Anti-KIR Antibodies Treat Lupus in Mice

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ABSTRACT
Epigenetically altered T cells cause lupus in genetically predisposed mice, and similar T cells are found in patients with active lupus. More recent reports demonstrate that the epigenetically altered cells comprise a novel CD4⁺CD28⁺ T cell subset characterized by co-overexpression of genes normally suppressed by DNA methylation, including those encoding CD11a, CD70, CD40L and the killer cell immunoglobulin-like receptor (KIR) gene family. Inducing a T cell DNA methylation defect in lupus-prone mice causes a similar subset and lupus. Since KIR genes are not expressed by normal T cells, we tested if antibodies to KIR proteins treat lupus in in mice. A cytotoxic antibody to murine KIR proteins was generated and injected into lupus-prone mice with a T cell DNA methylation defect. The antibody prevented the development of glomerulonephritis in the mice. This suggests that anti-KIR antibodies may be useful in the treatment of human lupus, although the long-term safety and efficacy remain to be established.

Keywords: Lupus; T cells; Epigenetics; Killer cell immunoglobulin-like receptors

INTRODUCTION
Systemic lupus erythematosus is an incompletely understood chronic, relapsing autoimmune disease with flares triggered by environmental agents that cause oxidative stress, such as infections and UV light, and characterized by the development of autoantibodies to cellular antigens and immune complex deposition in tissues such as the kidneys [1]. Despite advances in the development of therapeutic agents for the treatment of other autoimmune diseases, the treatment of lupus remains imperfect, and currently used immunosuppressive agents such as cyclophosphamide, azathioprine and mycophenolate mofetil are not completely effective and have significant toxicities [2]. The design of safer and more effective therapies will require a clearer understanding of the immune mechanisms causing flares of this disease.

Our group initially found that epigenetically altering human CD4⁺ T cells with DNA methylation inhibitors such as 5-azacytidine (5-azaC), procainamide or hydralazine causes demethylation and overexpression of normally silenced genes encoding proteins such as perforin, IFNγ, CD70, CD40L and CD11a [3]. Overexpression of these genes converts normal antigen specific "helper" T cells into autoreactive, cytotoxic pro-inflammatory cells [3]. Other studies demonstrated that similar epigenetically altered CD4⁺ T cells are present in patients with active lupus but not patients with inactive lupus or healthy controls, and that the number of epigenetically altered cells is directly proportional to disease activity as measured by the SLEDAI [3]. We also found that similarly altered murine CD4⁺ T cells also become autoreactive and overexpress methylation sensitive genes, and that the epigenetically altered cells are sufficient to cause a lupus-like disease when injected into syngeneic mice [4,5] or induced in transgenic models [6]. This suggests that the epigenetically altered human CD4⁺ T cells found in lupus patients contribute to lupus flares, and that therapeutics targeting these cells may treat the flares.

Later studies demonstrated that the killer cell immunoglobulin-like receptor (KIR) gene family is also expressed by 5-azaC treated CD4⁺ T cells and by CD4⁺ T cells from patients with active lupus. There are approximately 13 KIR genes [7], which are clonally expressed by NK cells but not normal T cells. However, inhibiting DNA methylation in CD4⁺ T cells activates all the KIR genes [8], and the KIR gene family is similarly expressed on CD4⁺ T cells from patients with active lupus [9]. More recent studies used multicolor flow cytometry to demonstrate that CD70, CD40L, CD11a and the KIR genes are all co-expressed...
on the same CD4+ CD28+ T cells, defining a novel CD4+CD28+CD11ahiCD40LhiCD70+KIR+ subset [10]. This subset is found in patients with active lupus but not in healthy subjects, and the size of the subset in lupus patients is directly related to disease activity as measured by the SLEDAI [10], suggesting that these cells may contribute to lupus flares. These observations also suggest that antibodies to a limited number of KIR proteins could deplete the epigenetically altered T cell subset, which expresses all the KIR genes, but only a clone of NK cells in lupus patients, providing a safe and effective treatment for this disease.

Mice also have KIR genes. In contrast to humans, mice have only three KIR genes, KIr3DL1, KirL1 and KirL2 which are expressed on NK1.1+ T cells [11]. Similar to humans though, 5-azaC also induces expression of the murine KIR genes as well as increasing CD70 and CD40L expression on CD4+ T cells. Further, the epigenetically altered T cells are sufficient to cause a lupus-like disease when injected into syngeneic mice [12-13]. This suggests that antibodies to murine KIR proteins may treat lupus in mice in which lupus flares are caused by a similar inducible T cell DNA methylation defect.

