events result in low protein levels of mannan-binding lectin in populations from southeast Africa and South America. J Immunol 1998;161:3169-175.

[10] Madsen HO, Garred P, Thiel S, Kurtzhals JA, Lamm LU, Ryder LP, Svejgaard A. Interplay between promoter and structural gene variants control basal serum level of mannan-binding protein. J Immunol. 1995;155:3013-3020.

[11] Garred P, Voss A, Madsen HO, Junker P. Association of mannose-binding lectin gene variation with disease severity and infections in a population-based cohort of systemic lupus erythematosus patients. Genes Immun 2001;2:442-450.

[12] Ogden CA, deCathelineau A, Hoffmann PR, Bratton D, Ghebrehiwet B, Fadok VA, Henson PM. C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. J Exp Med. 2001;194:781-795.

[13] Seelen MA, van der Bijl EA, Trouw LA, Zuiverloon TC, Munoz JR, Fallaux-van den Houten FC, Schlagwein N, Daha MR, Huizinga TW, Roos A. A role for mannose-binding lectin dysfunction in generation of autoantibodies in systemic lupus erythematosus. Rheumatology (Oxford) 2005;44:111-119.

[14] Palaniyar N, Nadesalingam J, Clark H, Shih MJ, Dodds AW, Reid KB.Nucleic acid is a novel ligand for innate, immune pattern recognition collectins surfactant proteins A and D and mannose-binding lectin.
J Biol Chem 2004;279:32728-32736.

[15] Shi L, Takahashi K, Dundee J, Shahroor-Karni S, Thiel S, Jensenius JC, Gad F, Hamblin MR, Sastry KN, Ezekowitz RA. Mannose-binding lectin-deficient mice are susceptible to infection with Staphylococcus aureus. J Exp Med. 2004;199:1379-1390.

[16] Nishino H, Shibuya K, Nishida Y, Mushimoto M. Lupus erythematosus-like syndrome with selective complete deficiency of C1q. Ann Intern Med 1981;95:322-324.

[17] Kirschfink M, Petry F, Khirwadkar K, Wigand R, Kaltwasser JP, Loos M. Complete functional C1q deficiency associated with systemic lupus erythematosus (SLE). Clin Exp Immunol 1993;94:267-272.

[18] Bowness P, Davies KA, Norsworthy PJ et al. Hereditary C1q deficiency

and systemic lupus erythematosus. QJM 1994;87:455-464.
[19] Walport MJ, Davies KA, Botto M. C1q and systemic lupus erythematosus. Immunobiology 1998;199:265-285.
[20] Berkel AI, Petry F, Sanal O et al. Development of systemic lupus erythematosus in a patient with selective complete C1q deficiency. Eur J Pediatr 1997;156:113-115.
[21] Slingsby JH, Norsworthy P, Pearce G et al. Homozygous hereditary C1q deficiency and systemic lupus erythematosus. A new family and the molecular basis of C1q deficiency in three families. Arthritis Rheum 1996;39:663-670.
[22] Hisano S, Matsushita M, Fujita T, Endo Y, Takebayashi S. Mesangial IgA2 deposits and lectin pathway-mediated complement activation in IgA glomerulonephritis. Am J Kidney Dis 2001;38:1082-1088.
[23] Steinfeld S, Penaloza A, Ribai P, Decaestecker C, Danguy A, Gabius HJ, Salmon I, Appelboom T, Kiss R.D-mannose and N-acetylglucosamine moieties and their respective binding sites in salivary glands of Sjogren's syndrome.
J Rheumatol. 1999;26:833-841.
[24] Santos I K, Costa C H, Krieger H, Feitosa M F, Zurakowski D, Fardin B, Gomes R B, Weiner D L, Harn D A, Ezekowitz R A, Epstein J E. Mannan-binding lectin enhances susceptibility to visceral leishmaniasis. Infect Immunol 2001;69:5212-5215.
[25] Takahashi R, Tsutsumi A, Ohtani K, Muraki Y, Goto D, Matsumoto I, Wakamiya N, Sumida T. Association of mannose binding lectin (MBL) gene polymorphism and serum MBL concentration with characteristics and progression of systemic lupus erythematosus. Ann Rheum Dis 2005;64:311-314.
[26] Malhotra R, Wormald MR, Rudd PM, Fischer PB, Dwek RA, Sim RB. Glycosylation changes of Ig associated with rheumatoid arthritis can activate complement via the mannose-binding protein. Nature Med 1995;1:237-243.
[27] Graudal NA, Madsen HO, Tarp U, Svejgaard A, Jurik G, Graudal HK. The association of variant mannose-binding lectin genotypes with radiographic outcome in rheumatoid arthritis. Arthritis Rheum 2000;43:515-521.
[28] Mullighan CG, Heatley S, Bardy PG et al. Lack of association between mannose-binding lectin gene polymorphisms and primary Sjogren's syndrome. Arthritis Rheum. 2000;43:2851-2852.

