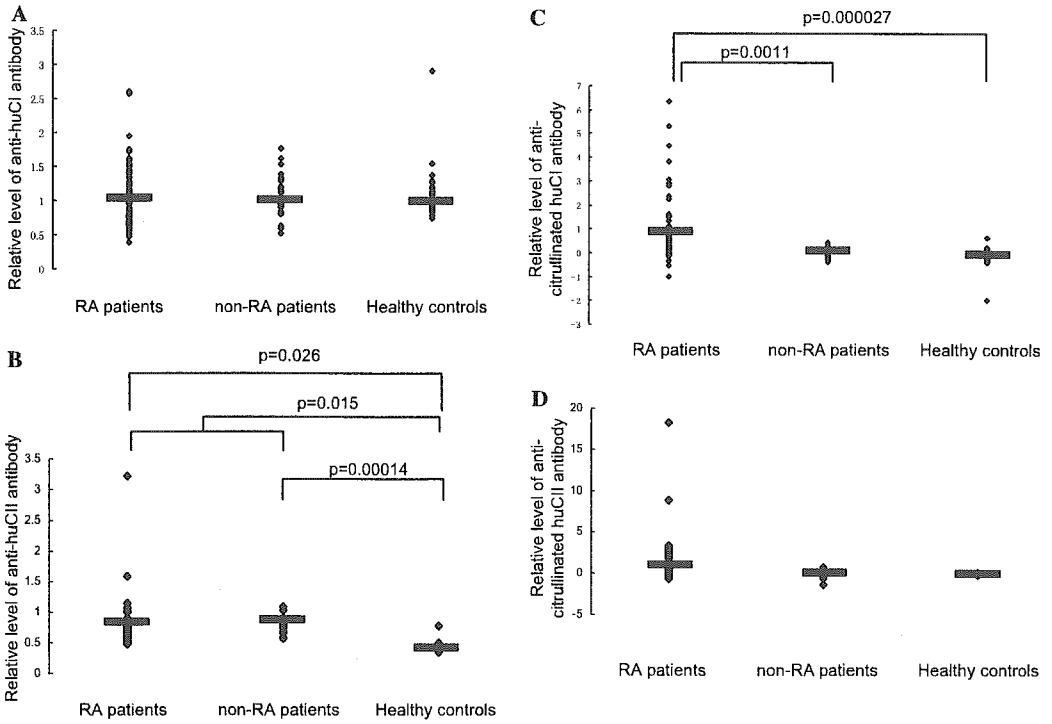

Fig. 3. Measurement of anti-collagen antibodies and anti-citrullinated collagen antibodies in RA patient sera and normal control sera. After coating, citrullination was produced by incubation with PADI2, followed by testing of the titers of anti-huCI antibodies and anti-citrullinated huCI antibodies (A,C, 117 RA sera, 37 non-RA patient sera, and 47 normal control sera). We also tested the titers of anti-huCII antibodies and anti-citrullinated huCII antibodies (B,D, 56 RA sera, 13 non-RA patient sera, and 9 normal control sera). The mean + 2 SD of healthy control values (A, >1.71; B, >0.72; C, >0.58; D, >0.12, respectively) was positive.

detection of insoluble proteins (including extracellular matrix proteins) as well as soluble proteins. This method also detects target proteins expressed at low levels, and is superior to other methods such as 2D-PAGE. One of several clones we identified was huCI peptides, which we examined in conjunction with other collagens known to be relevant to RA [9]. We confirmed citrullination of huCI by Western blotting, ELISA, and LC/MS/MS. We identified many citrullinated sites in huCI.

In the present study, we identified huCI peptide as a candidate substrate of citrullinated autoantigens by immunoscreening and found that anti-citrullinated huCI peptide antibody was specific to RA patients. huCI is one of the collagens that function as structural proteins, all of which have a characteristic triple helix structure with cyclic glycine and a high content of proline and hydroxyproline in their amino acid sequence. Among the collagens, CII has been the most studied, and there is evidence that it plays a pathologic role in RA. CII is major collagen in cartilage, and immunization with CII induces arthritis in mice and rats [6]. Bovine CII is also highly antigenic in transgenic mice that express HLA-DR1(*0101) and (*0401), which are associated with susceptibility to RA [5]. Anti-CII antibodies were observed in both RA (IgG, 41–72.4%) and non-RA (e.g., osteoarthritis and infective arthritis) (IgG, 36–88%) sera [3,31]. In the present study, anti-huCII antibody was also detected (41%) in RA patients. Compared to CII, there have been few reports indicating that CI plays a pathologic role in RA, although CI is widely expressed in bone, tendon, vascular tissues, synovial tissue, and various other tissues. In the present study, we observed no autoantibody recognizing non-citrullinated huCI in RA or control sera. In RA subjects, we observed that a marked increase in autoantibody positivity was associated with citrullination of huCI, but not with citrullination of huCII.

Although the present data indicate that anti-citrullinated huCI antibody is an RA-specific autoantibody, there are several issues that remain unresolved. First, collagen molecules form a triple helix with post-translational modification and their tertiary structure is believed to be a determinant of epitopes [13,15,20], although epitopes of anti-CCP antibodies are modified peptides. Second, it is not known how peptidylcitrullination alters antigenicity and breaks immunologic tolerance. The present findings, obtained by peptide-based immunoscreening and confirmation of recognition of citrullinated acid-extracted huCI molecules, provide a basis for further investigation to clarify the mechanisms of the roles of anti-citrullinated peptide antibodies in RA.

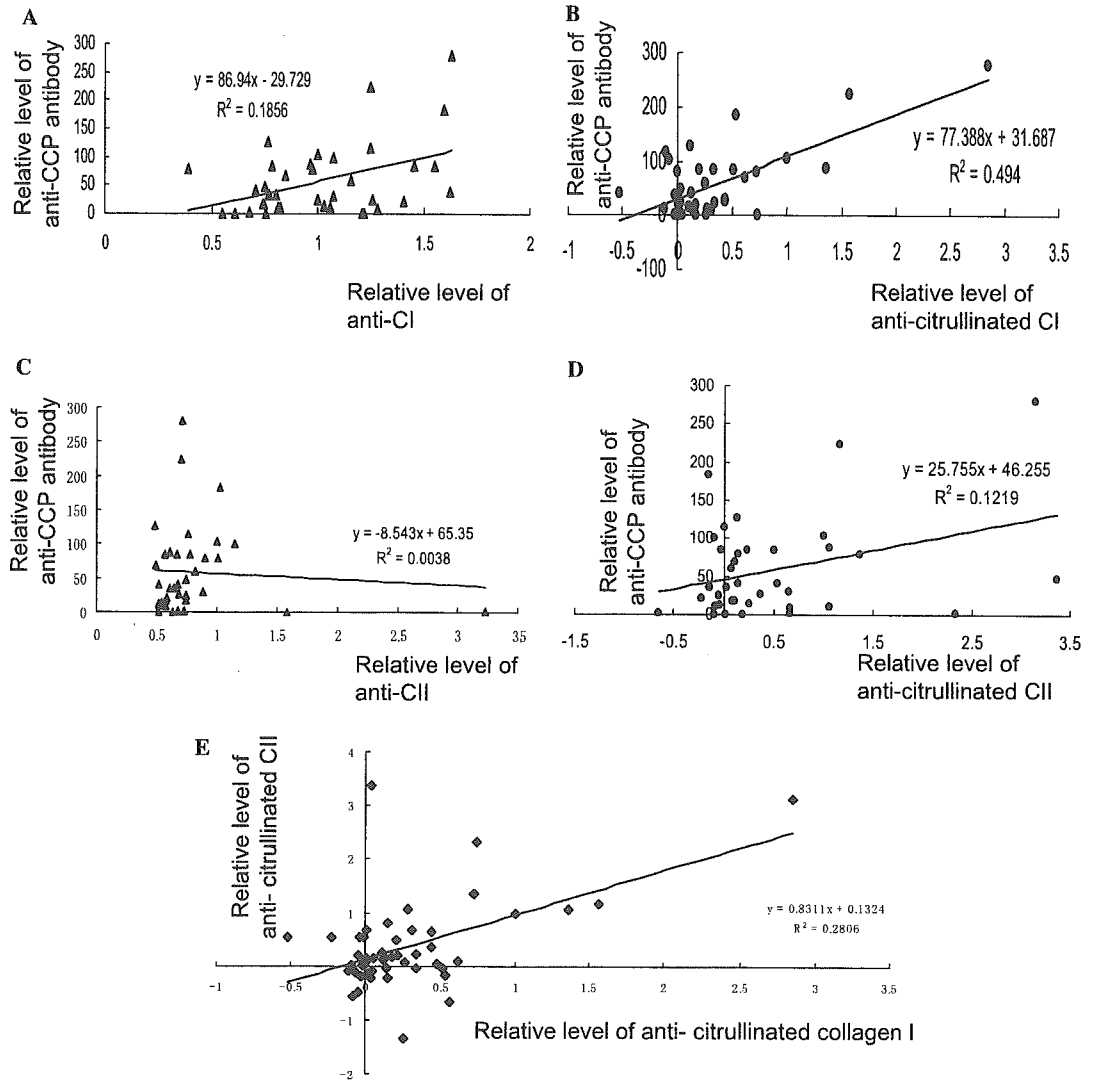

Fig. 4. Comparison of titers of anti-CCP and relative levels of anti-citrullinated collagen in RA sera. Comparison of (A) anti-huCI and (B) anti-citrullinated huCI levels with anti-CCP levels in RA sera. Comparison of (C) anti-huCII and (D) anti-citrullinated huCII levels with anti-CCP levels in RA sera. (E) Comparison of anti-citrullinated huCI levels with anti-citrullinated huCII levels in RA sera. There were no significant differences in any of these comparisons for any of the 37 RA samples. Regression line and correlation coefficient (R^2) are shown.

