LAG-3 is not responsible for selecting T helper cells in CD4-deficient mice

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Abstract

The product of the LAG-3 gene is a cell surface protein with significant homology to CD4. It has been suggested that it can serve as a functional equivalent of CD4 and account for the MHC class II-restricted responses which persist in CD4-deficient mice. To test this hypothesis, we have created CD4/LAG-3 double-deficient mice by successive homologous recombinations in embryonic stem cells. These animals turn out to be indistinguishable from CD4 single-deficient mice in their lymphocyte populations and responses that are controlled by MHC class II molecules. LAG-3 thus does not explain class II-restricted lymphocyte selection and function in the absence of CD4, strengthening the idea that these phenomena can occur independently of co-receptor signalling.

Introduction

Expression of the CD4 glycoprotein on a subset of T cells is highly correlated with recognition of antigens in the context of class II molecules of the MHC (1). Several lines of evidence point to a critical role for CD4 as a co-receptor in class II-mediated events. It plays a central role in positive selection of class II-restricted T cells in the thymus (2-4) and is also important, although not indispensable, in class II-mediated activation events in peripheral lymphoid organs and in T helper cell interactions with B cells (2).

There was some surprise, then, at results on CD4-deficient mice created by homologous recombination in embryonic stem (ES) cells (5,6). These animals had a sizeable population of double-negative (DN) CD4-CD8-TCRαβ+ T cells, actually larger than that of wild-type animals. This population was dependent on MHC class II molecules and was responsible for the significant class II-restricted T cell activity present in the mutant mice: proliferative response, help-dependent antibody production against virus or protein antigens and IFN-γ-mediated clearance of Leishmania infection (5,6).

What permits these class II-restricted, yet CD4-independent, cells to arise and function? Two possibilities were proposed. First, these cells might express receptors with relatively high affinity for their ligand, allowing CD4-independent selection and function. CD4-independent T cell activation had been reported previously and attributed to high-affinity receptors (7); positive selection in the absence of CD4 could be accounted for in this fashion by invoking a stochastic model of T cell development, but is not compatible with a strict instructional model. The alternative interpretation was that another molecule complemented the function of the absent CD4, a redundancy with some precedent in gene knockout experiments.

The LAG-3 molecule (8,9) was proposed as a CD4 substitute (5,6). LAG-3 is a transmembrane glycoprotein which belongs to the Ig superfamily. LAG-3 and CD4 have a similar structure, with four Ig-like extracellular domains, and show significant similarity in amino acid sequence (8,9). The two genes map very close to each other in the human and mouse genomes, and have a highly resemblant intron-exon structure (8; T. Miyazaki et al., in preparation). Furthermore, Baixeras et al. provided evidence that human LAG-3, like CD4, might engage in low-affinity interactions with MHC class II molecules (9). Thus, LAG-3 made an appealing candidate for complementing CD4 and explaining the partial phenotype of the CD4-knockout mice (5,6). We decided to explore this possibility by creating CD4/LAG-3 double-mutant mice.

Methods

Mice

We have described elsewhere mice carrying a knockout mutation of the LAG-3 gene (10). These mice derive from clone KE6 of ES cells, in which the LAG-3 gene was disrupted by recombination, leading to the insertion of a neomycin resistance cassette in lieu of exons 1-3 (Fig. 1A). The mutation
which one allele of the LAG-3 gene had been already disrupted, in which a 0.8 kb Kpnl-EcoRI fragment including a part of exon 5 is replaced by a PGK-Hyg cassette (shown as a black box) (12). Wavy lines denote plasmid sequences (pBluescript). The mutated gene resulting from the homologous recombination event is schematized on the bottom line. The SacI-BglII probe used for Southern blot analysis is shown, as are the 6.9 and 5.9 kb SacI fragments which hybridize to this probe, in wild-type and mutant genomic DNA respectively. Restriction enzymes: B, BamHI; C, ClaI; G, BglII; H, HindIII; K, KpnI; P, PstI; R, EcoRI; S, SacI; X, Xhol. (D) Southern Blot analysis of the CD4 allele. DNA was prepared from ES cell clones after electroporation of the construct shown in (C, middle line), digested with SacI and probed with 0.9 kb SacI-BglII fragment (C). The 6.9 kb wild-type band was replaced by a 5.9 kb mutant band in one of the six clones depicted here.

