Antigen-Specific Immune Tolerance Induced by Protein Antigens Conjugated with Polyethylene Glycol

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Summary

Protein antigens conjugated with polyethylene glycol (PEG) have been known to induce antigen-specific immune tolerance. To develop the application of PEG-protein antigen conjugate as a tool for immune intervention, the molecular and cellular mechanisms of peripheral immune tolerance induced by PEG-protein antigen conjugates need to be understood. We observed that the autoimmune-disease-prone New Zealand Black strain of mice is defective in that it does not develop the immune tolerance normally induced by PEG-ovalbumin conjugate. We applied the method of quantitative genetics to identify the critical gene responsible for the establishment of antigen-specific immune tolerance.

Introduction

Every living organism has a biological defense system against viruses and infectious microorganisms, which is called the "immune system". There are two types of immune systems, the innate immune system observed in all multi-cellular organisms, and the adaptive immune system found exclusively in vertebrate animals. The innate immune system eliminates pathogens by recognizing pathogen-associated molecular patterns. In contrast, the functions of the adaptive immune system are characterized by "specificity and memory", in which pathogens are recognized as non-self by highly sophisticated molecular machinery and are memorized to protect against repeated exposure. In vertebrates, two types of lymphocytes, T cells and B cells, are responsible for the adaptive immune system. These subsets of lymphocytes cooperate to produce antibodies that serve as highly specific molecular weapons to destroy the target pathogens. T cells and B cells need to be strictly controlled to avoid the auto-reactive immune response. Immune unresponsiveness towards particular molecular structures is called "immune tolerance". The adaptive immune system establishes immune tolerance towards "self" structures. The failure of self-tolerance leads to serious disorders. T cells discriminate between self and non-self structures and play a major role in establishing self-tolerance.

Ontogeny and differentiation of T cells are illustrated in Fig. 1 [1]. Immature lymphocytes

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committed to T cell lineage are differentiated from the hematopoietic stem cells in the bone marrow and enter into the cortex of the thymus. Subsequently, each T cell undergoes the somatic rearrangements of T cell receptor genes. Expression of the T-cell antigen receptor (TCR) molecules on the cell surface enables T cells to recognize antigenic peptides presented on the MHC molecules. A population of T cells with varieties of antigenic specificities is then subjected to the selection process. T cells with appropriate reactivity to the MHC plus self-peptides that are displayed on the thymic epithelial cells are positively selected. T cells with excess reactivity towards self-peptides are led to apoptotic cell death. This negative selection is the major cellular mechanism of the "central immune tolerance" established in the thymus. Subsequently, T cells in the thymus are further differentiated into $CD8^+$ cytotoxic T cells (Tc cells) and $CD4^+$ helper T cells (Th cells) based on their reactivity towards two types of MHC molecules, MHC-class I and MHC class II molecules, respectively.

The cellular and molecular mechanism of antibody production is illustrated in Fig. 2 [2]. A pathogenic microorganism is first trapped by the antigen-presenting cells (APCs), which are one of the constituents of innate immunity. Microorganisms are killed and digested into molecular components. Foreign peptides that originate from the pathogen are presented on the MHC molecule on APC, and then recognized by Th cells. B cells specific to the foreign molecule on the pathogen communicate with relevant Th cells. Induced by the stimulatory signals from Th cells, B cells then differentiate into plasma cells that produce a large amount of antibodies. This system is strictly regulated to avoid the production of harmful auto-reactive antibodies. This regulation is conducted by the mechanism of peripheral immune tolerance, the process of which, however, is not yet well understood.

The difficulty of studying the peripheral immune tolerance is caused by the lack of a suitable