The present sensitivity and specificity of anti-citrullinated huCI antibody were 32% and 99%, respectively, and they correlated strongly with those of anti-CCP. The specificity of anti-citrullinated huCI antibodies was nearly equal to that of anti-CCP antibody, but the sensitivity of anti-citrullinated huCI antibodies was significantly less than that of anti-CCP antibody. Although almost all subjects who were positive for anti-CCP antibody were also positive for anti-citrullinated huCI antibody, a few were positive for anti-citrullinated huCI antibody but not for anti-CCP antibody. Because anti-CCP recognizes a mixture of synthetic peptides containing citrulline, and because huCI molecules contain multiple arginine residues that are citrullinated, it appears likely that epitopes of anti-CCP antibodies comprise the majority of those of anti-citrullinated huCI, but not all of them.

In conclusion, we found that huCI is a substrate of PADIs and that citrullinated huCI strongly correlates with RA. However, the present results indicate that CI can become an autoantigen via citrullination by PADIs, and citrullination as post-translational modification appears to be an important factor in RA. In addition, the present results suggest that anti-citrullinated collagen antibodies comprise a subclass of anti-CCP. To produce autoantigens in RA patients, PADIs must modify their substrates, but the mechanisms of this modification are unclear. However, PADIs are also clearly present in

the extracellular region [36]. Also, PADIs may be activated in the extracellular region, because the calcium ion concentration is sufficiently higher in the extracellular region than in the cytoplasm or intracellular region [21]. We speculate that autoantibodies for citrullinated collagens react or cross-react with other citrullinated proteins that are locally produced at the site of rheumatoid inflammation of synovial tissue. We believe that anti-citrullinated huCI plays important roles in the development of RA. More study of the mechanisms of citrullination in vivo may provide findings that are applicable to RA therapy.

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ORIGINAL ARTICLE

Positional Effect of Amino Acid Replacement on Peptide Antigens for the Increased IFN-γ Production from CD4T Cells

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ABSTRACT

Background: Based on the fact that site-specific amino acid replacement on peptide antigens stimulated T cell clones to produce increased amount of IFN-γ, we investigated this structure-function relationship, using various peptide analogues.

Methods: We used three human Th0 clones (BC20.7,BC33.5 and BC42.1) that express distinct TCRα and TCRβ chains, but recognize the same TCR ligand ; *i.e.*, the same framework of peptide antigen BCGa p84-100 in the context of DRB1*1405. These T cells were stimulated with various peptide analogues, followed by determination of proliferative responses and IFN-γ production.

Results: Replacement of Leu at peptide position 2 (P2) by amino acids which are less hydrophobic than the wild type (Val, Ala) or those with similar structural or neutral charge (Thr, Ser), induced increased IFN-γ production from T cells. This phenomenon was associated with structural features of TCR, especially the length of CDR3 region of TCRα. Amino acid replacement at the other positions did not induce increased IFN-γ production.

Conclusions: Amino acid substitution at P2 frequently induces increased IFN-γ production in a clone-specific manner, which is associated with the structure of CDR3 in TCRVα chains.

KEY WORDS

analogue peptide, complementarity determining region 3, interferon gamma, peptide antigens, T-cell antigen receptor

INTRODUCTION

Recent studies showed that T cell activation is not an all-or-none type of event ; rather, qualitative changes in T cell responses can be induced by amino acid substitutions by either MHC molecules or antigenic peptides, *i.e.*, TCR ligands. Flexibility in recognition results in T-cell activation in the absence of a proliferative response, which is designated by the following terminology as demonstrated in previous studies by our group and others : partial agonism,[1] TCR antagonism,[2] anergy,[3] survival[4] and cytokine-specific up-regulation.[5,6]

Amino acid residues on antigenic peptides have been roughly divided into two groups ; one that is important for binding to TCR (T cell epitope), and the other that is important for binding to MHC (MHC anchor). However, the crystal structure of the human class II HLA-DR1 complexed with the influenza peptide reported by Stern *et al.*[7] demonstrated that all the amino acid residues of the influenza virus peptide physically made contact with both HLA and TCR, with the exception of only one residue at the amino terminus which is buried deeply in the groove of class II, hence, there is no possibility for interaction with TCR.

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In our previous studies, single amino acid substitutions on a group I allergen in the *Cryptomeria japonica* -derived peptide resulted in a significant increase in IFN-γ production, with no remarkable changes either in proliferative response or IL-4 production.[5] In this study, by using various analogue peptide species, we stimulated three human Th0 clones that express distinct TCRα and TCRβ chains, but recognize the same TCR ligand, and tried to determine the structure-function relationship that leads to increased IFN-γ production from T cells.

METHODS

SYNTHESIS OF PEPTIDES

The wild-type BCGa p 84-100 (EEYLILSARDVLAVVSK) and its analogue were synthesized using a solid-phase simultaneous multiple peptide synthesizer PSSM-8 (Shimadzu Corp., Kyoto, Japan), and were purified by C18 reverse-phase HPLC (Millipore).

T CELL CLONES

BCGa p84-100-specific T cell lines were established as described.[8] Three human CD4+ T cell clones (BC 20.7,BC33.5 and BC42.1) specific to BCGa p84-100+ DRA/DRB1*1405, yet bearing distinct TCRβ (BV13S3 ,BV6S1 and BV5S4, respectively ;)[8] established from PBMC of a BCG-primed healthy individual as described elsewhere,[4] were used throughout the study. T cells were fed 50 U/ml human rIL-2 and irradiated autologous PBMC prepulsed with the wild-type BCGa p84-100 on a weekly basis.

ASSESSMENT OF T-CELL RESPONSES

Antigen-induced proliferation of the T cell clones were assayed by culturing the T cells (3×10^4/well) in 96-well flat-bottomed culture plates in the presence of a peptide(s) and irradiated PBMC (1.5×10^5/well), using RPMI 1640 medium (Gibco, Grand Island, N.Y.) supplemented with 2 mM L-glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 10% heat-inactivated autologous plasma. For the proliferation assay, cells were cultured for 72 hr in the presence of 1 μCi/well of[^{3}H]thymidine during the final 16 hrs. Culture supernatants collected immediately before the addition of[^{3}H]thymidine were used to determine lymphokine concentrations, using hGM-CSF ELISA kits (R&D systems) and hIFN-γ ELISA kits (Otsuka,Tokyo,Japan).

DETERMINATION OF TCRA GENE USAGE BY T CELL CLONES

To determine TCRVA gene usage of the T cell clones that were cultured with irradiated autologous PBMC, RNA were extracted from the cell mixture, and converted to cDNA. TCRA variable region cDNA were amplified with anchored PCR as described previously for amplification of TCRA variable region cDNA.[9] A primary PCR was followed by two sequential nested PCR. TCRAC-specific primers used for primary PCR, nested PCR, and final PCR were CA4 (5'-CAG AAT CCT TAC TTT GTG AC), CA3 (5'-ATC GGT GAA TAG GCA GAC AG), and biotinylated CA5 (5'- CAC

Table 1 A panel of labeled TCRAV-specific oligonucleotide probes

AV gene	sequence	pool
AV4, 20	TGCTAAGACCACA/CCAGCC	A
AV11	TCTTCAGAGGGAGCTGTG	A
AV2	ATCCTTGAGAGTTTTACT	B
AV8a	CCATTCGAGCTGTATTTA	B
AV8b	GCATTCGAGCTTTATTTA	B
AV15	CATTTGCTGGATTTTCGT	B
AV17	GATCTTAGGAGCATCATT	B
AV21	TGGGGGCATCAGTGCTGA	B
AV3	GAGAAGAGGATCCTCAGG	C
AV5	ACTATTCTCCAGCATACT	C
AV10	CCGTGTCCATTCTTTGGA	C
AV13	GAGAGGAATACAAGTGGA	C
AV19	CAATTTTTGTTGGCTATT	C
AV24	AGCATCTGACGACCTTCT	C
AV25	TCCTTGAACATTTATTAA	C
AV26	CCTAGGGATATTGGGGTT	C
AV27	GAAAAAACTATACCATCT	C
AV29	CAGGCACCTTGTTGTGGC	C
AV32	ACTCATCACATCAATGTT	C
AV18	CTTTGGCAGCCCCATTAC	D
AV23	GAGACCCTCTTGGGCCTG	D
AV28	ACTAACTTTCGAAGCCTA	D
AV30	GGAGTGTGCATTCATAGT	D
AV7	GGAGGCACTA/GCAGGACAA	E
AV6	ACAGCTTCACTGTGGCTA	F
AV12	TGCCAGCCTGTTGAGGGC	F
AV14	GTGA/GTCTCCACCTGTCTT	F
AV1a	CTCCTGTTGCTCATACCA	G
AV1b	CTCCTGCTGCTCGTCCCA	G
AV1c	CTCCTGGAGCTTATCCCA	G
AV9	AAGCCCACCCTCATCTCA	G
AV16	GCCTCTGCACCCATCTCG	G
AV22	CTGATACTCTTACTGCTT	G
AV31	CCTCTCTGGACTTTCTAA	G

Oligomucleotide probes specific to TCRAV family genes. Degenerated probes were used to specify AV4 and AV20, AV7, and AV14 families. Three probes for AV1 family, and two probes for AV8 family were required to specify all members of each family. These probes were grouped into seven pools (pool A to G) depending on sequence similarity.