[29] Tsutsumi A, Ikegami H, Takahashi R, Murata H, Goto D, Matsumoto I, Fujisawa T, Sumida T. Mannose binding lectin gene polymorphism in patients with type I diabetes. Hum Immunol 2003;64:621-624.

[30] Jordan JE, Montalto MC, Stahl GL. Inhibition of mannose-binding lectin reduces postischemic myocardial reperfusion injury. Circulation. 2001 Sep 18;104:1413-1418.

[31] de Vries B, Walter SJ, Peutz-Kootstra CJ, Wolfs TG, van Heurn LW, Buurman WA. The mannose-binding lectin-pathway is involved in complement activation in the course of renal ischemia-reperfusion injury. Am J Pathol 2004;165:1677-1688.

[32] Madsen HO, Videm V, Svejgaard A, Svennevig JL, Garred P. Association of mannose-binding-lectin deficiency with severe atherosclerosis.
Lancet. 1998;352:959-960.

[33] Best LG, Davidson M, North KE, MacCluer JW, Zhang Y, Lee ET, Howard BV, DeCroo S, Ferrell RE.Prospective analysis of mannose-binding lectin genotypes and coronary artery disease in American Indians: the Strong Heart Study. Circulation. 2004;109:471-475.

[34] Ohlenschlaeger T, Garred P, Madsen HO, Jacobsen S. Mannose-binding lectin variant alleles and the risk of arterial thrombosis in systemic lupus erythematosus. N Engl J Med 2004;351:260-267.

[35] Rugonfalvi-Kiss S, Endresz V, Madsen HO, Burian K, Duba J, Prohaszka Z, Karadi I, Romics L, Gonczol E, Fust G, Garred P. Association of Chlamydia pneumoniae with coronary artery disease and its progression is dependent on the modifying effect of mannose-binding lectin. Circulation 2002;106:1071-1076.

[36] Garred P, Madsen HO, Halberg P et al. Mannose-binding lectin polymorphisms and susceptibility to infection in systemic lupus erythematosus. Arthritis Rheum. 1999;42:2145-2152.

[37] Seelen MA, Trouw LA, van der Hoorn JW, Fallaux-van den Houten FC, Huizinga TW, Daha MR, Roos A. Autoantibodies against mannose-binding lectin in systemic lupus erythematosus. Clin Exp Immunol 2003;134:335-43.

[38] Takahashi R, Tsutsumi A, Ohtani K, Goto D, Matsumoto I, Ito S, Wakamiya N, Sumida T. Anti-mannose binding lectin antibodies in sera of Japanese

patients with systemic lupus erythematosus. Clin Exp Immunol 2004;136:585-590.
[39] Mok MY, Jack DL, Lau CS, Fong DY, Turner MW, Isenberg DA, Lydyard PM Antibodies to mannose binding lectin in patients with systemic lupus erythematosus. Lupus 2004;13:522-528.
[40] Tsutsumi A, Sasaki K, Wakamiya N, Ichikawa K, Atsumi T, Ohtani K, Suzuki Y, Koike T, Sumida T. Mannose binding lectin gene: polymorphisms in Japanese patients with systemic lupus erythematosus, rheumatoid arthritis and Sjogren's syndrome. Genes Immun 2001;2:99-104.

FIGURE LEGENDS

Figure 1. Mannose binding lectin protein (MBL) and the MBL gene.

1-A: Four regions comprise the mannose binding lectin (MBL) peptide. The cysteine rich region contains cysteine residues that enable the peptides to form S-S bonds between peptides, and between triple helix components. The collagen domain (tandem repeat of Gly-X-Y sequences) of 3 peptides form a triple helix structure. Carbohydrate recognition domain binds to carbohydrates including mannose and N-acetylglucosamine.

