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The Role of Leukocyte Associated Immunoglobulin-Like Receptor-1 (LAIR-1) in Suppressing Collagen-Induced Arthritis

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Abstract

Several observations implicate a critical role for T cell dysregulation as a central problem in RA. We investigated a mechanism for suppressing T cell activation by stimulating a natural inhibitory receptor called leukocyte associated immunoglobulin-like receptor-1 (LAIR-1). The collagen-induced arthritis model and DR-1 transgenic mice were used to study the importance of LAIR-1 in autoimmune arthritis. Splenocytes from WT or LAIR-$1^{-/-}$ mice were stimulated with soluble anti CD3 antibody in the presence or absence of α1(II) and supernatants were collected for cytokine analysis. B6.DR1 mice were immunized with CII/CFA to induce arthritis and were treated with either the stimulatory monoclonal antibody to LAIR-1 or a hamster IgG control. Finally, B6.DR1/LAIR-$1^{-/-}$ and B6.DR1/LAIR-$1^{+/+}$ mice were challenged for CIA and mean severity scores were recorded thrice weekly. Using splenocytes or purified CD4+ cells that were sufficient in LAIR-1, CD3-induced cytokine secretion was significantly suppressed in the presence of collagen, while LAIR-1 deficient splenocytes had no attenuation. Treatment with a stimulatory monoclonal antibody to LAIR-1 also significantly attenuated CIA in the LAIR+/+ mice. When B6.DR1/LAIR-1−/− mice were immunized with CII they developed more severe arthritis and had a greater percentage of affected limbs than the wild type mice. These data demonstrate that collagen can suppress the T cell cytokine response through the action of LAIR-1. Treatment with stimulating LAIR-1 antibodies suppresses CIA while B6.DR1/LAIR-1 −/− mice develop more severe arthritis than wild type controls. These data suggest that LAIR-1 may be a potential therapeutic target for suppressing RA.

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1. Introduction

Rheumatoid arthritis (RA) is an inflammatory disorder of unknown etiology but characterized by autoimmunity. Currently there is no cure for RA. Current therapeutic strategies that induce general immunosuppression have significant side effects so our goal was to target specific biomolecular processes in the autoimmune pathway leading to RA. Activating naturally occurring inhibitory receptors might be a novel method for suppressing autoimmune arthritis. Although there are several immunoglobulin-like receptors on immune cells, one of these, LAIR-1 (Leukocyte Associated Immunoglobulin-Like Receptor-1, also called CD305) has a cytoplasmic domain that contains an immunoreceptor tyrosine based inhibition motif (ITIM) that appears to act as a negative regulator of immune cell signaling including activation of T cells. We believe that activating LAIR-1 may lead to diminished autoimmune activity and less severe disease in patients with RA.

LAIR-1 is a transmembrane glycoprotein inhibitory receptor consisting of a single extracellular immunoglobulin domain, a short stalk region, a transmembrane domain and a short cytoplasmic tail that contains two immunoreceptor tyrosine-based inhibitory motifs (1). LAIR-1 belongs to the inhibitory immunoglobulin superfamily and is structurally related to several family members whose genes are located in the leukocyte receptor complex on human chromosome 19 (1). LAIR-1 contributes to the regulation of the immune system by delivering inhibitory signals.

Although LAIR-1 is expressed on multiple immune cells, our focus in these experiments was on the CD4+T cell, because LAIR-1 can be upregulated on the CD4+ T cells during the inflammatory response and several observations implicate a critical role for T cell dysregulation as a central problem in RA. In this study, we examined the role of LAIR-1 in $CD4^+$ T cells in suppressing murine collagen-induced arthritis. We tested CD3-induced cytokine secretion and found that the expression of inflammatory cytokines was significantly suppressed in the presence of collagen (the ligand for LAIR-1), while LAIR-1 −/− cells were not similarly suppressed. Treatment with a stimulatory monoclonal antibody to LAIR-1 attenuated collagen-induced arthritis (CIA) in the mice. Finally, DR1/LAIR-1−/− mice that were immunized with CII developed more severe arthritis and had a greater percentage of limbs affected with arthritis than did the control mice in whom LAIR-1 was normally expressed (DR1/LAIR-1 +/+). These data suggest that LAIR-1 may be a potential therapeutic target for suppressing RA.

2. Methods and Materials

Preparation of Tissue Derived CII and Synthetic Peptides

The following nomenclature is used to define the antigens used in this study: CII = Type II collagen, CI = Type I collagen, A2 = a peptide containing the immunodominant determinant sequence of both bovine and human CII (GIAGFKGEQGPKGEB) (B stands for 4-hydroxyproline), α1(II) and α1(I) = the constituent protein chains of bovine CII or CI isolated by carboxymethyl-cellulose column chromatography at 45 °C.

Native CII was solubilized from fetal calf articular cartilage or murine articular cartilage by limited pepsin-digestion and purified as described earlier (2). The purified collagen was dissolved in cold 10mM acetic acid at 4 mg/ml and stored frozen at −70°C until used. Synthetic peptides representing collagenous sequences were supplied by New England Peptide Inc., (Gardner, Massachusetts) and were greater than 95% pure.

Antibodies

Antibodies used for this study included: monoclonal anti-murine LAIR-1 antibodies, (Affymetrix/eBioscience, San Diego, Ca) and Armenian hamster IgG isotype control for the anti LAIR-1 (Biolegend, San Diego, Ca). The Abs used for flow cytometry included: PacBlue-conjugated anti-CD4, PE-conjugated anti-IL-2, APC-conjugated anti-IFN-γ, FITC-conjugated anti-CD8, APC-conjugated anti-CD19, FITC-conjugated anti-CD11c, APC-conjugated anti-CD11b, APC-conjugated anti-DX5, APC-conjugated anti-GR-1 (BD Biosciences, San Jose, CA) and PE-conjugated anti-murine LAIR-1 antibodies, (Affymetrix/eBioscience, San Diego, Ca). All were used according to the manufacturer's recommendations.

$CD4^+$ T cell isolation/activation and hyridoma cells

Spleens were collected from DR1 mice that were either LAIR-1 sufficient or deficient and single-cell suspensions were prepared by mechanical disruption in complete DMEM medium (DMEM supplemented with 10 % FCS, 100 IU/ml of penicillin, 100 μg/ml streptomycin, 2.5 μM β-mercaptoethanol and 2 mM L-glutamine). In some experiments, $CD4^+$ naive T cells were isolated using a $CD4^+$ T cell isolation kit (Miltenyi Biotec, Auburn, Ca) based on a negative selection protocol. Flow cytometry was used to determine that the purity of the recovered $CD4^+$ T cells was >95%. In selected experiments the cells were cultured for varying lengths of time (30 seconds to 60 minutes) with antigen presenting cells (APCs: B6.DR1-positive splenocytes) that had been prepulsed with A2 peptide. To induce stimulation, cells were cultured with soluble anti-CD3 antibody for 24 hrs. To test for phenotype by flow cytometry, T hybridoma cells that we have previously described were cultured with α1(II) (3).

Flow cytometry

Mouse splenocytes were cultured with either α1(II), anti-CD3 or anti-LAIR-1 and the phenotype was determined by multiparameter flow cytometry using an LSRII flow cytometer (BD Biosciences, San Jose, CA). Cells were labeled with fluorochrome antibodies specific for CD4 or other lymphocyte subsets or murine LAIR-1. In some experiments using antibodies specific for IL-2 or IFN-γ, intracellular labeling was performed in order to detect the production and accumulation of cytokines within the endoplasmic reticulum after cell stimulation. Briefly, cells were activated and an inhibitor of protein transport (brefeldin A) was added to retain the cytokines within the cell. The cells were then fixed in paraformaldehyde and permeabilized. Anti-cytokine antibodies were added and the cells were analyzed by flow cytometer. When intracellular staining was implemented, plots were gated on $CD4^+$ T cells. A minimum of 10,000 cells was analyzed from each sample and the final analysis was performed using FlowJo software (Tree Star, Ashland, OR).