Table 2 TCRVA and TCRVB usage of BC clones

BC clone	TCRVA	TCRVB
20.7	(AV25S1)FCAGHNAG(AJ14S3)	(BV12S3)CASRQAGTAYE(BJ2S7)
33.5	(AV3S1)FCATERGQ(AJ13S2)	(BV6S1A1)CASSPTGTANT(BJ1S1)
42.1	(AV8S1A1)FCAASLDNY(AJ126)	(BV5S1A1)CASRRSTGE(BJ2S2)

TCRVA and VB usage are shown, with amino acid sequences in the N(D)N region.

TGG ATT TAG AGT CTC TC), respectively. A panel of labeled TCRAV-specific oligonucleotide probes (Table 1) were used to study TCRAV gene usage with PCR-ELISA.[10] First, seven pools of the AV-specific probes were hybridized with immobilized PCR products in microplates to find out positive wells. Then, the products were hybridized with individual AV probes in another set of plates to pin-point the AV genes predominantly used by the cDNA. To clone the entire variable region cDNA, cDNA were amplified with CA4 and reamplified with a nested primer, CA2 (5'-ACG CGT CGA CAC TGG ATT TAG AGT CTC TC). The products were subcloned into pBluscript II SK+ (Stratagene, La Jolla), and recombinant clones with the dominant VA gene were selected with dot blot DNA hybridization using corresponding VA-specific oligonucleotides. After sequence determination of these clones, dominant clones were selected as cDNA for the T cell clones.

RESULTS

TCRVA AND VB SEQUENCES

TCRVA and VB sequences of three T-cell clones BC 20.7, BC33.5 and BC42.1 are shown in Table 2. The N (D)N region sequences are shown as one-letter codes for amino acids, between V and J segments in parentheses. As described in our earlier studies, these T-cell clones recognize BCGa p84-100 (EEYLILSARDVLAVVSK ; with first anchor underlined), in the context of HLA-DRB1*1405.[4] It is especially important to note that N(D)N region consists of 8 and 11 residues at TCRVA and VB of BC20.7 and BC33.5, respectively, whereas that of BC42.1 consists of 9 and 9 residues, repectively.

STIMULATORY ACTIVITIES OF BCGA P84-100-DERIVED ANALOG PEPTIDE L87V TO BC20.7

To evaluate the effects of single amino acid substitutions, proliferation and lymphokine production in response to analogue peptides were determined and findings were compared with those seen with the wild-type peptide. Most of the analogue peptides that stimulated BC clones showed a pattern of lymphokine production similar to that for the wild-type peptide (not shown). However, IFN-γ production of BC20.7 was increased in response to several analogue peptides at high concentration (16 μM), especially peptide L87V in which Leu is replaced by Val at the 87 th residue of the peptide BCGa p 84-100, whereas neither T cell proliferation nor production of other lymphokines, showed any remarkable change ; *i.e.*, only the production of IFN-γ was affected for recognition of the analog peptide L87V. As shown in Figure 1, to determine whether or not the change of IFN-γ production was due to differences in the HLA-peptide or TCR-TCR ligand avidity between L87V and the wild-type peptide, responses of BC20.7 to several different concentrations of L87V were compared with those of the wild-type peptide. In the range of concentrations from 0.016 μM up to 16 μM, IFN-γ production in response to L87V constantly exceeded that of the wild-type peptide. Moreover, the plateau level of L87 V-driven IFN-γ production was significantly higher. Mean IFN-γ production of BC20.7 for L87V increased significantly in comparison to the wild-type, whereas no statistical differences were noted in proliferative responses between R21K and the wild-type at a range of 0.16 μM to 16 μM. The IL-4 production of BC20.7 for each analogue peptide was proportional to the proliferative response to each peptide, at a range of 0.0016 to 16 μM (not shown). In contrast, production of GM-CSF gradually increased, in a dose-dependent manner throughout the range of 0.016 to 16 μM. These data indicate that the plateau responses and proliferation of IFN-γ are not due to saturation of the TCR ligand on the APC surface.

STIMULATORY ACTIVITIES OF BCGA P84-100-DERIVED ANALOGUES TO THREE BC CLONES

All three T-cell clones were stimulated with analogues at 16 μM, with replacements at P1 (=86Y) through P9 (=94V). Table 3 summarizes the results, regarding proliferative responses and IFN-γ production. P1 (=86Y) replaced by Ala (A) indicates a peptide species EEALILSARDVLAVVSK. Relative IFN-γ responses are shown, where IFN-γ production was divided by proliferation. P1 replaced by A gave values of 96/100/98, indicating that BC20.7,BC33.5 and BC 42.1 exhibited 96%, 100% and 98% responses respectively, as compared with the wild-type. Asterisks indicate peptide species that did not exert full agonistic activity ; *i.e.*, peptide stimulation even at 16 μM did not give a plateau response.

Most of analogues that exhibited full agonistic activity, stimulated IFN-γ production at levels roughly similar to the wild-type peptide, *i.e.*, at around 100%. However, it is important to note that L87T,L87S,L87A, and L87V significantly($p<0.01$) induced increased levels of IFN-γ production of BC20.7 and BC33.5, but not of BC42.1. Such a clone-specific phenomenon was

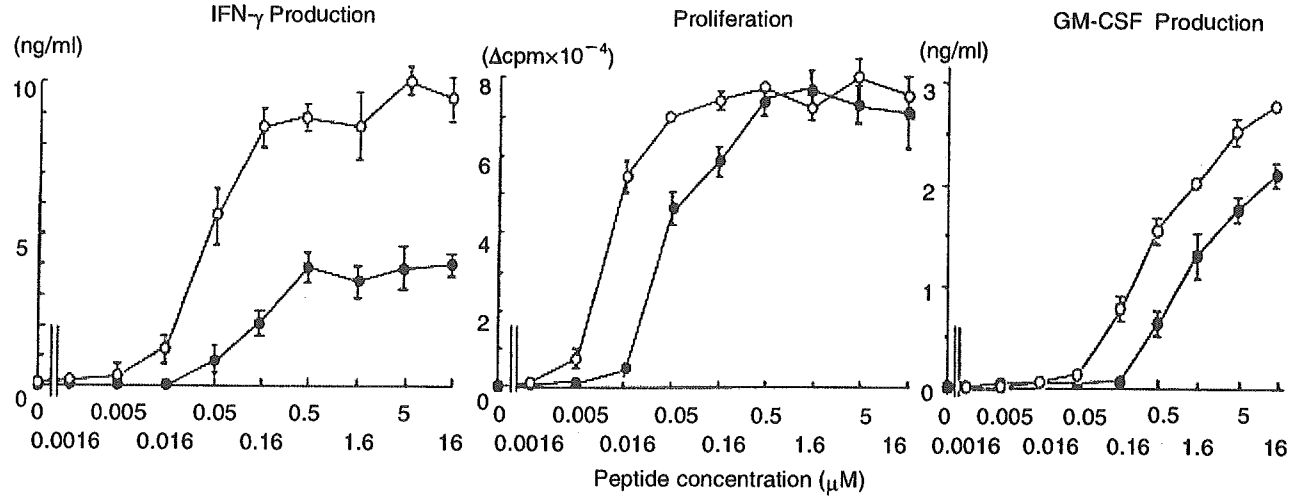

Fig. 1 IFN-γ production, GM-CSF production and proliferation of BC20.7 in recognition of either the wild-type peptide or L87V, at different concentrations. BC20.7 cells were cultured in triplicate with peptides and irradiated autologous PBMC, at the indicated concentrations. After 48-h incubation, supernatant fluids of triplicate cultures were collected. The remaining cells were pulsed with [^{3}H]-thymidine, harvested after 16h, and subjected to liquid scintillation counting. Closed circle, wild type peptide; open circle, L87V. Results are expressed as the geometric means ± standard error. IFN-γ production induced by L87V was significantly ($p<0.01$) higher than that induced by the wild-type peptide, at peptide concentrations ranging from 0.016 to 16 μM. On the other hand, plateau level of proliferation did not exhibit a significant difference, between 0.16 and 16 μM ($p>0.05$). GM-CSF production did not reach a plateau response even at 16 μM, without any statistical difference between L87V and the wild-type peptide, at 16 μM.

also observed when P5- and P8-substituted analogues were tested. Thus, S90E,S90G,S90M,D93Q,D93T and D93Y exhibited full agonism, in a clone-specific manner.