The large red arrow indicate the position of the 3 commonly observed amino acid changes caused by single nucleotide polymorphisms of the MBL gene.

1-B: The mannose binding lectin gene is composed of 4 exons and 3 introns. There are five common single nucleotide polymorphisms (SNP). Each of these SNP has a large effect on the serum concentration of MBL. H/L, X/Y, A/B, A/C, A/D are the commonly used nomenclatures for the alleles of these SNPs. These SNPs combine to make 6 common haplotypes, *HYA*,*LYA*, *LXA*, *HYD*, *LYB* and *LYC*, and strongly influence serum MBL concentration.

1-C: Possible relationship between mannose binding lectin and autoimmunity. The structure of mannose binding lectin (MBL) enables it to discriminate between self and invading microorganisms. MBL would not bind to normal viable cells. However, cells undergoing apoptosis will be recognized by MBL. MBL bound apoptotic cells are engulfed by phagocytes. MBL also binds to DNA. Therefore, MBL has a role in the clearance of potential autoantigens. In addition, MBL binds to various bacteria and viruses, and will aid in host defense through

opsonization or activation of the lectin pathway of the complement cascade. Thus, MBL may aid in protection against some unknown microorganism related to autoimmunity, and is also important when an individual is under immunosupression. It should be noted, that while MBL may be protective against occurrence of autoimmune diseases, it has a role in various tissue injuries, and may pathogenic in certain autoimmune conditions.

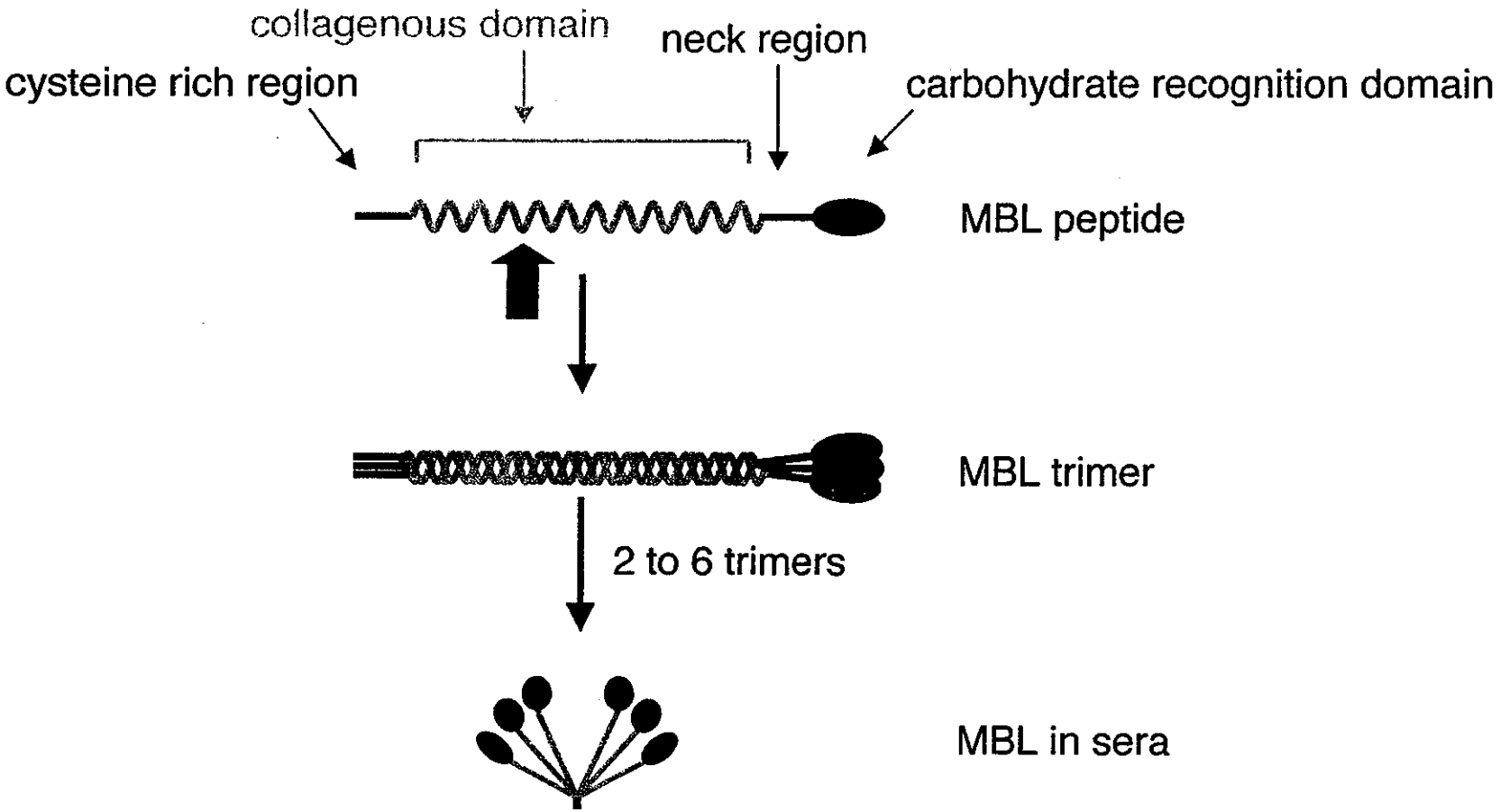
Table. Concentration of mannose binding lectin (MBL) in sera of Japanese individuals with different MBL haplotypes.