Generating B6.DR1/LAIR-1$^{-/-}$ (KO) mice

DBA/1 mice and B6 mice expressing the chimeric (human/mouse) DRB1*0101 construct were maintained in our onsite pathogen-free facility. The chimeric DRB1*0101 construct has been previously described, as has the production of Tg mice expressing these constructs (4). We chose the DBA/1 and the B6.DR1 transgenic mice because of their increased susceptibility to develop collagen-induced arthritis (CIA) (4). LAIR-1 KO (knockout) mice (5) were crossbred to B6.DR1 transgenic mice with a B6 background for 12 generations. Genomic DNA was obtained from blood samples and PCR used to identify mice homozygous for either the LAIR-1 −/− or LAIR +/+ and expressing the DR1 transgene. The LAIR-1 KO/B6.DR1 mice were found to have healthy phenotypes. All mice were fed standard rodent chow (Ralston Purina Co., St Louis, MO, USA) and water ad libitum. Sentinel mice were routinely tested for murine pathogens. Experiments were conducted in accordance with approved Institutional Animal Care and Use Committee (IACUC) protocol and the mice were 8 to 12 weeks old.

Immunizations and induction of arthritis

Mice were immunized with bovine CII for the induction of arthritis. CII was dissolved in 10 mM cold acetic acid and the CII was emulsified with complete Freund's Adjuvant (CFA) as previously described (2). Mice were immunized subcutaneously at the base of the tail with 100 μg of CII.

Measurement of the severity of arthritis

The severity of arthritis was determined by visually examining each forepaw and hindpaw and scoring them on a scale of 0 to 4 as described previously (2). Scoring was conducted by two examiners, one of whom was unaware of the identity of the treatment groups. Each mouse was scored thrice weekly beginning 3 weeks post immunization and continuing for 8 weeks. The mean severity score (sum of the severity scores for the group on each day/total number of animals in the group) and percent of arthritic limbs (sum of the number of arthritic limbs/total number of limbs per group × 100) was recorded at each time point.

Measurement of antibodies against collagen using ELISA

Mice were bled at 4 or 6 weeks after immunization and sera were analyzed for antibodies reactive with native homologous CII using a previously described modification of an ELISA (2). Serial dilutions of a standard serum were added to each plate. From these values, a standard curve was derived by computer analysis using a four-parameter logistic curve. Results are reported as units of activity, derived by comparison of test sera with the curve derived from the standard serum which was arbitrarily defined as having 50 units of activity. Reactivity to murine CII was not detected in sera obtained from normal mice.

Cytokine analysis

To measure cytokines, splenocytes from B6.DR1 mice were cultured with anti-CD3 antibody and supernatants were analyzed for cytokines. In other experiments, B6.DR1 mice were immunized with CII/CFA and draining lymph node cells were taken from the mice 10 days after immunization and were cultured with A2 peptide so that supernatants could be

analyzed for the presence of cytokines. Cells were incubated at 37 °C and harvested after 72 hours.

Each culture was set up using cells from three mice run in triplicates and supernatants were analyzed for the presence of IL-10, IL-4, IFN-γ and IL-17A using a Bio-plex mouse cytokine assay (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Values are expressed as picograms per ml and represent the mean values for each group taken from three separate experiments.

Western blots—Murine T hybridoma cells (1×10^7/10ml) were stimulated with α1(II) (50 μg/ml) or α-LAIR-1 (1mg/ml) for the indicated time periods. Samples were loaded and expression level of LAIR1 was examined with Western blot analysis using anti-LAIR1. Actin was used as control.

Statistical Analysis

Mean severity scores, antibody titers, and cytokine levels were compared using the Mann-Whitney test. The numbers of arthritic limbs were compared using Fischer's Exact Test.

3. Results

T cells and regulation of LAIR-1

Although previous reports suggest that surface expression of LAIR-1 can be regulated by stimulating the T cell receptor, it remained unknown whether LAIR-1 might be upregulated by stimulation with its natural ligand, collagen. Therefore, naive DBA/1 splenocytes were cultured with α1(II) and flow cytometry was used to evaluate the resulting expression of LAIR-1 on $CD4^+$ T cells. As shown in Figure 1, culture with α1(II) caused a significant upreguation of LAIR-1 (Panel A). Similarly, when murine T hybridoma cells were cultured with α1(II) (Panel B), the surface expression of LAIR-1 was clearly increased. In a third experiment, T hybridoma cells were stimulated with α1(II) and western blot analysis (Panel C) demonstrated increased expression of LAIR-1 following culture with α1(II). Taken together, these data indicate that α1(II) markedly upregulates the surface expression of the inhibitory receptor LAIR-1 on $CD4^+$ T cells.

T cell cytokines and LAIR-1

Inhibitory receptors such as LAIR-1 could become important tools for attenuating the inflammatory responses of $CD4^+$ T cells. In order to determine whether stimulation of LAIR-1 might inhibit T cell cytokine production, splenocytes from CII-immunized B6.DR1/$LAIR^{+/+}$ mice were cultured with the immunodominant A2 peptide to stimulate the TCR in the presence of either a hamster IgG isotype control or a monoclonal antibody stimulatory to LAIR-1 (Figure 2). When the supernatants were analyzed for cytokines, we found that cells cultured with the monoclonal antibody stimulatory to LAIR-1 had decreased expression of several T cell cytokines (Th1, Th2 and Th17) by the antigen stimulated T cells compared to the same cells treated with control hamster IgG.

To confirm that the antibody is stimulatory, splenocytes from arthritic mice were cultured with 10μg/ml of α -LAIR-1 for 24 hours and stained with DAPI (4',6-diamidino-2-phenylindole) using flow cytometry to confirm that >90% of the cells were viable at this concentration. Prior to culture, there were 22 ± 4% $CD4^+$ cells, 8.3 ± 1% $CD8^+$ cells, 60 ± 7% $CD19^+$ cells, as well as <5% of each of the $DX5^+$, $GR1^+$, $CD11b^+$, and $CD11c^+$ populations from the arthritic spleens. After 24 hours of culture with the antibody there was no change in any of the populations: 17 ± 3% CD4+ cells, 8.4 ± 2% $CD8^+$ cells, and 66 ± 2% $CD19^+$ cells. Mean fluoresence for LAIR-1+ splenocytes prior to culture was 1948 ± 477 and 3539 ± 581 after culture, ($p = 0.02$, $n = 3$). The increase in LAIR-1 was spread evenly among all immune cells tested. These data confirm that the antibody is not cytotoxic, but instead increases the LAIR-1. Moreover, LAIR-1 is upregulated on T cell hybridomas following culture with anti-LAIR-1 (Figure 2, lower right panel).

Next, we examined whether natural ligands for LAIR-1 might in a similar manner affect T-cell pro-inflammatory responses such as IL-17A, IFN-γ and IL-2. Using an *in vitro* assay with splenocytes from mice that were either sufficient or deficient in LAIR-1 but which had not been immunized we cultured with anti-CD3 in the presence of absence of CII to determine their ability to produce each of these cytokines (Figure 3, left side). There was no difference in cytokine production in the $LAIR^{-/-}$ and $LAIR^{+/+}$ groups if cultured with media alone. When murine $CD4^+$ T cells were stimulated with $CD3^+$ antibodies, there was increased production of IL-2, IFN-γ, and IL-17A in both the $LAIR^{-/-}$ and $LAIR^{+/+}$ groups compared to baseline levels. When the cells were stimulated with both anti CD3 antibodies and α1(II), there was a statistically significant suppression of IL-2, IFN-γ and IL-17A in the $LAIR^{+/+}$ groups but not in the $LAIR^{-/-}$ groups (Figure 3, left side). These data demonstrate that activation of LAIR-1 can suppress T cell inflammatory cytokine production (IL-2, IFN-γ, and IL-17A) in the presence of $LAIR^{+/+}$, but not in $LAIR^{-/-}$ (KO) cells. Purified T hybridoma cells (Figure 3, upper right) and purified CD4+ cells (lower right) demonstrated the same level of suppression in the presence of α1(I).