DISCUSSION

It is not very easy to identify TCR genes used by T cell clones, since they are usually cultured with irradiated autologous PBMC that includes polyclonal T cells. Random cloning of TCR cDNA derived from the cultured cells is minimally helpful in the identification, unless a large number of clones are examined. This problem was circumvented by the use of PCR-ELISA that was developed for TCRBV use,[9] and established in the present report for TCRVA usage. This technique allowed us to quantitate TCRV gene usage in the cDNA samples, and thus to identify the TCRV gene used by the T cell clones.

Three T-cell clones used in the present study recognize the same TCR ligand, as proven in our previous study. This is based on the fact that these clones recognize BCGa p84-100(^{84}EEYLILSARDVLAVVSK^{100}) in the context of DRB1*1405, and react to truncated peptides in a similar fashion.[11] Both BC20.7 and BC33.5 have 8 and 11 residues at N(D)N region of TCRVA and VB, respectively, whereas BC42.1 alone exhibits a different pattern, *i.e.*, 9 residues at N(D)N regions of TCRVA and VB. When peptide antigen is presented by class II MHC molecules, the N-terminal half of antigenic peptide is recognized mainly by CDR3 of TCRVA, whereas the C-terminal half is recoginized by CDR3 of TCRVB, which corresponds to N(D)N regions.[12] Interestingly, certain amino acid replacements on P2 induced increased IFN-γ production in BC20.7 and BC33.5 but not in BC42.1 cells, whereas those on P8 exhibited full agonism in BC 42.1 cells alone. It is thus likely that structural features of VACDR3 and VBCDR3 are responsible for specific responses induced by P2 and P8 analogues, respectively. Shuffling of N(D)N sequences between BC 42.1 and BC 20.7, or between BC 42.1 and BC 33.5 is underway to address this point.

Only L87T,L87S,L87A,and L87V induced IFN-γ enhancement. These arrangements are either smaller hydrophobic (A and V), or structurally similar neutral amino acids (T and S), indicating that close contact between P2 and TCRVA is taking place. Indeed, such a phenomenon is also seen in B-cell somatic hypermuation.[13] Thus, B-cell V region mutation in immunoglobulin heavy chain genes shows higher affinity than the germ-line sequence, usually associated with Gly, Ala, Val, Ser, Thr, or Cys, *i.e.*, small hydrophobic or small neutral residues. Apparently these mutations are not associated with static charges, but can affect either hydrogen bonding, van der Waar's force, or hydrophobic interactions.

In our previous studies using cedar pollen-derived peptides, T to V replacement on P2 also induced IFN-

Table 3 Increased IFN-γ production induced by peptide analogues

Replaced by	Relative IFN-γ response (BC20.7 / BC33.5 / BC42.1)								
	P1 =86Y	P2 =87L	P3 =88I	P4 =89L	P5 =90S	P6 =91A	P7 =92R	P8 =93D	P9 =94V
K	*/*/*	*/*/*	*/*/*	*/*/*	*/*/*	*/*/*	108/115/90	*/*/*	*/*/*
E	*/*/*	*/*/*	*/*/*	*/*/*	*/105/94	*/*/*	*/*/*	*/*/*	*/*/*
Q	*/*/*	88/79/90	*/*/*	92/79/81	*/*/*	*/*/*	*/*/*	*/*/87	*/*/*
N	*/*/*	105/97/95	*/*/*	88/92/97	*/*/*	*/*/*	*/*/*	*/*/*	*/*/*
T	*/*/*	177/210/86	110/92/81	*/*/*	77/97/108	*/*/*	*/*/*	*/*/95	85/96/91
S	*/*/*	155/187/90	95/95/99	*/*/*	100/100/100	81/87/97	*/*/*	*/*/*	93/75/99
G	*/*/*	110/98/79	90/100/92	*/*/*	*/*/115	77/69/93	*/*/*	*/*/*	94/99/100
A	96/100/98	189/202/94	105/94/83	107/93/83	88/104/110	100/100/100	*/*/*	*/*/*	80/81/92
V	91/91/85	271/259/92	91/84/86	105/96/86	99/100/101	90/76/85	*/*/*	*/*/*	100/100/100
L	93/88/102	100/100/100	100/90/101	100/100/100	*/*/*	*/*/*	*/*/*	*/*/*	*/*/*
Y	100/100/100	*/*/*	*/*/*	*/*/*	*/*/*	*/*/*	*/*/*	*/*/98	*/*/*
M	89/93/91	*/*/*	96/99/103	89/70/85	*/91/*	*/*/*	*/*/*	*/*/*	*/*/*
W	90/103/109	*/*/*	*/*/*	*/*/*	*/*/*	*/*/*	*/*/*	*/*/*	*/*/*

Positions 1 – 9 (P1 – P9) of BCGa p84-100 (EEYLILSARDVLAVVSK; with P1 underlined), was replaced by indicated amino acids. T cells were stimulated with peptide species at 16 μM. To obtain relative IFN-γ response values, plateau responses of IFN-γ (pg/ml) were first divided by plateau responses of proliferation (cpm). Then, the following calculation was performed: relative IFN-γ responses=100x[IFN-γ/proliferation to analogues]/[IFN-γ/proliferation to the wild-type BCGa p84-100]. The denominator was 0.0533. *Peptide that did not induce fully agonistic proliferation.

γ enhancement, whereas proliferation remained the same. Therefore, although not generalized, mutual replacement on G, A, V, L, S, or T at P2, tends to induce IFN-γ-specific enhancement. Such observations also have been reported in another study with different peptide species.[14] In this sense, analogue-induced clonal anergy is often observed, especially when residue replacement is made on P7 or P8.[11] Moreover, truncation of the C-terminal moiety of antigenic peptides, in general, exhibit TCR antagonism.[15] In other words, if a rule that applies to altered polyclonal novel responses induced by peptide analogues is established, it will lead us to novel therapeutic interventions using peptide analogues. Our observations on P2 replacement which is associated with increased IFN-γ production are imperative to furthering our understanding.

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

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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

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

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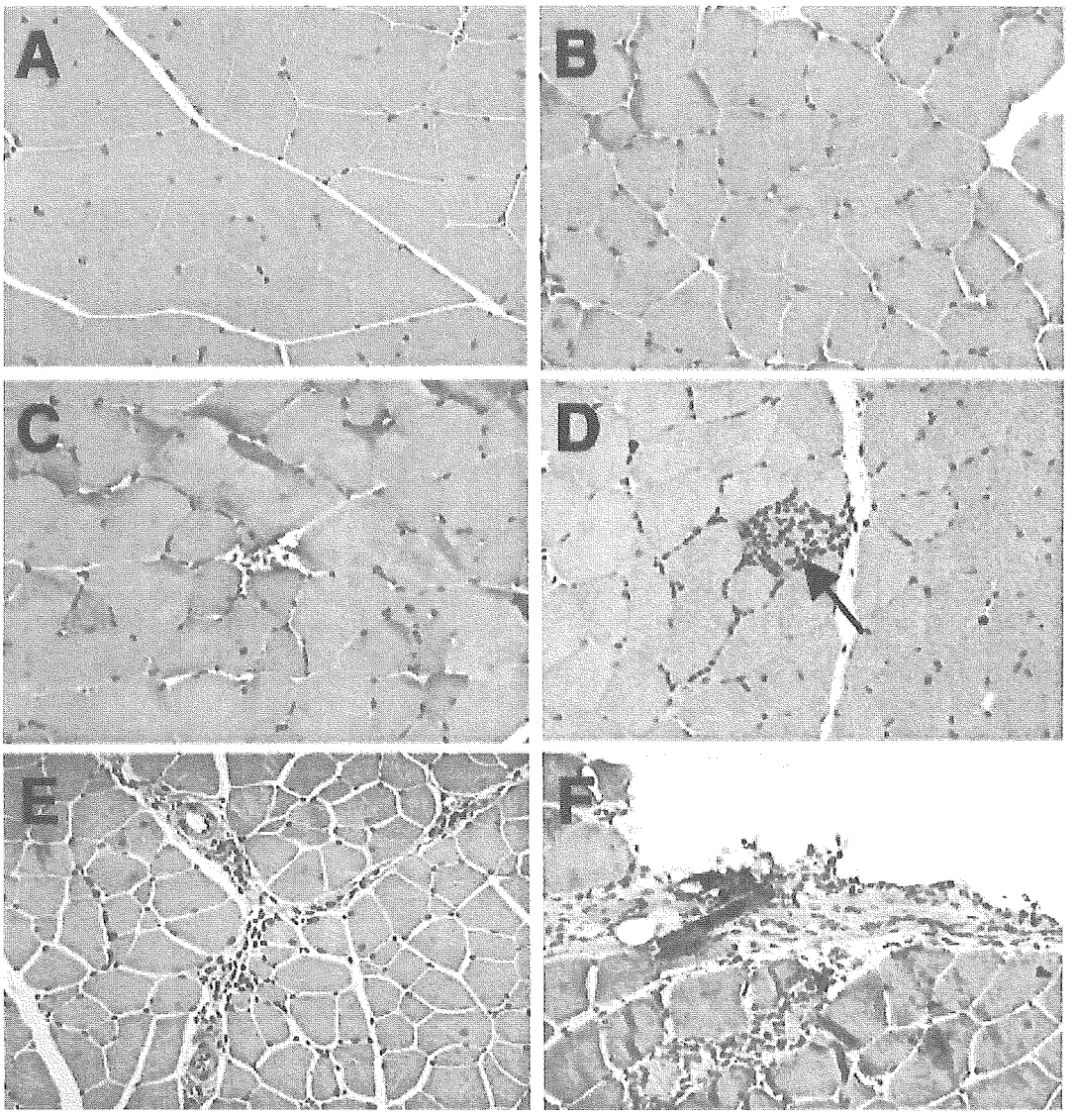

