	Systemic sclerosis (n=11)	Rheumatoid arthritis (n=11)	Healthy controls (n=11)
Mean (SD) age at examination, years	57·7 (11·8)	59·1 (12·0)	52·7 (10·6)
Median (IQR) disease duration, years	10·0 (7·0–11·0)	9·0 (1·5–14·0)	..
Postmenopausal	7 (64%)	7 (64%)	7 (64%)
Current smokers	1 (9%)	1 (9%)	1 (9%)
Hypertensive	1 (9%)	2 (18%)	1 (9%)
Hypercholesterolaemic	2 (18%)	3 (27%)	2 (18%)
Receiving corticosteroids	5 (45%)	5 (45%)	..

Data are number of participants unless otherwise stated.

Table 1: Clinical characteristics of study participants

these tests to examine the quantity and function of CEP in patients with systemic sclerosis.

Methods

Patients

We studied 11 female patients with systemic sclerosis, all of whom met the American College of Rheumatology preliminary criteria.[10] Patients with symptoms that overlapped those of other connective-tissue diseases were excluded. Controls matched for age and sex were 11 female patients with rheumatoid arthritis, as a systemic inflammatory disease control, and 11 healthy women. All patients with rheumatoid arthritis met the American College of Rheumatology classification criteria[11] and had evidence of active disease according to the published criteria.[12] We studied only women because the prevalence of systemic sclerosis is five to ten times higher in women than in men, and the number and function of CEP are potentially influenced by sex because physiological new blood formation occurs prominently in the endometrium. Patients were selected from those who visited our outpatient clinic during a 2-month period (November and December, 2002) and were willing to donate blood samples for this study. All peripheral-blood samples were obtained on several occasions between November, 2002, and February, 2003. This study was approved by the Keio University Institutional Review Boards, and written informed consent was obtained from all participants.

Procedures

For quantification of CEP, peripheral-blood mononuclear cells were isolated from 20 mL heparinised venous blood by Lymphoprep (Nycomed Pharma AS, Oslo, Norway) density-gradient centrifugation. CD34-positive cells were enriched from peripheral-blood mononuclear cells by an immunomagnetic technique with a monoclonal antibody to CD34 coupled to magnetic beads (CD34 MicroBeads; Miltenyi Biotech, Bergisch Gladbach, Germany). The CD34-cell-enriched fraction was then incubated with a monoclonal antibody to VEGFR-2 (KDR-1; Sigma, St Louis, MO, USA) and biotin-conjugated goat antibody to mouse IgG $F(ab')_2$ (Immunotech, Marseille, France), followed by additional staining with a fluorescein-isothiocyanate-conjugated monoclonal antibody to CD34 (AC136), phycoerythrin-conjugated monoclonal antibody to CD133 (AC133/2; Miltenyi Biotech), and streptavidin-PC5 (Immunotech). Control cells were also prepared by incubation with fluorescence-labelled isotype-matched monoclonal antibodies. The cells were then analysed by three-colour flow cytometry with a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Viable cells were identified by gating on forward and side scatters, and the expression of CD133 and VEGFR-2 was assessed on gated CD34-positive cells. CEP were identified as cells positive for CD34, VEGFR-2, and CD133.[5] The total number of viable cells in the CD34-cell-enriched fraction was assessed from its ratio to the FlowCount microbeads (Beckman-Coulter, Hialeah, FL, USA), and we calculated the absolute numbers of cells in 20 mL peripheral blood that were positive for: CD34; both CD34 and CD133; or CD34, CD133, and VEGFR-2 (CEP); and the number positive for CD34 and VEGFR-2 but negative for CD133. A consistent detector sensitivity, compensation setting, and scatter gate set were used to analyse all samples. Because of variability in staining intensity across the samples, the cut-off setting was decided on in individual samples on the basis of the staining intensity of control cells incubated with a series of isotype-matched monoclonal antibodies. All procedures were done by the same operator without knowledge of the sample identity. The coefficients of variation for five separate assays of CEP in the samples from two healthy individuals were 14·2% and 10·7%.

CD133-positive cells were isolated from at least 5×10^7 peripheral-blood mononuclear cells by the MACS sorting system with magnetic beads coupled with monoclonal antibody to CD133 (Miltenyi Biotech). Flow-cytometric analysis showed that the cell fractions positive for CD133 consistently contained more than 85% such cells. The CD133-positive cells were resuspended in Dulbecco's modified Eagle's medium (Sigma) and cultured on fibronectin-coated chamber slides (Beckton-Dickinson) overnight. Human umbilical-vein endothelial cells (Clonetics, San Diego, CA, USA) were used as a mature endothelial-cell control. The adherent cells were then fixed with 4% paraformaldehyde, and incubated for 30 min with rabbit polyclonal antibodies to VEGFR-2 or Tie-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in combination with mouse monoclonal antibodies to CD31 (WM-59), VEGFR-2 (KDR-1; both Sigma), CD45 (PD7/26), von Willebrand factor (2F2-A9; both Dako, Carpinteria, CA, USA), CD146 (P1H12), or vascular endothelium cadherin (BV6; both Chemicon International, Temecula, CA, USA). The cells were subsequently incubated with rabbit-specific IgG conjugated to AlexaFluor 568 and mouse-specific IgG conjugated to AlexaFluor 488 (Molecular Probes, Eugene, OR, USA). To assess the uptake of acetylated LDL, cultured adherent cells were incubated with acetylated LDL labelled with 1,1'-dioctadecyl-3,3,3',3'-tetramethy-

lindocarbocyanine (Molecular Probes) for 1 h at 37°C, then with a rabbit antibody to VEGFR-2 and rabbit-specific IgG conjugated to AlexaFluor 488. The negative controls were cells incubated with normal mouse IgG and rabbit IgG instead of the primary antibodies. Nuclei were stained with TO-PRO-3 (Molecular Probes). The stained cells were examined with a confocal laser fluorescence microscope (LSM5 PASCAL; Carl-Zeiss, Göttingen, Germany). At least ten cells that stained for VEGFR-2 were investigated for expression of CD31, vascular-endothelium cadherin, CD146, von Willebrand factor, CD45, and Tie-2 and for uptake of acetylated LDL.

The concentrations of vascular endothelial growth factor in heparinised platelet-poor plasma and of basic fibroblast growth factor, hepatocyte growth factor, and erythropoietin in serum were measured by specific ELISA kits (Quantikine, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

To assess the potential of CEP to differentiate into mature endothelial cells in response to angiogenic stimuli, the cells were cultured as described previously[13-15] with several modifications. CD133-positive cells and CD133-negative cells were separated from more than 5×10^7 peripheral-blood mononuclear cells by the MACS sorting system. CD133-positive cells were then plated with CD133-negative cells at a ratio of 1 to 50 on fibronectin-coated chamber slides at an overall density of 5×10^3 cells/μL. The cells were cultured in endothelial cell basal medium 2 (Clonetics) supplemented with MV SingleQuots containing fetal bovine serum, vascular endothelial growth factor, basic fibroblast growth factor, epidermal growth factor, insulin-like growth factor 1, heparin, and ascorbic acid for 5 days. Because CEP are known to form late-outgrowth colonies after 9 days in vitro,[15] proliferation of CEP is negligible in this short-term culture. The cells were fixed and incubated with mouse antibody to VEGFR-2 (KDR-1) or monoclonal antibody to von Willebrand factor (2F2-A9) followed by incubation with AlexaFluor 568 mouse-specific IgG and then with fluorescein-isothiocynate-conjugated mouse monoclonal antibody to CD45 (PD7/26). The negative controls were cells incubated with normal mouse IgG instead of the primary antibody. The cells were examined with a confocal laser fluorescence microscope. The individual staining was carried out in two to four wells, and the mean number of cells that lacked CD45 expression but expressed VEGFR-2 or von Willebrand factor was counted. The proportion of mature CEP obtained in this in-vitro maturation treatment was calculated as the ratio of CD45-negative, von-Willebrand-factor-positive cells to CD45-negative, VEGFR-2-positive cells.

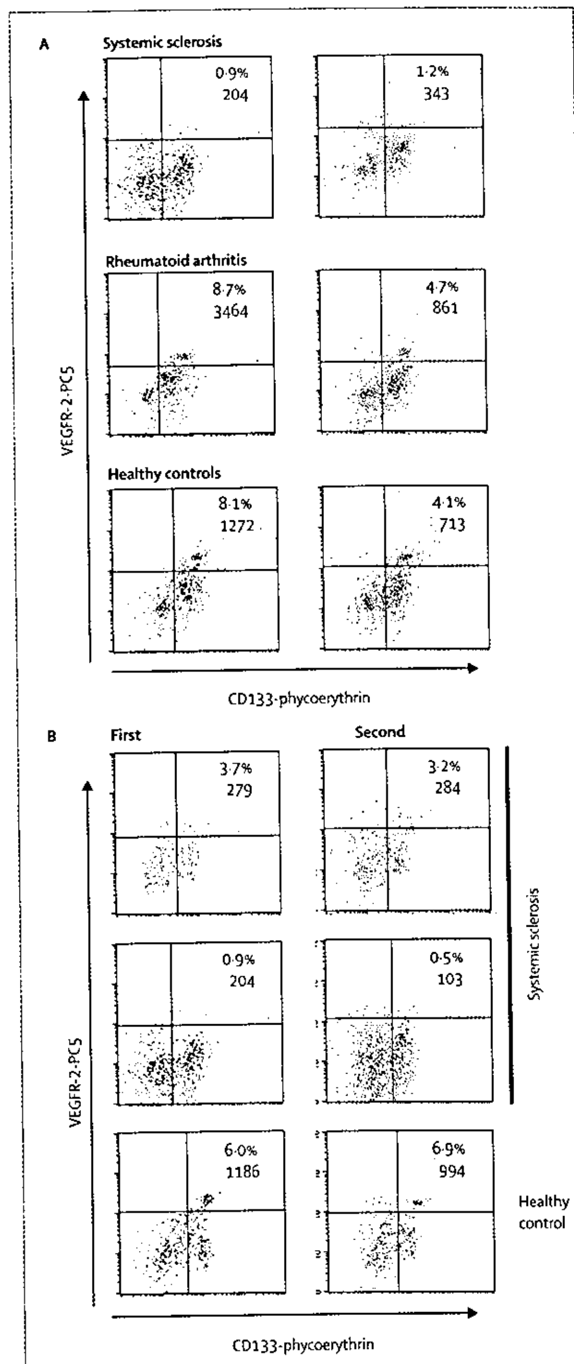

Figure 1: Detection of CEP
A: Detection of CEP by flow cytometry in two representative participants from each study group. The upper right section of individual dot-plot images indicates CD34-positive, CD133-positive, VEGFR-2-positive CEP. The percentage of CEP in the gated CD34-positive cells and the absolute number of CEP in 20 mL peripheral blood are shown in each panel. B: Serial measurement of CEP beyond a 3-month period in two patients with systemic sclerosis and one healthy control.