Haplotypes	n	MBL concentration (mg/l)	Standard deviation
HYA/LYA	15	1.411	0.548
HYA/HYA	18	1.177	0.481
LYA/LYA	1	1.210	-
HYA/LXA	5	0.830	0.321
LYA/LXA	5	0.584	0.338
HYA/LYB	24	0.333	0.310
LYA/LYB	9	0.119	0.056
LXA/LYB	3	0.013	0.023
LYB/LYB	16	0.002	0.008
total	96	0.622	0.634

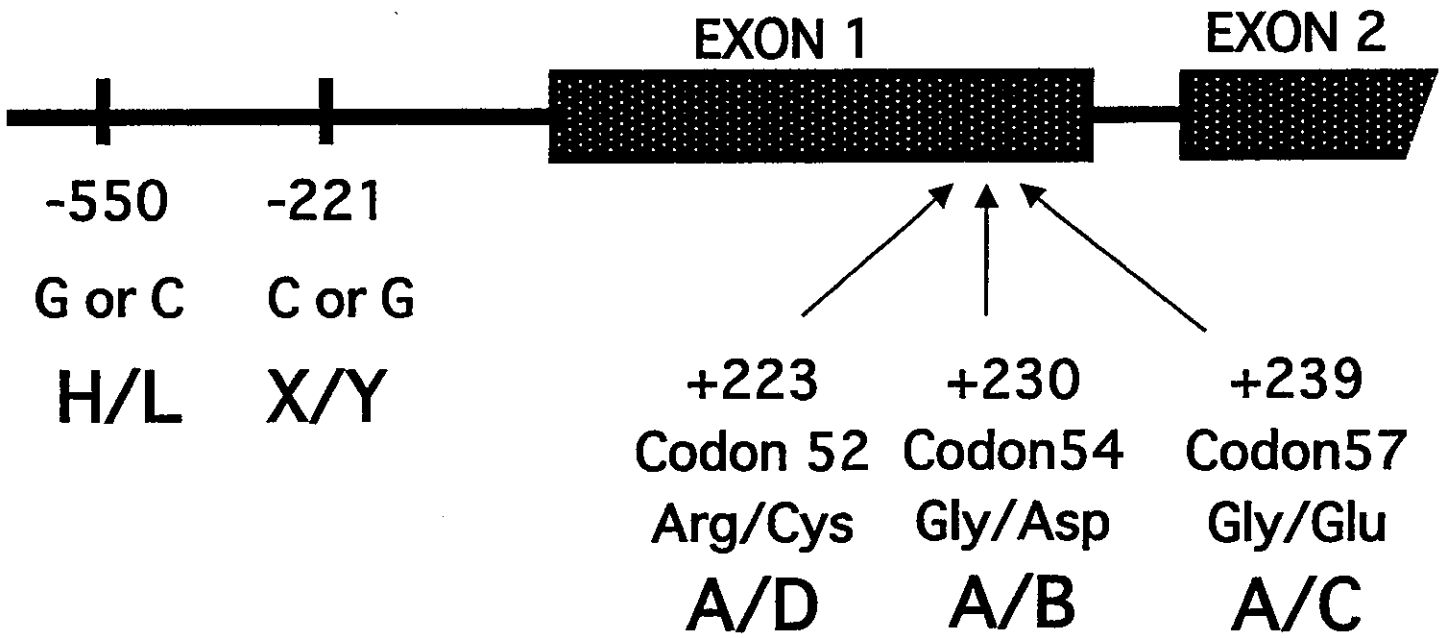
See text and figure for definition of haplotypes.

