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Cutting Edge: Ectopic Expression of CD40 Ligand on B Cells Induces Lupus-Like Autoimmune Disease

Tetsuya Higuchi,*† Yuichi Aiba,* Takeshi Nomura,‡ Junichiro Matsuda,§ Keiji Mochida,§ Misao Suzuki,¶ Hitoshi Kikutani,‖ Tasuku Honjo,§ Kiyoshi Nishioka,‖ and Takeshi Tsubata2*†

CD40 ligand (CD40L) is ectopically expressed on B cells in patients with systemic lupus erythematosus (SLE) and lupus-prone BXSB mice. To assess the role of the ectopic CD40L expression in development of SLE, we have established transgenic mice expressing CD40L on B cells. Some of the 12- to 14-mo-old CD40L-transgenic mice spontaneously produced autoantibodies such as antinuclear Abs, anti-DNA Abs, and anti-histone Abs. Moreover, approximately half of the transgenic mice developed glomerulonephritis with immune-complex deposition, whereas the kidneys of the normal littermates showed either no pathological findings or only mild histological changes. These results indicate that CD40L on B cells causes lupus-like disease in the presence of yet unknown environmental factors that by themselves do not induce the disease. Thus, ectopic CD40L expression on B cells may play a crucial role in development of SLE. The Journal of Immunology, 2002, 168: 9–12.

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease manifesting inflammatory damage in a variety of organs including glomerulonephritis (1, 2). The etiology of SLE involves both genetic and environmental factors. SLE is characterized by production of autoantibodies to various nuclear components (1, 2). Lupus-like disease is induced by abnormalities in either regulators of B cell Ag receptor signaling such as Lyn (4–6), Src homology domain 2-containing protein tyrosine phosphatase (7), and FcγRIIB (8), or regulators of apoptosis such as Bim (9). However, none of the abnormalities of these molecules is shown so far in SLE patients.

CD40 is a member of the TNFR family and is expressed in cells such as B cells, macrophages, and dendritic cells (10, 11). Its ligand, CD40 ligand (CD40L), is a member of the TNF ligand family, expressed mainly on activated T cells. CD40/CD40L plays a pivotal role in cell activation. In B cells, interaction with CD40L promotes proliferation and survival of B cells, Ig isotype switching, and germinal center reaction (11). In patients with SLE, CD40L has been reported to be overexpressed on T cells and ectopically expressed on B cells (12, 13). Ectopic expression of CD40L on B cells is also observed in lupus-prone BXSB mice (14). Clegg et al. (15) have demonstrated that constitutive CD40L expression on T cells induces thymic atrophy due to enhanced apoptosis of thymocytes. They also showed inflammatory bowel disease and thickening of the glomerular capillary wall in the transgenic mice and suggested that these pathological changes may be due to abnormal T cell selection in thymus. However, autoantibody production or inflammatory changes in the glomeruli were not demonstrated in these mice.

We have established transgenic mouse lines expressing CD40L ectopically on B cells. At 8–12 wk of age, CD40L-transgenic mice show increase of both B cell number and serum Ig level by 2-fold and 5-fold, respectively. B cells are resistant to apoptosis induced in vitro, probably due to constitutive CD40 signaling in B cells. However, B cells are not spontaneously activated in these mice, because almost all the B cells are quiescent in vivo and show normal expression of surface markers for maturation and activation of B cells, such as IgD, IgM, CD23, and CD86. Remarkably, these mice show normal T cell development in thymus and exhibit only mild inflammatory bowel disease in contrast to the finding in transgenic mice expressing CD40L on T cells. In this study we demonstrate that CD40L-transgenic mice spontaneously produce autoantibodies such as anti-DNA Abs and develop lupus-like glomerulonephritis as they age. These findings indicate that ectopic expression of CD40L on B cells may be a crucial factor in lupus-like autoimmune disease.

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Address correspondence and reprints requests to Dr. Takeshi Tsubata, Department of Immunology, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan. E-mail address: tsubata.imm@mri.tmd.ac.jp

Abbreviations used in this paper: SLE, systemic lupus erythematosus; CD40L, CD40 ligand; PAS, periodic acid Schiff.


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expression of CD40L on B cells can induce lupus-like disease without inducing abnormal thymocyte development.