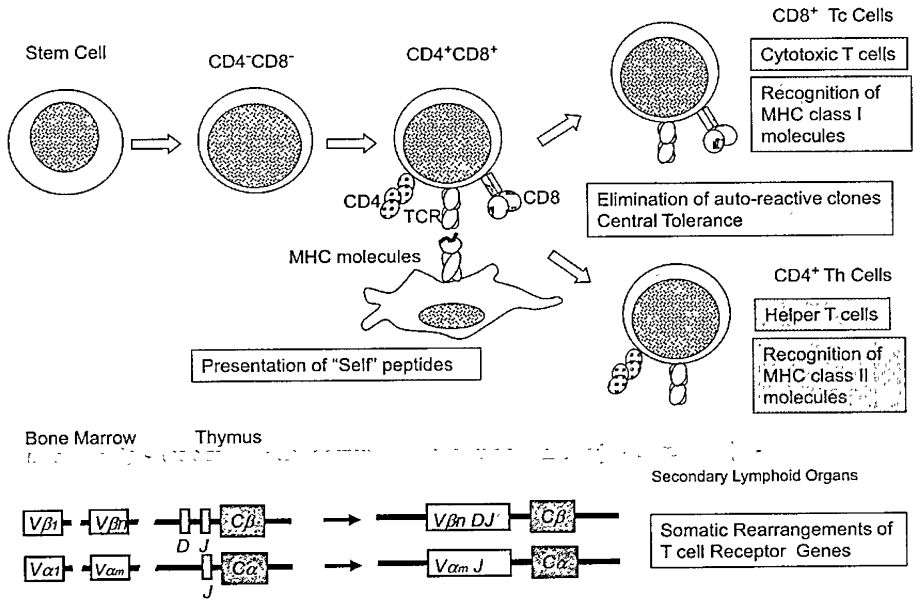

Fig. 1 Ontogeny and differentiation of T lymphocytes. Progenitor cells committed to T lymphocytes are generated from the hematopoietic stem cells in the bone marrow and enter into the cortex of the thymus, where differentiation and selection of the T cells occur (See text).

experimental model. We propose an approach to studying the mechanism of peripheral immune tolerance using tolerogenic PEG-protein antigen conjugates. Our proposal is a method of quantitative genetics applied to the defective immune tolerance induction observed in an autoimmune-prone strain of mice.

PEG-Protein antigen conjugate as a model immunological tolerogen

Polyethylene glycol (PEG) is a linear synthetic polymer produced by the polymerization of ethylene oxide. PEG has a structure in which hydrophobic ethylene units are repeatedly joined by polar ether bonds with high flexibility. PEG is uniquely soluble in both water and organic solvents. When introduced into the circulation of animals, PEG exerts no toxicity and no immunogenicity to elicit an immune response. It has long been known that protein antigens conjugated with PEG serve as immunological tolerogens when administered to experimental animals [3]. We have been using hen egg albumin (ovalbumin, hereafter OVA) as a model antigen and have been studying the antigen-specific immune tolerance induced in mice by the tolerogenic PEG conjugate of OVA(PEG-OVA, Fig. 3)[4,5].

In normal mouse strains such as C57BL/6, OVA is recognized as a foreign protein and elicits antibody production. In contrast, PEG-OVA does not elicit antibody production. Moreover, the administration of PEG-OVA to C57BL/6 mice induces OVA-specific immune tolerance. Figure 4 illustrates the experimental procedures used to demonstrate the tolerogenic capacity of PEG-OVA. A group of mice received three weekly intraperitoneal pretreatments with PEG-OVA and subsequently received a challenge with immunogenic unconjugated native OVA. As a control, another group of mice received three weekly intraperitoneal pretreatments with phosphate-