**Diagnosis of CD4 gene**

To construct the CD4 targeting vector carrying the hygromycin-resistant gene (Hyg') fragment, we used the CD4 targeting vector previously constructed by Killeen et al. (11) and modified it by replacing the neomycin resistance gene by a Hyg' fragment. Briefly, a 0.6 kb Kpnl-EcoRI fragment, including the neomycin resistance gene and part of the CD4 exon 5, was excised from plasmid pCD4K7/2BTK and replaced by a 1.2 kb ClaI-BglII fragment from plasmid pH458 (12) which contains the Hyg' coding region driven by the PGK promoter. The resulting constructs are schematized in Fig. 1(C, middle line). ES cells (2×10^5) from K6 clone, in which one allele of the LAG-3 gene had been already disrupted by homologous recombination (see above), were transfected by electroporation of 15 μg Sspl linearized CD4 targeting vector in 500 μl of ES culture medium at 400 V/125 μF. After electroporation, cells were seeded on 10 cm gelatinized dishes in standard ES culture medium: DMEM (high glucose) supplemented with 1 mM sodium pyruvate, 0.4 μg/ml gentamycin, 15% FCS, 1000 U/ml LIF and 0-1 mM β-mercaptoethanol. The source of LIF was culture supernatant of COS cells transfected with LIF expression plasmid pC10-GR DIA/LIF (13). Selection with 150 μg/ml hygromycin was imposed after 48 h. Surviving colonies were transferred into 48-well gelatinized plates after 10 days, at which time selection was removed. After expanding each colony, screening for homologous recombination was performed by Southern blot analysis after a SspI digest, probed with the 0.9 kb SacI-BglII fragment (Fig. 1C). The identification of one such homologous recombination event is depicted in Fig. 1(D).

Four targeted ES clones were injected into 5.5-day-old C57BL/6 blastocysts as described (14). Male chimeric mice were mated to C57BL/6 females. The CD4 and LAG-3 mutations were transmitted and co-segregated in the progeny from one line, indicating that two mutations had taken place.

**Fig. 1.** Gene disruptions. (A) Disrupted LAG-3 gene. The wild-type LAG-3 gene is shown on the top line, above a representation of the disrupted gene, in which a neomycin resistance gene (slashed box) replaces exons 1-3. Note that we have not identified the 5' and 3' ends of LAG-3 mRNA. Exons 4-6 are located in a 5.8 kb XhoI-HindIII fragment, but have not been mapped accurately within this fragment. The position of the probe used for Southern blot analysis is indicated, as are the 6.4 and 5.2 kb BamHI fragments which hybridize to this probe, with wild-type and mutant DNA respectively. (B) Southern blot analysis of the LAG-3 knockout mutation. BamHI-digested tail DNA from a set of mice was probed with the BamHI/HindIII 3.5 Kb fragment as shown in (C). (C) Disruption of the CD4 gene. A partial restriction enzyme map of the CD4 gene is presented on the top line, in the region of exons 4-6 (boxed). The second line represents the construct used to disrupt the gene, in which a 0.3 kb Kpnl-EcoRI fragment including a part of exon 5 is replaced by a PGK-Hyg cassette (shown as a black box) (12). Wavy lines denote plasmid sequences (pBluescript). The mutated gene resulting from the homologous recombination event is schematized on the bottom line. The SacI-BglII probe used for Southern blot analysis is shown, as are the 6.9 and 5.9 kb SacI fragments which hybridize to this probe, in wild-type and mutant genomic DNA respectively. Restriction enzymes: B, BamHI; C, ClaI; G, BglII; H, HindIII; K, KpnI; P, PstI; R, EcoRI; S, SacI; X, Xhol. (D) Southern Blot analysis of the CD4 allele. DNA was prepared from ES cell clones after electroporation of the construct shown in (C, middle line), digested with SacI and probed with 0.9 kb SacI-BglII fragment (C). The 6.9 kb wild-type band was replaced by a 5.9 kb mutant band in one of the six clones depicted here.
on the same chromosome. Animals carrying this chromosome were intercrossed to produce homozygous null mice. Screening of mice for the CD4 mutation was achieved by PCR (using primers AGCTTTCGATGGAGGGCGGT and AACCAGCCTCGTCTGGCTAAGA, which amplify a 290 bp fragment of the Hpy1 gene) and for the LAG-3 mutation by Southern blot (Fig. 1B).

**Antibodies and flow cytometry**

Thymus or spleen cells were stained with saturating levels of mAb and analysed using a ELITE flow cytometer as described (15).

**T cell priming assay in vivo**

Mice were immunized in the rear footpads with 50 μg chicken ovalbumin (OVA) emulsified in complete Freund’s adjuvant (CFA). After 10 days, popliteal draining lymph node cells were harvested and challenged in culture with varying doses of OVA. Proliferation was assessed by incorporation of [3H]thymidine in the last 6 h of a 4 day culture period.

**T-dependent antibody production in vivo**

Mice were immunized with 50 μg keyhole limpet haemocyanin (KLH) in CFA (i.p.) and boosted 21 days later with 10 μg KLH in PBS (i.p.). Seven days after the boost, sera were collected and the anti-KLH antibody evaluated by ELISA with an anti-lgG second-step reagent (Jackson Immunoresearch, West Grove, PA) as described (16).

**Results and discussion**

To explore the function of murine LAG-3, we had previously generated mutant mice lacking this gene; LAG-3 expression was abolished in these mice but they exhibited, as judged from multiple analyses, normal MHC class II-restricted immune functions (10).