Statistical methods

No formal sample-size calculation was done before the study began. Since the number and function of CEP are potentially influenced by the weather, we thought that all samples should be obtained in one winter season. This constraint limited our sample size to 11 patients in

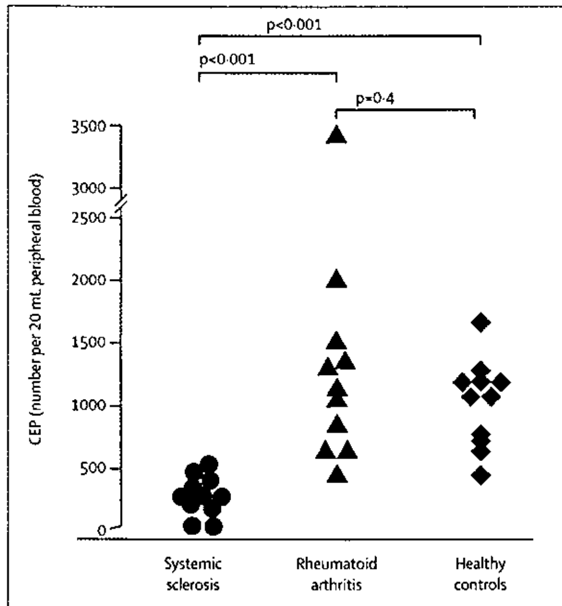

Figure 2: **Absolute CEP numbers in 20 mL peripheral blood from each participant**
p values by Kruskal-Wallis test followed by groupwise comparisons with Wilcoxon's rank sum test.

each group. Categorical variables were compared by use of the χ^2 test. The Shapiro-Wilk test was used to assess the normality of continuous data. Comparison between any two groups was by unpaired *t* test for normally distributed data or non-parametric Mann-Whitney test for non-normally distributed data. Multiple group comparison was done by one-way ANOVA for data with normal distribution. The Kruskal-Wallis test was used for data with non-normal distribution. When the p value for this overall comparison was significant (<0·05), post-hoc pairwise comparisons were done with the Dunnett's *t* test for data with normal distribution or Wilcoxon's rank sum test for data with non-normal distribution. The p values of the post-hoc pairwise comparisons were subsequently adjusted by Bonferroni correction. Correlation between two variables was tested by Pearson's test.

Role of the funding source

The sponsor of the study had no role in the study design; collection, analysis, or interpretation of data; writing of the report; or the decision to submit it for publication.

Results

The clinical characteristics of the participants are summarised in table 1. There was no difference in age at examination among the three groups. The disease duration was similar in patients with systemic sclerosis and those with rheumatoid arthritis, and both groups included patients with early and late disease. All the participants were female, and factors potentially affecting the number of CEP, including age, menstruation status, smoking, hypertension, and hypercholesterolaemia,[16] were matched among the three groups. No participant had diabetes mellitus or coronary-artery disease or was taking statins. No patient with systemic sclerosis or rheumatoid arthritis had received cytotoxic drugs or ciclosporin at any time during their illness, but five patients with systemic sclerosis and five with rheumatoid arthritis were receiving low-dose corticosteroids (<10 mg daily) at the time of blood sampling. Of the patients with systemic sclerosis, six had diffuse cutaneous involvement. The mean modified Rodnan's total skin score[17] at examination was 11·4 (SD 6·6). All patients had Raynaud's syndrome and seven had pitting scars. Two patients had active fingertip ulcers on several digits, and in one amputation of the right foot was necessary owing to progressive gangrene.

Flow-cytometric analyses for the staining of CD133 and VEGFR-2 on the gated CD34-positive cells in representative participants are shown in figure 1. CEP, identified as cells positive for both CD133 and VEGFR-2, were clearly visible in samples from two patients with rheumatoid arthritis and two healthy controls but were scarcely detected in samples from two patients with systemic sclerosis. The absolute numbers of CEP were much lower in patients with systemic sclerosis than in those with rheumatoid arthritis and healthy controls (figure 2) but similar in patients with rheumatoid

	Systemic sclerosis (n=11)	Rheumatoid arthritis (n=11)	Healthy controls (n=11)	p (for overall comparison)
Absolute number of cells in 20 mL peripheral blood				
Mean (SD) CD34-positive	12557 (9974)*	15 225 (10135)	25892 (9552)	0·009
Mean (SD) CD34-positive, CD133-positive	4668 (3691)†	8109 (5468)	13768 (7023)	0·002
Median (IQR) CD34-positive, CD133-positive, VEGFR-2-positive (CEP)	274 (178–395)‡§	1154 (653–1524)	1074 (713–1186)	<0·0001
Median (IQR) CD34-positive, CD133-negative, VEGFR-2-positive	79 (23–218)	76 (37–180)	53 (31–64)	0·3
Median (IQR) circulating concentrations of angiogenic factors				
Vascular endothelial growth factor, ng/L	34·5 (11·0–53·1)*¶	76·0 (59·4–112·5)†	10·0 (10·0–16·1)	<0·0001
Basic fibroblast growth factor, ng/L	33·0 (21·7–36·1)‡	24·0 (19·4–26·9)‡	9·0 (8·6–9·7)	<0·0001
Hepatocyte growth factor, ng/L	1330 (1062–1466)†	1337 (1064–1441)†	741 (691–900)	<0·0001
Erythropoietin, IU/L	9·7 (3·5–11·1)	13·0 (7·7–15·8)*	6·2 (5·2–8·2)	0·01

p for the overall group comparison was assessed by one-way ANOVA for data with normal distribution or the Kruskal-Wallis test for data with non-normal distribution. When post-hoc pairwise comparisons followed by the Bonferroni correction were made, patients with systemic sclerosis or rheumatoid arthritis differed significantly different from healthy controls at: *p<0·05; †p<0·01; or ‡p<0·001. Patients with systemic sclerosis differed significantly different from those with rheumatoid arthritis at: §p<0·001; or ¶p<0·05.

Table 2: **Absolute numbers of CEP and concentrations of angiogenic factors in circulation of participants**

arthritis and healthy controls (table 2). Repeated measurements for five patients with systemic sclerosis and three healthy controls found stable numbers of CEP beyond a 3-month period in all cases (figure 1). In the patients with systemic sclerosis, the CEP number was not associated with the disease subset (diffuse *vs* limited), disease duration, or total skin score, but it was significantly lower in the seven patients with pitting scars than in the four without this feature (204 [IQR 110–276] *vs* 403 [326–479], p=0·03 by Mann-Whitney test). In addition, active fingertip ulcers were observed exclusively in patients with the lowest and second lowest numbers of CEP, which were less than 5% of the mean in healthy controls. The absolute numbers of cells positive for the three markers of interest in the three groups are also listed in table 2. The numbers of circulating CD34-positive and CD34-positive, CD133-positive cells were significantly lower in patients with systemic sclerosis or rheumatoid arthritis than in healthy controls. This difference may reflect the impaired haemopoiesis that is reported in patients with rheumatoid arthritis.[18] The number of CD34-positive, CD133-negative, VEGFR-2-positive cells containing circulating mature endothelial cells[5] was slightly but not significantly higher in patients with systemic sclerosis or rheumatoid arthritis than in healthy controls.

The CEP phenotype was assessed by fluorescence double-immunostaining of adherent CD133-positive cells of three patients with systemic sclerosis and three healthy controls. Representative images obtained from a healthy control are shown in figure 3. More than 80% of the adherent CD133-positive, VEGFR-2-positive cells coexpressed CD31 and Tie-2 and were positive for uptake of acetylated LDL, but they lacked expression of the haemopoietic marker CD45. This feature was seen in the control human umbilical-vein endothelial cells and was consistent with previous reports.[5,6] By contrast, expression of vascular-endothelium cadherin and a mature endothelial-cell marker CD146 in CEP was faint and not distributed on the cell surface. Furthermore, CEP lacked expression of von Willebrand factor, another marker of mature endothelial cells. There was no difference in the phenotype of CEP between patients with systemic sclerosis and healthy controls.