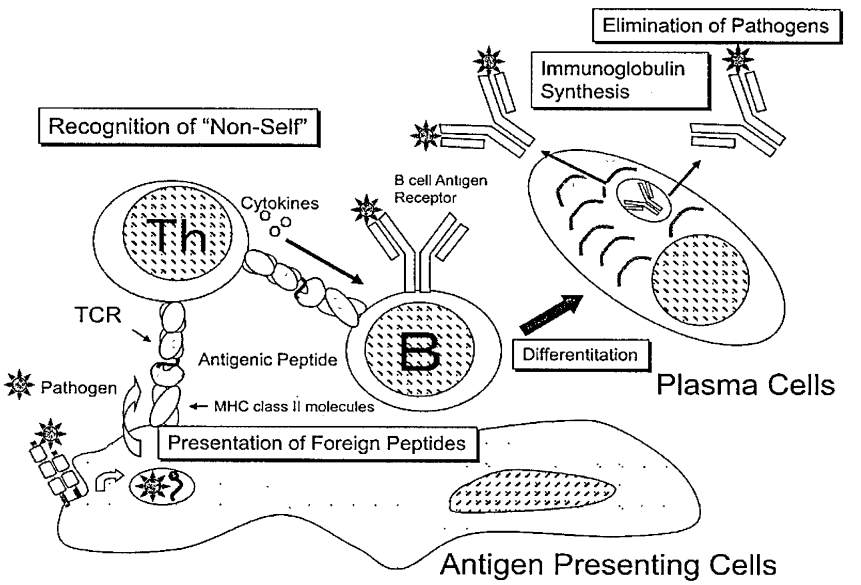

Fig. 2 Regulation of the antibody production in the peripheral immune system. Foreign peptides produced by the intracellular digestion of the microorganisms are presented by the MHC molecules on the surface of the antigen-presenting cells. Antibody molecules highly specific to the pathogenic microorganisms are generated by the plasma cells differentiated from B cells. Th cells are responsible for the regulation of the B cell differentiation. Immune tolerance of the Th cells to the self peptides avoid the production of autoreactive antibodies.

buffered saline (PBS). Figure 5 shows the time course of anti-OVA antibody production in the two groups of mice, those pretreated with PEG-OVA and those pretreated with PBS. In the control mice, the titer of anti-OVA reached a peak level exceeding 3 x 10^4 four weeks after the second immunization. However, in mice pretreated with PEG-OVA, the levels of the antibody were merely marginal as compared with the detection limit (< 10^2). It has been demonstrated that the effect of PEG-OVA is specific to the immune response to OVA. We prepared PEG-OVA with various degrees of modification with PEG and studied the immunogenicity and tolerogenic capacity of PEG-OVA. By increasing the degree of conjugation with PEG, the immunoreactivity of PEG-OVA to the anti-OVA antibody was decreased. It was possible to prepare PEG-OVA with

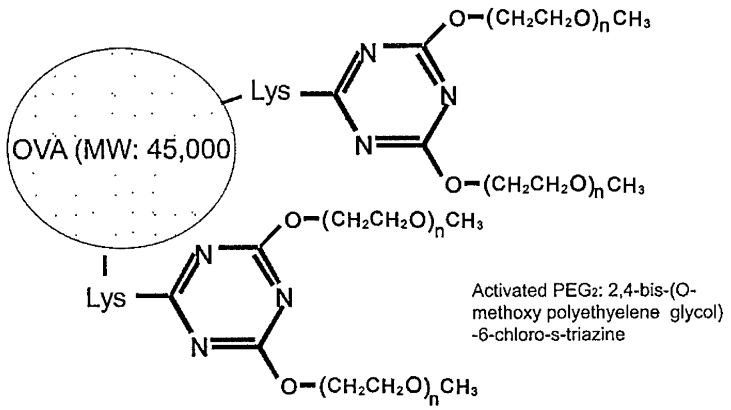

Fig. 3 Ovalbumin (OVA) conjugated with polyethylene glycol (PEG) as a model tolerogen. Polyethylene glycol (MW: 5,000) is conjugated with the amino groups of the lysine residues of ovalbumin via the triazine ring using the PEG-modifier, Activated PEG_2 [2,4-bis-(O-methoxy polyethylene glycol)-6-chloro-s-triazine].

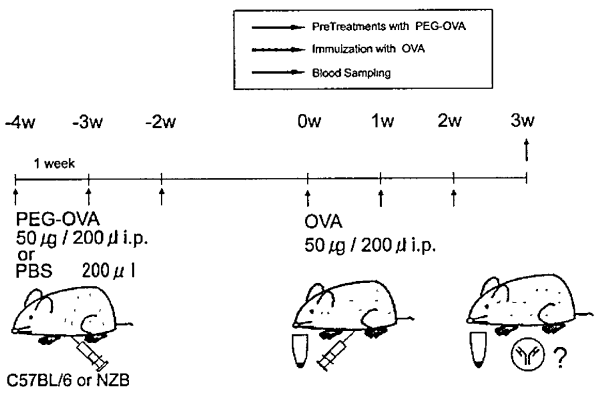

Fig. 4 Experimental model of peripheral immune tolerance induction. Two groups of mice receive weekly intraperitoneal injections of ovalbumin conjugated with polyethylene glycol (PEG-OVA). Two weeks after these treatments, mice are immunized with unconjugated OVA to stimulate the production of anti-OVA antibodies.