In a third set of experiments to explore the role of natural ligands for LAIR-1 in modulation of cytokine responses, splenocytes from naïve (unimmunized animal) $LAIR^{+/+}$ and $LAIR^{-/-}$ mice were cultured for 24hrs with and without α1(II), then stimulated with soluble anti CD3 for another 24 hrs. The cells were stained for CD4, IL-2 and IFN-γ and flow cytometry was used to demonstrate that α1(II) can suppress $CD3^+$-induced T cell inflammatory cytokine production in the presence of $LAIR^{+/+}$ but not in $LAIR^{-/-}$ cells (Figure 4).

Flow-jo software was used to calculate the numbers (percentages) of positive cells. They were as follows: IFN- γ^{pos} LAIR +/+ = 0.185 ± 0.152; IFN- γ^{pos} LAIR +/+ and α1(II) = 0.0057 ± 0.0045; IFN- γ^{pos} LAIR −/− = 0.254 ± 0.018; IFN- γ^{pos} LAIR −/− and α1(II) = 0.285 ± 0.023; $p = 0.045$ when comparing the LAIR +/+ with and without α1(II). $P < 0.001$ when comparing LAIR +/+ and α1(II) with LAIR−/− and α1(II).

The IL-2 ^{pos} LAIR +/+ = 0.293 ± 0.05, IL-2 ^{pos} LAIR +/+ and α1(II) = 0.050 ± .005, IL-2 ^{pos} LAIR −/− = 0.299 ± 0.10, IL-2 ^{pos} LAIR −/− and α1(II) = 0.303 ± 0.045. $P = 0.009$ comparing the IL-2 ^{pos} LAIR +/+ cells with and without α1(II).

Taken together, these data indicate that stimulation of LAIR-1 might be an effective way to attenuate autoimmune arthritis.

Treatment of B6.DR1 mice with anti-LAIR-1 antibody

In our quest to use mouse models to develop treatments that will eventually translate into therapies for human RA, we used the collagen-induced arthritis model to treat mice *in vivo* with a stimulating LAIR-1 antibody and evaluate disease severity and antibody and cytokine responses. B6.DR1 transgenic mice were immunized with CII/CFA and treated intraperitoneally with either anti-LAIR-1 IgG, or a matched IgG control. We found that the mice receiving the stimulating anti-LAIR antibody had significant suppression of the severity of the arthritis compared to littermate controls administered the control antibody (Figure 5, upper panel). The severity of the arthritis was statistically significant. (On day 37 after immunization severity scores were 1.5 ± 1.9 vs. 6.4 ± 2.4, $p = 0.007$; Fig 4). When serum taken from the mice at four weeks after immunization was tested for levels of antibodies against CII, there was a decrease in anti-CII antibodies in the group of mice that received anti-LAIR IgG antibody compared to the littermate control group of mice that received the IgG control (33 ± 7.9 units vs 62 ± 16 units, $p = 0.04$).

In a second experiment, either anti-LAIR-1 or control antibody was injected directly into the ankle joint of the right hindpaw of B6.DR1 mice for a total of seven treatments (Figure 5, lower panel). Each right hindpaw was scored for the severity of arthritis. As shown in figure 5 the final severity scores were significantly different.

Similarly, we found that the final severity scores for forepaws were as follows: Lair-1 treated mice 0.4 ± 0.9; hamster IgG treated mice 2.6 ± 1.4; untreated mice 3.0 ± 2, (** $p = 0.008$) comparing Lair-1 treated to either hasmster IgG treated or untreated, suggesting that the antibody had effects on other joints despite intrasynovial administration into the hindpaws.

We found that arthritis severity scores for the anti-LAIR-1 treated group were significantly different from scores taken from mice treated with either the control hamster IgG or untreated. Taken together these data suggest that an antibody that stimulates LAIR-1 effectively attenuates autoimmune arthritis.

To confirm that the antibody does not deplete cell populations *in vivo*, two B6.DR1 mice were injected intraperitoneally with 0.5mg of the anti-LAIR-1 IgG and two days later, spleens were removed and tested for various cell populations using flow cytometry. As noted previously, in wild type mice, there were 22 ± 4% $CD4^+$ cells, 8.3 ± 1% $CD8^+$ cells, 60 ± 7% $CD19^+$ cells, as well as <5% of each of the $DX5^+$, $GR1^+$, $CD11b^+$, and $CD11c^+$ populations. After injection there were 20 ± 3% $CD4^+$ cells, 6 ± 3% CD8+ cells, 58 ± 4% CD19+ cells, as well as <5% of each of the $DX5^+$, $GR1^+$, $CD11b^+$, and $CD11c^+$ populations. These data demonstrate that the antibody did not induce a significant change in the numbers of lymphoid cells present *in vivo*.

DR1/LAIR$^{-/-}$ mice and collagen induced arthritis

In an effort to determine the extent to which LAIR-1 affects autoimmune arthritis *in vivo*, we obtained $LAIR^{-/-}$ mice and intercrossed them with B6.DR1 mice. Littermate controls

were selected that were either B6.DR1 LAIR-$1^{-/-}$ or B6.DR1 LAIR-$1^{+/+}$ and the mice were challenged with CII/CFA and observed for the development of arthritis. As shown in Figure 6, B6.DR1/$LAIR^{-/-}$ (KO) mice developed arthritis that was more severe with more arthritic limbs than B6.DR1 littermate controls ($p \leq 0.05$ beginning on day 35 after immunization), (Figure 5).

Splenocytes were collected from both groups of mice for cytokine analysis. The splenocytes were cultured with A2 peptide (3 μmol/ml) or no antigen, then supernatants were analyzed for cytokines. $LAIR^{-/-}$ mice were found to have greater Th1, Th2 and Th17 cytokine responses (IFN-gamma, IL-17A, IL-10 and IL-4) than $LAIR^{+/+}$ mice (Table I).

Antibody subclass analysis in immunized LAIR-$1^{+/+}$ and LAIR-$1^{-/-}$ mice

Since the absence of an important inhibitory receptor might have broad effects on the resulting autoimmunity, we tested whether antibody subclasses influenced by inflammatory cytokines vary between the $LAIR^{+/+}$ and the LAIR-$1^{-/-}$ mice. Serum samples were collected from each mouse six weeks after immunization with CII/CFA and analyzed for quantity of CII-specific Ab by ELISA (Figure 7).

When the mean antibody titers for each group of mice were examined, we found that the total collagen-specific IgG was significantly different between the two groups of mice, with IgG levels being significantly higher in the LAIR-$1^{-/-}$ mice. ($p = .001$). Although the $IgG2_c$ and IgG3 subclasses analyzed from the LAIR-$1^{-/-}$ sera showed a trend toward higher levels when compared with wild type controls, the most strikingly significant difference was noted in the $IgG2_b$ group, a subclass known to capable of fixing complement and influenced by IFN-γ ($p = 0.008$). Taken together these data confirm that the absence of the inhibitory LAIR-1 accentuates both arthritis and the complement-fixing $IgG2_b$ antibody subclass titers to collagen in the collagen-induced arthritis model following immunization with CII/CFA.

4. Discussion

The proper functioning of the immune system is based on a fine balance between activation and inhibition. Although immune suppression can lead to the threat of infection by pathogens and immunodeficiency, insufficient inhibition can lead to damage to self or autoimmunity (6). Inhibitory immune receptors play a crucial role in this balance. We have studied the inhibitory receptor, LAIR-1 and demonstrate that collagen stimulates LAIR-1 to suppress T cell cytokine responses. Furthermore, antibodies that stimulate LAIR-1 will suppress murine autoimmune arthritis if administered *in vivo* and mice genetically deficient in the LAIR-1 receptor mice develop more severe arthritis than wild type controls. These data suggest that LAIR-1 may be a potential therapeutic target for suppressing RA.