Angiogenic factors are known to potentiate the proliferation and recruitment of CEP from the bone marrow.[19,20] To test whether the lower numbers of CEP in patients with systemic sclerosis were due to the impaired production of angiogenic factors, circulating concentrations of vascular endothelial growth factor, basic fibroblast growth factor, hepatocyte growth factor, and erythropoietin were measured in the three study groups (table 2). Concentrations of all these angiogenic factors were higher in patients with systemic sclerosis than in healthy controls, as reported previously,[21,22] and differences in vascular endothelial growth factor, basic fibroblast growth factor and hepatocyte growth factor were significant. The concentrations of basic fibroblast growth factor and hepatocyte growth factor were similar in patients with systemic sclerosis and in those with rheumatoid arthritis, but the concentration of vascular endothelial growth factor was significantly higher in patients with rheumatoid arthritis than in those with systemic sclerosis. When relations between circulating concentrations of individual angiogenic factors and the CEP number were examined, only the concentration of

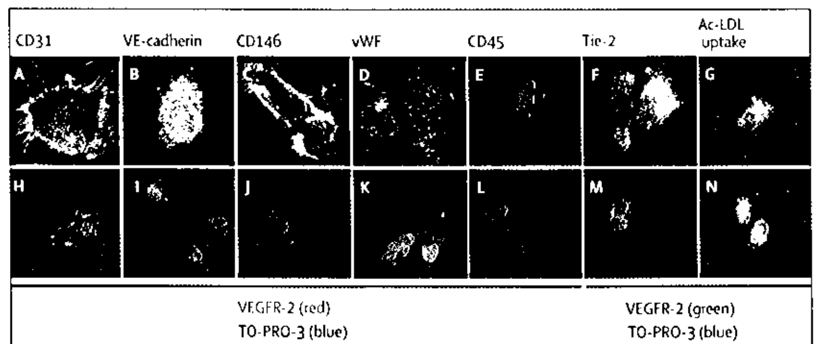

Figure 3: **Phenotypic characterisation of CEP**
Human umbilical-vein endothelial cells (A–G) and adherent CD133-positive cells from a healthy control (H–N) were fixed, stained with antibodies to VEGFR-2 (A–E and H–L; red, and F, G, M, and N; green) in combination with various antibodies, and examined with a confocal laser fluorescence microscope. VE=vascular endothelium; vWF=von Willebrand factor; Ac-LDL=acetylated LDL. Nuclei were stained with TO-PRO-3. Original magnification, ×630.

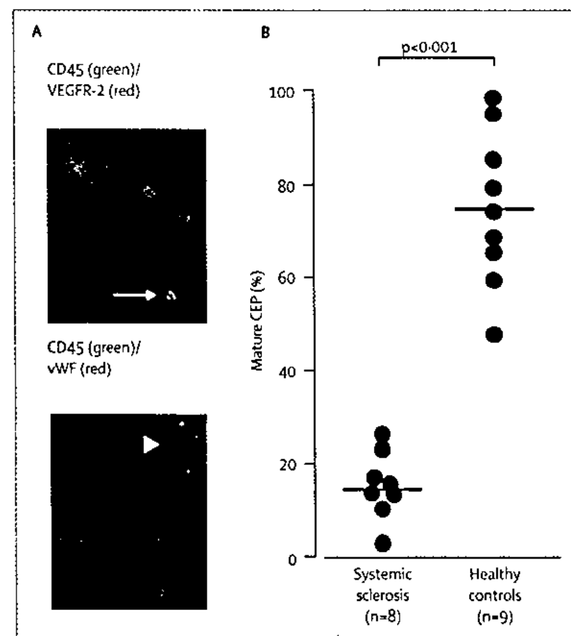

Figure 4: **In-vitro differentiation of CEP into mature endothelial cells in response to angiogenic stimuli**
A: CD45-negative, VEGFR-2-positive cells originated from CEP (arrow), and CD45-negative, von-Willebrand-factor-positive cells were mature endothelial cells that differentiated from CEP (arrowhead). CD45-positive cells lacking expression of VEGFR-2 or von Willebrand factor (vWF) were derived from circulating monocytes. Original magnification, ×100. B: Proportion of CEP with maturation potential in response to in-vitro angiogenic treatment. Comparisons were made by unpaired t test. Bar indicates mean value.

basic fibroblast growth factor was negatively correlated with the CEP number ($r=-0\cdot76$, $p=0\cdot006$).

In a preliminary experiment, we showed that CEP were induced to express von Willebrand factor by the in-vitro maturation treatment; therefore, the upregulated expression of this factor was used to assess the maturation potential of CEP as a marker for mature endothelial cells. Freshly isolated CD133-positive cells were cultured with a combination of angiogenic factors in the presence of CD133-negative cells, which were a required source of additional stimuli necessary for in-vitro maturation. We could not obtain sufficient CD133-positive cells for this assay from three patients with systemic sclerosis who had extremely low numbers of CEP. None of the adherent cells from three healthy donors expressed von Willebrand factor after overnight culture, indicating negligible contamination of mature endothelial cells in these cultures. After a 5-day culture, nearly all the adherent cells expressed CD45, and these were likely to have originated from monocytes in the CD133-negative cell fraction. By contrast, CD45-negative, VEGFR-2-positive cells and CD45-negative, von-Willebrand-factor-positive cells, which originated from CEP, represented less than 1% of the total adherent cells (figure 4). The proportion of CEP that differentiated in vitro into endothelial cells expressing von Willebrand factor was significantly lower in eight patients with systemic sclerosis than in nine healthy controls (mean 15·3% [SD 7·2] *vs* 74·5% [16·6], $p<0\cdot001$ by unpaired *t* test).

Discussion

We found that patients with systemic sclerosis had much lower numbers of CEP than healthy controls. Patients with extremely low CEP numbers were likely to have profound vascular involvement, such as pitting scars and fingertip ulcers. An overall deficiency in CD34-positive cells and CD34-positive, CD133-positive cells in systemic sclerosis and rheumatoid arthritis suggests impairment of haemopoiesis in these connective-tissue diseases; however, a deficiency in CEP was detected in systemic sclerosis but not in rheumatoid arthritis. Although the CEP phenotype was similar for cells isolated from patients with systemic sclerosis and healthy controls, CEP in patients with systemic sclerosis were functionally impaired and resistant to the in-vitro maturation treatment. In view of the important role of CEP in postnatal vasculogenesis, our findings strongly suggest that the failure of new blood-vessel formation in response to vascular damage in patients with systemic sclerosis can be explained by defective vasculogenesis. Because of the lack of specific markers to distinguish CEP from mature endothelial cells and certain haemopoietic cells accurately, the cells expressing CD34, CD133, and VEGFR-2 analysed in our assays might include non-endothelial precursors or miss a subset of primitive cells with capacity to differentiate into endothelial cells.

The rapid endothelialisation of denuded injured vessels is essential to avoid fatal complications, such as bleeding and thrombosis. When vascular injury occurs, many growth factors are rapidly produced by the damaged vessels and adherent activated platelets to promote vascularisation.[6,19] After acute vascular insult, including burns and coronary-artery bypass grafting, there is a rapid rise in plasma concentrations of vascular endothelial growth factor followed by mobilisation of CEP during the first 6–12 h; and the concentration of vascular endothelial growth factor returns to the basal value within 48–72 h.[6] In a setting of defective vasculogenesis such as systemic sclerosis, this tissue response might be accelerated and prolonged, and sustained production of the growth factors would induce the proliferation of vascular smooth-muscle cells and fibroblasts in the intima and excessive deposition of extracellular matrix. This process might be increased by activation of inflammation and coagulation systems, which is secondarily induced by endothelial damage and dysfunction. This pathogenetic process might not be restricted to vessel walls but might also activate fibroblasts in the surrounding tissues to induce excessive fibrosis in the dermis and other organs. Patients with rheumatoid arthritis, who have raised concentrations of circulating angiogenic factors similar to those in patients with systemic sclerosis, show prominent formation of new blood vessels on the synovium, which is now thought to be a preferential therapeutic target.[23]

The mechanism that causes the deficiency of CEP in systemic sclerosis is unknown. CEP in peripheral blood could be destroyed or their production in the bone marrow impaired. Several in-vitro studies with serum antibodies from patients with systemic sclerosis have found that endothelial-cell apoptosis is induced by antibodies to endothelial cells through antibody-dependent cell-mediated cytotoxicity[24] or via interaction with a specific cell-surface molecule.[25] In addition, cytomegalovirus is thought to be involved in the pathogenesis of systemic sclerosis, since it can infect endothelial cells.[26] CEP and mature endothelial cells share many phenotypic and functional properties,[5] so CEP might be a preferential target of these cytotoxic activities.

However, the high concentrations of vascular endothelial growth factor, basic fibroblast growth factor, and hepatocyte growth factor we found in the circulation of patients with systemic sclerosis strongly suggest that CEP and their stem cells in the bone marrow did not respond adequately to these angiogenic factors. An imbalance between angiogenic and angiostatic factors is a possible cause, but the concentration of endostatin, an angiostatic factor generated from collagen type XVIII, is not increased in patients with systemic sclerosis compared with healthy controls.[21] However, the possibility that other angiostatic factors suppress

maturation of CEP or their stem cells cannot be excluded. Furthermore, continuous endothelial injury might lead to eventual depletion of CEP, as suggested in patients with multiple risk factors for atherosclerosis,[16] although two patients with early systemic sclerosis (disease duration <3 years) in our study had low CEP numbers (279 and 463 per 20 mL). Further studies of a large number of patients with early and late disease are needed to test this possibility. An alternative hypothesis is that CEP, their stem cells, or both, are functionally altered and are intrinsically hyporesponsive to angiogenic stimuli, since the CEP in patients with systemic sclerosis showed impaired maturation potential in vitro induced by a combination of angiogenic stimuli. The negative correlation between the circulating concentrations of basic fibroblast growth factor and the number of CEP in patients with systemic sclerosis supports this hypothesis. Similar CEP dysfunction has also been reported in diabetes mellitus, a disease known to cause vasculopathy similar to that of systemic sclerosis.[27] Further functional characterisation of the CEP in patients with systemic sclerosis is necessary for these possibilities to be examined, but the low frequency of CEP in the circulation, especially in these patients, makes these studies difficult.