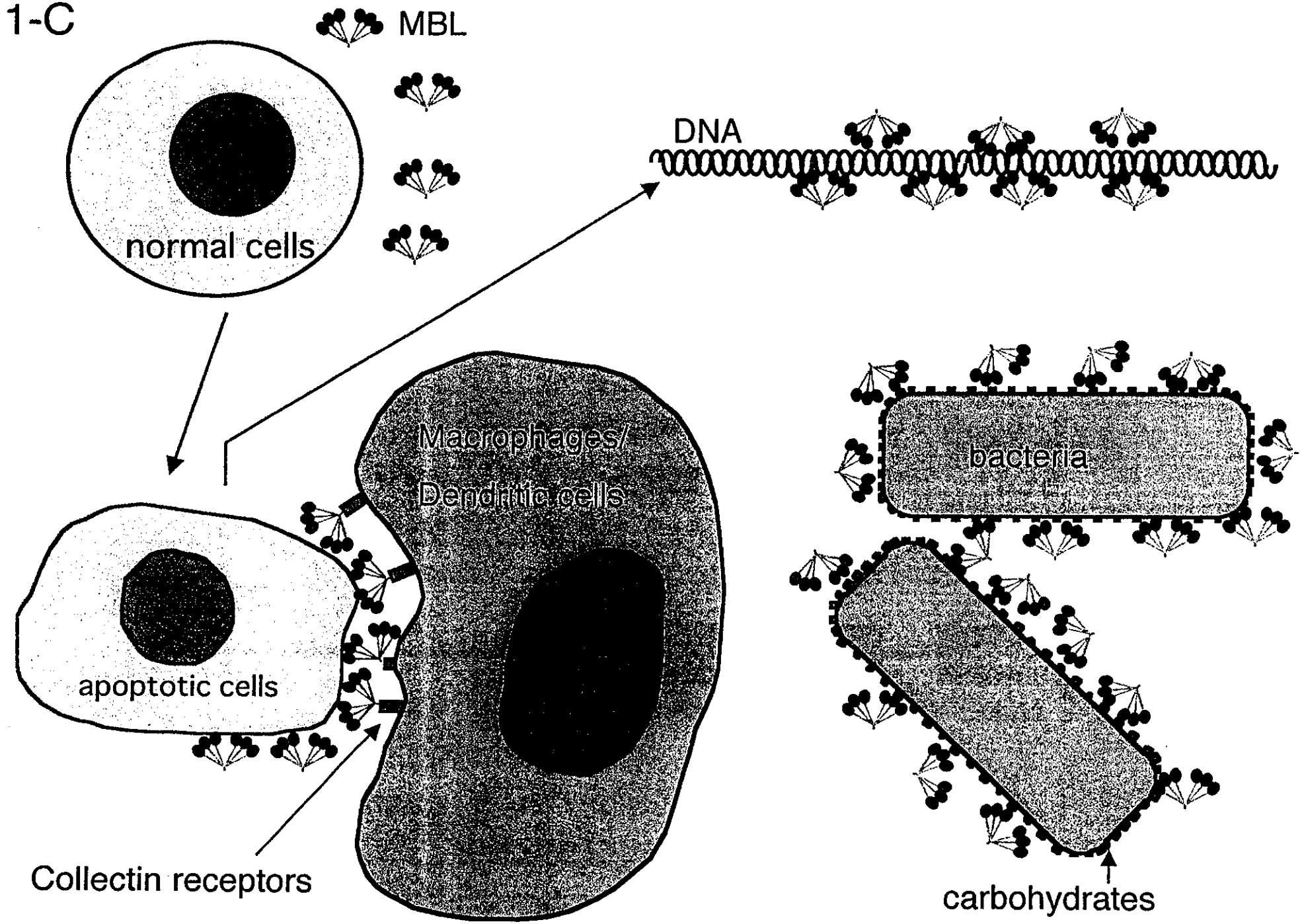
From Tsutsumi et al. [40]

1-A


collagenous domain
neck region
cysteine rich region
carbohydrate recognition domain
MBL peptide
MBL trimer
2 to 6 trimers
MBL in sera