PATIENTS AND METHODS

Cell lines and monoclonal anti-KIRL1 antibodies

The murine KY2 KIR+ NK1.1 cell line was generously donated by Dr. Fumio Takei (Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, BC V6T 2B5, Canada). The cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum and IL-2 as described [14].

Mouse anti-mouse KIRL1 antibodies were generated by Thermo Fisher Pierce Scientific. Briefly, Balb/c mice were immunized with a synthetic KIRL1 peptide (SVQVSAEHHESGSHV) conjugated to KLH [15]. Splenocytes from the immunized mice were fused with the Balb/c Sp2/0-Ag14 hybridoma, and then supematants from growth-positive wells were screened by ELISA for antibodies to the free peptide. Hybridoma supernatants were further tested for their ability to bind KY2 cells. Positive lines were cloned and a selected hybridoma, clone 2A11, was grown for antibodies to mouse T cells was measured by flow cytometry. Briefly, 2A11 antibody with or without 2-mercaptoethanol and stained with Imperial dye (Thermo Fisher) or transferred to PVDF membranes (EMD Millipore, Billerica, MA). Non-specific binding sites on the membranes were blocked using 5% non-fat dry milk blotting solution (Bio-Rad). IgM was detected using HRP-goat anti-mouse IgM (Santa Cruz Biotechnology, Dallas TX) and 4CN (4-chloro-1-naphthol) HRP color development kit (BioRad). The molecular weight of the proteins was assessed using Kalidioscope pre-stained molecular weight markers (BioRad). MOPC 104e, a murine IgM antibody (Sigma), was used as a positive control.

Antibodies and flow cytometric analyses

PE-Hamster anti-mouse CD154 (CD40L) and PECy5-rat anti-mouse CD4 antibodies were from Pharmingen (San Diego CA). Affinity purified mouse anti-mouse KIR antibody 2A11 was labeled with FITC using a FITC-Fast conjugation kit (ABCam, Cambridge, MA) and Zeba Desalt spin columns (Thermo Fisher) according to the manufacturers’ instructions. Controls included isotype and fluorochrome matched antibodies, single antibodies and ‘full minus one’ (FMO) staining controls in which one of each of the fluorochrome-conjugated antibodies was serially omitted while retaining the rest. The cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ) or an iCyte Synergy flow cytometer (Sony Biotechnology, San Jose CA) and WINLIST V.8 software (Vertly Software House (http://www.vsh.com)).

Cytotoxicity assay

The ability of the 2A11 antibody to kill NKT cells was measured using KY2 cells and the Cytotox 96R colorimetric assay (Promega, Madison, WI) according to the manufacturer’s instructions. Briefly, KY2 cells were plated into 96 well round bottom microtiter plates. The 2A11 antibody with or without Guinea pig complement (Sigma) was added to the wells and the cultures incubated for 30 min at 37°C in a 5% CO2 balanced air incubator. Samples of the supernatants were transferred to a fresh 96 well flat bottom plate, mixed with the assay substrate and buffer then incubated for 30 minutes at ambient temperature in the dark. The reaction was stopped and absorbance read immediately at 490 nm using a Spectromax Spectrophotometer (Molecular Devices). Cell numbers, complement and antibody concentrations were optimized for the assay. Controls included anti-Thy antibody as a positive cytotoxicity control, and non-cytotoxic mouse IgM (MOPC104e, from Pharmigen) as a negative control. Complement-mediated cytotoxicity of the 2A11 monoclonal anti-mouse KIRL1 antibody to mouse T cells was measured by flow cytometry. Briefly, T cells were purified by negative selection from SJL mouse splenocytes using EasySep mouse T cell isolation kits (StemCell Technologies, Cambridge, MA). Purified CD4+ T cells were treated with 2A11 alone or with guinea pig...
complement for 30 minutes at 37°C. The cells were washed with PBS containing 10% bovine serum albumin buffer and incubated with PE-goat anti-mouse IgM or PE CY5-anti-CD4 (PharMingen) on ice for 30 minutes. The cells were washed, fixed in 2% paraformaldehyde and analyzed by FACS.

Mice
SJL/J mice were purchased from Jackson Laboratories (Bar Harbor, ME). SJL double transgenic (dnMEK+/CD2rtTA+) mice were bred at the University of Michigan animal facility as previously described [16]. All animals were housed in filter-protected cages and provided with irradiated standard 5053 rodent diet (Lab Diet, PMI Nutrition International, Brentwood, MO), and water ad libitum. All mice were bred and maintained in a specific pathogen-free facility by the Unit for Laboratory Animal Medicine at the University of Michigan in accordance with National Institutes of Health and American Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International Guidelines. All procedures were approved by the University of Michigan Institutional Animal Care and Use Committee.