Conventional treatment for vascular involvement in patients with systemic sclerosis is limited to non-specific vasodilator agents, and little success has been achieved by these therapies, which solely target vascular dysfunction.[28] The findings of this study suggest that strategies that mobilise CEP into the circulation might have therapeutic potential in the vasculopathy of systemic sclerosis. Granulocyte colony-stimulating factor is known to mobilise CEP from the bone marrow.[5] Moreover, although the concentrations of vascular endothelial growth factor and basic fibroblast growth factor are already high in patients with systemic sclerosis, a further increase obtained by the administration of these factors, either as recombinant proteins or by gene transfer,[29,30] could augment vasculogenesis as a therapeutic option for patients with severe ischaemic disease. Statins stimulate CEP kinetics and increase the number of CEP in circulation.[31] Therapeutic effects of these potential strategies will depend on the mechanism for the defective vasculogenesis in systemic sclerosis.

Thus, our findings lead us to propose a new theory that insufficient vascular repair machinery through defective vasculogenesis contributes to the vascular involvement in systemic sclerosis. Since the number of patients examined in this study was small, further studies are necessary to confirm our theory. Investigation of functional changes in postnatal vasculogenesis in patients with systemic sclerosis could be useful for clarifying the pathogenesis of this disease and developing novel therapeutic interventions for ischaemic complications in patients with systemic sclerosis.

Contributors

M Kuwana had the original idea for the study, designed the study, and participated in experimental procedures and data analysis. Y Okazaki did most of the experimental procedures. H Yasuoka and Y Kawakami participated in some of the experimental procedures and the data analysis. Y Ikeda recruited the patients and supervised the study. All authors contributed to the writing of the report.

Conflict of interest statement

None declared.

Acknowledgments

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β_2-glycoprotein I: antiphospholipid syndrome and T-cell reactivity☆

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Abstract There is increasing evidence showing that recurrent thrombosis and intrauterine fetal loss in antiphospholipid syndrome (APS) are attributable to antiphospholipid (aPL) antibodies. We have recently identified autoreactive CD4+ T cells to β_2-glycoprotein I (β_2GPI) that promote production of pathogenic antiphospholipid antibodies. β_2GPI-specific CD4+ T cells preferentially recognize the antigenic peptide containing the major phospholipid (PL)-binding site in the context of DR53. T-cell helper activity that stimulates B cells to produce IgG anti-β_2GPI antibodies is mediated through IL-6 and CD40–CD154 interaction. β_2GPI-specific T cells respond to reduced β_2GPI and recombinant β_2GPI fragments produced in a bacterial expression system but not to native β_2GPI, indicating that the epitopes recognized by β_2GPI-specific T cells are 'cryptic' determinants, which are generated at a subthreshold level by the processing of native β_2GPI under normal circumstances. Although β_2GPI-specific T cells are detected in both APS patients and healthy individuals, these autoreactive T cells are activated in vivo in APS patients but not in healthy individuals. These findings indicate activation of β_2GPI-specific T cells and subsequent production of pathogenic anti-β_2GPI antibodies can be induced by the exposure of such T cells to cryptic peptides of β_2GPI efficiently presented by functional antigen-presenting cells (APC). Delineating the mechanisms that induce the efficient processing and presentation of cryptic determinants of β_2GPI as a consequence of antigen processing would clarify the etiology that initiates the autoantibody response in APS.
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Abbreviations: APC, antigen-presenting cells; aPL, antiphospholipid; APS, antiphospholipid syndrome; β_2GPI, β_2-glycoprotein I; PL, phospholipid; TCR, T-cell receptor.

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Introduction

Antiphospholipid syndrome (APS) is an autoimmune disorder characterized by arterial and venous thrombosis as well as recurrent pregnancy loss in association with circulating antiphospholipid (aPL) antibodies [1]. aPL antibodies often target plasma proteins with the capacity to bind anionic phospholipids (PLs) rather than PL itself. β_2-glycoprotein I (β_2GPI) and prothrombin are identified to be the major aPL antigens [2]. β_2GPI-dependent aPL antibodies are strongly associated with thrombosis and other clinical features of APS [3] and thus are included as one of the criteria for the classification of APS [4]. β_2GPI is a plasma glycoprotein that binds various kinds of negatively charged substances, including PLs, lipoproteins, activated platelets and activated endothelial cells [5–7]. β_2GPI possesses five complement control protein repeats (domains I–V), and the major PL-binding site has been identified as a positively charged area located at amino acid positions 281–288 in domain V [8,9]. The physiological function of β_2GPI has not been established to date, but it has been suggested that the protein may play a scavenging role for exposed anionic materials, including PL-expressing foreign particles [10] and oxidized lipoprotein [11]. Pathogenicity of aPL antibodies in APS patients has been demonstrated in the experimental model, into which normal mice are infused with an IgG fraction of APS patients' sera [12,13]. A precise mechanism for thrombophilia caused by aPL antibodies still remains unclear, but many hypotheses have been proposed to date [14]. For example, anti-β_2GPI antibodies bind to endothelial cell surfaces by recognizing the adhered β_2GPI and induce endothelial cell activation, resulting in the up-regulation of procoagulant and inflammatory processes [15]. On the other hand, anti-β_2GPI antibodies bind to oxidized low-density lipoprotein through adhered β_2GPI and promote its uptake by macrophages, resulting in the promotion of atherosclerosis [16].

Although antigenic specificity and functional characteristics of aPL antibodies have been extensively analyzed, little information is available about the cellular mechanisms that induce the production of pathogenic aPL antibodies. However, anti-β_2GPI antibodies in APS patients are mainly of the IgG and IgA isotypes [17], suggesting that its production requires antigen-specific T-cell help. In this regard, adoptive transfer of peripheral blood lymphocytes derived from an APS patient to severe combined immune deficiency mice results in a continuous increase in circulating aPL antibodies and the APS manifestation [18]. Animals immunized with foreign β_2GPI develop manifestations of the APS along with anti-β_2GPI antibody production [19,20]. The APS phenotype can be transferable to naïve mice by infusing bone marrow cells from the β_2GPI-immunized mice [21]. In this model, T cell-depleted bone marrow cells do not induce anti-β_2GPI antibody production in naïve recipients. These findings all together indicate the active involvement of the T cells responsive to β_2GPI in the production of pathogenic anti-β_2GPI antibodies. In this report, we summarize updates on autoreactive T cells to β_2GPI and their pathogenic roles in APS.

Identification of autoreactive CD4$^+$ T cells to β_2GPI

Because β_2GPI is a plasma protein abundantly present in the circulation (~200 μg/ml), antigen-presenting cells (APCs) constantly take up β_2GPI, process it and present the β_2GPI-derived peptides to T cells. Therefore, we hypothesized that T-cell tolerance to β_2GPI is tightly maintained under normal circumstances through negative selection in the thymus as well as through a post-thymic mechanism of peripheral tolerance. To test this hypothesis, we compared the proliferative response of peripheral blood T cells from APS patients in cultures supplemented with 10% fetal bovine serum containing 20 μg/ml bovine β_2GPI and in cultures with 10% fetal bovine serum depleted of bovine β_2GPI and found no difference in the T-cell proliferation between these circumstances [22]. In addition, purified human β_2GPI at concentrations of 1–200 μg/ml did not induce proliferation in T cells cultured in medium containing β_2GPI-free fetal bovine serum. These findings support the theory that T-cell tolerance to the native form of β_2GPI exists even in patients with APS. On the other hand, two groups reported the presence of T cells reactive with native β_2GPI in peripheral blood from patients with SLE or APS. In a report by Davies et al. [23], a borderline T-cell response to native β_2GPI was detected at an antigen concentration of 30 μg/ml (20 μg/ml β_2GPI contained in the culture medium with heat-denatured normal human serum plus 10 μg/ml of purified human β_2GPI), but this result was based on the comparison with a culture with heat-denatured normal human serum containing β_2GPI at a concentration of 20 μg/ml. Visvanathan and McNeil [24] and Visvanathan et al. [25] proposed that T cells reactive with native β_2GPI in APS patients stimulated circulating monocytes to produce tissue factors when stimulated with native

β_2GPI in vitro, whereas little or no tissue factor was induced when T cells from patients without the APS or healthy controls were tested. Later, this investigator group retracted their report because they were unable to reproduce their own findings [26]. These studies lack important data regarding specific recognition of β_2GPI-derived peptides in the context of HLA molecules by the responding T cells.

Previous studies in our laboratory indicate that structural modification of autoantigens could induce expression of antigenic self-determinants recognized by pathogenic autoreactive T cells in other autoimmune diseases [27,28]. According to this idea, we prepared chemically reduced β_2GPI, trypsinized β_2GPI fragments and a series of recombinant β_2GPI fragments produced in a bacterial expression system (rβ_2GPI) and used them to stimulate T cells in bulk peripheral blood mononuclear cell cultures [22]. As a result, we reproducibly detected T-cell responses to reduced β_2GPI and rβ_2GPI, but not to trypsinized β_2GPI fragments, in the majority of APS patients with anti-β_2GPI antibodies. T cells reactive with modified β_2GPI had a CD4$^+$ helper phenotype and were restricted mainly by HLA-DR. In addition, these β_2GPI preparations induced IgG anti-β_2GPI antibody production in peripheral blood mononuclear cell cultures in vitro. Anti-β_2GPI antibodies synthesized in this in vitro culture system had similar features of pathogenic anti-β_2GPI antibodies in APS patients because they recognized β_2GPI immobilized on cardiolipin-coated plastic plates and prolonged diluted activated partial thromboplastin time.

