Mycoplasma Superantigen Is a CDR3-dependent Ligand for the T Cell Antigen Receptor

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Summary

Superantigens are defined as proteins that activate a large number of T cells through interaction with the Vβ region of the T cell antigen receptor (TCR). Here we demonstrate that the superantigen produced by Mycoplasma arthritidis (MAM), unlike six bacterial superantigens tested, interacts not only with the Vβ region but also with the CDR3 (third complementarity-determining region) of TCR-β. Although MAM shares typical features with other superantigens, direct interaction with CDR3-β is a feature of nominal peptide antigens situated in the antigen groove of major histocompatibility complex (MHC) molecules rather than superantigens. During peptide recognition, Vβ and Vα domains of the TCR form contacts with MHC and the complex is stabilized by CDR3–peptide interactions. Similarly, recognition of MAM is Vβ-dependent and is apparently stabilized by direct contacts with the CDR3-β region. Thus, MAM represents a new type of ligand for TCR, distinct from both conventional peptide antigens and other known superantigens.

In the first step of a specific immune response, T lymphocytes are activated by interaction between the TCR and peptide antigen bound to the MHC. Specific peptide recognition is achieved by the unique structure of TCRs, formed by somatic rearrangement of their composite gene segments (Vα, Jα, Vβ, Dβ, and Jβ). Most contacts with peptide are formed by very diverse CDR3 (third complementarity-determining region) regions that are encoded by the V-D-J junctional region (1, 2). During recombination, a second level of diversity is provided by the trimming and addition of nucleotides (N-additions) at the junctions (3). As a result of the huge diversity of TCRs created, one peptide antigen activates only a small number of T cells bearing specific receptors. However, several groups of microorganisms have developed a way to activate large numbers of T cells. They produce superantigens, potent stimulators of T cells, that form contacts with lateral surfaces of both the MHC and TCR. In superantigen recognition, the complexity of the TCR is ignored and interaction occurs between the Vβ region and the superantigen. In this way, a single superantigen can activate ~10% of all T cells (4–6).

Superantigens can be produced by bacteria, viruses, and even plants (7–9). Their biological role remains unclear, except for several mouse mammary tumor virus (MMTV) superantigens that are essential during the viral life cycle. By activating T and B cells in neonatal mice, MMTV superantigens form a pool of dividing cells that amplify the viral load and allow viral persistence until maturation of the main target site, the mammary tissue. The endogenous form of MMTV superantigen, acquired by integration of the viral gene into the mouse genome, has the opposite role. Mice expressing endogenous superantigens are protected from viral infection because of deletion of the superantigen-reactive T cell Vβ subsets in early development (10, 11). Other superantigens also cause proliferation and expansion of T cells with a responding Vβ phenotype, often followed by deletion of the targeted subset. It has been hypothesized that nonspecific stimulation of large numbers of T cells by superantigens could include self-reactive T cells and lead to autoimmunity (5). A recent report suggests involvement of a human endogenous retrovirus (HERV-K)-encoded superantigen in induction of autoimmune diabetes (12). Unlike MMTV-encoded superantigens that are products of the 3’ LTR, this superantigen seems to be encoded by the env gene. It activates the Vβ7+ subset of T cells, which was previously found to be enriched in pancreatic infiltrates of diabetic patients (13).

Abbreviations used in this paper: MAM, Mycoplasma arthritidis mitogen; MMTV, mouse mammary tumor virus; SE, staphylococcal enterotoxin.

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The superantigen produced by *Mycoplasma arthritides* (MAM) does not have significant homology to the primary sequence of other known bacterial or viral superantigens (14). Nevertheless, MAM shares several features common to many superantigens, including sequence presentation by MHC class II to responding CD4 and CD8 T cells, lack of restriction of the presenting MHC class II alleles, lack of an antigen processing requirement, and the ability to stimulate T cells expressing specific Vβ genes (for review see reference 15). However, functional differences between MAM and other superantigens have been noted. MAM is a relatively weak mitogen for polyclonal human T cells, but it is an efficient stimulator of B cells and induces IgM production better than do staphylococcal superantigens (16). It has been suggested that the poor mitogenic activity of MAM reflects a lower frequency of MAM-reactive T cells among peripheral lymphocytes compared with other superantigens (16, 17).