To determine whether LAG-3 was indeed substituting for CD4 in the CD4-deficient animals, we decided to create LAG-3/CD4 double-knockout mice. Because the genes are very closely linked, the usual approach of crossing the single mutants to generate a double mutant was not feasible. Therefore, we elected to mutate the CD4 gene in ES cells in which the LAG-3 gene had already been disrupted by homologous recombination. The hygromycin resistance gene was inserted within a segment of the cloned CD4 gene, in the same configuration as used by Killeen et al. (11) in their initial mutagenesis. This plasmid DNA was then electroporated into the ES clone KE6 (10), in which the LAG-3 gene had already been disrupted by recombinational insertion of the neomycin resistance gene. Four of the resulting 130 hygromycin-resistant colonies were found to have undergone the desired homologous recombination event at the CD4 locus. The hygromycin-resistant colonies were intercrossed to produce homozygous null mice. Seven days after the boost, sera were collected and the anti-KLH antibody evaluated by ELISA with an anti-lgG second-step reagent (Jackson Immunoresearch, West Grove, PA) as described (16).

**Fig. 2.** Increased DN TCRβ+ population in CD4° as well as LAG°CD4° mice. (A) Lymph node cell suspensions were prepared from wild-type (heterozygote), CD4-deficient and LAG/CD4-double-deficient mice, and were stained with reagents specific for CD4, CD8 and TCRβ, and analysed by flow cytometry. The three panels display the CD4/CD8 profiles after gating on TCRβ+ cells, all for 50,000 total lymphocytes. (B) Quantitative measurements from several such stainings of lymph node or spleen cells. For lymph node (LN, left), each bar represents the proportion of DN cells among TCRβ+ cells in an individual mouse. For spleen, we calculated the absolute number of CD4°CD8° TCRβ+ cells per spleen.

The double-mutant line with the original CD4 single-mutant line (11), thus yielding LAG°CD4° mice, as well as phenotypically wild-type controls.

We first quantitated, by three-color flow cytometry, the size of the DN TCRβ+ population, observed to be expanded in previous studies of CD4 single-deficient mice. As expected and illustrated in Fig. 2(A), we did find a marked increase in the population of TCRβ+CD4+CD8− cells in CD4° animals. Such an increase was also detected in LAG°CD4° animals. Measurements from several such experiments are tabulated in Fig. 2(B): it is clear that the prevalence of TCRβ+CD4°CD8− cells is not affected by the absence of LAG-3, both in relative proportion of cells (in lymph node) and in absolute numbers (in spleen).

These observations were extended to the thymi of the same set of mice. As shown in Fig. 3, there was an increased population of DN TCRβ+ cells in thymi of CD4° as well as LAG°CD4° animals.

We then evaluated the capacity of the mutant mice to mount MHC class II-restricted responses, using an assay known to give completely negative results in class II-deficient mice (14) but positive results in CD4° animals, albeit at reduced levels (4−6): mice were challenged with KLH to explore their capacity to mount an antibody response to a T-dependent antigen (Fig. 4B). Here again, a reduced but very significant response...
was observed in the mutant mice—both the CD4\textsuperscript{0/0} and LAG\textsuperscript{0/0}CD4\textsuperscript{0/0} animals. These experiments indicate, then, that class II-dependent B cell help (which implies T cell priming as well as class II-restricted T–B collaboration) is maintained in the LAG\textsuperscript{0/0}CD4\textsuperscript{0/0} as efficiently as in the CD4\textsuperscript{0/0} mutants.

The results from our analyses thus give a clear, if negative, answer to the question posed at the outset: LAG-3 does not substitute for CD4 and is not responsible for the residual class II-selected and restricted cells and immune functions that persist in CD4\textsuperscript{0/0} mice. We did not formally demonstrate the class II-dependence of the DN TCR\textsuperscript{β+} cells observed in the LAG\textsuperscript{0/0}CD4\textsuperscript{0/0} mice, as had been done with CD4\textsuperscript{0/0} animals \cite{5,6}. However, the cell-type distributions and immune response patterns are too similar in CD4\textsuperscript{0/0} and LAG\textsuperscript{0/0} CD4\textsuperscript{0/0} mice to leave any doubt in this respect.

Of course, the possibility remains that another, hitherto unidentified, molecule has a function redundant to that of CD4. Yet, by removing from contention the only plausible candidate proposed to date, we have rendered more likely the alternative interpretation, that the increased population of DN cells with class II-restricted function results from co-receptor-independent thymic selection. These represent a substantial number of cells, ~20% of the CD4\textsuperscript{+} cells found in a normal mouse. The existence of such a mode of selection cannot readily be accounted for by instructional models of positive selection \cite{17-19}, which posit that the initial commitment to the CD4 lineage requires that the cell undergoing selection must sense the class of MHC molecule that engages its TCR, most readily through co-receptor co-engagement. It is, in contrast, fully compatible with stochastic views \cite{15,20-22}: selection into the CD4 lineage in the absence of co-engagement and signalling by the CD4 molecule would correspond to those cells whose receptor has enough intrinsic affinity for MHC targets to achieve selection without co-receptor participation. The observations are also compatible with the '50/50' models which have been recently proposed \cite{23,24} in which selection into the CD4 lineage would behave in a stochastic fashion, while commitment to the CD8 lineage would require strict instructional signals.

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Abbreviations

- CFA: complete Freund's adjuvant
- DN: double negative
- ES: embryonic stem
- KLH: keyhole limpet haemacytin
- OVA: ovalbumin
CD4/LAG-3 double knockout

References