1-B


EXON 1
EXON 2
-550
-221
G or C
C or G
H/L
X/Y
+223
+230
+239
Codon 52
Codon54
Codon57
Arg/Cys
Gly/Asp
Gly/Glu
A/D
A/B
A/C


1-C
MBL
normal cells
DNA
Macrophages/
Dendritic cells
bacteria
apoptotic cells
Collectin receptors
carbohydrates

Clinical and Experimental Immunology (in press)

TCRVα14^{+} NKT cells function as effector T cells in collagen-induced arthritis mice

Yasuyuki Ohnishi, PhD *,†, Akito Tsutsumi, MD, PhD *, Daisuke Goto, MD, PhD *, Satoshi Itoh, MD, PhD *, Isao Matsumoto, MD, PhD *, Masaru Taniguchi, MD, PhD ††, and Takayuki Sumida, MD, PhD *

*: Department of Internal Medicine, Institute of Clinical Medicine, University of Tsukuba, Ibaraki, Japan

†: Mitsubishi Chemical Safety Institute Ltd., Ibaraki, Japan

††: RIKEN Research Center for Allergy and Immunology, Yokohama, Japan

Keywords: NKT cells, collagen-induced arthritis, effective, cytokine

Corresponding author: Dr. Takayuki Sumida, Department of Internal Medicine, Institute of Clinical Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba City, Ibaraki 305-8575, Japan.

FAX No. 81-29-853-3222 TEL No. 81-29-853-3221

e-mail: tsumida@md.tsukuba.ac.jp

Summary

Natural killer (NK) T cells are a unique, recently identified cell population and are suggested to act as regulatory cells in autoimmune disorders. In the present study, designed to investigate the role of NKT cells in arthritis development, we attempted to induce arthritis by immunization of type II collagen (CIA) in Jα281 knock out (NKT-KO) and CD1d knock out (CD1d-KO) mice, which are depleted of NKT cells. From the results, the incidence of arthritis (40%) and the arthritis score (1.5 ± 2.2 and 2.0±2.7) were reduced in NKT-KO and CD1d-KO mice compared to those in respective wild type mice (90%, 5.4±3.2 and 2.0±2.7, $P<0.01$). Anti-CII antibody levels in the sera of NKT-KO and CD1d-KO mice were significantly decreased compared to the controls (OD values; 0.32±0.16 and 0.29±0.06 vs. 0.58±0.08 and 0.38±0.08, $P<0.01$). These results suggest that NKT cells play a role as effector T cells in CIA. Although the cell proliferative response and cytokine production in NKT-KO mice after the primary immunization were comparable to those in wild type mice, the ratio of activated T or B cells were lower in NKT-KO mice than wild type mice after secondary immunization (T cells: 9.9±1.8% vs

16.0±3.4%, P<0.01, B cells: 4.1±0.5% vs 5.1±0.7%, P<0.05), suggesting that inv-NKT cells contribute to the pathogenicity in the development phase of arthritis. In addition, IL-4 and IL-1_ mRNA expression levels in the spleen during the arthritis development phase were lower in NKT-KO mice, while the IFN-_ mRNA expression level was temporarily higher. These results suggest that inv-NKT cells influence cytokine production in arthritis development.

In conclusion, inv-NKT cells may promote the generation of arthritis, especially during the development rather than initiation phase.

Introduction

Natural killer T cells represent a novel lymphoid lineage distinct from conventional T cells, B cells, and NK cells. As invariant NKT cells (inv-NKT), whose TCR is a single invariant Vα14Jα281 chain, can secrete both Th1 and Th2 cytokines, it was suggested that inv-NKT cells play a role in immunomodulative function. Deficient or defective NKT cells are associated with certain autoimmune diseases [1-3], as well as with several animal models of autoimmune disease [4, 5]. Recent studies showed that inv-NKT cell activation protected against type 1 diabetes in NOD mice [6], EAE [7], and MRL lpr/lpr mice [8]. Chiba *et al.* reported that collagen-induced arthritis (CIA) was suppressed by NKT cell activation with OCH, an analog of _-GalCer [9]. However, the natural function of inv-NKT cells on the development of arthritis remains unclear.

