IL-4– and IFN gamma–producing T cells studied by lineage ablation of IL-4–producing cells. Cell 1993;75:985-95.
7. Coffman RL, Lebman DA, Rothman P. Mechanism and regulation of immunoglobulin isotype switching. Adv Immunol 1993;54:229-70.
8. Sanderson CJ. Interleukin-5, eosinophils, and disease. Blood 1992;79: 3101-9.
9. Ho IC, Hodge MR, Rooney JW, Glimcher LH. The proto-oncogene c-maf is responsible for tissue-specific expression of interleukin-4. Cell 1996;85:973-83.
10. Zheng W, Flavell RA. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell 1997;89: 587-96.
11. Kim JI, Ho IC, Grusby MJ, Glimcher LH. The transcription factor c-Maf controls the production of interleukin-4 but not other Th2 cytokines. Immunity 1999;10:745-51.
12. Zhang DH, Cohn L, Ray P, Bottomly K, Ray A. Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene. J Biol Chem 1997;272:21597-603.
13. Lavenu-Bombled C, Trainor CD, Makeh I, Romeo PH, Max-Audit I. Interleukin-13 gene expression is regulated by GATA-3 in T cells: role of a critical association of a GATA and two GATG motifs. J Biol Chem 2002;277:18313-21.
14. Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP. Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annu Rev Immunol 1999;17:189-220.
15. Rogge L, D'Ambrosio D, Biffi M, Penna G, Minetti LJ, Presky DH, et al. The role of Stat4 in species-specific regulation of Th cell development by type I IFNs. J Immunol 1998;161:6567-74.
16. Rogge L, Barberis-Maino L, Biffi M, Passini N, Presky DH, Gubler U, et al. Selective expression of an interleukin-12 receptor component by human T helper 1 cells. J Exp Med 1997;185:825-31.
17. Parronchi P, Mohapatra S, Sampognaro S, Giannarini L, Wahn U, Chong P, et al. Effects of interferon-alpha on cytokine profile, T cell receptor repertoire and peptide reactivity of human allergen-specific T cells. Eur J Immunol 1996;26:697-703.
18. Shibuya H, Nagai T, Ishii A, Yamamoto K, Hirohata S. Differential regulation of Th1 responses and CD154 expression in human CD4+ T cells by IFN-alpha. Clin Exp Immunol 2003;132:216-24.
19. Schandene L, Del Prete GF, Cogan E, Stordeur P, Crusiaux A, Kennes B, et al. Recombinant interferon-alpha selectively inhibits the production of interleukin-5 by human CD4+ T cells. J Clin Invest 1996;97:309-15.
20. Terabe M, Park JM, Berzofsky JA. Role of IL-13 in regulation of anti-tumor immunity and tumor growth. Cancer Immunol Immunother 2004; 53:79-85.
21. Geppert TD, Lipsky PE. Accessory cell independent proliferation of human T4 cells stimulated by immobilized monoclonal antibodies to CD3. J Immunol 1987;138:1660-6.
22. Hirohata S. Human Th1 responses driven by IL-12 are associated with enhanced expression of CD40 ligand. Clin Exp Immunol 1999;115:78-85.
23. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell 2000;100:655-69.
24. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 2003;4:330-6.
25. Stock P, Akbari O, Berry G, Freeman GJ, DeKruyff RH, Umetsu DT. Induction of T helper type 1-like regulatory cells that express Foxp3 and protect against airway hyper-reactivity. Nat Immunol 2004;5:1149-56.
26. Wu CY, Gadina M, Wang K, O'Shea J, Seder RA. Cytokine regulation of IL-12 receptor beta2 expression: differential effects on human T and NK cells. Eur J Immunol 2000;30:1364-74.
27. Hibbert L, Pflanz S, De Waal Malefyt R, Kastelein RA. IL-27 and IFN-α signal via Stat1 and Stat3 and induce T-Bet and IL-12Rβ2 in naive T cells. J Interferon Cytokine Res 2003;23:513-22.
28. Kelly BL, Locksley RM. Coordinate regulation of the IL-4, IL-13, and IL-5 cytokine cluster in Th2 clones revealed by allelic expression patterns. J Immunol 2000;165:2982-6.
29. Cousins DJ, Lee TH, Staynov DZ. Cytokine coexpression during human Th1/Th2 cell differentiation: direct evidence for coordinated expression of Th2 cytokines. J Immunol 2002;169:2498-506.
30. Bohjanen PR, Okajima M, Hodes RJ. Differential regulation of interleukin 4 and interleukin 5 gene expression: a comparison of T-cell gene induction by anti-CD3 antibody or by exogenous lymphokines. Proc Natl Acad Sci U S A 1990;87:5283-7.
31. Jung T, Schauer U, Rieger C, Wagner K, Einsle K, Neumann C, et al. Interleukin-4 and interleukin-5 are rarely co-expressed by human T cells. Eur J Immunol 1995;25:2413-6.
32. Tendler CL, Burton JD, Jaffe J, Danielpour D, Charley M, McCoy JP, et al. Abnormal cytokine expression in Sezary and adult T-cell leukemia cells correlates with the functional diversity between these T-cell malignancies. Cancer Res 1994;54:4430-5.
33. Parronchi P, De Carli M, Manetti R, Simonelli C, Sampognaro S, Piccinni MP, et al. IL-4 and IFN (alpha and gamma) exert opposite regulatory effects on the development of cytolytic potential by Th1 or Th2 human T cell clones. J Immunol 1992;149:2977-83.
34. Demeure CE, Wu CY, Shu U, Schneider PV, Heusser C, Yssel H, et al. In vitro maturation of human neonatal CD4 T lymphocytes. II. Cytokines present at priming modulate the development of lymphokine production. J Immunol 1994;152:4775-82.
35. Andreone P, Gramenzi A, Loggi E, Favarelli L, Cursaro C, Margotti M, et al. In vitro effect of indomethacin and interferon-α on Th1 and Th2 cytokine synthesis in patients with chronic hepatitis C. Cytokine 2004; 26:95-101.
36. Farrar JD, Asnagli H, Murphy KM. T helper subset development: roles of instruction, selection, and transcription. J Clin Invest 2002;109:431-5.
37. Seder RA, Paul WE, Davis MM, Fazekas DS. The presence of interleukin 4 during in vitro priming determines the lymphokine-producing potential of CD4+ T cells from T cell receptor transgenic mice. J Exp Med 1992;176:1091-8.
38. Swain SL, Weinberg AD, English M, Huston G. IL-4 directs the development of Th2-like helper effectors. J Immunol 1990;145:3796-806.
39. Cogan E, Schandene L, Crusiaux A, Cochaux P, Velu T, Goldman M. Brief report: clonal proliferation of type 2 helper T cells in a man with the hypereosinophilic syndrome. N Engl J Med 1994;330:535-8.
40. Simon HU, Seelbach H, Ehmann R, Schmitz M. Clinical and immunological effects of low-dose IFN-α treatment in patients with corticosteroid-resistant asthma. Allergy 2003;58:1250-5.