FIGURE 1. Histological changes found in the muscle of murine EAM. Quadriceps femoris muscle of normal mice and immunized mice on day 7 showed no inflammatory changes (*A* and *B*, respectively). On day 14, mild cellular infiltration in the muscle tissue was shown (*C*). Muscle tissues of EAM mice on 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

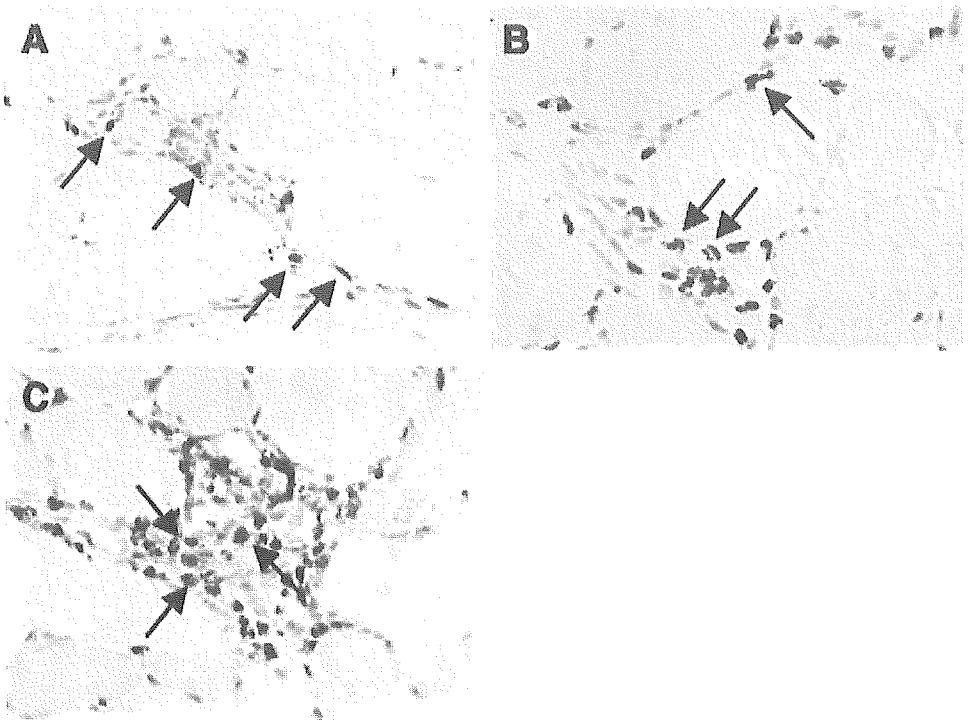

FIGURE 2. Infiltration of CD4 and CD8 T cells and macrophages in the muscles of EAM mice. Frozen sections of the quadriceps femoris muscle of EAM mice on day 21 were examined by immunohistochemistry using mAb against CD4 (*A*), CD8 (*B*), and F4/80 (*C*). The arrows indicate $CD4^+$, $CD8^+$, and $F4/80^+$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

mean ± SEM. The difference between two groups of mice was considered significant at $p < 0.05$.

Results

Development of EAM

SJL/J mice were immunized with purified rabbit myosin fraction and CFA on days 0, 7, and 14. On days 0, 7, 14, and 21, the quadriceps femoris muscles of these mice were histologically examined with H&E staining. All muscle specimens of normal SJL/J mice and immunized mice on day 7 showed normal appearance with no inflammatory changes (Fig. 1, *A* and *B*, respectively), whereas those of mice immunized with rabbit myosin fraction showed mild mononuclear cell infiltration at day 14 (Fig. 1*C*). On day 21, a significant number of mononuclear cells were infiltrated among the muscle fibers (endomysium; Fig. 1*D*), at perivascular areas (perimysium; Fig. 1*E*), and epimysium (Fig. 1*F*). Scattered lesions with aggregates of infiltrated mononuclear cells were formed, in which atrophic or necrotic muscle fibers were noted (arrow in Fig. 1*D*). Injection of PBS and CFA into SJL/J mice did not show infiltration of inflammatory cells in the quadriceps femoris muscles (data not shown).

To determine the subsets of infiltrating mononuclear cells in the quadriceps femoris muscles of EAM mice, we performed immunohistochemical analysis using mAbs against CD4, CD8, and F4/80. $CD4^+$ T cells were mainly located in the perimysium and some were found in the endomysium (Fig. 2*A*). $CD8^+$ T cells were predominantly detected in the endomysium and surrounded nonnecrotic muscle fibers (Fig. 2*B*). $F4/80^+$ macrophages were located in the endomysium as well and were especially present around the necrotic muscle fibers (Fig. 2*C*). Because these histological findings of inflammatory cell infiltration patterns resembled those of affected muscle lesions in IIM patients (34–36), we decided to use the EAM mice as an experimental model of IIM.

To evaluate a time course of cellular infiltration into the muscles, we counted the numbers of infiltrated $CD4^+$, $CD8^+$, and $F4/80^+$ cells on days 0, 7, 14, and 21 by immunohistochemical method. The majority of the infiltrating cells on day 14 were $F4/80^+$ macrophage (Fig. 3). In contrast, the number of $CD4^+$ and $CD8^+$ T cells was not increased until day 14, and they had significantly migrated into the muscles on day 21. These results were similar to previously reported data (37).

CX3CL1 and CX3CR1 expression in the muscle of EAM mice

We examined the expression of CX3CL1 in the muscle of normal SJL/J mice and EAM mice by immunohistochemistry. In the quadriceps femoris muscles of normal mice, no CX3CL1 expression was detected (Fig. 4, *A* and *G*). In contrast, CX3CL1 was expressed on infiltrated mononuclear cells predominantly in the endomysium and vascular endothelial cells of EAM mice on day 14 (Fig. 4, *B* and *H*, respectively) and day 21 (Fig. 4, *C* and *I*, respectively).

We next examined the expression of CX3CR1 on the infiltrated mononuclear cells in the quadriceps femoris muscle of EAM mice by double immunohistochemical staining. Some $CD4^+$ T cells expressed CX3CR1 (Fig. 5, *A–C*). The majority of $CD8^+$ T cells and most of the $F4/80^+$ macrophages expressed CX3CR1 (Fig. 5, *D–F* and *G–I*, respectively).

Effect of anti-mouse CX3CL1 mAb on EAM mice

To analyze the effect of anti-CX3CL1 mAb administration on EAM mice, we evaluated the histological changes in quadriceps femoris muscle using H&E staining. The incidence of inflammatory cell infiltration in control Ab-treated mice was 100% ($n = 10$). Treatment with anti-CX3CL1 mAb did not change the incidence of cellular infiltration (100%; $n = 10$). EAM mice treated with control Ab showed mononuclear cell infiltration with atrophy and necrosis of muscle fibers (Fig. 6*A*). In comparison, anti-CX3CL1 mAb-treated EAM mice showed milder histological changes (Fig. 6*B*). Analysis of histological scores of inflammatory changes in the quadriceps femoris muscles indicated that treatment with anti-CX3CL1 mAb significantly reduced inflammatory cell infiltration in the muscles of EAM mice compared with treatment with control Ab (Fig. 6*C*). Moreover, anti-CX3CL1 mAb treatment reduced the number of necrotic muscle fibers in muscles (Fig. 6*D*). A similar result was obtained in another independent set of experiments.