Antigen recognition profiles of β_2GPI-reactive T cells

To further characterize T cells responsive to modified β_2GPI in APS patients, CD4$^+$ T-cell clones specific to rβ_2GPI were generated from APS patients with a high level of serum anti-β_2GPI antibodies [29]. Ten out of 12 β_2GPI-reactive T-cell clones were able to induce production of anti-β_2GPI antibodies from autologous B cells in response to rβ2GPI. As expected, β_2GPI-specific T-cell clones responded to reduced β_2GPI but not to native β_2GPI. These T-cell clones responsive to modified β_2GPI were really β_2GPI-reactive because they specifically recognized synthetic peptides encoding the amino acid sequences of human β_2GPI. Ten of the 14 T-cell clones (71%) recognized a synthetic peptide encompassing amino acid residues 276–290 of β_2GPI (p276–290) containing the major PL-binding site. When a panel of L-cell transfectants expressing a single HLA class II molecule was used as APCs, recognition of p276–290 was restricted by the DRB4*0103 (DR53). This restriction profile may explain the previously reported associations of anti-β_2GPI antibodies with the DR4, DR7 and DR9 haplotypes [30], all of which are in linkage disequilibrium with DR53. On the other hand, a small number of β_2GPI-specific T-cell clones reactive with epitopes in domain I/II, domain IV and p247–261 in domain V was also generated from APS patients, indicating heterogeneous antigenic specificities in β_2GPI-specific T cells. Recently, Ito et al. [31] analyzed the T-cell epitopes on β_2GPI using β_2GPI-specific T-cell lines established from APS patients and healthy individuals by stimulating with a mixture of 40 synthetic peptides covering the entire amino acid sequence of β_2GPI. They identified four distinct epitopes, including p64–83, p154–174, p226–246 and p244–264, but T-cell lines recognizing the peptide containing the major PL-binding site were not generated. It is possible that such peptide was not efficiently presented to T cells in the context of the HLA-DR molecule because synthetic peptides containing the major PL-binding site may be removed by its capacity to bind anionic PLs expressed in abundance on apoptotic cell surfaces in an in vitro culture. Until now, we have generated nearly 100 T-cell lines responsive specifically to rβ_2GPI from APS patients and healthy donors carrying DR53 and found that >90% of them recognize p276–290. As shown in Fig. 1, p276–290 is unique because it contains the entire major PL-binding site (amino acids 281–288) as well as a previously reported DR53-binding motif [32]. Interestingly, Gharavi et al. [33] reported that the immunization of normal mice with a human β_2GPI peptide encompassing amino acid residues 274–288 (termed GDKV peptide), which also contains

Human β_2GPI sequence	276 290
p276-290	KVSFFCKNKEKKCSY
DR53-binding motif	K--I----KI
Major PL-binding site	CKNKEKKC
GDKV peptide (p274-288)	GDKVSFFCKNKEKKC
Mouse β_2GPI sequence	
Mouse p274-288	GDKIHFYCKNKEKKC
H-2E^d-binding motif	F--K-L--K

Figure 1 Amino acid sequence of p276–290, a dominant T-cell epitope on β_2GPI. p276–290 contains the previously reported DR53-binding motif and the major PL-binding site. The GDKV peptide was used to immunize normal mice by Gharavi et al. [33]. Mouse version of the GDKV peptide and H2–E^d-binding motif are also shown. A box denotes the major PL-binding site.

the major PL-binding site, induced the production of anti-β_2GPI antibodies possessing thrombotic properties. Production of the IgG isotype of anti-β_2GPI antibodies in their in vivo system indicates that this response is a result of T-cell-dependent isotype switch. Therefore, it is likely that the GDKV peptide binds H2–E^d molecule and prime xeno-reactive T cells to the human sequence. This initial response may subsequently diversify to the mouse sequence by cross-reactivity. We found that many of our T-cell clones responsive to p276–290 generated from APS patients also recognized the GDKV peptide. It is of note that a dominant T-cell epitope on β_2GPI is shared between APS patients and an experimental model for APS.

T cells use clonally unique T-cell receptors (TCRs) to recognize peptide epitopes. β_2GPI-specific CD4$^+$ T-cell clones responsive to p276–290 commonly had the TCR β-chains with a rearranged Vβ7 segment and a TGxxN/Q motif in their CDR3 sequence [34]. This is consistent with another finding obtained from polymerase-chain reaction and single-strand conformation polymorphism analysis combined with in vitro stimulation of peripheral blood T cells with rβ2GPI, in which the Vβ7 segment with the TGxxN/Q motif or minor variations was oligoclonally expanded after stimulation of peripheral blood T cells with rβ_2GPI in the majority of APS patients. Surprisingly, β_2GPI-reactive T cells in three independent APS patients used an identical Vβ7$^+$ TCRβ chain that was shown to recognize p276–290. In addition, the Vβ8$^+$ TCR with a distinct motif PxAxxD/E, which is presumed to recognize the epitope other than p276–290 of β_2GPI, was detected in some APS patients. Depletion of Vβ7$^+$ or Vβ8$^+$ T cells from the peripheral blood mononuclear cell cultures significantly inhibited in vitro anti-β_2GPI antibody production in response to rβ_2GPI, confirming that T cells carrying these TCR β-chains are the predominant repertoire that induces anti-β_2GPI antibody production.

Effector function of β_2GPI-reactive T cells

CD4$^+$ T cells are functionally classified according to their cytokine expression profiles. In this regard, all 12 β_2GPI-reactive T-cell clones produced IFN-γ and had a Th1- or Th0-like cytokine expression profile [29]. It is now known that the Th1/Th2 antagonism in humans is not as strict as reported in mice and that T-cell function is associated with individual cytokine profiles rather than Th1/Th2 phenotypes. When potential associations between cytokine profiles and the capacity to promote anti-β_2GPI antibody production were examined in individual β_2GPI-reactive T-cell clones, an expression level of IL-6 was positively correlated with the helper activity, but expression of IFN-γ, IL-2, IL-4 or IL-10 was not. The mechanisms for the T and B cell collaboration regulating the production of anti-β_2GPI antibodies were further examined using an in vitro assay system consisting of β_2GPI-reactive CD4$^+$ T-cell clones and autologous peripheral blood B cells [29]. In this assay system, anti-IL-6 monoclonal antibody blocked T cell-dependent anti-β_2GPI antibody production, but anti-IFN-γ monoclonal antibody did not, indicating IL-6 as a major B cell-activating factor produced by β_2GPI-specific T

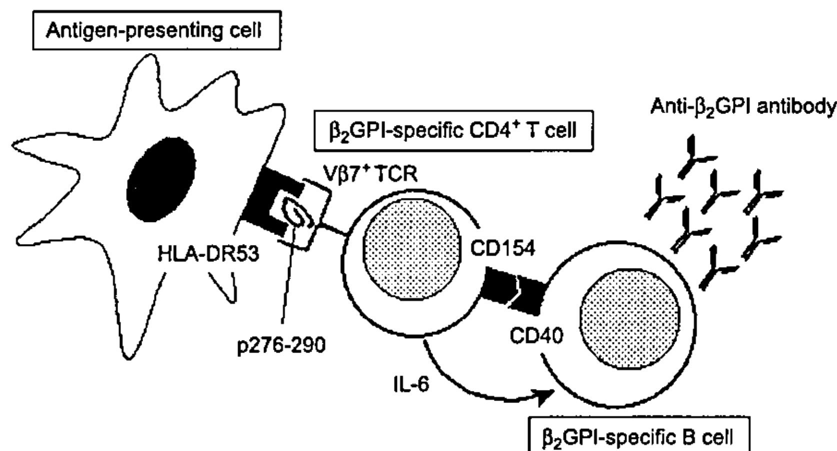

Figure 2 Schematic presentation of the cellular mechanism that induces anti-β_2GPI antibody production in APS patients. β_2GPI-specific CD4$^+$ T cell is activated by the recognition of p276–290 complexed with an HLA-DR53 molecule expressed on APC via Vβ7$^+$ TCR. Activated β_2GPI-specific T cell stimulates specific B cell to produce anti-β_2GPI antibodies through the expression of CD154 and IL-6.

cells. However, the effect of IL-6 required the CD40–CD154 engagement because anti-CD154 monoclonal antibodies almost completely inhibited anti-β_2GPI antibody production induced by β_2GPI-reactive CD4$^+$ T-cell clones. Taken together, CD4$^+$ T cell-dependent B-cell activation depends on two types of stimuli; T cell-derived IL-6 and CD40–CD154 engagement (Fig. 2).

Mechanisms for induction of autoantibody response to β_2GPI

Circulating β_2GPI-reactive T cells are detectable not only in APS patients with serum anti-β_2GPI antibodies but also in some healthy individuals [22,31]. In addition, analysis of TCR β-chains for β_2GPI-reactive T cells indicated that APS patients and healthy responders used similar TCR β-chains [34]. These findings indicate that β_2GPI-reactive T cells are a component of the normal T cell repertoire. To evaluate the activation status of β_2GPI-reactive T cells in APS patients and healthy responders, a precursor frequency of β_2GPI-reactive T cells was determined using limiting-dilution analysis [35]. As shown in Fig. 3, frequency of β_2GPI-reactive T cells in circulating T cells was significantly increased in APS patients compared with healthy individuals, indicating that the production of aPL antibodies in APS patients is a result of the activation of β_2GPI-reactive T cells.

Taken together with the finding that β_2GPI-specific CD4$^+$ T cells responded to reduced β_2GPI and rβ_2GPI but not to native β_2GPI, it is likely that p276–290 and other epitopes recognized by β_2GPI-specific T cells are 'cryptic' determinants. The concept of the 'cryptic' self-determinant was proposed by Lehmann et al. [36] and Sercarz et al. [37] and extended by other investigators [38,39]. As shown in Fig. 4, extracellular antigens are taken up by APCs and degraded into peptide fragments by endosomal proteases. This process is known as antigen processing. Only the peptides with the capacity to bind HLA class II molecules are expressed on the cell surface in the form of the peptide–HLA class II complex and presented to T cells (a process termed antigen presentation). Of numerous peptides predicted by the amino acid sequences of the autoantigen, a small proportion of peptides is presented to T cells as a consequence of antigen processing and antigen presentation. Those peptides are a representative of the autoantigen and termed dominant self-determinants, whereas the remaining peptides, which are not efficiently presented and are normally hidden from the immune system, are termed cryptic self-determinants. There is increasing interest in the possibility that crypticity is an important characteristic of epitopes recognized by the autoreactive T cells and thus is relevant to autoimmune pathogenesis [37–39]. T cells recognizing dominant self-determinants in APCs undergo deletion in the thymus or inactivation in the periphery. On the other hand, T cells specific for cryptic self-determinants are a component of the normal T-cell repertoire but normally do not encounter antigenic peptides in the periphery. These T cells might become activated and autoaggressive if the previously cryptic self-determinants were presented at a higher concentration. This concept represents the major hypothesis for the pathogenesis of autoimmune diseases. Our findings regarding the T-cell response to β_2GPI in APS patients are consistent with this cryptic epitope model because β_2GPI-reactive T cells recognize the epitope peptides that are not generated from native β_2GPI but are generated from structurally modified β_2GPI, exist in the normal T-cell repertoire and are activated in vivo in APS patients but not in healthy individuals.