Inhibition of CX3CL1 (Fractalkine) Improves Experimental Autoimmune Myositis in SJL/J Mice[1]

Fumihito Suzuki,* Toshihiro Nanki,[2]* Toshio Imai,† Hirotoshi Kikuchi,‡ Shunsei Hirohata,‡ Hitoshi Kohsaka,* and Nobuyuki Miyasaka*

Idiopathic inflammatory myopathy is a chronic inflammatory muscle disease characterized by mononuclear cell infiltration in the skeletal muscle. The infiltrated inflammatory cells express various cytokines and cytotoxic molecules. Chemokines are thought to contribute to the inflammatory cell migration into the muscle. We induced experimental autoimmune myositis (EAM) in SJL/J mice by immunization with rabbit myosin and CFA. In the affected muscles of EAM mice, CX3CL1 (fractalkine) was expressed on the infiltrated mononuclear cells and endothelial cells, and its corresponding receptor, CX3CR1, was expressed on the infiltrated CD4 and CD8 T cells and macrophages. Treatment of EAM mice with anti-CX3CL1 mAb significantly reduced the histopathological myositis score, the number of necrotic muscle fibers, and infiltration of CD4 and CD8 T cells and macrophages. Furthermore, treatment with anti-CX3CL1 mAb down-regulated the mRNA expression of TNF-α, IFN-γ, and perforin in the muscles. Our results suggest that CX3CL1-CX3CR1 interaction plays an important role in inflammatory cell migration into the muscle tissue of EAM mice. The results also point to the potential therapeutic usefulness of CX3CL1 inhibition and/or blockade of CX3CL1-CX3CR1 interaction in idiopathic inflammatory myopathy. *The Journal of Immunology, 2005, 175: 6987–6996.*