Collagen, an extracellular matrix protein, is the ligand for LAIR-1, unlike other ITIM-bearing receptors which tend to react with transmembrane proteins (1, 7). The discovery that collagens are functional ligands for LAIR-1 (8) revealed a novel role for collagen in the regulation of immune function. Subsequently C1q (which has a collagenous tail) has been reported to successfully stimulate LAIR-1 and cause immune suppression in monocytes and dendritic cells (9, 10). Collagens are the most abundant type of proteins in vertebrates and

the hallmark of collagens is their triple-helical structure. All collagens are composed of three polypeptide chains, termed α chains, that are characterized by repeating glycine-X-Y sequences. Amino acids in positions X and Y are often proline and 4-hydroxyproline (B), respectively (7). The integrity of the collagen triple helix is dependent on the hydroxylation of prolines by prolyl 4-hydroxylase (P4H) and investigators have demonstrated that LAIR-1 is more likely to bind to the collagen triple helix peptides containing multiple Gly-Pro-Hypro repeats. In general, the GPB content of the synthetic peptides appeared the dominant determinant for LAIR-1 binding, although other residues impact binding as well (11).

Although it has been previously believed that the tertiary structure of collagen was necessary to trigger LAIR-1, we found that denatured α chains of type I or type II collagen were as capable of activating LAIR-1 as their native counterparts (11). Lebbink and coworkers using designed synthetic trimeric peptides encompassing the entire triple-helical domain of human collagens II and III determined that smaller linear peptides with no tertiary structure had no effect on LAIR-1, while selected synthetic trimeric peptides could both bind and stimulate LAIR-1 at functional binding sites (11). These data suggest that although some tertiary structure of the collagen appears to be required for activation of LAIR-1, the complete triple helical structure is not necessary. Although investigators have demonstrated that collagen is the functional ligand for LAIR-1, we cannot rule out the possibility that the induction of LAIR-1 expression could be induced by collagen binding to other receptors, leading to activation of the cell and generation of LAIR-1 expression.

In contrast to typical inhibitory receptors, whose expression is restricted to one type of immune cell, LAIR-1 is found on many cells in the immune system. These cells include $CD4^+$ T cells, $CD8^+$ T cells, monocytes, granulocytes, dendritic cells, NK cells, NKT cells and a subset of B cells (5). Although multiple cells are likely involved in the pathogenesis of arthritis and stimulation of LAIR-1 undoubtedly has broad effects *in vivo*, we have chosen to focus on the $CD4^+$ T cell, because LAIR-1 can be upregulated on $CD4^+$ T cells, and $CD4^+$ T cells, especially Th1 and Th17 cells, play a prominent role in the initiation of systemic immune responses in RA. it has been shown that T cells are important in the pathophysiology of RA (12). The success of Abatacept also shows that targeting T cells are an effective therapeutic strategy in treating RA (13)

We demonstrate that LAIR-1 can be upregulated on the surface of the $CD4^+$ T cell following culture with collagen. This observation is novel, although other investigators have reported regulated surface expression of LAIR-1 following other types of stimulation of the T cell receptor (14). One group of investigators showed that *in vitro* stimulation of the TCR decreased LAIR-1 expression upon activation (14), while the opposite result was reported by Maasho and coworkers (i.e.T cell stimulation *in vitro* increased surface expression of LAIR-1) (15). In another study, naive $CD4^+$ and $CD8^+$ T cells and effector $CD8^+$ T cells had higher LAIR-1 expression than memory T cells (15).

Receptors that inhibit pro-inflammatory Th1 and Th17 cytokine production could potentially modulate autoimmune arthritis. Since T-cell inflammatory responses (IL-17A, IFN-γ and IL-2) were significantly inhibited by the addition of collagen to the cultures in the presence of LAIR-1, we chose to look at LAIR-1 stimulation *in vivo*. The wide array of

immunological tools available for mice makes murine models of RA useful tools to explore mechanisms of disease inhibition more rapidly and with greater sophistication. Antibodies to LAIR-1 were administered to mice *in vivo* and were found to significantly attenuate autoimmune arthritis. Our data suggests that this antibody is stimulatory because LAIR-1 is upregulated on T cell hybridomas following culture with anti-LAIR-1, although we cannot rule out the possibility that the antibody blocks LAIR ligands. Similarly, when LAIR-$1^{-/-}$ mice were crossed to B6.DR1 transgenic mice (arthritis susceptible haplotype) (4) and were immunized with CII/CFA, the LAIR-$1^{-/-}$/DR1 mice developed arthritis that is more severe, with more arthritic limbs than B6.DR1 littermate controls. Because LAIR-1 is found on many cells in the immune system, including $CD4^+$ T cells, $CD8^+$ T cells, monocytes, granulocytes, dendritic cells, NK cells, NKT cells and a subset of B cells (5) we believe that multiple cells are likely involved in LAIR-1-induced suppression of arthritis and that the in vivo effects undoubtedly involve multiple immune cells. Although our data on autoimmune arthritis clearly demonstrate that the absence of LAIR-1 causes more severe arthritis, other investigators have shown that C57Bl/6 LAIR-$1^{-/-}$ mice were not different from LAIR-$1^{+/+}$ mice regarding development of spontaneous autoimmune disease or exacerbation of development of colitis or EAE (5).

Given that the ligand for LAIR-1 is collagen, the antigen used in the CIA model, we recognize that other murine models of arthritis would be useful to analyze the importance of LAIR-1. In preliminary experiments we have shown that the spontaneous arthritis of IL-1 receptor antagonist (Ra) deficient mice (SAD) in which there is a genetic IL-1Ra **deficiency** on the BALB/c background (16), can be treated with a known LAIR-stimulating ligand (CII), and cause attenuation of arthritis (unpublished data). Although more work will be needed to determine that stimulation of LAIR-1 is the primary mechanism of action in these experiments, these data suggest that therapies directed at LAIR-1 may be broadly applicable in autoimmune arthritis.

Recently it has been observed LAIR-1 expression was significantly decreased in circulating $CD4^+$ T cells in RA patients compared to both OA patients and healthy individuals (14). These results suggest that manipulating the expression of LAIR-1 may be a novel way to improve the treatment of RA (14). Others have reported increased levels of LAIR-2 (a soluble LAIR-1 inhibitor) in rheumatoid arthritis (17). Correlations between downregulation of an inhibitory receptor and the predisposition for autoimmune diseases such as systemic lupus erythematosus (SLE), type 1 diabetes (T1D) and multiple sclerosis (MS) have also been reported (18). Similarly, disrupted LAIR-1–mediated immune silencing leads to a loss of self-tolerance in pDCs and B cells from patients with SLE (19). Although inhibitory receptors are thought to be important in balancing immune responses and the general assumption is that lack of inhibition predisposes for autoimmune diseases, much work remains to be done to establish the molecular pathways by which these phenomena occur and what other molecules are required.

In our study, the total collagen-specific IgG was significantly higher in the LAIR-$1^{-/-}$ mice compared to the LAIR-$1^{+/+}$ mice. Although the $IgG2_c$, and IgG3 subclasses analyzed from the LAIR-$1^{-/-}$ sera showed a trend toward higher levels when compared with wild type controls, the most striking differences were noted in the $IgG2_b$ group, a subclass known to

capable of fixing complement and influenced by IFN-γ. These results suggest that genetic deletion of LAIR-1 significantly enhances the inflammatory response. On the other hand, a trend towards lower serum levels of IgG1 were noted in the B6.DR1 LAIR-$1^{-/-}$ mice. Tang *et al* similarly reported that C57BL/6 LAIR $-1^{-/-}$ mice had lower serum levels of IgG1 compared to wild type mice (5).

In summary, we have found that the surface expression of LAIR-1 increases following stimulation with collagen. We hypothesize that the expression of LAIR-1 can be upregulated *in vivo* to influence the severity of autoimmune arthritis (20, 21) and that LAIR-1 can be stimulated *in vivo* to suppress T cell activity and inflammation in general. Treatment with either anti-LAIR-1 or the LAIR-1 ligand may give impetus to effectively reduce inflammatory cytokine secretion in T cells of patients with RA. On the other hand, decreased signaling by inhibitory receptors may lead to an over activation of the immune system contributing to, or even being the cause of, autoimmune diseases (22). We believe that therapies directed toward stimulating LAIR-1 hold great promise for downregulating unwanted inflammation (1) and that this natural inhibitory molecule can be exploited to suppress the tissue injury occurring in autoimmune arthritis.