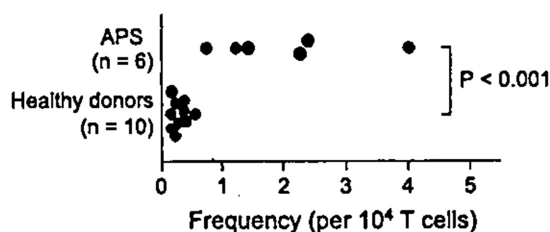

Figure 3 Precursor frequencies of β_2GPI-reactive T cells in peripheral blood from APS patients and healthy donors. The frequency of β_2GPI-reactive T cells was determined by limiting-dilution analysis, and the results are shown as the number of β_2GPI-reactive T cells per 10^4 peripheral blood T cells. All APS patients and healthy donors are responders to rβ_2GPI in bulk peripheral blood T-cell cultures. Comparison was made using Student's *t*-test.

The fundamental question is how normally cryptic p276–290 becomes visible to the immune system and elicits a sustained pathogenic response in APS patients. T cells responsive to cryptic self-determinants would not encounter antigenic peptides in the periphery under normal circumstances; it is likely that a pathogenic autoreactive T-cell response is induced by the de novo presentation of a previously cryptic p276–290 under special conditions (Fig. 5). Potential mechanisms that reveal cryptic self-determinants in APCs include modulation of antigen processing and/or increased antigen delivery to the processing compartment [38]. In this regard, reduction of β_2GPI and expression of recombinant β_2GPI in a bacterial expression system induce expression of cryptic peptides that are able

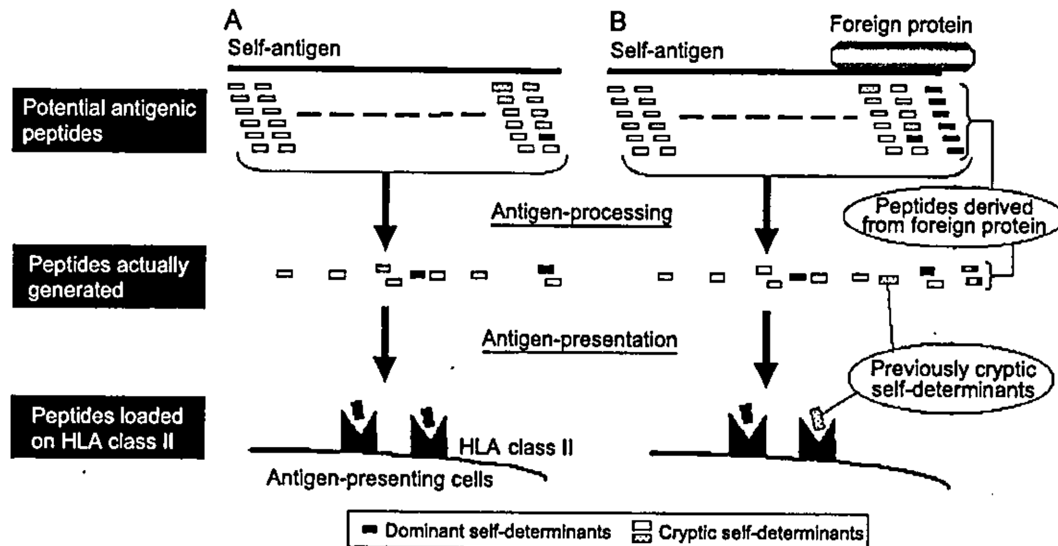

Figure 4 Expression of dominant and cryptic self-determinants as a consequence of the antigen processing and antigen presentation. (A) A self-antigen is taken up by APCs and degraded by endosomal proteases into peptide fragments. Although there are numerous potential antigenic peptides, a small proportion of peptides is actually generated by antigen processing, and only a few peptides with the capacity to bind HLA class II molecules are expressed on the cell surface in the form of the peptide–HLA class II complex and presented to T cells. The peptides expressed on APC membranes are termed dominant self-determinants, whereas the remaining peptides that are normally hidden from the immune system are termed cryptic self-determinants. (B) Modulation of antigen processing by the complex formation of a self-antigen with a foreign protein, one of the potential mechanisms that reveal previously cryptic self-determinants. Antigen processing of a self-antigen complexed with a foreign protein results in the generation of previously cryptic self-determinants as well as peptides derived from the foreign protein. If newly generated cryptic self-peptides were capable of binding HLA class II molecules, those peptides would be expressed on the cell surface in the form of the peptide–HLA class II complex and elicit specific T-cell response.

to stimulate β_2GPI-specific T cells. Although factors that induce the expression of p276–290 as a consequence of antigen processing in APS patients are unknown, several lines of evidence suggest that p276–290 is revealed when β_2GPI is complexed with anionic surfaces. PL-bound β_2GPI, but not PL or β_2GPI alone, induces a high level of anti-β_2GPI antibodies and lupus anticoagulant activity in normal mice without adjuvant [40]. Moreover, immunization of β_2GPI-bound apoptotic cells into normal mice induces the production of anti-β_2GPI antibodies [41]. The major PL-binding site located on the surface of a β_2GPI molecule should be easily accessed by proteases in endocytic compartments during antigen processing, and therefore, the peptides containing the intact major PL-binding site would not be generated from native β_2GPI. In contrast, the binding of β_2GPI to anionic surfaces may protect the major PL-binding site from protease attack by masking the site, resulting in the appearance of the previously cryptic peptide containing the entire major PL-binding site. A similar mechanism is reported by Simitsek et al. [42], who found that antibody-binding to the antigen suppresses the generation of some epitopes and boosts that of others. Remarkably, both suppressed and boosted epitopes were present within a protein domain that was 'fingerprinted' by the antibody, while epitopes that lay outside this domain were not affected. Based on these findings, they speculated that the antibody, by binding and stabilizing a protein domain, might influence the accessibility of

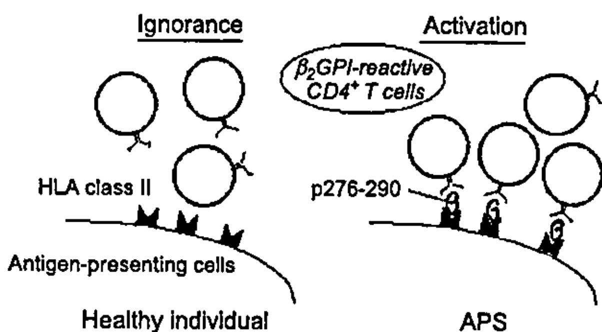

Figure 5 Activation of β_2GPI-reactive CD4$^+$ T cells in the normal T-cell repertoire by the de novo presentation of a previously cryptic p276–290. T cells recognizing cryptic p276–290 would not encounter antigenic peptides in the periphery in healthy individuals (immunologic 'ignorance'), whereas pathogenic β_2GPI-reactive CD4$^+$ T cells are activated upon recognition of a previously cryptic p276–290 expressed on functional APCs in APS patients.

the site to proteases during antigen processing. Taken together, modulation of antigen processing would influence processing of autoantigens that are bound by high-affinity ligands. Because β_2GPI is a plasma protein abundant in the circulation, excessive exposure to anionic surfaces, such as microorganisms and apoptotic cells, may induce the formation of a large quantity of β_2GPIs bound to anionic surfaces in vivo. In this regard, associations between various types of infections and the production of aPL antibodies have been reported [43], and infection is one of the major precipitating factors contributing to the development of catastrophic APS [44]. However, to initiate the pathogenic autoimmune response, additional factors, such as impaired regulatory function and nonspecific inflammation mediated by cytokines and toll-like receptor ligands, are apparently necessary in conjunction with the expression of the previously cryptic epitope.

Another possibility for the presentation of previously cryptic p276–290 is that APS patients might have been exposed to foreign proteins that cross-react with β_2GPI. It has been reported that immunization with foreign β_2GPI induces production of a high titer of anti-β_2GPI antibodies as well as development of clinical features of APS in normal mice [19,20]. In this model, it is presumed that immunization of mice with human β_2GPI first leads to the production of antibody to human (foreign) β_2GPI, followed by the appearance of autoantibodies reactive with murine (self) β_2GPI. The mechanism is thought to be the generation of cross-reactive B cells, which are initially primed by a foreign protein, then bind a self-antigen and process and present self-peptides to T cells [45]. Because B cells that acquire antigen via surface immunoglobulin require approximately 1000- to 10,000-fold less antigen for subsequent T-cell response, B cells may have the ability to efficiently concentrate small quantities of determinants that are previously cryptic [46]. In this regard, Blank et al. [47] reported that bacterial peptides homologous to β_2GPI induced pathogenic anti-β_2GPI antibodies along with the APS manifestation in normal mice.