Materials and Methods

Mice

C57BL/6 and MRL/lpr mice were purchased from Sankyo Labo Service (Tokyo, Japan). CD40-deficient mice were described previously (16). The CD40L-transgenic mice were established by injecting the DNA fragment containing the mouse CD40L cDNA, a VPRE promoter, the IgH intron enhancer, and the Ig κ enhancer into C57BL/6 fertilized eggs. These mice were maintained in our animal facility. Cross-breeding of these mice was performed and the presence of each transgene was identified by tail DNA PCR assays.

Detection of antinuclear Ab

Cells of mouse myeloma line J558L were smeared on slide glass and fixed with 95% ethanol. After blocking with PBS containing BSA, sequentially diluted sera were applied and incubated for 1 h at room temperature. After washing, cells were reacted with FITC-conjugated anti-mouse IgG Ab (Southern Biotechnology Associates, Birmingham, AL) and observed under fluorescence microscopy.

ELISA analysis

Total amount of IgG was measured by standard sandwich ELISA analysis using anti-mouse IgG Ab (Southern Biotechnology Associates). For detection of autoantibodies, ssDNA was prepared by boiling solution containing bovine thymus DNA (Nacalai Tesque, Kyoto, Japan) for 5 min, followed by immersing on an ice bath. dsDNA was generated by sonicating grade I calf thymus DNA (Sigma-Aldrich, St. Louis, MO), followed by digestion with SI nuclease. ELISA plates were coated with either 10 μg/ml ssDNA or the same concentration of histone type H-I from calf thymus (Sigma-Aldrich). Alternatively, ELISA plates were coated with 10 μg/ml dsDNA in the presence of poly-t-lysine. Plates were blocked with PBS containing 0.5% BSA (Sigma-Aldrich), and sequentially diluted sera were incubated in plates. After further incubation with alkaline phosphatase-labeled anti-mouse IgG Ab (Southern Biotechnology Associates), plates were developed by phosphatase substrate (Sigma-Aldrich). The absorbance was measured by an ELISA reader (Molecular Devices, Menlo Park, CA) at 405 nm and analyzed with Delta Soft 3 (BioMetallics, Princeton, NJ). Concentrations of anti-DNA Abs were determined relative to the standard curves of anti-CD40L transgenic mice (Fig. 1). In contrast, these autoantibodies were not produced in normal littermates except for a small proportion.

Detection of proteinuria

The protein level of mouse urine was semiquantitatively analyzed as described previously (17).

Histopathological and immunohistochemical analysis

Mice were sacrificed and kidneys were perfused with saline. Each kidney was divided into two pieces. One piece was fixed in 10% formalin, paraffin-embedded, and sectioned before staining either with H&E or by periodic acid Schiff (PAS) procedure. The severity of glomerulonephritis was graded according to the degree of deposition of PAS-positive substance, hypercellularity, and sclerotic change in glomeruli as described previously (18, 19), with slight modification. The other piece of the kidney was quickly frozen in OCT compound (Sakura Finetechanical, Tokyo, Japan), sectioned, and stained with FITC-conjugated anti-mouse IgG Ab, anti-mouse IgM Ab, or anti-mouse C3 Ab (Southern Biotechnology Associates).

Statistics

The results were analyzed by nonparametric Mann-Whitney’s U test.

Flow cytometry

Cells were stained with FITC-labeled anti-B220 mAb (BD PharMingen, San Diego, CA) and biotin-conjugated anti-mouse CD40L mAb (BD PharMingen), followed by reaction with PE-conjugated streptavidin (DAKO, Glostrup, Denmark). In some experiments, spleen cells were cultured with or without 10 μg/ml anti-CD40 mAb HM40-3 (a gift from Dr. H. Yagita, Juntendo University) for 12 h in RPMI 1640 medium supplemented with 10% FCS, 50 μM 2-ME, and 1 mM glutamine before staining.

Results

To assess autoantibody production in CD40L-transgenic mice, we obtained sera from 12- to 14-mo-old CD40L-transgenic mice and their nontransgenic littermates. The concentrations of total serum IgG in CD40L-transgenic mice and their normal littermates were 6.2 ± 2.16 and 3.2 ± 0.89 (mean ± SD of six mice), respectively. When we tested for IgG class antinuclear Abs, anti-DNA Abs, and antihistone Abs, all of which are frequently produced in SLE (1, 2), from one-third to one-half of CD40L-transgenic mice showed production of these autoantibodies (Fig. 1). In contrast, these autoantibodies were not produced in normal littermates except for a small proportion.