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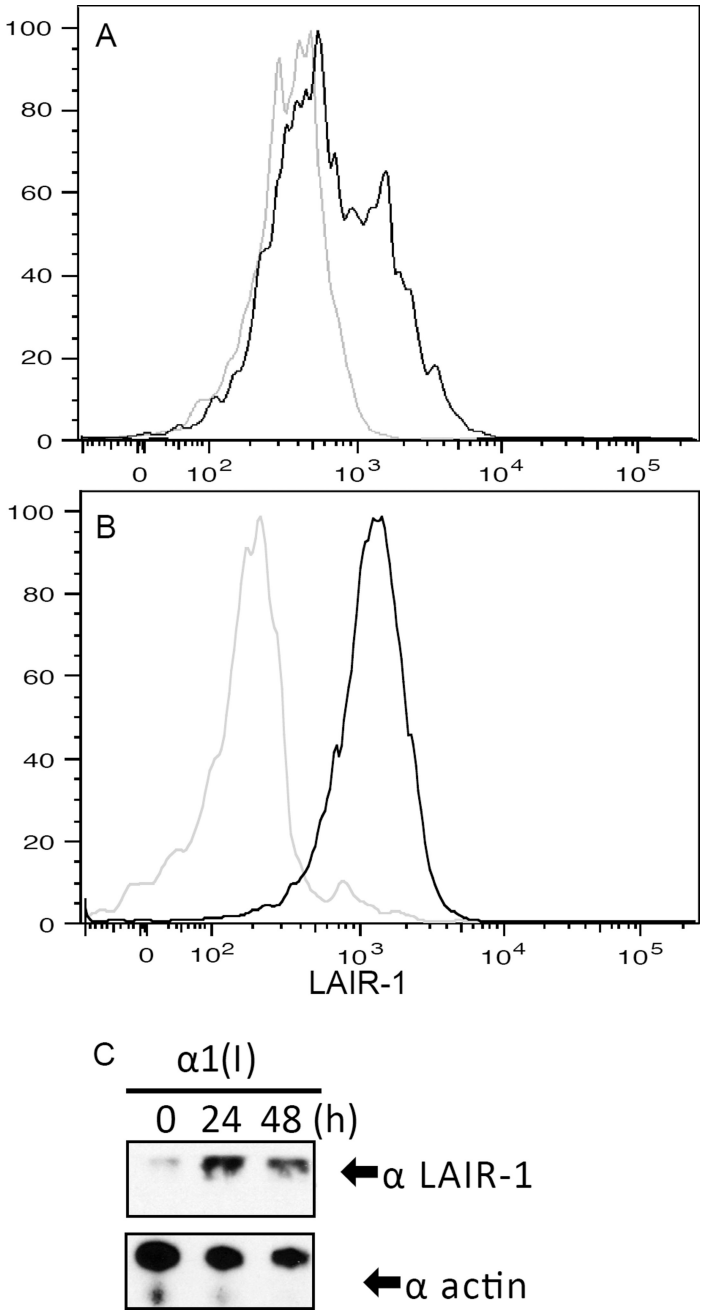

Figure 1. Induction of LAIR-1 in T cells

(Panel A) Naïve DBA/1 splenocytes were cultured with α1(II) for 24 hours. The expression of LAIR-1 was examined by flow cytometry using a PE conjugated anti-LAIR-1 antibody. Histograms are gated on $CD4^+$ T cells. The data are representative of three separate experiments. Mean fluorescence (MF) for cells cultured with media alone (light grey line) was 392±32 while MF for cells cultured with α1(II) (black line) was 1048 ± 45, $p \leq 0.001$ (Panel B) *Murine T hybridoma cells* (2×10^6/ml) were stimulated with type II collagen (α1(II), 50 μg/ml) for the indicated time period. The expression of LAIR1 was examined by

flow cytometry as in Panel A. Mean fluorescence = 1208 ± 53 compared to 185 ± 24 as a mean of three separate experiments.

(Panel C). *Induction of LAIR1 by type II collagen*. Murine T hybridoma cells (1×10^7/10ml) were stimulated with α1(II) (50 μg/ml) for the indicated time period. Samples were loaded and expression level of LAIR1 was examined with Western blot analysis using anti-LAIR-1. Actin was used as control. Data shown are representative of three separate experiments.

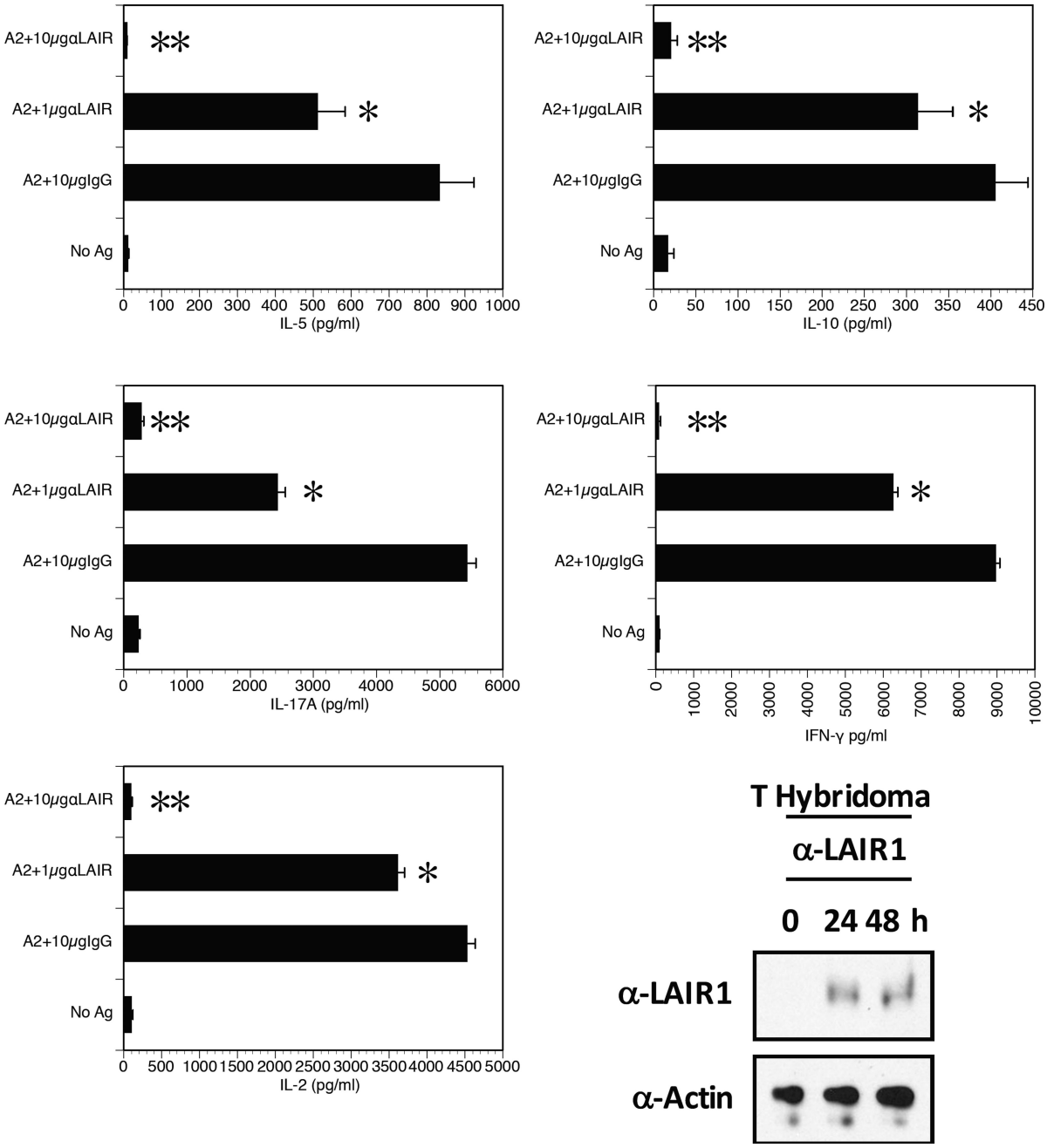

Figure 2. Suppression of the cytokine response by antigen stimulated T cells using an antibody which stimulates LAIR-1

Splenocytes from CII-immunized B6.DR1/LAIR$^{+/+}$ mice were cultured with the A2 peptide (3μmol/ml) or no Ag, in the presence of the indicated concentrations of either hamster IgG or a monoclonal antibody to LAIR-1. Values indicated represent the mean ± SEM (pg/ml) of three separate experiments. *p ≤ 0.01, **p≤ 0.001. Cytokine responses from cells cultured with A2 peptide in the absence of antibody were not different from data obtained with control IgG.