Idiopathic inflammatory myopathy (IIM),[3] including polymyositis and dermatomyositis, is characterized by chronic inflammation of the voluntary muscles associated with infiltration of inflammatory cells, including CD4 and CD8 T cells and macrophages, in the skeletal muscle (1–3). Infiltrated CD4 and CD8 T cells express cytotoxic molecules, such as perforin and granzyme granules, and the T cells and macrophages express inflammatory cytokines, such as TNF-α and IFN-γ (4–8). Therefore, the infiltrated inflammatory cells might play an important role in the pathogenesis of IIM. The inflammatory cell migration into the muscle is thought to involve the interaction of chemokines and chemokine receptors (9–14).

Chemokines are involved in leukocyte recruitment and activation at the site of inflammatory lesion (15). Approximately 50 chemokines have been identified to date, and they are classified into four subfamilies, C, CC, CXC, and CX3C chemokines, based on the conserved cystein motifs (16). Although the majority of chemokines are small secreted molecules, CX3CL1 (fractalkine) is expressed on the cell surface as a membrane-bound molecule (17, 18). The membrane-bound CX3CL1 is expressed on endothelial cells stimulated with TNF-α, IL-1, and IFN-γ (19–21), induces adhesion of the leukocytes, and supports leukocyte transmigration into tissue (22, 23). The soluble form of CX3CL1 is generated by proteolytic cleavage at a membrane-proximal region of the membrane-bound CX3CL1 by TNF-α-converting enzyme (a disintegrin and metalloproteinase domain 17) and a disintegrin and metalloproteinase domain 10 (24, 25), and is known to induce leukocyte migration (23). In contrast, CX3CR1, a unique receptor for CX3CL1, is expressed on peripheral blood CD4 and CD8 T cells that express cytotoxic molecules and type 1 cytokines (26, 27). CX3CR1 is also expressed on monocytes/macrophages, NK cells, and dendritic cells (28, 29).

Based on the infiltration of CTLs and macrophages into the affected muscles in patients with IIM, we speculated that the CX3CL1-CX3CR1 interaction might contribute to the inflammatory cell migration. In the present study we induced experimental autoimmune myositis (EAM) in SJL/J mice and examined CX3CL1 and CX3CR1 expression in the affected muscle of EAM mice. Furthermore, we studied the effect of CX3CL1 inhibition on EAM mice.

Materials and Methods

Induction of EAM

Male 5-wk-old SJL/J mice were purchased from Charles River Japan. Purified myosin from rabbit skeletal muscle (6.6 mg/ml; Sigma-Aldrich) was emulsified with an equal amount of CFA (Difco Laboratories) with 3.3 mg/ml *Mycobacterium butyricum* (Difco Laboratories). Mice were immunized intracutaneously with 100 μl of emulsion into four locations (total, 400 μl) on the back on days 0, 7, and 14. On day 21, the mice were killed, and the quadriceps femoris muscles were harvested. The muscle tissues were frozen immediately in chilled isopentane precooled in liquid nitrogen, and then 6-μm-thick cryostat sections were prepared at intervals of 200 μm. The sections were stained with H&E or used for immunohistochemistry. The experimental protocol was approved by the institutional animal care and use committee of Tokyo Medical and Dental University.