Treatment of mice
A lupus-like disease was induced in selected groups of female double transgenic (dnMEK+/CD2rtTA+) SJL mice by administering 4 mg/ml doxycycline (DOX) (Sigma, St. Louis, MO) and 5% glucose in their drinking water. The DOX-treated animals were injected intraperitoneally (i.p.) twice weekly with saline, 0.5 mg normal mouse IgM, or 0.5 mg affinity purified 2A11 monoclonal mouse-anti-mouse-KIRL1, using 8 mice per group for a total of 24 mice. Blood was collected at 0, 4, 8 and 12 weeks of treatment and analyzed for anti-dsDNA antibody by ELISA [17]. This dose was based on reports on anti-T cell antibodies in the treatment of lupus by others (PMID: 3886436). Protein and hemoglobin in mouse urine were measured at each time point by Chemstrip 7 dipstick (Roche, Madison, WI). One week following the final injection the mice were euthanized and the kidneys were prepared for histology.

For in vitro studies, SJL splenocytes were stimulated with Concanavalin A then cultured with or without 5-azaC as previously described [18]. The cells were then washed twice in standard buffer (PBS containing 1% bovine serum albumin (BSA) and 0.001% sodium azide) at 4°C. Non-specific binding was blocked by incubating the cells 1 hour on ice in standard buffer containing 10% BSA. The cells were stained in the dark for 1 hour, washed, then fixed in 2% paraformaldehyde and stored in the dark at 4°C.

Histopathology
The kidneys were bisected. One half of the tissue was fixed in 10% neutral buffered formalin, routinely processed and paraffin embedded. Five micron sections were deparaffinized and stained with hematoxylin and eosin. The remaining half of the tissue was embedded in optimal cutting temperature compound (O.C.T., Thermo Fisher), snap frozen and stored at -80°C. Five micron sections were cut from the frozen tissue and fixed for 10 minutes in ice cold acetone. Non-specific sites were blocked with 10% FBS/PBS then the sections were stained for mouse IgG deposits using a 1:50 dilution of biotin-goat anti-mouse IgG (Fcspecific) antibody (US Biologicals)/FITC-Streptavidin (BD Pharmingen).

Elisa
Mouse anti-dsDNA IgG antibodies were measured by ELISA as previously described (17). Briefly, 96 well flat bottom microtiter plates (Costar, Corning, NY) were coated overnight at 4°C with 10 µg/ml dsDNA in PBS, pH 7.2. Various dilutions of mouse sera or murine monoclonal IgG anti-dsDNA antibody (Clone BV16-13, Millipore) standard were added and the plates incubated overnight at 4°C. Bound anti-dsDNA antibody was measured at 450 nm using HRP-goat anti-mouse IgG-Fc-specific antibody (Bethyl Labs, Montgomery, TX) and OneStep Ultra TMB substrate (Thermo, Rockford, IL). The KIRL1 peptide used to immunize the mice was added where indicated as an inhibitor of antibody binding.

Statistical analysis
Student’s t-test, chi-square, two-tail Fisher Exact test and linear regression were used as appropriate to determine the significance of differences between groups using SYSTAT software on a Dell PC Optiplex 745 microcomputer and by GraphPad Prism V.6 software. The median and SD of populations identified by flow cytometry were calculated using the statistical functions within the FlowJo software (FlowJo, Ashland Oregon, USA).

Theory/calculation
If the epigenetically altered T cell subset is important in the pathogenesis of lupus, cytotoxic antibodies to the subset should treat autoimmunity in mice in which lupus can be induced by a mechanisms similar to that involved in human lupus.

RESULTS
Anti-KIR antibodies
To determine the role of KIR+ T cells in murine lupus we first generated monoclonal antibodies in mice to mouse KIR proteins as described in Methods. The 2A11 anti-KirL1 monoclonal antibody was analyzed for molecular weight and isotype by reducing and non-reducing PAGE and Western Blot gel electrophoresis (Figure 1A). The antibody was an IgM but had a molecular weight of approximately 260 kD suggesting it was dimeric rather than the typical pentameric form, exhibited by MOPC104e, usually observed for this isotype. Monoclonal 2A11 had kappa light chains (not shown). Figure 1A also shows that reduction of the disulfide bonds with 2-ME results in a 75 kD heavy chain consistent with IgM. The specificity of the 2A11 antibody for KIR proteins was confirmed by staining the murine KY2 NK1.1+ cell line with FITC-conjugated 2A11. 2A11 antibody bound KIR+ KY2 NK cells (Fig 1B). This binding to KY-2 cells was blocked by pre-incubating the 2A11 antibody with immunizing KIR peptide (Figure 1C) confirming that the 2A11 antibody was specific for KIR proteins.
methylation in CD4+ T cells activates KIR gene expression [8].
So we tested binding of the 2A11 anti-KIR antibody to 
untreated and 5-azaC-treated murine CD4+ T cells. Figure 1D 
shows that 2A11 detects low levels of KIR on CD4+ SJL T cells, 
and that 5-azaC treatment caused a significant increase in the 
KIR expression detectable by FITC-2A11 binding. Taken 
together our results show that the murine hybridoma antibody 
recognized KIR proteins on NKT and CD4+ T cells in mice.