(Lower right panel) Murine T hybridoma cells (1×10^7/10ml) were stimulated with anti-LAIR-1, 1 mg/ml) for the indicated time period. Samples were loaded and expression level

of LAIR1 was examined with Western blot analysis using anti-LAIR-1. 150 mg of sample per lane was loaded and expression level of LAIR1 was examined with Western blot analysis using anti-LAIR1 antibody (produced in Armenian Hamster, 1:500 dilution). Actin was used as control. These data are representative of three separate experiments and confirm that the antibody is not cytotoxic, but instead increases the LAIR-1.

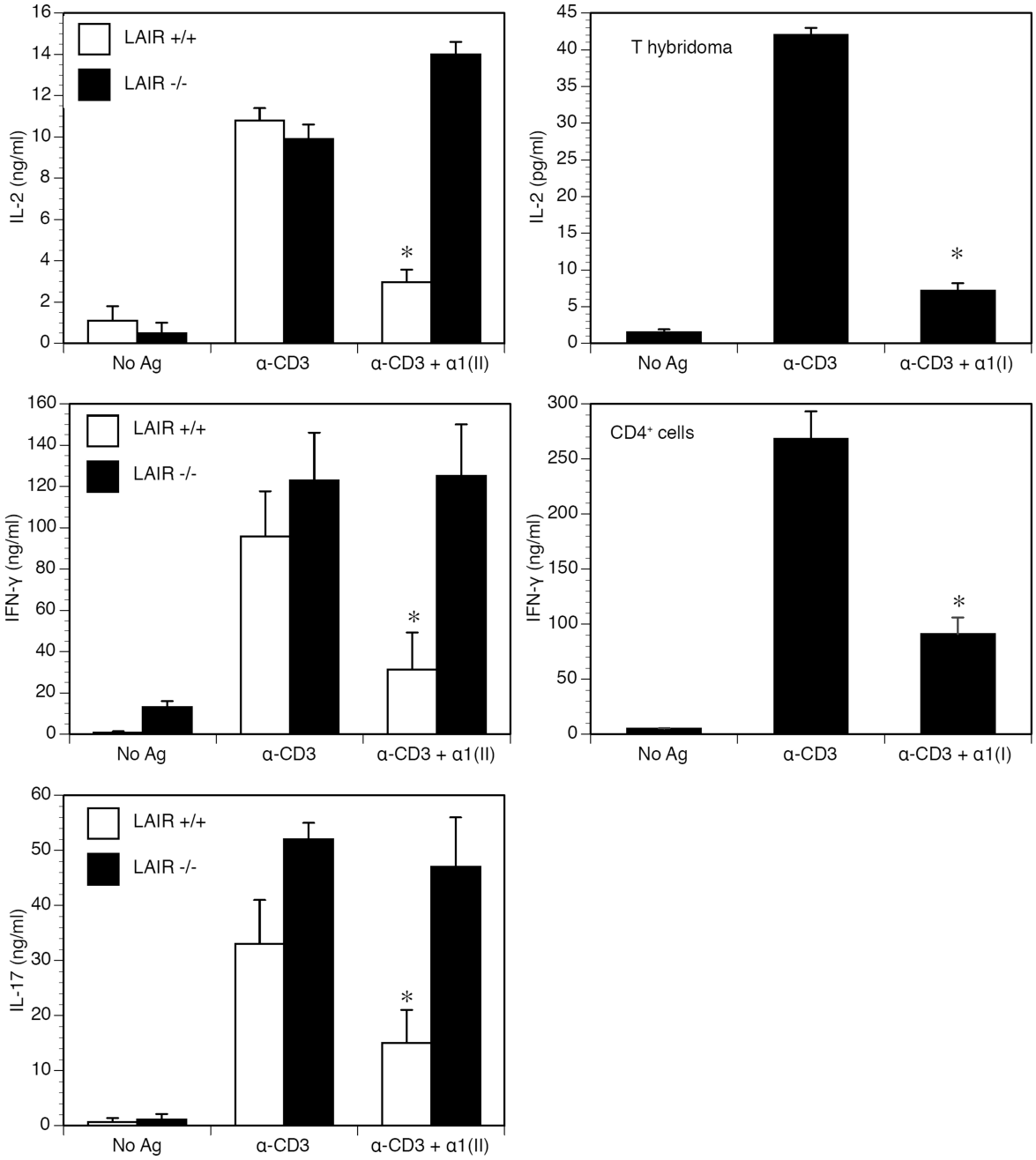

Figure 3. In vitro analysis of LAIR-1 mediated cytokine suppression

Splenocytes from naïve LAIR$^{+/+}$ and LAIR$^{-/-}$ mice were cultured for 24hrs with and without α1(II) and stimulated with soluble anti CD3 antibody for another 24 hrs. Similarly T hybridoma cells and purified CD4+ T cells from from naïve LAIR$^{+/+}$ were cultured for 24hrs with and without α1(I) and stimulated with soluble anti CD3 antibody for another 24 hrs. The supernatants were collected and the concentration of cytokines was measured using Multiplex ELISA. The data shown represent the means of three separate experiments. CD3 responses of wild type cells when compared with and without collagen are significantly different, * p≤ 0.001 for IFN-γ, IL-17A, and IL-2.