Immunohistochemistry

Immunohistological staining was performed as described previously (26, 30) with some modifications. Briefly, 6-μm-thick sections were air-dried and fixed in cold acetone at −20°C for 3 min. After air-drying at room

*Department of Medicine and Rheumatology, Tokyo Medical and Dental University Graduate School, Tokyo, Japan; †KAN Research Institute, Kyoto, Japan; and ‡Department of Internal Medicine, Teikyo University School of Medicine, Tokyo, Japan

Received for publication January 4, 2005. Accepted for publication September 1, 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 in part by a grant-in-aid from the Japan Intractable Diseases Research Foundation; the Fund for Intractable Diseases Research by Atsuko Ouchi from Tokyo Medical and Dental University; the Ministry of Health, Labor, and Welfare; and the Ministry of Education, Science, Sports, and Culture, Japan.

[2] Address correspondence and reprint requests to Dr. Toshihiro Nanki, Department of Medicine and Rheumatology, Tokyo Medical and Dental University Graduate School, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. E-mail address: nanki.rheu@tmd.ac.jp

[3] Abbreviations used in this paper: IIM, idiopathic inflammatory myopathy; EAM, experimental autoimmune myositis; PTX, pertussis toxin.

0022-1767/05/$02.00

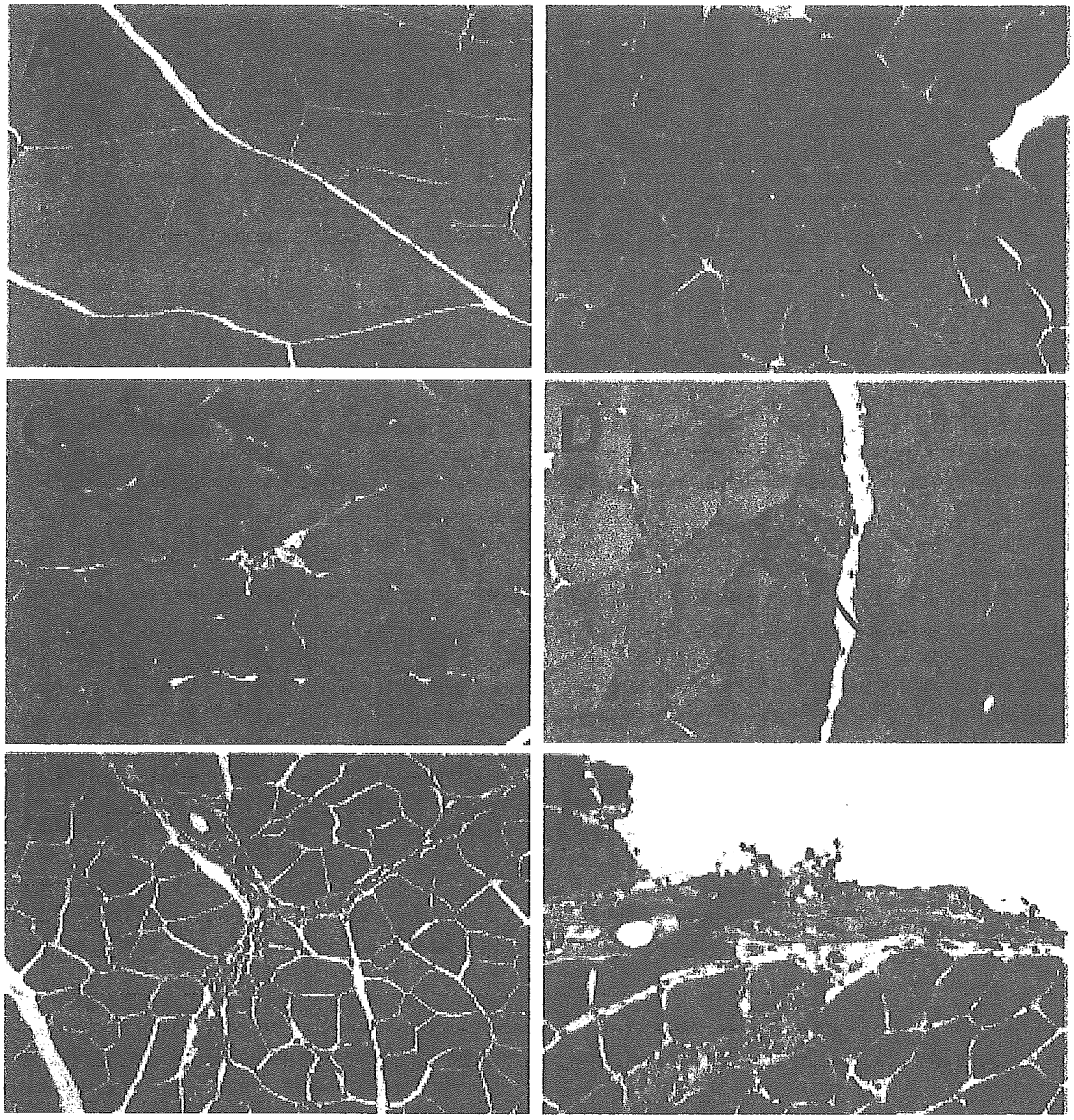

FIGURE 1. Histological changes found in the muscle of murine EAM. Quadriceps femoris muscle of normal mice and immunized mice on day 7 showed no inflammatory changes (*A* and *B*, respectively). On day 14, mild cellular infiltration in the muscle tissue was shown (*C*). Muscle tissues of EAM mice on day 21 showed cellular infiltration in the endomysium (*D*), perimysium (*E*), epimysium (*F*), and necrotic muscle fibers (arrow in *D*). H&E staining was used. Original magnification, ×200.