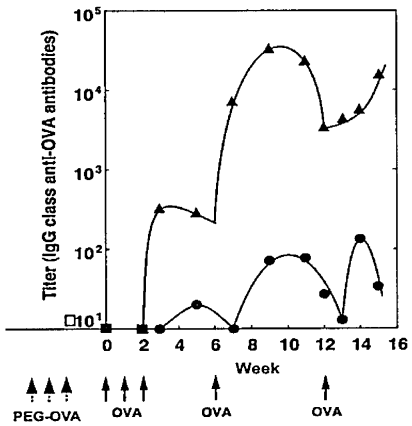

Fig. 5 Time cources of the production of anti-OVA antibodies in mice pre-treated with either ovalbunin conjugated with PEG (PEG-OVA) (▲) or phosphate-buffered saline (PBS) (●).

no reactivity towards the antibody. The loss of the ability to be recognized by anti-OVA antibody may be attributed to the loss of immunogenicity, i.e., the capacity to elicit an anti-OVA immune response. Nevertheless, the tolerogenic capacity of PEG-OVA did not correlate with the loss of immunoreactivity. There was an optimum degree of conjugation with PEG to attain the highest tolerogenic capacity. Tolerogenic PEG-OVA was found to retain the immunoreactivity to the anti-OVA antibody. This observation was intriguing since the irreversible denaturation of PEG-OVA with guanidine hydrochloride resulted in the loss of both immunoreactivity and the tolerogenic capacity of PEG-OVA [5]. Classical studies with mice suggested that OVA-specific Th cells were made tolerant by pretreatment with PEG-OVA. However, our studies on the tolerogenic capacity of PEG-OVA suggested the potential involvement of B cells in the establishment of peripheral immune tolerance as induced by PEG-OVA.

Defective immune tolerance in autoimmune-prone NZB mice

We observed that, in contrast to normal strain of mice such as BALB/c and C57BL/6, the autoimmune-disease-prone New Zealand Black (NZB) strain of mice did not develop the tolerance normally induced by PEG-OVA. NZB mice spontaneously produce anti-erythrocytes autoantibodies and have been studied as a model of autoimmune hemolytic anemia [6]. In NZB mice, pretreatment with PEG-OVA resulted in an enhanced immune response as demonstrated by the elevated titers of anti-OVA antibodies (Fig. 6). The results confirmed that PEG-OVA pretreatment induced tolerance against OVA in normal mice, whereas the same pretreatment enhanced the immune response, instead of tolerance, in autoimmune-prone mice. This observation is a clue identifying the key genetic element responsible for the establishment of peripheral immune tolerance.

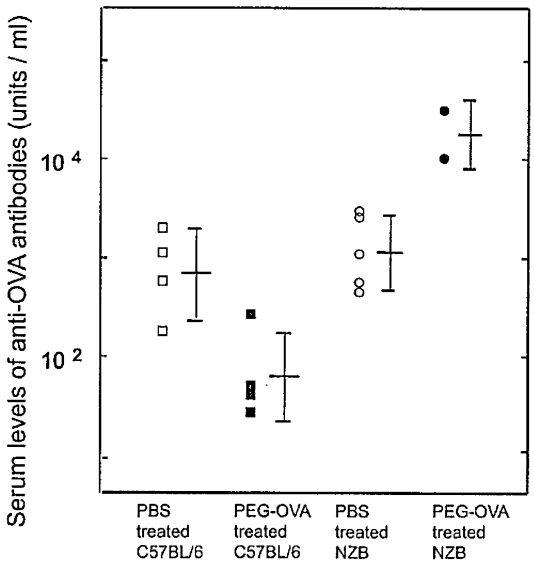

Fig. 6 Defective immune tolerance in New Zealand Black (NZB) mice. In normal C57BL/6 mice, pretreatment with PEG-OVA resulted in the reduction of anti-OVA antibodies as in Fig. 5. In the autoimmune-disease-prone NZB mice, the same treatment enhanced the titer of anti-OVA antibodies.