Figure 1: (A) The molecular weight and isotype of the 2A11 hybridoma 
protein were analyzed on 4-20% gradient SDS-PAGE gel 
electrophoresis and Western Blot. The 2A11 hybridoma line secretes 
an approximately 260 kD IgM antibody. Reduction of the disulfide 
bonds with 2-ME resulted in a 75 kD heavy chain consistent with IgM 
isotype (B) KIR+ KY2 NKT cells were stained with FITC-conjugated 
anti-NK1.1 or FITC-conjugated 2A11 anti-KIR (solid lines) then 
analyzed by flow cytometry. Controls included a FITC-conjugated 
isotype matched myeloma protein (dotted line) (C) Peptide inhibition 
of 2A11 binding to NKT cell line (KY2) Left: FITC-2A11 binding to 
KY2 cells. Vertical bar separates positive from negative staining. Right: 
Same as left figure but a 10 molar excess of KIR (immunizing) peptide 
was added (D) SJL mouse splenocytes were stimulated with 
Concanavalin-A then cultured with or without 5-azaC as described in 
materials and methods. PE-Cy5-CD4+ T cells were gated and KIR 
expression was measured as in B.

Complement-dependent killing of KIR+ cells by the 2A11 
antibody was tested using KIR+ KY2 cells and 5-azaC-treated 
syngeneic CD4+ T cells as targets. Figure 2A shows that the 
2A11 antibody was not cytotoxic to KY2 cells by itself but lysed 
the cells in the presence of complement. Similarly, 2A11 
binding to cultured, 5-azaC treated SJL splenic CD4+ T cells was 
detectable by FACS (Figure 2B). The combination of the 2A11 
antibody and complement caused a significant (p=0.017) 
decrease in the number of KIR+ CD4+ T cells (Figure 2C).

Figure 2: The 2A11 antibody plus complement kills KIR-bearing cells 
(A) Cytotoxicity of the mouse anti-mouse-KIR antibody 2A11 was 
measured as described in Methods. Murine KY2 NKT cells were 
incubated with 2A11 antibody with or without Guinea pig 
complement for 30 minutes at 37°C in a 5% CO2 balanced air 
incubator, supernatants collected, mixed with substrate and 
cytotoxicity measured. Controls included anti-Thy antibody as a 
positive cytotoxicity control, and non-cytotoxic mouse IgM 
(MOPC104e) as a negative control (B) CD4+ SJL splenic T cells were 
incubated with the 2A11 antibody. The cells were washed and binding 
detected using PE-anti-mouse IgM (C) CD4+ T cells were incubated 
with complement then stained with PE-Cy5 anti-CD4 and PE anti-
mouse IgM as in (B). The results shown are a representative of 3 
experiments.

Anti-KIR antibodies in vivo
The effects of the 2A11 anti-KIR antibody on the development 
of autoimmunity were then tested in a transgenic murine lupus 
model with an inducible T cell DNA methylation defect similar 
to that found in T cells from patients with active lupus [13]. 
Adding doxycycline to the drinking water of these mice activates 
expression of a dominant negative MEK selectively in T cells, 
preventing Dnmt1 upregulation during mitosis, causing DNA 
hypomethylation in the daughter cells, methylation-sensitive 
gene overexpression and a lupus-like disease [13]. Female 
transgenic mice receiving doxycycline were injected i.p. with 0.5 
mg of the anti-KIR antibody or a control IgM antibody twice a 
week for 12 weeks. This dose was found to optimally deplete 
KIR+ T cells in pilot experiments. The mice were then sacrificed 
and the kidneys analyzed for evidence of a lupus-like 
glomerulonephritis. Figure 3A shows that doxycycline induces 
an inflammatory glomerulonephritis characterized by leukocyte 
infiltration and karryorhectic debris (arrows). Figure 3B shows 
that a control IgM antibody has no effect on the development of 
an inflammatory glomerulonephritis in these mice. In contrast, 
Figure 3C shows that the anti-KIR antibody prevents 
development of the inflammation.
Evidence for an immune complex mediated glomerulonephritis in these mice was sought using fluorochrome conjugated antibodies to mouse IgG. Figure 4A shows a kidney section from a mouse receiving doxycycline stained with FITC-conjugated anti-IgG, and demonstrates that doxycycline induces an immune complex glomerulonephritis. Figure 4B similarly shows that administration of normal mouse IgM has no effect on the immune complex deposition. In contrast, Figure 4C shows that the anti-KIR antibody prevents immune complex deposition in these mice. The anti-KIR antibody also caused a decrease in proteinuria relative to the control IgM treatment (normal mouse IgM \(65.7+12.5 \text{ mg/dL}\) vs. 2A11 27.14+13.8 mg/dL, n=7/group mean+SEM).