temperature, the slides were rehydrated in PBS for 2 min three times, and then the endogenous peroxidase activity was blocked by incubation in 1.0% H_2O_2 in PBS for 10 min, followed by rinsing for 2 min three times in PBS. Nonspecific binding was blocked with 10% normal rabbit serum in PBS for 30 min. For CD4, CD8, and F4/80 staining, the sections were incubated with 5 μg/ml rat anti-mouse CD4 mAb (GK1.5; Cymbus Biotechnology), 2 μg/ml rat anti-mouse CD8a mAb (53-6.7; BD Pharmingen), 5 μg/ml rat anti-mouse F4/80 mAb (C1:A3-1; Serotec), or normal rat IgG in Ab diluent (BD Pharmingen) overnight at 4°C. The samples were then washed three times in PBS for 5 min each time and incubated with biotin-conjugated rabbit anti-rat IgG (DakoCytomation) for 30 min at room temperature with 5% normal mouse serum. To analyze a time course of cell infiltration, numbers of $CD4^+$, $CD8^+$, and $F4/80^+$ cells in six randomly selected fields at ×200 were counted from three EAM mice on days 0, 7, 14, and 21.

For mouse vascular endothelial cell staining, we used a tyramide signal amplification kit (NEL700A; PerkinElmer). After blocking with 10% normal rabbit serum, the sections were incubated with 5 μg/ml rat anti-mouse vascular endothelial cadherin Ab (11D4.1; BD Pharmingen) or normal rat IgG overnight at 4°C. The samples were then washed three times in PBS for 5 min each time and incubated with biotin-conjugated rabbit anti-rat IgG for 30 min at room temperature with 5% normal mouse serum. After washing three times in PBS for 5 min each time, the sections were incubated with streptavidin-HRP for 30 min at room temperature and washed in PBS three times for 5 min each time. The samples were incubated with biotinyl tyramide amplification reagent at room temperature for 5 min, then washed three times in PBS for 5 min each time, and incubated again with streptavidin-HRP for 30 min. After washing three times in PBS for 5 min each time, diaminobenzidine tablets (Sigma-Aldrich) were used for visualization. The sections were counterstained in hematoxylin for 30 s and washed in tap water for 5 min.