Here we demonstrate that the MAM represents a new type of ligand for the TCR, intermediate between superantigens and conventional peptide antigens, because it forms contacts both within the Vβ region and the CDR 3-β region of the TCR.

**Materials and Methods**

Human T Cell Clones. CD4+ clones were obtained after stimulation of fresh PBL with MAM by limiting dilution in 96-well plates. Every 2 wk clones were stimulated by syngeneic irradiated non-T PBL as previously described (18) and maintained in RPMI 1640 with 10% FCS and 5% IL-2 (Pharmacia Biotech, Piscataway, N.J.).

Proliferative Response. 2 × 10^4 T cells and 1.5 × 10^4 irradiated (15,000 rads) lymphoblastoid 8866P cells were incubated with 10-fold dilutions of superantigens (from 1 to 10^-4 ng/ml) in triplicate microwells for 48 h, then labeled overnight with [3H]-thymidine and harvested. Reactivity was scored as cpm greater than background. Superantigens were purchased from Toxin Technology, Sarasota, FL; MAM, purified to homogeneity, was twofold over background. Several Superantigens. Ten human T cell clones derived from T cell clones N17 and J17 were amplified with primers specific for human Vβ 8, Vβ 12, or Vβ 17.1 (mouse SEC 15,957 20,429 14,608 2,038

**Results**

MAM Reactivity Is Not Determined by TCR Vβ Alone. To characterize MAM-reactive TCRs, we tested a panel of 16 human T cell clones for proliferative responses to 7 different superantigens (Table 1, Fig. 1). Clonal T cells were incubated with APCs in the presence of gradual 10-fold dilutions of each superantigen, then pulsed with [3H]-thymidine and harvested. Among T cell clones using the same Vβ genes, similar patterns of reactivity were observed with most superantigens, whereas reactivity to MAM often differed (Fig. 1, shaded field). T cell clones expressing either Vβ 5.1, Vβ 8, Vβ 12, or Vβ 17 could respond to MAM, but within each of these Vβ subsets there were both reactive

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*Background: 1,239 CPM
†Background: 394 CPM

Numbers show mean CPM of triplicates. Each T cell clone was tested at least four times. Data on 16 clones are summarized in Figure 1.
Hodtsev et al. and nonreactive (MAM -) clones. Lack of reactivity to MAM was not dose dependent, as we were unable to find concentrations of MAM that would activate MAM - clones (Table 1). The MAM - cells were competent to respond to TCR-mediated signals from other superantigens (Fig. 1). In addition, the sequences of TCR Vβ regions from MAM + and MAM - T cell clones were identical, ruling out the possibility that Vβ allelic variants resulted in differences in MAM reactivity.

Strikingly, all T cell clones that did not respond to MAM used the TCR Jβ2.1 gene segment, whereas MAM-reactive clones used other Jβ2 segments (Fig. 2). Alignment of the CDR3 regions showed that a tyrosine (Y), present within the conserved motif Q(Y/F)FG, correlated with responsiveness to MAM. Nonreactive clones had phenylalanine (F) at this position. The same correlation was observed in polyclonal cell lines obtained after repeated stimulation of fresh T cells with MAM. Dominant clones within these cultures (Fig. 2; SEL) represent T cells expanded in vitro after stimulation with MAM. All Vβ17 MAM + and SEL clones have isoleucine at position 94 in addition to Y within the Q(Y/F)FG motif in their CDR3-β region.

Staining data indicated that in the polyclonal cell lines only the Vβ17 + subset was expanded in response to MAM, to ~50% of all T cells, in agreement with previous data (21). The minor MAM-responsive phenotypes (Vβ5, Vβ8, and Vβ12) were not expanded in these polyclonal cell lines. However, within these minor Vβ subsets, dominant clones were also found after three to five stimulations with MAM. We hypothesize that these clones represented relatively infrequent cells within the Vβ5, Vβ8, or Vβ12 subsets, which may depend on the other TCR elements for

### Table 1

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**Figure 1.** Proliferative response of human T cell clones to bacterial superantigens. The shaded boxes represent examples of clones differing in MAM reactivity despite shared Vβ usage (Vβ5.1, 8, 12.2 and 17.1). Vβ13.1 and 13.2 differ primarily in the HVR4 region, a component of the lateral TCR surface that interacts with superantigens (24, 37). We confirm that TCRs expressing the two closely related Vβ13 gene segments have few superantigen reactivities in common. Key to symbols: -, no reactivity with 1 ng/ml or any concentration; +, reactivity with 10⁻¹ ng/ml or greater; ++, reactivity with 10⁻² ng/ml or greater; ++++, reactivity with 10⁻³ ng/ml or greater. See Table 1 for example of raw data.