FIGURE 1. Spontaneous autoantibody production in CD40L-transgenic mice. Sera were obtained from 12- to 14-mo-old female CD40L-transgenic mice (CD40L Tg) and age-matched normal littersmates (LM) maintained under conventional conditions. Sera from 4-mo-old female MRL/lpr mice were used as positive controls. A–C, IgG class antinuclear Abs were detected by indirect immunofluorescence. Titers of antinuclear Abs were determined by maximal fold dilution of sera that generate positive staining (A). Representative staining by sera from CD40L-transgenic mice (B) and sera from the normal littersmates (C) are shown. D, Concentrations of IgG class anti-ssDNA Abs in sera were measured by ELISA. Horizontal bars represent mean values. E and F, Detection of IgG class anti-dsDNA Abs (E) and IgG class anti-histone Abs (F) by ELISA.
few mice producing a low level of the autoantibodies. In some
CD40L-transgenic mice, the level of serum anti-DNA IgG was
comparable to that in MRL/lpr mice used as positive controls.
These results indicate that some of the CD40L-transgenic mice
produce autoantibodies characteristic for SLE.

Because glomerulonephritis is often associated with SLE (1, 2),
we next measured the urine protein level in 12- to 14-mo-old
CD40L-transgenic mice and their normal littermates. The level of
urine protein was elevated in some of the CD40L-transgenic mice
(Fig. 2A), suggesting the presence of nephritis in these mice. In
stead, histopathological analysis revealed that approximately half
of the CD40L-transgenic mice showed apparent glomerulonephritis
with diffuse deposition of PAS-positive substance and hyper-
cellularity in glomeruli (grade 2 and 3) (Fig. 2, B and C). Some of
them were accompanied with sclerotic and/or severe proliferative
changes (grade 3). In contrast, normal litters showed either no
pathological findings (grade 0) or only marginal changes (grade 1)
(Fig. 2, B and D). We observed similar pathological changes in
another line of CD40L-transgenic mice (data not shown). Immu-
nofluorescence staining revealed deposition of IgG (Fig. 2E), C3
(Fig. 2F), and IgM (data not shown) in the glomeruli of CD40L-
transgenic mice but not those of the normal litters (Fig. 2, G
and H). These results indicate that CD40L-transgenic mice spon-
taneously develop glomerulonephritis with immune-complex depo-
sition, which is characteristic for SLE (20-23).

Finally, we assessed CD40L expression on CD40L-transgenic B
cells. We failed to detect CD40L expression on spleen B cells from
CD40L-transgenic mice by flow cytometry, whereas CD40L ex-
pression was detectable on B cells from CD40-deficient CD40L-
transgenic mice (Fig. 2F). This indicates that CD40L expression on
B cells is down-modulated by interaction with CD40 in agreement
with a previous observation (24). This conclusion is supported by
the observation that CD40L expression become detectable by
 treatment with anti-CD40 mAb (Fig. 2F), probably by blocking
interaction between CD40 and CD40L. Thus, ectopic CD40L ex-
pression on B cells is not detectable, although it is crucial for
inducing lupus-like disease.

Discussion

We demonstrate that approximately half to one-third of the trans-
genic mice expressing CD40L ectopically on B cells produce au-
toantibodies including anti-DNA Abs and exhibit glomerulone-
phritis with immune-complex deposition at 12–14 mo of age,
indicating that some of the CD40L-transgenic mice develop lupus-
like disease. Because not all the CD40L-transgenic mouse develop