To investigate the role of inv-NKT cells on arthritis in the CIA mouse model, NKT-cell-deficient mice were analyzed. Arthritis was suppressed and anti-CII antibody levels were reduced in these mice. In addition, the ratios of activated T and B cells were lower, and IL-4 and IL-1_ mRNA expression was lower, in the deficient mice during the development phase

rather than induction phase of arthritis, while IFN-_ mRNA expression was temporarily higher. Therefore, we concluded that inv-NKT cells could promote the generation of arthritis and that they affected arthritis development rather than immunological initiation.

Materials and Methods

Mice. Male C57BL/6 mice aged 10-12 weeks old were purchased from Charles River Japan Inc. (Yokohama, Japan). Male NKT cell (TCR Jα281) knock out mice (NKT-KO) [10] and male CD1d knock out mice (CD1d-KO) [11], in which the genetic background was C57BL/6, were used in this study. NKT-KO and CD1d KO mice were established after backcrossing 10 generations or more to B6 mice, respectively, and were kindly provided by Prof. M.Taniguchi (RIKEN Research Center for allergy and Immunology, Yokohama, Japan). The study design was approved by the Ethical Committee of the University of Tsukuba.

Reagents. Chicken type II collagen (CII) was purchased from Sigma-Aldrich Corp. (ST. Louis, USA). CII was dissolved in 0.1M acetic acid

and diluted with 0.01M PBS (pH 7.4). Incomplete Freund's Adjuvant (IFA) and heat-inactivated *M. tuberculosis* (H37Ra) were purchased from Difco Laboratories (Detroit, USA).

Induction of collagen-induced arthritis (CIA) and evaluation of clinical severity. C57BL/6, NKT-KO and CD1d-KO mice were immunized intradermally at the base of the tail with 100 μg chicken CII emulsified in IFA containing 250 μg of inactivated *M. Tuberclosis* (H37Ra). On Day 21 the animals were boosted with an intradermal injection [12]. The animals were observed at 2- or 4-day intervals and evaluated for the severity of arthritis by scoring each paw. The observation period was set for 28 days after the booster immunization, because the clinical scores did not worsen after that in our preliminary experiment using a small number of animals. The scores ranged from 0 to 3 (0, no swelling or redness; 1, swelling or redness in one joint; 2, two joints or more involved; 3, severe arthritis of the entire paw and joints). The score for each animal was the sum of the score for all four paws.

Measurement of anti-CII antibody. C57BL/6, NKT-KO and CD1d-KO mice were sacrificed 28 days after the booster injection and sera were collected. Anti-CII IgG antibodies (anti-CII IgG Abs) were measured by ELISA [13].

Briefly, each mouse serum was diluted 100,000 times with blocking buffer (Block Ace, Dainippon Pharmaceuticals Co., Osaka, Japan) and was incubated in a type II collagen-coated well for 1hr at 37°C. The wells were washed 3 times with washing buffer (0.01M Tris-HCl containing 0.05% Tween 20) and treated with biotinylated goat anti-mouse IgG antibody (Zymed Laboratories, Inc., South San Francisco, USA, diluted 4000 times with the blocking buffer) for 2hr at room temperature. After washing, avidin-alkaline phosphatase (EY laboratories, Inc., San Mateo, USA) diluted 4000 times with the blocking buffer was added to each well and incubated for 1 hr at room temperature. After washing, color development was carried out by an ELISA amplification system (Invitrogen Co., Carlsbad, USA) and the optical density was determined at 490 nm.

Measurement of the primary CII-specific response *ex vivo*. C57BL/6 and NKT-KO mice were immunized intradermally with 100 μg chicken CII

emulsified in IFA containing 250 μg of inactivated *M. Tuberclosis* (H37Ra). Nine days after immunization, spleens were removed. The red blood cells were removed from the splenocytes by treatment with 0.16M Tris-NH_4Cl solution, and $2x10^5$ cells were restimulated in triplicates with several concentrations of chicken CII (6.25, 12.5, 25, and 50 _g/mL) for 72 hr and the proliferative response was estimated using the BrdU ELISA system (Cell Proliferation ELISA kit, Roche Diagnostics GmbH, Mannheim, Germany). IFN-γ and IL-4 concentrations in the culture supernatants were measured by ELISA using an immunoassay kit (Biosource International Inc., Camarillo, USA).