For mouse CX3CL1 staining, the endogenous peroxidase activity was blocked by incubation in 1.0% H_2O_2 in methanol, and then the sections were incubated overnight at 4°C with goat anti-mouse CX3CL1 Ab (sc-7227; Santa Cruz Biotechnology) or normal goat IgG in Ab diluent at 5 μg/ml. The samples were then washed three times in PBS for 5 min each time and incubated with biotin-conjugated rabbit anti-goat IgG (DakoCytomation) for 30 min at room temperature with 5% normal mouse serum. After washing three times in PBS for 5 min each time, the sections were incubated with peroxidase-conjugated streptavidin (DakoCytomation) for 30 min at room temperature and washed three times for 5 min each time. For enhancing the expression of CX3CL1 on endothelial cells, a tyramide signal amplification kit was

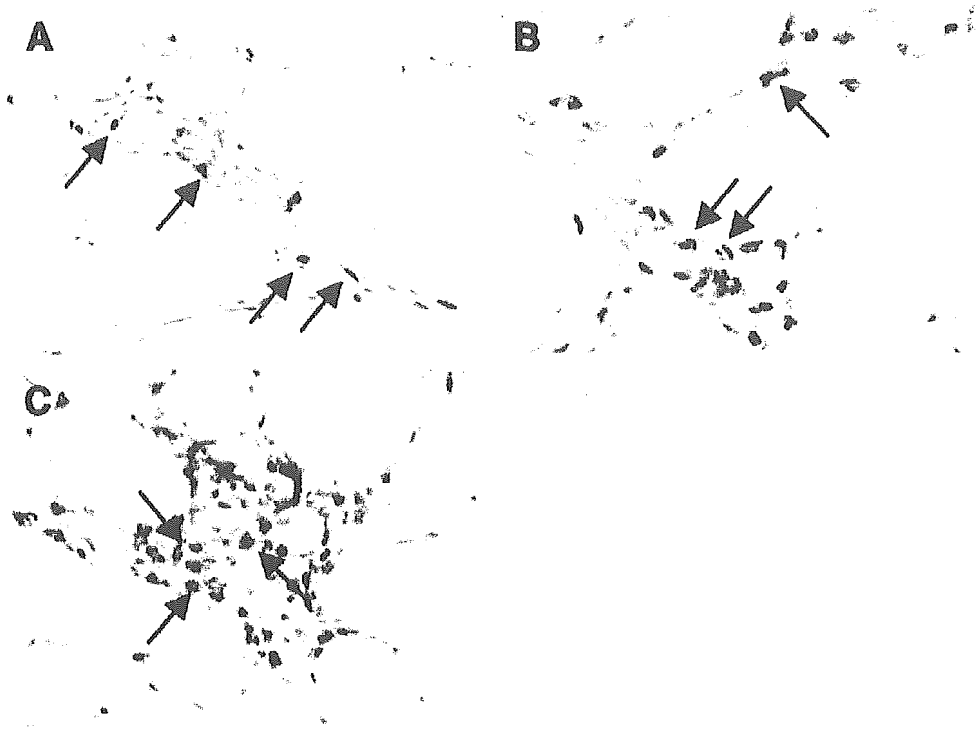

FIGURE 2. Infiltration of CD4 and CD8 T cells and macrophages in the muscles of EAM mice. Frozen sections of the quadriceps femoris muscle of EAM mice on day 21 were examined by immunohistochemistry using mAb against CD4 (*A*), CD8 (*B*), and F4/80 (*C*). The arrows indicate $CD4^+$, $CD8^+$, and $F4/80^+$ cells. Original magnification, ×200.

used as described above. Diaminobenzidine tablets were used for visualization. The sections were counterstained in hematoxylin for 30 s and washed in tap water for 5 min.

For CD4, CD8 or F4/80, and CX3CR1 double staining, the sections were incubated overnight at 4°C with 5 μg/ml rat anti-mouse CD4 mAb (GK1.5), 5 μg/ml rat anti-mouse CD8 mAb (53-6.7), 5 μg/ml rat anti-mouse F4/80 mAb (C1:A3-1), or normal rat IgG in Ab diluent. Subsequently, the samples were washed three times for 5 min each time in PBS and incubated with Alexa Fluor 488-conjugated goat anti-rat IgG (Molecular Probes) at 5 μg/ml for 1 h at room temperature. For CX3CR1 staining, the sections were washed three times in PBS for 5 min each time and then incubated with rabbit anti-mouse CX3CR1 Ab (30) or normal rabbit IgG at 5 μg/ml in Ab diluent for 2 h at room temperature. Next, the samples were washed three times for 5 min each time in PBS and incubated with Alexa Fluor 568-conjugated goat anti-rabbit IgG (Molecular Probes) at 5 μg/ml for 1 h at room temperature. The slides were examined using fluorescent microscopy (BZ-Analyzer; Keyence).