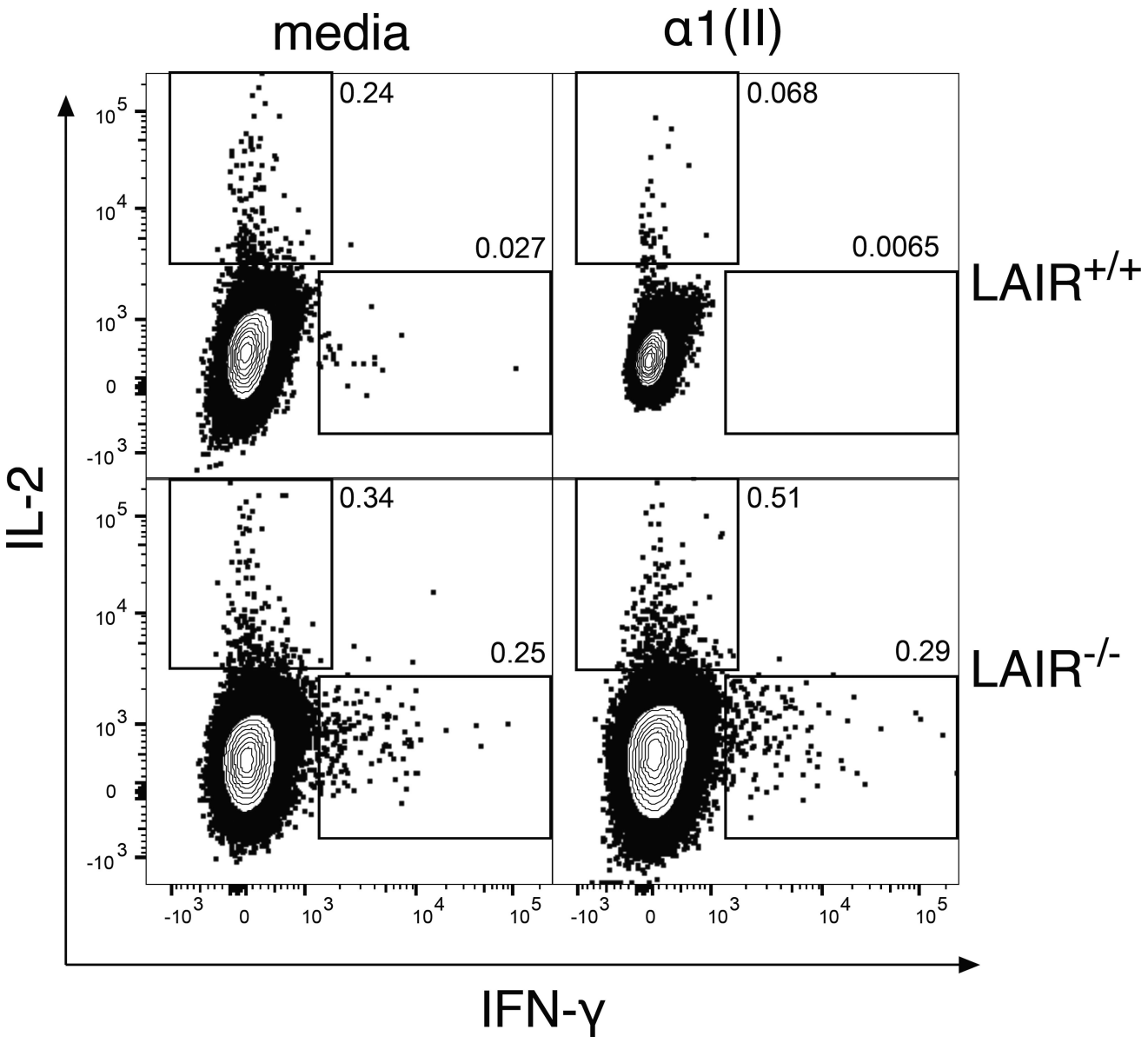

Figure 4. Flow cytometric analysis of LAIR-1-mediated cytokine suppression

Splenocytes from naïve LAIR$^{+/+}$ and LAIR$^{-/-}$ mice were cultured for 24hrs with and without α1(II), then stimulated with soluble anti CD3 antibody for another 24 hrs. Data shown are representative of six separate analyses and scatter plots are gated on CD4^{+} T cells.

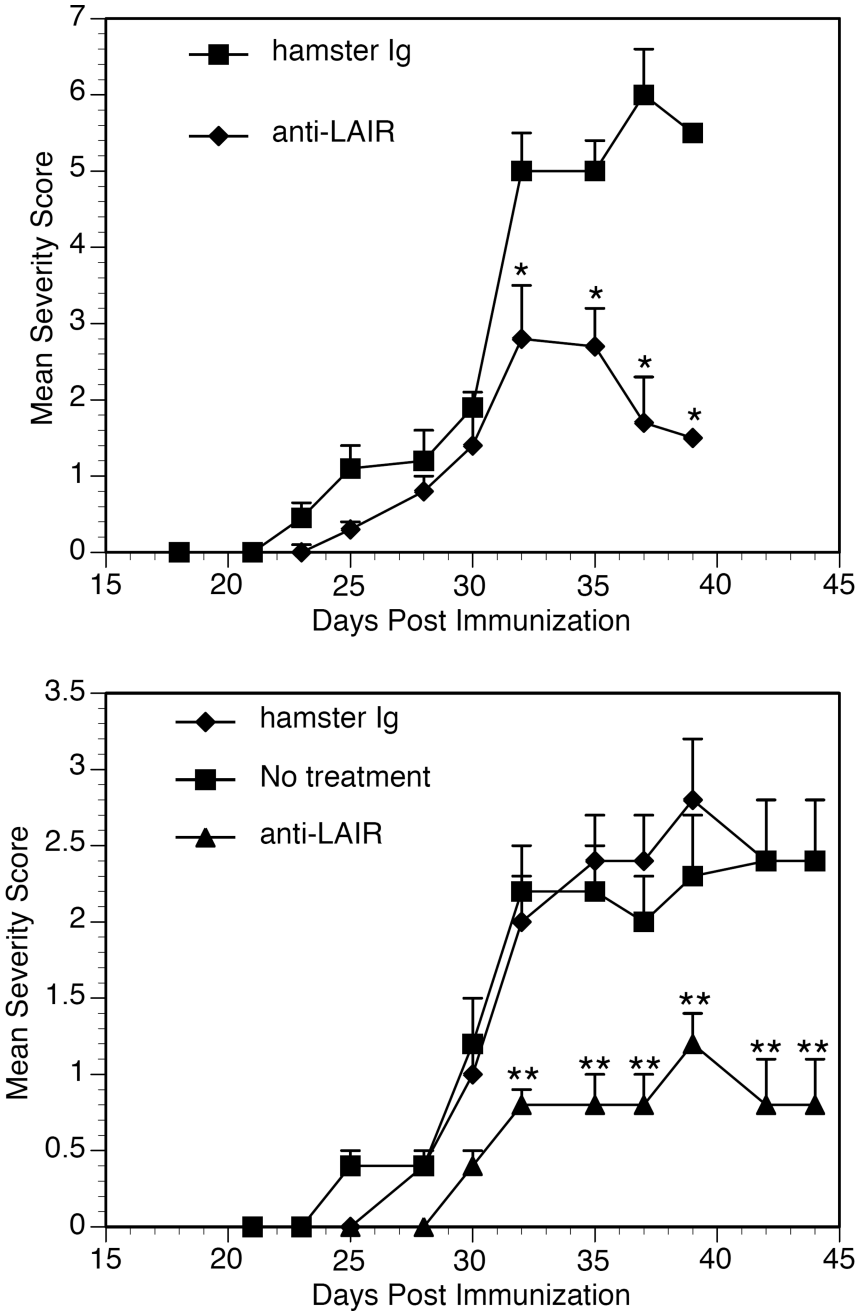

Figure 5. LAIR-1 can mediate suppression of CIA

(Upper panel) Two groups of littermate B6.DR1 mice were immunized with type II collagen/CFA. On days 1 and 11, each mouse was treated intraperitoneally with either 0.2 mg of a monoclonal IgG antibody stimulating LAIR-1 (diamonds, n=4), or 0.2 mg of a hamster IgG control (squares, n=7). On day 19 post immunization, each mouse was treated with 0.1mg of each antibody intraperitoneally. The mean severity scores were significantly different between the two groups beginning at day 32 (* = p≤ 0.05). An untreated control group was no different than the mice treated with the hamster IgG control (final severity scores 6.2 ± 2.2).

(Lower panel) In a second experiment, three groups of B6.DR1 mice were injected intrasynovially into each right ankle joint with either 10 μg of a stimulating antibody to LAIR-1 (diamonds n = 5) or 10 μg of the control hamster IgG (squares n = 5), or were untreated (circles, n = 8). Treatments were given on days 3, 7, 10, 14, 16, 21, and day 23 after immunization. Data shown are the mean severity scores of the right hindpaws for each group of animals. The arthritis severity scores for the LAIR-1 treated group were significantly different from both the hamster IgG scores and the untreated scores beginning day 32, $p \leq 0.005$.