**DISCUSSION**

Our previous studies demonstrated that human CD4+ T cells epigenetically altered with DNA methylation inhibitors overexpress genes normally suppressed by DNA methylation, and become autoreactive to self-class II MHC molecules alone [19], and that patients with active lupus have similar cells [3]. Furthermore we demonstrated that similar epigenetically altered CD4+ murine T cells also become autoreactive and are sufficient to cause a lupus-like disease in lupus-prone mice [20]. This suggests that depleting this subset may be beneficial to lupus patients.

Array-based expression profiling of the epigenetically altered human T cells revealed that multiple genes are overexpressed, including the KIR genes [8]. The KIR genes are clonally expressed by NK cells but not by normal T cells. However, CD4+ T cells epigenetically altered with DNA methylation inhibitors in vitro aberrantly co-express the KIR gene family on the same T cell, as do CD4+ T cells from patients with active lupus [8,9]. More recent studies demonstrate that the T cell
genes activated by DNA demethylation in human lupus patients, including the KIR genes, are co-expressed by a previously unrecognized CD3+CD4+CD28+KIR +CD11ahiCD70+CD40Lhi T cell subset, suggesting that the subset may be a target for new approaches to the treatment of lupus. The observation that the KIR genes are expressed clonally on NK cells but not on normal T cells, and that epigenetically altered CD4+ T cells overexpress many if not all the KIR genes, also suggests that antibodies to a limited number of KIR genes may deplete the altered T cell subset while only affecting a limited number of NK cell clones, minimizing potential side effects.

To test this hypothesis we generated hybridomas to a murine KIR peptide and selected the 2A11 IgM anti-KIR antibody. We then tested if the antibody could prevent the development of lupus in a murine model in which lupus-like autoimmunity is caused by epigenetically altered T cells, similar to those contributing to human lupus [13]. Our results showed that the anti-KIR antibody prevented the development of glomerulonephritis. The antibody also decreased proteinuria, although the decrease was of borderline significance. The anti-KIR antibody also decreased overall IgG levels in the mice, consistent with an anti-inflammatory effect.

Somewhat paradoxically, the anti-KIR antibody increased anti-DNA antibody levels in the recipient mice. However, others have reported that anti-KIR antibodies cause NK cell apoptosis [21], and apoptotic chromatin induces anti-DNA antibody production [22]. The NK apoptosis likely is responsible for the increase in the anti-DNA antibodies. The clinical significance of the anti-DNA antibodies is uncertain as no other pathologic changes in the mice were seen. It should also be noted that human NK cells express KIR genes clonally [23]. Normal T cells do not express KIR genes, while CD4+ T cells epigenetically altered with DNA methylation inhibitors express the whole KIR gene family [8]. This suggests that an antibody to a limited number of KIR genes would deplete the human KIR+ T cell subset while only affecting subset of NK cells. Importantly, previous studies used antibodies to stimulatory KIR antibodies to treat multiple myeloma in humans, and found no evidence for the development of clinically evident autoimmunity, supporting safety of the antibodies [24]. Antibodies to inhibitory KIR proteins were also well tolerated in a patient with acute myelogenous leukemia [25].

The limitations of this study are that mice are an imperfect model for all human due to the possibility of unrecognized species-specific differences in pathologic immune responses. None the less, the results support the hypothesis that anti-KIR antibodies may be similarly useful in the treatment of human lupus.

CONCLUSIONS

These studies demonstrate therapeutic efficacy of anti-KIR antibodies in a murine lupus model caused by a mechanism closely resembling that contributing to human lupus. Anti-KIR antibodies were well tolerated in our mice as shown above and humanized anti-KIR antibodies are well tolerated in patients with multiple myeloma. This suggests that anti-KIR antibodies may prove to be safe and beneficial in human lupus patients.

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