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**FIGURE 2.** CD40L-transgenic mice develop glomerulonephritis with immune-complex deposition. A, Semiquantitative measurement of urine protein. Urine from 12- to 14-mo-old female CD40L-transgenic mice (CD40L Tg) and age-matched normal littersmates (LM) maintained under conventional conditions was spotted on filter paper. Protein level was measured semiquantitatively by staining with bromophenol blue. The grade of proteinuria was defined as follows: grade 6, equivalent to 30 mg/ml BSA; grade 5, 10 mg/ml BSA; grade 4, 3.3 mg/ml BSA; grade 3, 1.1 mg/ml BSA; grade 2, 0.37 mg/ml BSA; grade 1, 0.12 mg/ml BSA. B–D, Histopathological analysis of kidneys. Sections of kidneys from 12- to 14-mo-old CD40L-transgenic mice and age-matched normal littersmates were stained with PAS or H&E and observed under microscopy. The severity of glomerulonephritis was graded from 0 to 3 according to the degree of deposition of PAS-positive substance, hypercellularity, and sclerosis in glomeruli (B). Representative histopathology of CD40L-transgenic glomeruli exhibiting severe proliferative glomerulonephritis (C) and glomeruli of normal littersmates (D) are shown. E–H, Immunohistochemical analysis of kidneys. Frozen sections of kidneys from 12- to 14-mo-old CD40L-transgenic mice (E and F) and age-matched normal littersmates (G and H) were stained with FITC-conjugated anti-mouse IgG Ab (E and G) or FITC-conjugated anti-mouse C3 Ab (F and H). Sections were observed under fluorescence microscopy. I and J, Detection of CD40L on B cells. CD40L-transgenic mice were crossed with CD40-deficient mice to generate CD40−/− CD40L-transgenic mice. Either freshly isolated spleen cells (I) or those treated with anti-CD40 Ab (J, thick line) or no Ab (J, dotted line) from indicated mice were stained with FITC-labeled anti-B220 Ab and biotin-labeled anti-CD40L Ab, followed by reaction with PE-labeled streptavidin. CD40LTg-CD40−/−, CD40L-transgenic mice; CD40LTg-CD40+/−, CD40−/− CD40L-transgenic mice; LM, normal littersmates. Expression of CD40L on B220+ cells was analyzed by flow cytometry. Freshly isolated spleen cells (I) or anti-CD40 mAb- treated spleen cells (J) stained with FITC-labeled anti-B220 Ab and PE-labeled streptavidin were analyzed as staining controls (thin lines). Representative data of more than three experiments are shown.
lupus-like disease. CD40L expression appears to require environmental factors to induce lupus-like disease. Requirement of environmental factors is also suggested in development of SLE in human (3). Nonetheless, the normal littermates show either no abnormality or only mild histological changes in kidneys, although they are maintained in the same environment as CD40L-transgenic mice. These results indicate that ectopic expression of CD40L on B cells causes SLE-like disease in the presence of yet unknown environmental factors, which by themselves do not induce the disease. Thus, ectopic CD40L expression on B cells demonstrated in SLE patients (12) and SLE-prone BXXB mice (14) may be crucial in the pathogenesis of SLE. It will be interesting to systematically examine CD40L expression on B cells from a large number of SLE patients. In CD40L-transgenic mice, CD40L expression is not detected by flow cytometry due to its down-modulation in the presence of CD40 (24). Thus, a sensitive assay to detect CD40L is required for further analysis of CD40L expression on SLE B cells.

The total IgG level is markedly increased in young CD40L-transgenic mice compared with normal littermates. However, sera from 12- to 14-mo-old CD40L-transgenic mice contain only twice as much IgG as those from the normal littermates due to gradual increase of IgG in normal but not CD40L-transgenic mice as they age. Thus, the autoantibody production in CD40L-transgenic mice may not be ascribed to polyclonal B cell activation, but may involve abrogation of self-tolerance. Because CD40L blocks apoptosis of B cells induced by Ag receptor ligation (11), it might be possible that CD40L induces survival of self-reactive B cells, which otherwise undergo apoptosis by interaction with self-Ags.

Recent studies demonstrated that the serum level of BALL/TALL-1/BlyS, a member of TNF family activating the same signaling molecules that are activated by CD40L (25), is increased in patients with SLE (26, 27). As is the case for CD40L, overexpression of BALL/TALL-1/BlyS can induce autoantibody production and development of lupus-like disease in mice (28, 29). Moreover, inactivation of this molecule by soluble receptor blocks development of autoimmune disease in lupus-prone (New Zealand Black × New Zealand White)F1 mice (30), as is the case for inactivation of CD40L by treatment with anti-CD40L Ab (31). These findings suggest that BALL/TALL-1/BlyS as well as CD40L play a role in the pathogenesis of SLE. However, BALL/TALL-1/BlyS induces a marked increase in the B cell number (28, 29), which is not normally seen in SLE patients, whereas CD40L-transgenic mice show only 2-fold increase in B cell number. Moreover, the disease activity of SLE does not correlate with the level of BALL/TALL-1/BlyS (26). Further analysis is thus required for fully elucidating the role of CD40L and BALL/TALL-1/BlyS in the pathogenesis of SLE.

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