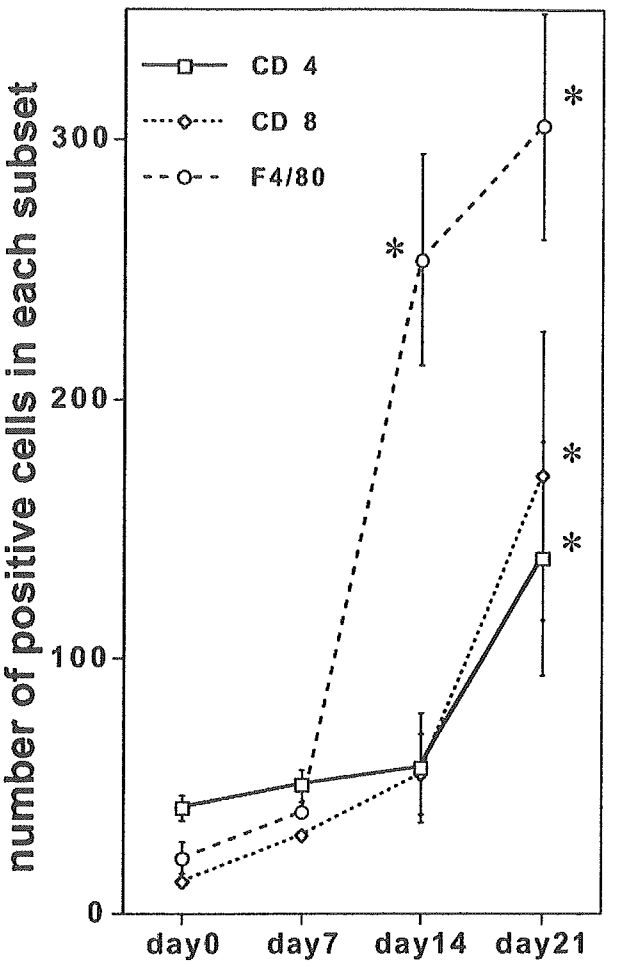

FIGURE 3. Time course of inflammatory cell infiltration into the muscle tissue of EAM mice. The numbers of infiltrating $CD4^+$, $CD8^+$, and $F4/80^+$ cells into the quadriceps femoris muscles were counted by immunohistochemistry. Data represent the mean ± SEM. *, $p < 0.05$.

We next examined the effect of anti-CX3CL1 mAb treatment on the numbers of each subset of infiltrating cells. The numbers of $CD4^+$, $CD8^+$, and $F4/80^+$ cells in quadriceps femoris muscles were counted and compared between mice treated with control Ab and those with anti-CX3CL1 mAb. Anti-CX3CL1 mAb treatment significantly reduced the number of infiltrated $CD4^+$ T cells by ~30% (Fig. 7*A*), $CD8^+$ T cells by ~50%, and $F4/80^+$ macrophages by up to 50% (Fig. 7, *B* and *C*).

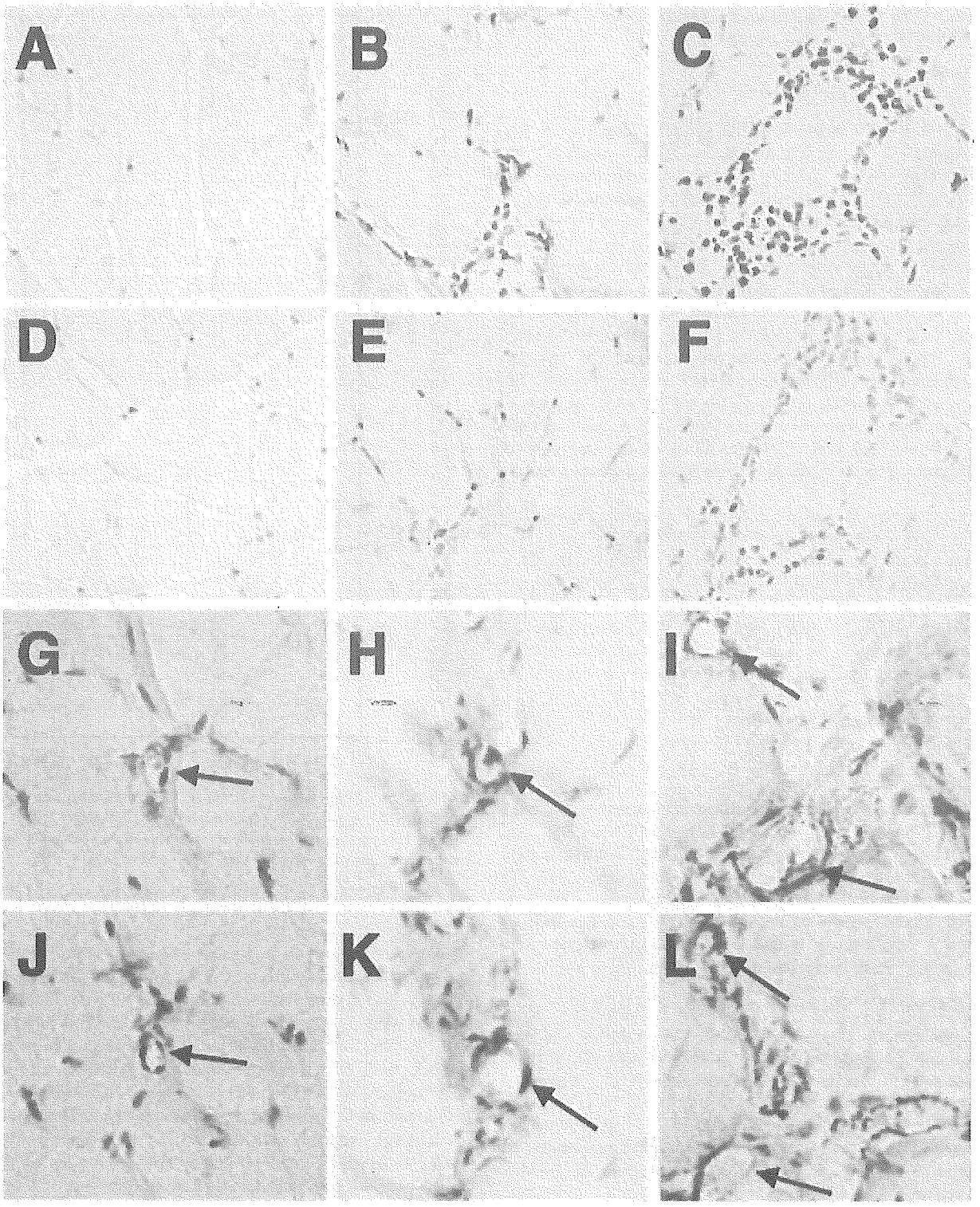

FIGURE 4. CX3CL1 expression in the muscles of EAM mice. Expression of CX3CL1 was examined by immunohistochemistry in normal mice (*A* and *G*) and EAM mice on day 14 (*B* and *H*) and day 21 (*C* and *I*). Vascular endothelial cadherin expression in the normal mice (*J*) and EAM mice on day 14 (*K*) and 21 (*L*) was also examined using serial sections with *G*, *H*, and *I*, respectively. Stainings with isotype control Ab for CX3CL1 are shown (*D*, normal mice; *E*, EAM on day 14; *F*, EAM on day 21). Arrows indicate vascular endothelial cadherin-positive endothelial cells (*J–L*), and corresponding endothelial cells (*G–I*). Original magnification, ×400.

We finally examined the effects of anti-CX3CL1 mAb treatment on the expression of cytokines and cytotoxic molecule in the quadriceps femoris muscle of EAM mice by quantitative RT-PCR. Although the relative quantities of TNF-α, IFN-γ, and perforin mRNA were very low in normal SJL/J mice, they were significantly up-regulated in EAM mice that received control Ab treatment ($p < 0.05$). Furthermore, treatment with anti-CX3CL1 mAb strikingly reduced mRNA expression (Fig. 8).