Summary

Accumulating evidence in APS patients as well as in experimental APS indicates an important role of β_2GPI-specific CD4$^+$ T cells in anti-β_2GPI antibody production and in the pathogenesis of APS. Further studies examining the mechanisms that induce the efficient processing and presentation of cryptic determinants of β_2GPI as a consequence of antigen processing would clarify the etiology of APS. In addition, elimination or inactivation of pathogenic β_2GPI-specific CD4$^+$ T cells could be a therapeutic strategy that inhibits anti-β_2GPI antibody production and prevents thrombosis and fetal loss in patients suffering from APS [48].

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Autoreactive CD8+ Cytotoxic T Lymphocytes to Major Histocompatibility Complex Class I Chain–Related Gene A in Patients With Behçet's Disease

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Objective. **To detect and characterize the autoreactive CD8+ T cells to major histocompatibility complex class I chain–related gene A (MICA), a stress-inducible antigen preferentially expressed on the epithelium and endothelium, in patients with Behçet's disease (BD).**

Methods. **A candidate for the antigenic MICA peptide was selected based on its predicted binding affinity for HLA–B51 and proteasomal cleavage sites. Peripheral blood T cells from 14 patients with BD and 15 healthy controls were repeatedly stimulated with the MICA peptide, and the specific T cell response was measured by peptide-induced interferon-γ. Cytotoxic T lymphocyte activity was examined by chromium-51 release from an HLA–B51–transfected B cell line in the presence of the MICA peptide.**

Results. **A 9-mer peptide AAAAAIFVI (termed MICA transmembrane [MICA-TM]) was selected as a candidate for the antigenic peptide presented by HLA–B51. A specific T cell response to MICA-TM was detected in 4 patients with BD (29%) but in none of the 15 healthy donors. All 4 responders had HLA–B51 and active disease, and the specific T cell response was lost after the BD-related symptoms disappeared. The MICA-induced T cell response was specifically inhibited by anti–HLA class I antibody or by CD8+ cell depletion. MICA-reactive T cells recognized an HLA–B51–transfected B cell line pulsed with MICA-TM or a B cell line transfected with both HLA–B51 and MICA in the absence of exogenous peptides. Finally, MICA-stimulated T cell lines lysed the HLA–B51–expressing B cell line in the presence of MICA-TM.**

Conclusion. **HLA–B51–restricted cytotoxic T lymphocytes autoreactive to MICA may be involved in the pathogenesis of BD.**

Behçet's disease (BD) is an inflammatory disorder characterized by uveitis, oral and genital aphthous ulcers, and skin lesions (1). The etiology of BD remains unclear, but BD has several unique epidemiologic and clinical features (1,2), as follows: the high-prevalence area is from southern Europe to Japan along the Silk Route; susceptibility is strongly associated with HLA–B51; and microbial infection, surgical procedures, and mechanical stimulation often precede the disease flare. These findings indicate that both genetic and environmental factors contribute to the pathogenic process of BD. This is supported by the finding that HLA–B51–transgenic mice show enhanced neutrophil function but fail to express the BD phenotype (3). Because a primary role of HLA class I antigens such as HLA–B51 is to present endogenous peptides to CD8+ T cells, the lack of the disease phenotype in this model can be explained by the absence of an HLA–B51–restricted pathogenic peptide that would activate the disease-relevant CD8+ T cells.

Recent advances in the understanding of HLA class I–binding peptide motifs have enabled us to detect and characterize autoreactive CD8+ cytotoxic T lymphocytes (CTLs) involved in the pathogenesis of autoimmune diseases (4). For example, in patients with

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primary biliary cirrhosis, disease-relevant CTLs to the E2 component of pyruvate dehydrogenase complexes were detected using synthetic peptides selected according to their binding affinity to HLA–A2 (5). Because CD8+ T cell infiltration is often detected in BD lesions (2), we hypothesized that HLA–B51–restricted CTLs play some role in the BD pathogenesis by targeting a self antigen selectively expressed in the affected tissues. We selected major histocompatibility complex class I chain–related gene A (MICA) as a candidate for this autoantigen, because this protein is preferentially expressed on epithelial and endothelial cells in a stress-dependent manner (6,7). To test our hypothesis, we examined the HLA–B51–restricted CTL response to MICA in patients with BD.

PATIENTS AND METHODS

Patients and controls. We studied 14 patients with BD (5 men and 9 women) who fulfilled the diagnostic criteria defined by the International Study Group (8). Fifteen healthy individuals (9 men and 6 women) served as controls. The patients with BD were grouped as having active disease (6 patients) or inactive disease (8 patients) at the time of blood examination. Active disease was defined as the presence of characteristic BD symptoms, including severe oral/genital ulcers and ocular involvement, that required introduction of treatment with corticosteroids and/or cyclosporine. All samples were obtained after the patients gave their written informed consent, as approved by the institutional review board.

HLA–B51 genotyping. HLA–B51 was detected by polymerase chain reaction using sequence-specific primers and sequence-based typing (9).

Selection and synthesis of MICA peptides. MICA is a polymorphic antigen located near the HLA–B gene on chromosome 6 (10). A potential antigenic MICA peptide derived from the amino acid sequence of MICA*009, which is in strong linkage disequilibrium with HLA–B51 (10), was selected based on the following online resources: a program that ranks peptides based on their predicted half-time dissociation coefficient for HLA class I molecules (http://www.bimas.dcrt.nih.gov/molbio/hla_bind/index.html), and the PAProC program, which predicts proteasomal cleavage sites during antigen processing (http://www.uni-tuebingen.de/uni/kxi/). The selected peptides were synthesized using a solid-phase method based on fluorenylmethoxycarbonyl chemistry and purified using reverse-phase high-performance liquid chromatography. The purity of all peptides was >90%.

B cell lines expressing MICA and HLA–B51. Complementary DNAs encoding HLA–B51 (B*5101) and MICA (MICA*009) were introduced into C1R, a human B cell line deficient in HLA–A and HLA–B, by gene transfer using GenePulser (BioRad, Hercules, CA). The B*5101 and MICA*009 alleles were chosen because this combination is the most common in Japanese patients with BD (10,11). C1R transfectants stably expressing HLA–B51 (C1R/B51) and MICA (C1R/MICA) were generated by limiting dilution. A C1R cell line expressing both MICA and HLA–B51 (C1R/MICA/B51) was generated by the transient expression of HLA–B51 in C1R/MICA by gene transfer. Expression of HLA–B51 and MICA was evaluated by flow cytometry using a monoclonal antibody (mAb) to HLA class I (Sigma, St. Louis, MO) and a polyclonal antibody to MICA (kindly provided by Dr. Minoru Kimura, Tokai University) (12).

Generation of a MICA-stimulated T cell line. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood using Lymphoprep (Nycomed Pharma, Oslo, Norway) density-gradient centrifugation, and resuspended in Iscove's modified Dulbecco's medium containing 10% autologous platelet-poor plasma, 2 m*M* L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. A T cell line was established by the repetitive stimulation of peripheral blood T cells with a potential antigenic MICA peptide or a control peptide as previously described (13), with some modifications. Briefly, PBMCs (3×10^6) were cultured with the peptide (2 μM) in 24-well plates in a humidified atmosphere of 5% CO_2 at 37°C. Interleukin-2 (IL-2) (50 units/ml; Biogen, Cambridge, MA) was added to the culture on day 3. On days 7 and 14, viable cells (3×10^5) were restimulated with 3×10^6 irradiated (60 Gy) autologous PBMCs preincubated with the peptide (2 μM) and IL-2. On day 21, the cells were harvested and used as a MICA-stimulated T cell line.

Interferon-γ (IFNγ) release assay. The antigenic specificity of individual MICA-stimulated T cell lines was examined as previously described (13). Briefly, a MICA-stimulated T cell line (5×10^4) was cultured in duplicate with stimulators (10^5) pulsed with the MICA peptide (2 μM, unless otherwise indicated) and IL-2 (30 units/ml) in 96-well U-bottomed plates. Autologous Epstein-Barr virus–transformed lymphoblastoid B cell line cells (LBLs) or C1R transfectants were used as stimulators. After incubation for 18 hours, the culture supernatants were collected and applied to an enzyme-linked immunosorbent assay to measure the IFNγ concentration. In some experiments, anti–HLA class I, anti–HLA–DR, anti–HLA–DQ, anti–HLA–DP, or an isotype-matched control mAb was added at the initiation of the culture. MICA-stimulated T cell lines depleted of CD4+ or CD8+ cells using a magnetic bead–conjugated anti-CD4 or anti-CD8 mAb (Dynal, Oslo, Norway), respectively, were also used as responders.

Cytotoxicity assay. Cytotoxic activity of the MICA-stimulated T cell line was examined by chromium-51 (^{51}Cr) release assay (13). Briefly, a ^{51}Cr-labeled C1R/B51 cell line was incubated with or without a potential antigenic MICA peptide (2 μM) and cocultured with a MICA-stimulated T cell line at an effector-to-target ratio of 10:1 or 1:1 for 24 hours. Radioactivity in the culture supernatants was counted using a γ counter. All cultures were examined in triplicate, and the percent of specific lysis was calculated as the ^{51}Cr released in association with the peptide stimulation divided by the maximal ^{51}Cr release obtained by lysing cells with 0.5% Triton X.

Statistical analysis. Comparisons between 2 groups were performed using the chi-square test or the Mann-Whitney U test as appropriate.

RESULTS

Selection of MICA peptides. A potential HLA–B51–restricted antigenic MICA peptide was determined

by scanning the amino acid sequence of the MICA*009 protein for a 9-mer peptide containing the HLA–B51–binding motif that would be generated as a consequence of proteasomal cleavage during antigen processing. This analysis identified a peptide encoding a transmembrane region encompassing amino acids 294–302 (AAAAAIFVI; MICA-TM). Notably, MICA-TM includes part of a triplet-repeat microsatellite polymorphism consisting of 6 alanine residues, which is reported to be associated with BD (10). Another peptide encompassing amino acids 70–78 (GKDLRMTLA; MICA70) was selected as a control peptide based on its having a low binding affinity to HLA–B51 and being generated after processing.