Analysis of responsible gene for defective tolerance in the progeny of F_2-intercross between C57BL/6 and NZB strains of mice

In order to identify the loci of the responsible genes, we crossed (C57BL/6 x NZB) F_1 hybrid to obtain a total of 191 female F_2 intercross mice. These mice were pretreated with PEG-OVA and subsequently immunized with OVA to test their capacity to induce tolerance. Figure 7 illustrates the distributions of the titers of anti-OVA antibodies in (C57BL/6 x NZB) F_2 intercross mice. F_2 intercross mice showed widely distributed titers. Some of the F_2 intercross mice had low or undetectable levels of antibodies showing the normal tolerance induction, while others showed abnormally high titers comparable to those observed in the parental NZB mice. Figure 8 illustrates a strategy of linkage analysis of the responsible gene in F_2 intercross mice. F_1 mice are heterozygote with respect to all the chromosomal polymorphic genes. In the process of meiosis to produce gametes in the F_1 hybrid mice, crossover of the chromatids occurs, resulting in the recombination of chromosomal genomic DNA. The resulting F_2 intercross mice are mosaic with respect to the genotypes of all the genes, either heterozygous or homozygous for the parental alleles. By studying the associations between the genotypes of the polymorphic microsatellite markers and the phenotypes of the F_2 intercross mice, the loci of the critical genes responsible for the defective immune tolerance in NZB mice will be revealed. Figure 9 shows the preliminary results of the genome-wide scan for the loci regulating the defective tolerance in F_2 intercross mice. Defective immune tolerance in NZB mice appeared to be controlled by multiple genes, and three markers showed potential linkage (data not shown). The highest lodscore was observed at the marker *D1Mit159* on the telomeric region of mouse chromosome 1.

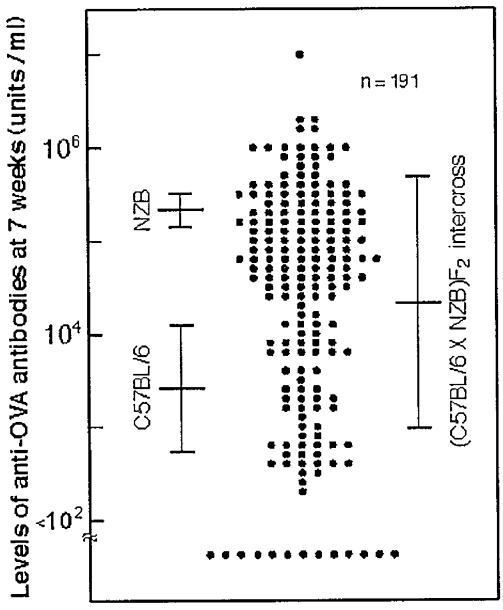

Fig. 7 Distributions of anti-OVA antibodies in (C57BL/6 x NZB) F_2 intercross mice. A progeny of 191 (C57BL/6 x NZB) F_2 intercross mice received three intraperitoneal pretreatments with PEG-OVA and subsequently immunized with un-conjugated OVA as in Fig. 6. Mean levels of anti-OVA antibodies observed in the parental C57BL/6 and NZB strains are shown by the horizontal bars together with the vertical bars showing the standard deviations.

Perspective of the future approach

It has been known for many years that PEG-protein antigen conjugates induce tolerance of Th cells specific to the protein antigen [3]. This observation once attracted wide attention. PEG-allergen conjugates may be used in the treatment of various allergic diseases. In gene therapy, PEG-conjugates may be useful in preventing problematic immune responses against the vector for gene delivery and against the products of introduced genes. Nevertheless, the lack of knowledge of the mechanism of Th cell tolerance as induced by PEG conjugates prevented their potential application as tools for immune intervention. As complex cellular interactions are involved in the regulation of immune response, conventional methods of cellular immunology have not been proven to be useful in uncovering the mechanism of peripheral immune tolerance.

We are taking a unique approach by focusing on the measurable quantitative phenotype, i.e., the level of antibody production, and by applying the method of quantitative genetics on F_2 intercross mice. The NZB strain of mice has long been studied due to its genetic predisposition to autoimmune reaction. The importance of this strain was supported by the finding that an F_1 hybrid between NZB and NZW develops severe autoimmune phenotypes typical of human systemic lupus erythematosus (SLE) [7]. There have been numerous linkage studies focusing on the autoimmune phenotype of NZB and related strains. However, the NZB gene that play the pivotal role in the autoimmune predisposition has remained unknown. The results of our preliminary genome-wide quantitative trait loci mapping demonstrated that the locus of the responsible gene was linked to a marker on the telomeric region of chromosome 1. The telomeric region of mouse chromosome 1 demands attention, as it is syntenic to the corresponding region