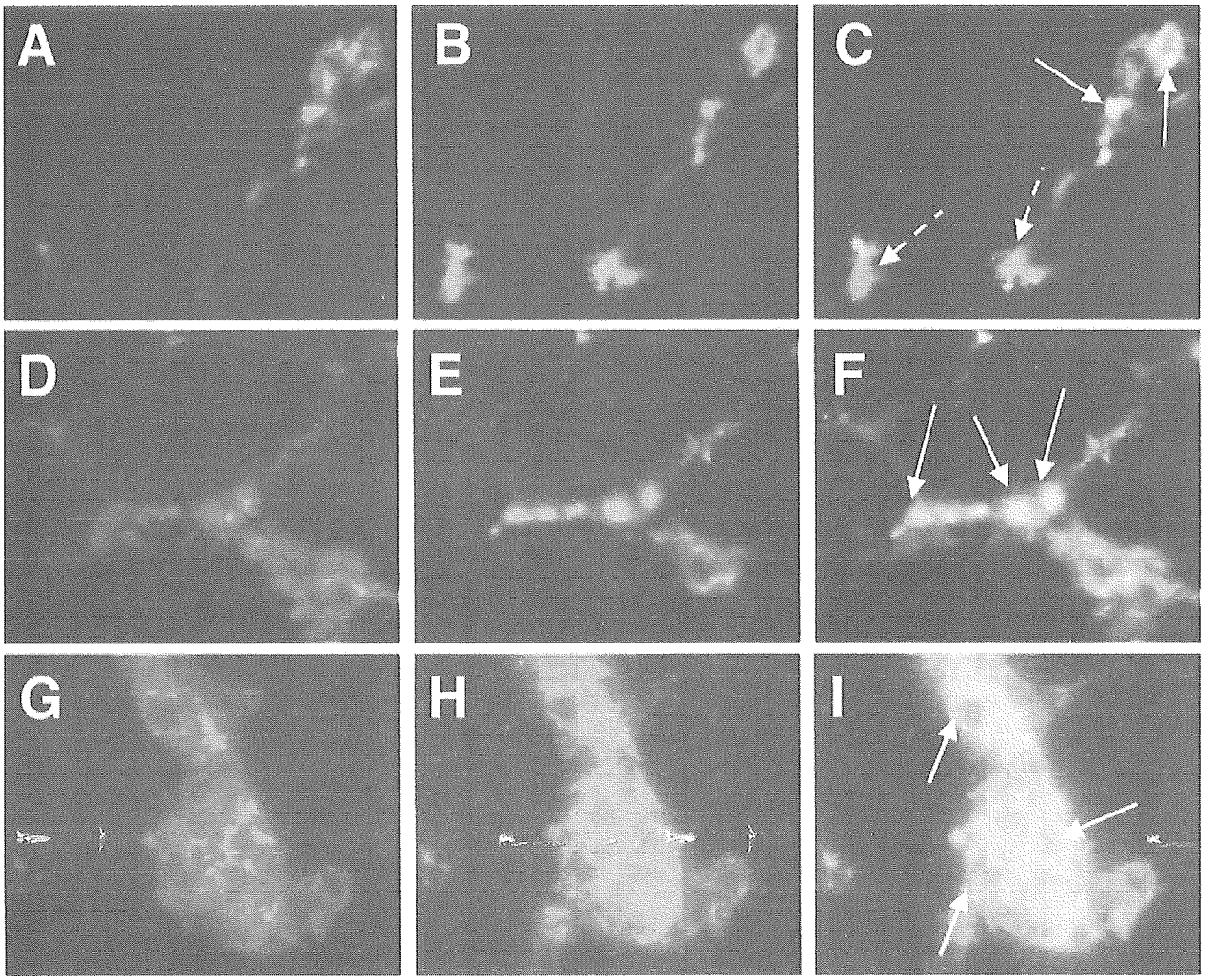

FIGURE 5. CX3CR1 expression on $CD4^+$, $CD8^+$, or $F4/80^+$ cells in the EAM muscle. Muscle tissues from EAM mice were double stained with CD4, CD8, or F4/80, and CX3CR1, and analyzed with fluorescent microscopy (*A*, CX3CR1; *B*, CD4; *C*, merged *A* and *B*; *D*, CX3CR1; *E*, CD8; *F*, merged *D* and *E*; *G*, CX3CR1; *H*, F4/80; *I*, merged *G* and *H*). Solid arrows indicate double-positive cells. Dotted arrows indicate CX3CR1-negative CD4 T cells. Original magnification, ×200.

Considered together, the above results indicate that treatment with anti-CX3CL1 mAb reduced infiltration of CD4 and CD8 T cells and macrophages and reduced the expression of various inflammatory cytokines and cytotoxic molecule in muscles.

Discussion

The major findings of the present study were the following. 1) CX3CL1 was expressed on infiltrated mononuclear cells and vascular endothelial cells, and its corresponding receptor, CX3CR1, was expressed on infiltrated inflammatory cells in the muscles of EAM. 2) Treatment with anti-CX3CL1 mAb ameliorated histological inflammatory changes in EAM mice, reduced the numbers of infiltrated CD4 and CD8 T cells and macrophages, and reduced the expression of TNF-α, IFN-γ, and perforin in the muscles. These results suggest that CX3CL1-CX3CR1 interaction seems to play an important role in inflammatory cell migration into the muscles of EAM mice.

Development of EAM in SJL/J mice by immunization with rabbit purified skeletal myosin fraction and CFA was previously reported (37–40). We modified the method by increasing the amount of immunized myosin and CFA and the addition of *Mycobacterium butyricum*. This modification shortened the period required for the development of myositis from 5 wk, which was thought to be appropriate for the induction (38), to 3 wk. Moreover, although pertussis toxin (PTX) injection into the peritoneal cavity increased the severity of inflammatory changes in the muscle (31), and thus, PTX was administered in the previous models (31, 36, 38), our modified method induces significant myositis without PTX injection. The EAM mice showed inflammatory cell infiltration in the endomysium, perimysium, and epimysium with muscle fiber necrosis. Immunohistochemical analysis showed that the invading cells surrounding nonnecrotic muscle fibers in the endomysium were mainly CD8 T cells, whereas macrophages were predominantly detected in necrotic fibers, and CD4 T cells were located in perimysium. Moreover, quantitative RT-PCR showed up-regulation of expression of TNF-α, IFN-γ, and perforin mRNA in the muscle of EAM mice. These findings in EAM mice are similar to those reported in IIM patients (4–8, 34–36).

Inflammatory cell migration into the affected muscle of IIM is thought to involve chemokine-chemokine receptor interaction

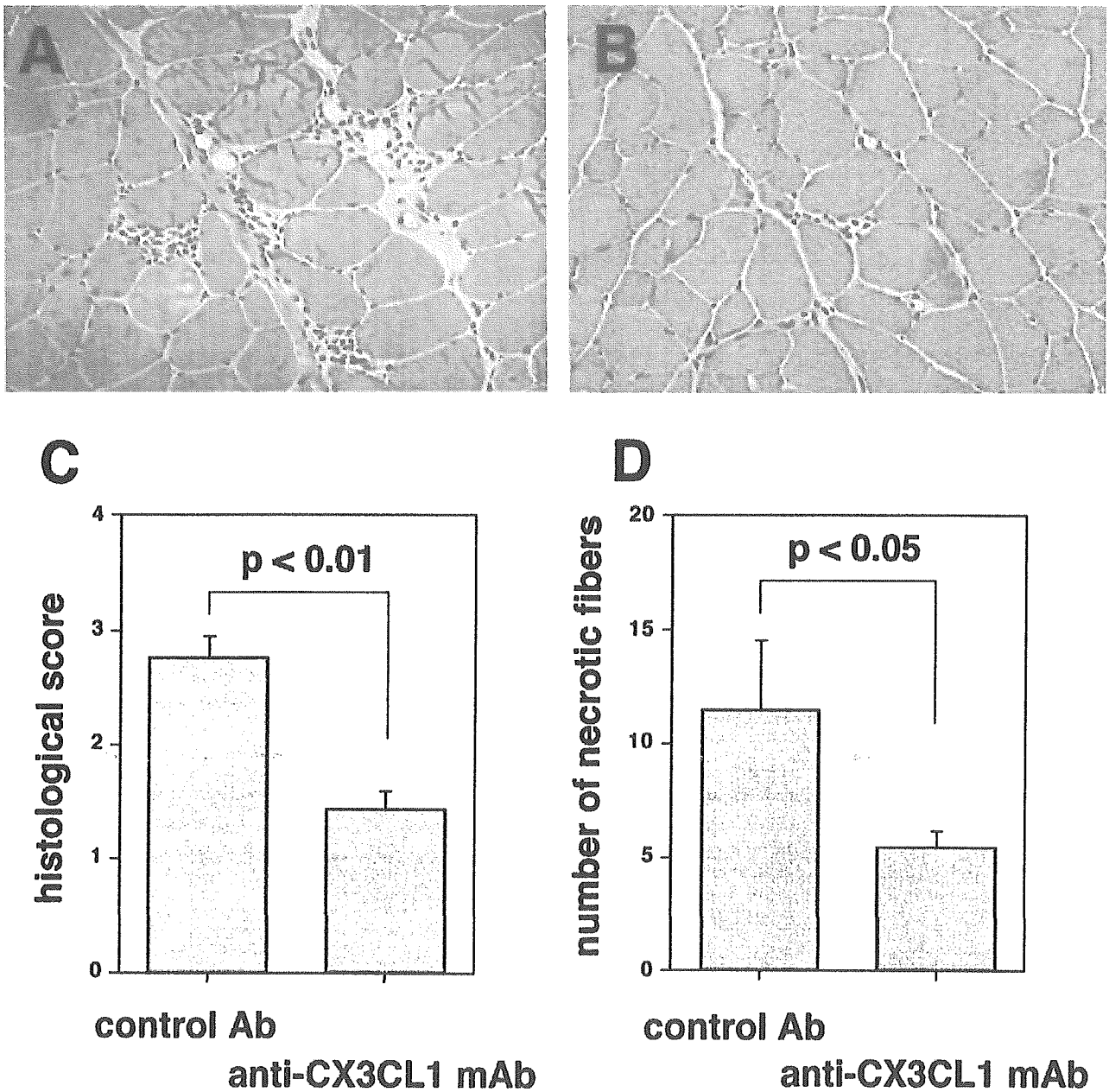

FIGURE 6. Inhibition of inflammatory changes in the muscle by treatment with anti-CX3CL1 mAb. Five hundred micrograms of hamster anti-mouse CX3CL1 mAb or control Ab was injected into the peritoneal cavity three times per week from day 0 for 3 wk. On day 21, the quadriceps femoris muscles of EAM mice were examined with H&E staining, histological scores were evaluated, and the numbers of necrotic fibers were counted. Mice treated with control Ab showed inflammatory cell accumulation (*A*). Mice treated with anti-CX3CL1 mAb showed milder inflammatory changes (*B*). Representative photomicrographs of histology from 10 animals in each group are shown. Histological scores of inflammatory changes in quadriceps femoris muscles were evaluated (*C*). The numbers of necrotic fibers were counted in the muscle tissues (*D*). Data represent the mean ± SEM.