Detection of MICA-reactive T cells. Because a MICA-TM–specific T cell response was not detected directly from the peripheral blood of patients with BD, we repeatedly stimulated peripheral blood T cells with MICA-TM before they were subjected to the assay. From a representative BD patient, a T cell line generated by repetitive stimulation with MICA-TM showed a profound response to MICA-TM but not to MICA70 (Figure 1A). This response was specifically blocked by an anti–HLA class I mAb (Figure 1B). When serial concentrations of MICA-TM were used, a significant response was observed at a peptide concentration of 10^{-2} μM or higher (Figure 1C). In contrast, T cell lines repeatedly stimulated with MICA70 failed to respond to MICA70 or MICA-TM. These findings indicate the presence of HLA class I–restricted T cells responsive to MICA-TM in this patient.

The same method was used to screen 14 patients with BD and 15 healthy controls for the MICA-TM–specific T cell response. Figure 2 shows MICA-TM–specific IFNγ production, which was calculated by subtracting the IFNγ concentration obtained for cultures with MICA70 from that obtained for cultures with MICA-TM, in individual subjects. A MICA-TM–specific T cell response was detected in 4 patients with BD (29%) but in no healthy controls ($P = 0.03$). Notably, all the responders were HLA–B51-positive and had active disease. All 6 patients with active disease were not receiving corticosteroids or immunosuppressants at the time of the blood examination, indicating that a difference in the medical therapy did not influence the results. We repeated the assay in 11 patients with BD, including 3 with active disease, and consistent results were obtained regarding the presence or absence of the specific response. In all 4 responders, the MICA-TM–specific T cell response was not detectable at followup, when all of the BD-related symptoms were gone (>6 months after

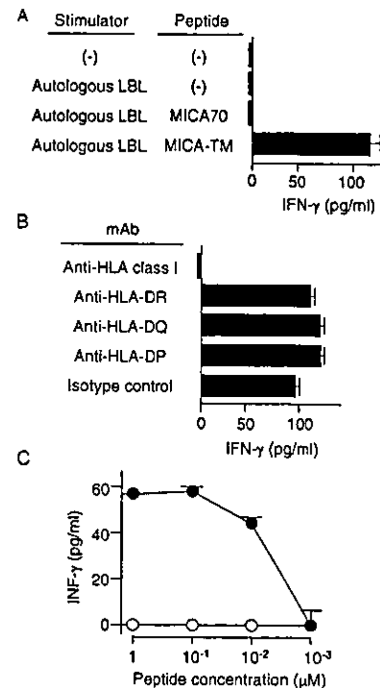

Figure 1. T cell response to major histocompatibility complex class I chain–related gene A (MICA) in a representative patient with active Behçet's disease (BD). **A,** The peptide-specific response of a MICA-stimulated T cell line was assessed by interferon-γ (IFNγ) release in response to autologous lymphoblastoid B cell line cells (LBLs) pulsed with MICA transmembrane (MICA-TM) or MICA70. **B,** A MICA-stimulated T cell line was cultured with MICA-TM in the presence of anti–HLA class I, anti–HLA-DR, anti–HLA-DQ, anti–HLA-DP, or an isotype-matched control monoclonal antibody (mAb). **C,** A MICA-stimulated T cell line was cultured with serial concentrations of MICA-TM (solid circles) or MICA70 (open circles). Results shown are the mean and SD. Analogous findings were obtained from 5 independent experiments using peripheral blood samples from 4 BD responders.

the first evaluation). Thus, a T cell response to MICA-TM was specifically detected in BD patients in association with HLA–B51 and active disease status.

Characterization of MICA-reactive T cells. To examine whether the MICA-TM–induced T cell response was HLA–B51–restricted, a series of C1R transfectants expressing HLA–B51, MICA, or both were used as stimulators in the IFNγ release assay. Wild-type C1R expressed low levels of HLA class I (presumably HLA–C) and MICA, but an up-regulated expression of HLA class I and MICA was detected in C1R/B51 and C1R/MICA, respectively (Figure 3A). At least 10% of the C1R/MICA/B51 cells consistently expressed both MICA and HLA–B51. When MICA-stimulated T cell lines generated from 3 patients with BD were cultured with C1R or C1R/B51 in the presence or absence of

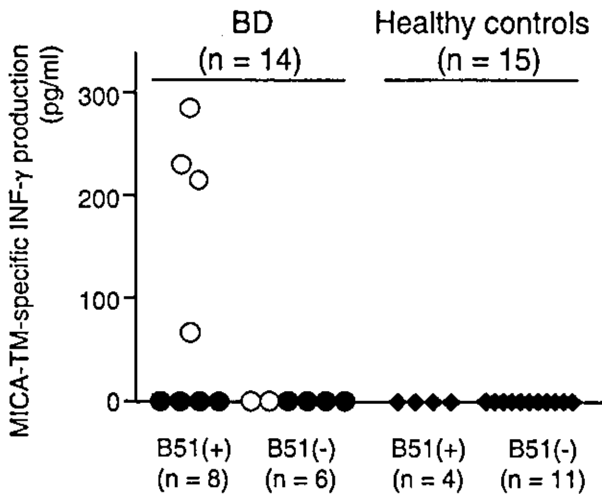

Figure 2. T cell response specific to MICA in 14 patients with BD and 15 healthy controls in the presence or absence of HLA-B51. The MICA-TM-specific IFNγ production of individual MICA-stimulated T cell lines was calculated by subtracting the IFNγ concentration obtained in cultures with MICA70 from that obtained in cultures with MICA-TM. Open circles denote BD patients with active disease; solid circles denote BD patients with inactive disease. See Figure 1 for definitions.

MICA-TM or MICA70, a T cell response was detected solely in the culture with C1R/B51 pulsed with MICA-TM (Figure 3B). C1R/MICA lacking HLA-B51 expression failed to induce the T cell response. The response induced by MICA-TM was abolished by anti-HLA class I mAb. In addition, the depletion of CD8+ T cells, but not CD4+ T cells, from MICA-stimulated T cell lines cancelled the specific T cell response (data not shown). Finally, MICA-stimulated T cell lines responded to C1R/MICA/B51 in the absence of the exogenous peptide, and this response was blocked by anti-HLA class I mAb. These findings together indicate that MICA-reactive CD8+ T cells recognize MICA-TM, which is endogenously generated as a consequence of processing, in the context of HLA-B51.

To evaluate whether MICA-reactive CD8+ T cells have cytotoxic activity, MICA-stimulated T cell lines were cultured with ^{51}Cr-labeled C1R/B51 pulsed with or without MICA-TM (Figure 3C). The target cell lysis was observed specifically in the presence of MICA-TM, indicating that MICA-reactive T cells exhibited CTL activity.

DISCUSSION

Previous studies examining the role of autoimmunity in BD pathogenesis focused on CD4+ T cells and found T cells that were autoreactive to heat shock protein 60 (Hsp60) and retinal S antigen in BD patients, especially those with ocular involvement (2). Interestingly, Hsp60 is a stress-inducible antigen similar to MICA and is overexpressed in the oral mucosa and skin lesions of patients with BD (14). Here, we demonstrate the presence of HLA-B51-restricted CTLs autoreactive to MICA in patients with BD. The relevance of the MICA-reactive CTLs to the pathogenesis is supported

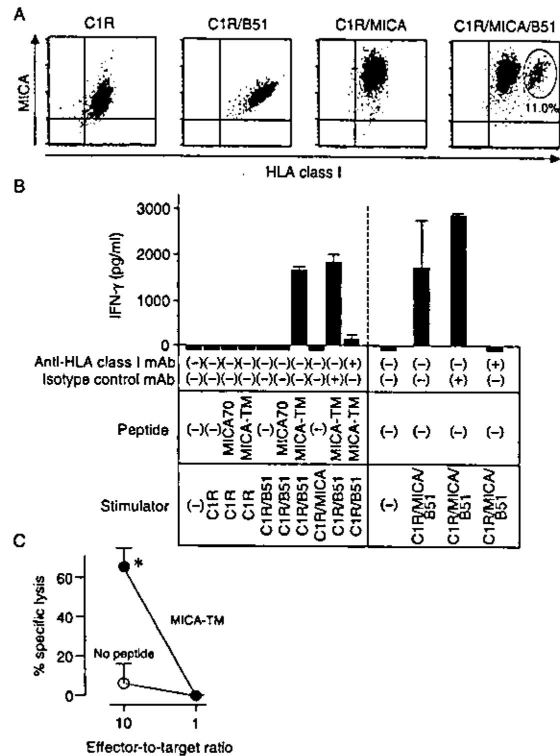

Figure 3. The HLA-B51-restricted antigen recognition profile and cytotoxic activity of MICA-stimulated T cell lines. **A,** Flow cytometric analysis for the expression of HLA class I and MICA in C1R, a C1R transfectant expressing HLA-B51 (C1R/B51), a C1R transfectant expressing MICA (C1R/MICA), and a C1R transfectant expressing both molecules (C1R/MICA/B51). C1R/MICA and C1R/B51 were clones generated by limiting dilution, and C1R/MICA/B51 was a C1R/MICA cell line transiently transfected with HLA-B51. **B,** Responses of a MICA-stimulated T cell line to a series of C1R transfectants in the presence or absence of MICA-TM or MICA70. The T cell response was measured by IFNγ production. Anti-HLA class I monoclonal antibody (mAb) or an isotype-matched control mAb was added at the initiation of some cultures. Results shown are the mean and SD. **C,** Cytotoxic activity of a MICA-stimulated T cell line evaluated by chromium-51 release assay. C1R/B51 preincubated with or without MICA-TM was used as a target. Results shown are the mean and SD. * = $P < 0.05$ by Mann-Whitney U test, versus no peptide at an effector-to-target ratio of 10:1. A representative result of 3 independent experiments is shown. See Figure 1 for definitions.