Treatment with anti-mouse CX3CL1 mAb

A mAb against murine CX3CL1 was generated from Armenian hamsters immunized with recombinant murine CX3CL1 by a standard method. One mAb, 5H8-4, was selected for additional studies. The specificity was examined by ELISA using a panel of murine CXC (MIP-2, keratinocyte-derived chemokine, and CXCL9, 10, 12, and 13), CC (CCL1–7, 9–12, 17, 19–22, 25, 27, and 28), C (XCL1), and CX3C (CX3CL1) chemokines. The mAb reacted specifically with murine CX3CL1. Five hundred micrograms of hamster anti-mouse CX3CL1 mAb (5H8-4) or control Ab (hamster IgG; ICN Pharmaceuticals) was injected into the mouse peritoneal cavity three times per week from day 0 for 3 wk. The injection of anti-CX3CL1 mAb did not affect the number of PBMC (data not shown).

The severity of inflammatory changes was classified using five grades according to the classification of Kojima et al. (31) with some modification: score 0, no inflammation; score 1, mild endomysial inflammatory changes; score 2, severe endomysial inflammatory changes; score 3, perimysial inflammatory changes in addition to score 2; and score 4, diffuse extensive lesion. If multiple lesions were found in one muscle specimen, 0.5 point was added to the indicated score. To evaluate the severity of inflammation using a different aspect, we counted the number of necrotic muscle fibers, and $CD4^+$, $CD8^+$, and $F4/80^+$ cells in continuous three sections. Each section examined six random fields at ×400. The evaluation of histopathological inflammatory changes was performed in a blind fashion for the experimental group identity.

Real-time RT-PCR

Total RNA was prepared from a 100 mg muscle block using RNA extraction solution, Isogen (Nippon Gene), and treated with DNase I (Invitrogen Life Technologies). The first-strand cDNA was synthesized using oligo$(dT)_{12-18}$ primers (Pharmacia Biotech) and SuperScript II reverse transcriptase (Invitrogen Life Technologies).

The relative quantitative real-time PCR was performed using SYBR Green I on ABI PRISM 7000 (Applied Biosystems) according to the instructions provided by the manufacturer. The cDNA was amplified with primers for TNF-α (5′, GTA CCT TGT CTA CTC CCA GGT TCT CT; 3′, GTG TGG GTG AGG AGC ACG TA), IFN-γ (5′, CCT GCG GCC TAG CTC TGA; 3′, CCA TGA GGA AGA GCT GCA AAG), perforin (5′, CCA CGG CAG GGT GAA ATT C; 3′, GGC AGG TCC CTC CAG TGA), and GAPDH (5′, ATG CAT CCT GCA CCA CCA A; 3′, GTC ATG AGC CCT TCC ACA ATG). These primers were designed using the ABI Primer Express Software program (Applied Biosystems). The reaction buffer contained the following components: 25 μl of SYBR Green PCR Master Mix (Applied Biosystems), 300 nM forward and reverse primers, 50 ng cDNA template, and RNA-free distilled water up to 50 μl of total volume. The PCR was conducted using the following parameters: 50°C for 2 min, 95°C for 10 min, and 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. GAPDH mRNA was used as an internal control to standardize the amount of sample mRNA. A validation experiment demonstrated approximately equal efficiencies of the target and reference. Thus, the relative expression of real-time PCR products was determined using the ΔΔCt method that compares the mRNA expression levels of the target gene and the housekeeping gene (32, 33). One of the control samples was chosen as a calibrator sample.

Statistical analysis

Differences in the score of tissue inflammation, number of necrotic muscle fibers, number of migrated cells, and relative expression levels of TNF-α, IFN-γ, and perforin between control Ab- and anti-mouse CX3CL1 mAb-treated EAM mice, and the relative expression levels of TNF-α, IFN-γ, and perforin between normal and EAM mice were examined for statistical significance using Mann-Whitney's *U* test. All data were expressed as the