(9–14). In the present study we focused on the role of CX3CL1-CX3CR1 interaction in the inflammatory cell migration. We showed the expression of CX3CR1 on some CD4 T cells and most CD8 T cells in EAM mice. It has been reported that CTLs including both $CD4^+$ and $CD8^+$ T cells invade the muscle fibers in IIM patients (3). These cells possess cytotoxic molecules, such as perforin and granzyme B, which are released into muscle cells (4, 5). Furthermore, type 1 cytokines, such as TNF-α and IFN-γ, were expressed in the inflammatory lesions of IIM patients (6–8). These findings suggest that the cytotoxic molecules and type 1 cytokines play important roles in the inflammatory lesions in IIM patients. In contrast, we reported previously that peripheral blood $CX3CR1^+$ T cells express cytotoxic molecules and type 1 cytokines (26, 27). Therefore, the interaction of CX3CL1 and CX3CR1 could induce the migration of T cells, which express cytotoxic molecules and type 1 cytokines, into the affected muscles.

The infiltrated macrophages into the affected muscle also express inflammatory cytokines (9, 41). They express TNF-α and IL-1β, which could stimulate T cells, macrophages, and

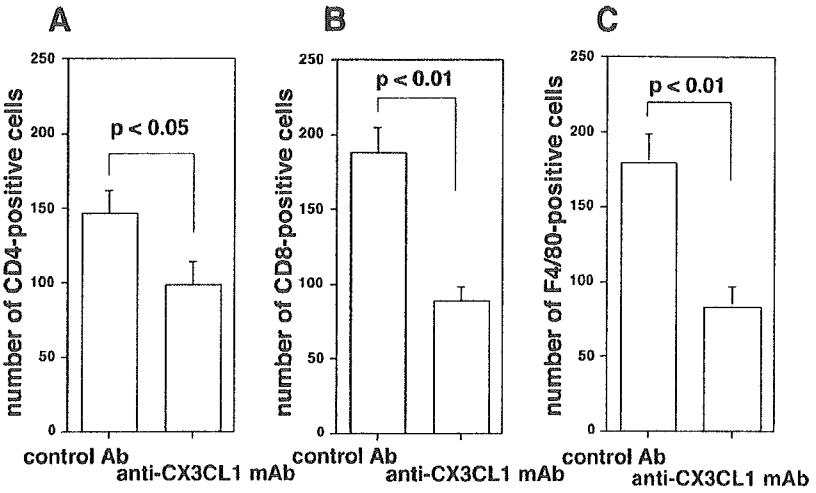

FIGURE 7. Decreased numbers of infiltrating cells of each subset by anti-CX3CL1 mAb treatment. Numbers of infiltrating $CD4^+$, $CD8^+$, and $F4/80^+$ cells were counted in the quadriceps femoris muscles from the experiment shown in Fig. 6. Data represent the mean ± SEM.

endothelial cells to produce various inflammatory cytokines, chemokines, and adhesion molecules. Moreover, these cytokines might have myocytotoxic effects (42–44). Our results showed that the majority of the $F4/80^+$ macrophages expressed CX3CR1 in the muscle of EAM mice. Thus, the CX3CL1-CX3CR1 interaction might also play an important role in macrophage migration into the affected muscle in addition to T cell migration.

CX3CL1 was expressed on infiltrated mononuclear cells in the affected muscles of EAM mice. Because CX3CL1 expression was located in the endomysium, infiltrated macrophages and/or CD8 T cells may express CX3CL1 in the muscles. Furthermore, we showed that CX3CL1 was also expressed on vascular endothelial cells in the EAM muscle tissue on days 14 and 21, but not in normal mice. It was reported that CX3CL1 was expressed on endothelial cells activated with TNF-α and IFN-γ in vitro (19–21). Expressed CX3CL1 on endothelial cells might recruit $CX3CR1^+$ cells, including macrophages and T cells, into muscle. These cells, in turn, express TNF-α and IFN-γ, which induce additional CX3CL1 expression on endothelial cells and also on recruited inflammatory cells. The enhanced expression of CX3CL1 may induce additional inflammatory cell migration. Consequently, these amplification cascades could contribute to the expansion of pathological changes in EAM mice. In fact, inhibition of CX3CL1 reduced the numbers of migrated CD4 and CD8 T cells and macrophages in the affected muscles of EAM mice and also reduced the expression of TNF-α, IFN-γ, and perforin. These results suggest that CX3CL1 blockade reduces the migration of inflammatory cells, which express cytotoxic molecules and cytokines, into the muscles. Thus, inhibition of CX3CL1-CX3CR1 interaction might be a potentially suitable therapeutic strategy for treatment of IIM.

Our data showed that mRNA expression of TNF-α, IFN-γ, and perforin was almost totally inhibited by anti-CX3CL1 mAb treatment, although the numbers of infiltrated monocytes were decreased by up to 50%. Recently it was reported that stimulation with CX3CL1 enhanced production of proinflammatory cytokines such as IFN-γ as well as the release of cytolytic granules by T cells (45). Thus, blockade of CX3CL1 might inhibit not only cellular migration, but also cytokine and cytotoxic molecule expression, by stimulation with CX3CL1 in the EAM muscle. Alternatively, because $CX3CR1^+$ T cells express type 1 cytokine and cytotoxic molecules (23, 26, 27), and $CX3CR1^{high}$ positive monocytes greatly produce inflammatory cytokines compared with $CX3CR1^{low}$ positive monocytes (46–48), treatment with anti-CX3CL1 mAb may selectively inhibit the migration of such specific T cells and macrophages. Therefore, anti-CX3CL1 mAb might be able to inhibit the expression of cytokine and cytotoxic molecules effectively in muscles, but additional study is required.

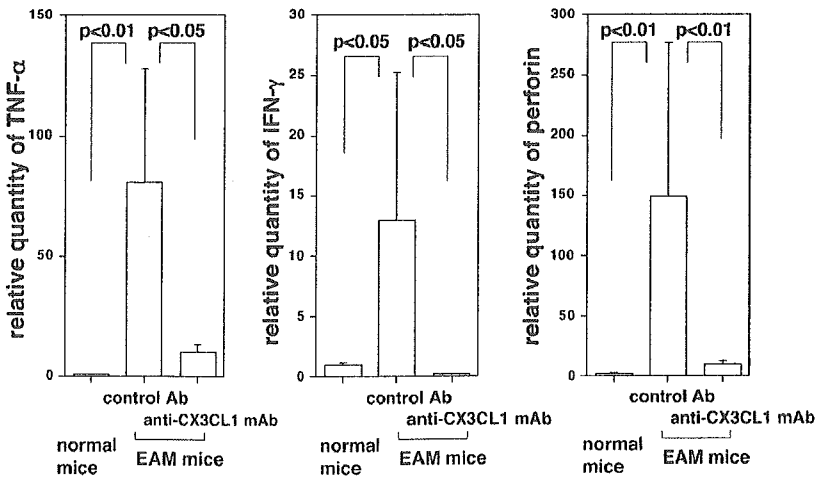

FIGURE 8. Reduction of TNF-α, IFN-γ, and perforin expression by anti-CX3CL1 mAb treatment. Expression of TNF-α, IFN-γ, and perforin mRNA in quadriceps femoris muscles from normal mice (n = 10) and from the experiment shown in Fig. 6 were measured using real-time RT-PCR. Data represent the mean ± SEM.

We recently reported that inhibition of CX3CL1 ameliorated collagen-induced arthritis in mice, probably by suppression of inflammatory cell migration into the synovium (30). Others reported that anti-CX3CR1 Ab treatment blocked inflammatory cell infiltration in the glomeruli, prevented crescent formation, and improved renal function in the Wistar-Kyoto crescentic glomerulonephritis model (49). Furthermore, the gene deletion of CX3CR1 resulted in an ~50% decrease in the formation of atherosclerotic lesions and the number of infiltrated macrophages in the lesion in experimental atherosclerosis mice (50, 51). These results together with our findings suggest that blockade of CX3CL1-CX3CR1 interaction might be therapeutically useful for several diseases associated with inflammatory cell infiltration. In this study we propose that such treatment is also suitable for IIM. To our knowledge, this is the first report demonstrating that a chemokine inhibitor could reduce the severity of myositis.

In conclusion, we demonstrated in the present study that inhibition of CX3CL1 significantly improved histopathological changes in the muscles of EAM mice, suggesting that blockade of CX3CL1 might be therapeutically beneficial for IIM.

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Disclosures

The authors have no financial conflict of interest.

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