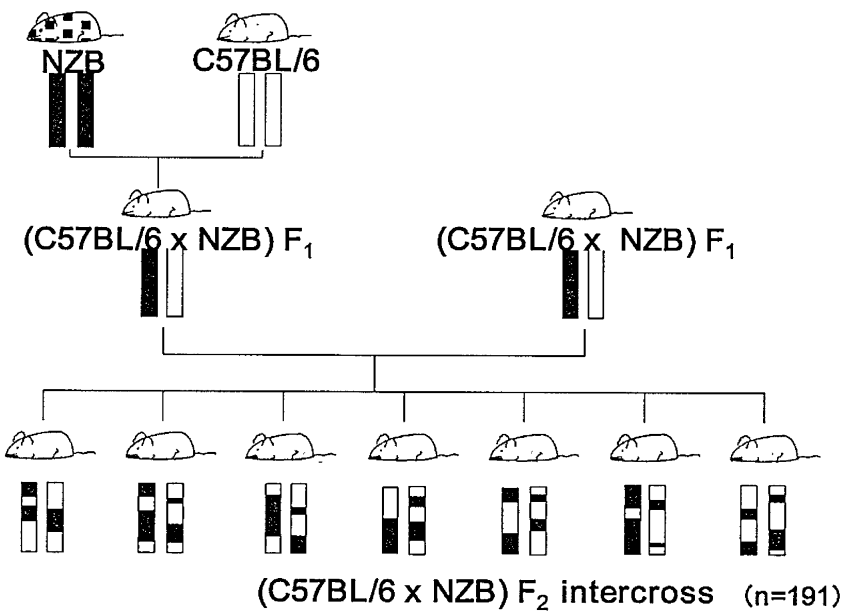

Fig. 8 Strategy of the genetic linkage studies on the defective immune tolerance in New Zealand Black (NZB) mice. A progeny of mice is generated by crossing the F_1 hybrid of normal C57BL/6 and autoimmune-disease-prone NZB strains. The resulting F_2 intercross mice are heterogeneous with respect to genotypes of all the chromosomal loci being either homozygous or heterozygous for the parental alleles.

of human chromosome 1, where numerous autoimmune-susceptible loci have been mapped by linkage studies [8]. We are currently trying to develop a method to handle large numbers of F_2 intercross mice in order to dissect the critical chromosomal regions involved in the regulation of peripheral immune tolerance. The results of our studies will not only lead to the application of PEG-protein antigen conjugates but will also contribute to the understanding of the genetic basis of autoimmune predisposition.

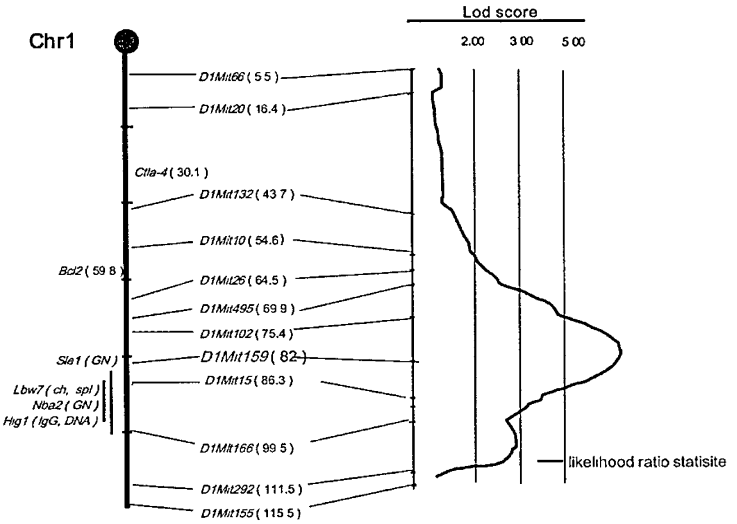

Fig. 9 Major chromosomal region linked to the defective immune tolerance of NZB mice as induced by PEG-OVA. The highest lodscore of genetic linkage was observed at the marker *D1Mit159* located on the telomeric region of mouse chromosome 1. The region of human chromosome 1 syntenic to this region is known to contain autoimmune susceptibility loci.

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