Flow cytometric analysis. Fluorescein isothiocyanate (FITC)-labeled anti-TCR_ mAb (clone H57-597), anti-CD45R(B220) mAb (clone RA3-6B2), and PE-labeled anti-CD69 mAb (clone H1.2F3) were purchased from eBioscience, Inc. (San Diego, USA). Rat anti-mouse Fc_R II/III mAb (clone 2.4G2, BD Biosciences, San Diego, USA) was used as the Fc block. Splenocytes were collected from eight C57BL/6 male mice and eight NKT-KO male mice 5 days after the booster immunization and were treated with 0.16M Tris-NH_4Cl solution. The cells were stained with mAbs and propidium iodide (PI, BD Biosciences, San Diego, USA), and were

analyzed by flow cytometry using EPICS XL-MCL (Beckman Coulter, Inc., Fullerton, USA).

Quantitative RT-PCR. The spleen was removed from three of the C57BL/6 male mice and three of the NKT-KO male mice 5, 10, 15, and 30 days after the booster immunization and they were preserved in RNA*later* (QIAGEN GmbH, Hilden, Germany). Total RNA was extracted by TriZol reagent (Invitrogen Co., Carlsbad, USA) and no genomic DNA contamination was confirmed using the GAPDH primer. First strand cDNA was synthesized using SuperScript III First Strand System (Invitrogen Co., Carlsbad, USA). The relative expression levels of IFN-_, IL-1_, and IL-4 mRNA were determined by Taqman RT-PCR technology (ABI PRISM 7700, Applied Biosystems, Foster city, USA). The target gene copy number of each sample was standardized by GAPDH gene expression. The primer-probe set for each cytokine and GAPDH was purchased from Applied Biosystems (Assay-on demand system).

Statistical analysis. Statistical analysis was carried out using the Fisher's exact test for the incidence of arthritis, and the Student's or Welch t-test for arthritis score, anti-CII antibody titer, percentage of CD69-positive cells,

and relative cytokine mRNA expression.

Results

CIA in NKT-KO and CD1d-KO mice

To evaluate the association of Vα14-Jα281 NKT cells with the development of CIA, two different KO mice, Jα281-KO (NKT-KO) and CD1d-KO mice, were used and the incidence and severity of arthritis in each were compared to genetically matched C57BL/6 mice. In NKT-KO mice, the incidence (40%) and arthritis score (1.5 ± 2.2) were significantly reduced compared with the control mice (90%, 5.4 ± 3.2, $p<0.01$)(Fig. 1a, b). In CD1d-KO mice, the incidence (40%) and arthritis score (2.0 ± 2.7) were also significantly reduced compared with control mice (90%, 6.4 ± 4.2, $p<0.01$)(Fig. 1c, d). Judging from these findings, we hypothesize that inv-NKT cells function as effector T cells.

Anti-CII antibody in sera from NKT-KO and CD1d-KO mice

Serum anti- CII IgG Abs were also significantly decreased in NKT-KO and CD1d-KO mice (OD value: 0.32 ± 0.16 and 0.29 ± 0.06 respectively) compared with their controls (OD value: 0.58 ± 0.08 and 0.38 ± 0.08) ($P<0.01$) (Fig. 2). The decrease in pathogenic anti-CII Ab levels is one

reason why arthritis is suppressed in the NKT-cell-deficient mice.

Primary anti-CII response in NKT-KO mice

The immune system of mice was stimulated with adjuvant including microbial antigens as well as CII for the induction of CIA. NKT cells were stimulated with microbial antigens in the context of CD1d, an MHC class-I like molecule on APC [14]. Therefore, it is possible that the suppression of the incidence and severity of arthritis in NKT-KO mice is attributable to the reduction of the immune response to microbial antigens, leading to a reduced response to CII. To address this possibility, the degree of cell proliferation in NKT-KO mice was compared with those in C57BL/6 mice when stimulated with chicken CII after *in vivo* immunization with CII and inactivated *M. Tuberculosis*. The results of the cell proliferation assay, mean OD values and S.D. at 6.25, 12.5, 25, and 50 _g/mL of CII, were 0.11±0.06, 0.16±0.04, 0.24±0.04, and 0.42±0.08 in C57BL/6 mice, and 0.08±0.09, 0.13±0.10, 0.20±0.08, and 0.32±0.14 in NKT-KO mice, respectively (Fig 3a). This indicates that the cell response to CII is not significantly different between NKT-KO and C57BL/6 mice at any antigen concentration tested, suggesting that the suppression of CIA in