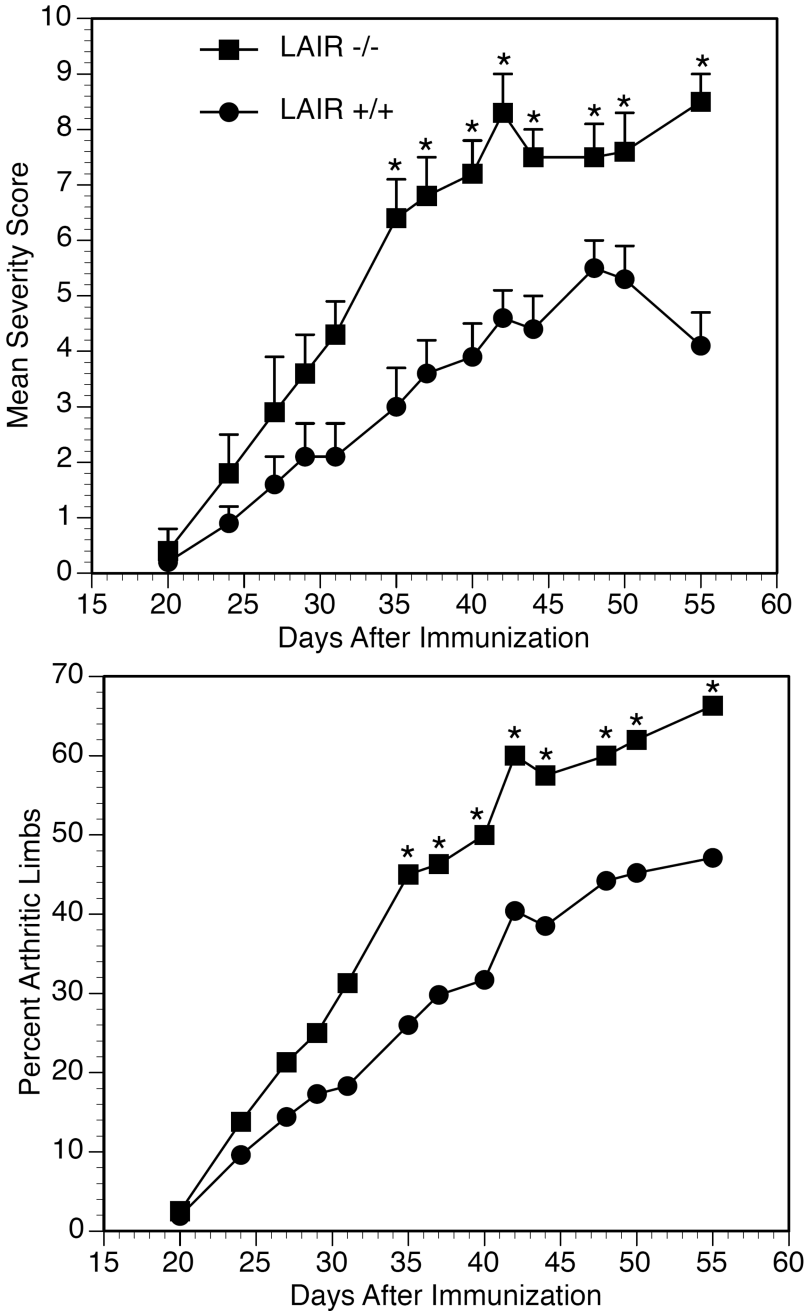

Figure 6. LAIR-1 −/− Mice have severe arthritis

B6.DR1 LAIR$^{-/-}$ mice and B6.DR1 wild type mice were immunized with CII/CFA; B6.DR1 littermate controls (diamonds, n=26) and B6.DR1/LAIR$^{-/-}$ (squares, n=20). Each mouse was scored three times weekly beginning 2 weeks post immunization. Our data shows that B6.DR1/LAIR$^{-/-}$ mice develop arthritis that is more severe (upper panel) beginning on day 35 after immunization (* $p \leq 0.05$). The LAIR$^{-/-}$ mice also have more arthritic limbs (lower panel) than B6.DR1 littermate controls beginning day 35, (* $p<0.05$). This data is representative of two independent experiments.

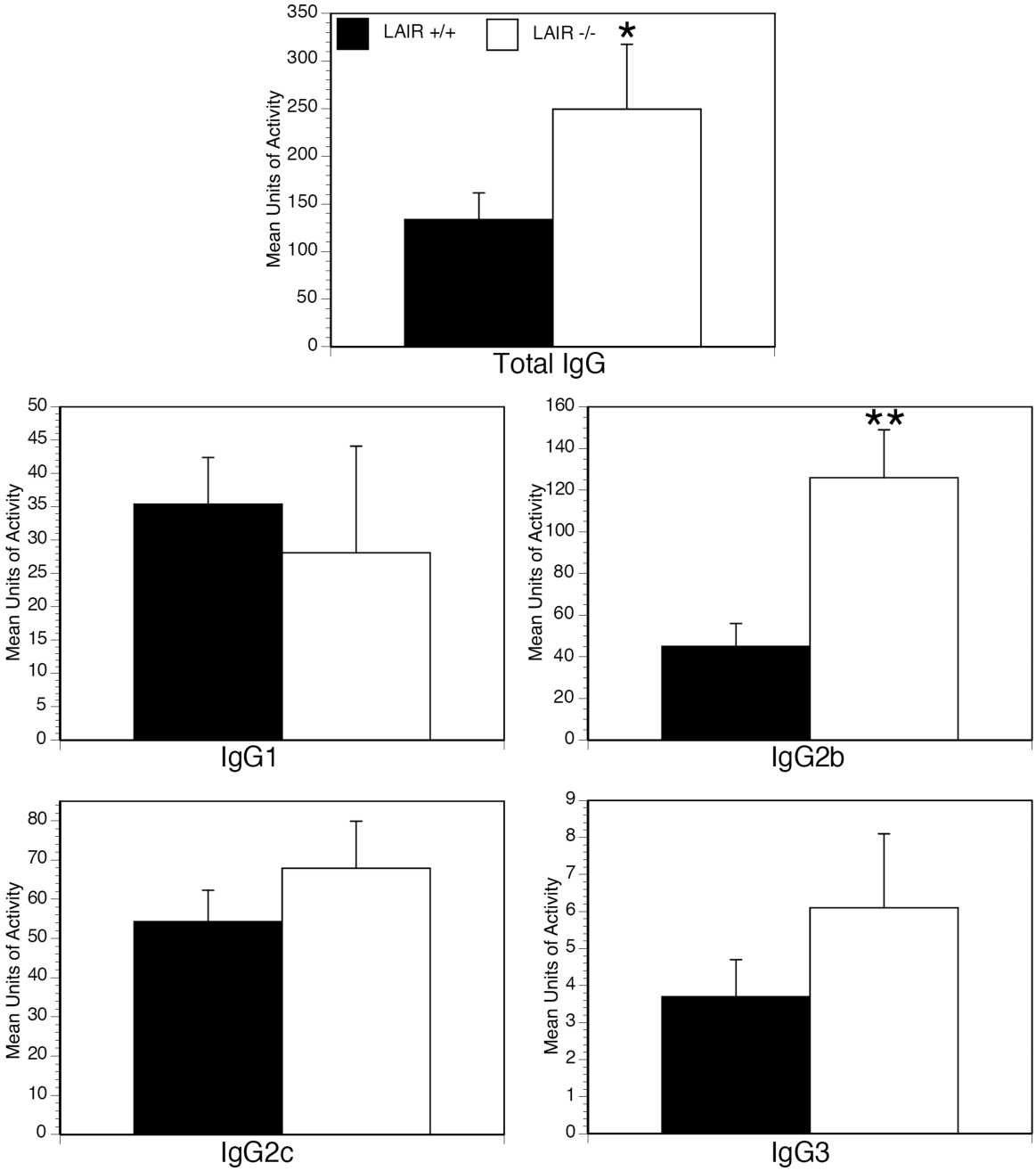

Figure 7. Production of Ab specific for CII by LAIR$^{-/-}$ compared to LAIR$^{+/+}$ Mice

LAIR$^{+/+}$ and LAIR$^{-/-}$ littermates were immunized with 100 μg of bovine CII emulsified in CFA as described in *Materials and Methods*. Serum samples were collected six weeks after immunization and analyzed for quantity of CII-specific Ab by ELISA. Units of Ab were calculated using standard reference sera. Data are expressed as mean titers per group (n=13 for LAIR$^{-/-}$ mice, n=23 for LAIR$^{+/+}$ mice) ± SEM. This data is representative of two independent experiments.

Table I

Cytokine responses in arthritic LAIR −/− mice

Splenocytes from CII-immunized B6.DR1/LAIR$^{+/+}$ and B6.DR1/LAIR$^{-/-}$ mice were cultured with A2 peptide (3μmol/ml) or no Ag, then supernatants were analyzed for cytokines. LAIR$^{-/-}$ mice were found to have greater Th1, Th2 and Th17 cytokine responses than LAIR$^{+/+}$ mice.

Cytokines pg/ml					
		IFN-γ	**IL-17A**	**IL-4**	**IL-10**
LAIR$^{+/+}$	No Ag	130 ±20	218±24	3±2	16 ± 6
	A2 Peptide	2,004±77	12,209±181	15±7	59 ± 2
LAIR−/−	No Ag	126±17	59±25	3±3	11 ± 4
	A2 Peptide	21,161±70 *	20,595 *	48 ± 7 *	631 ± 15 *

Values indicated represent the mean ± SEM (pg/ml) of three separate experiments.

* $p \leq 0.01$