**Figure 2.** TCR α and β chains from human T cell clones with different MAM reactivity. The sequences represent MAM-reactive clones (+), MAM nonreactive clones (−), and dominant clonal sequences (SEL) obtained from three polyclonal cell lines (K3, J5, D3) that were repeatedly stimulated with MAM, respectively, 3 ×, 5 ×, or 3 × consecutively at 2-wk intervals. The ratio shown after the name of the cell line indicates the number of identical CDR3-β sequences over the total number of sequences obtained for the indicated Vβ subset. Vα and Vβ nomenclature is according to reference 63, and Jα is according to EMBL/Genbank/DDBJ accession No. M94081.
MAM reactivity. Consistent with this view, V\(\alpha2\) and V\(\alpha8\) were frequently observed in clones of minor V\(\beta\) phenotypes (Fig. 2). Thus, reactivity may depend on V\(\alpha\) usage with these minor V\(\beta\) phenotypes, as previously described for low affinity TCR V\(\beta\)-superantigen interactions (29–31).

However, the major MAM-responsive V\(\beta17\) phenotype is not \(\alpha\) chain–dependent. To prove this point, we transfected \(\beta\) chains derived from clones N17 and J17 into two mouse T hybridoma cell lines, DS 23.27 and YL\(\beta\)-, that are \(\beta\) chain–deficient (Table 2). Surface expression of the endogenous mouse TCR-\(\alpha\) chain, either V\(\alpha2\) or V\(\alpha3.1\), respectively, is rescued by TCR complex formation with the transfected \(\beta\) chain. \(\beta\) chains N17 and J17 retained their differential MAM reactivity regardless of which \(\alpha\) chain they were paired with (Table 2).

Presentation of superantigens to T cell clones can be sensitive to minor differences among MHC class II alleles (32) and the nature of the peptide in the MHC class II groove (33). Therefore, we tested the reactivity of MAM\(^+\) and MAM\(^-\) clones with three superantigens presented by lymphoblastoid cell lines expressing different MHC class II alleles (Fig. 3). In each of four groups (V\(\beta17\), V\(\beta8\), V\(\beta12\), and V\(\beta5\)), MAM\(^+\) and MAM\(^-\) clones retained their specific reactivity regardless of the MHC class II alleles expressed on APCs. The magnitude of the proliferative responses differed from one APC type to another, probably due to different levels of expression of MHC class II or accessory molecules, but the pattern of reactivity remained the same. MAM\(^+\) clones were always MAM-reactive and MAM\(^-\) clones did not respond to MAM presented by other APCs.

TCR-\(\beta\) Chain CDR 3 Mutants. Sequence alignment of TCR-\(\beta\) chains implicated a single residue (Y/F) of the CDR 3 region in determining MAM reactivity (Fig. 2). To test whether Y103 of clone N17 (MAM\(^+\)) and the equivalent residue, F106, of clone J17 (MAM\(^-\)) determine MAM reactivity, we attempted to reconstitute reactivity of J17 by mutagenesis in the CDR 3-\(\beta\) region (Fig. 4). Wild-type and mutant TCRs were expressed in DS23.27 cells and tested for IL-2 production in response to several superantigens. A single amino acid substitution (JY, F106Y) at position 106 was insufficient to restore reactivity to MAM. A second candidate amino acid was isoleucine in position 94. This position is encoded by the V\(\beta\)-Db junctional region and in all V\(\beta17\) clones reactive with MAM, it was represented by isoleucine (Fig. 2). Therefore, we introduced a second substitution at position D94 of J17. Strikingly, the combination of D94I and F106Y (transfectant JIY) resulted in full reconstitution of MAM reactivity (Fig. 4) while the single change, D94I, was insufficient (JIF).

To demonstrate that other regions of CDR 3 were not involved, we tested three deletion mutants including either SEC2 and see to MAM\(^+\) and MAM\(^-\) T cell clones. In each group of T cell clones (V\(\beta17\), V\(\beta8\), V\(\beta12\), and V\(\beta5\)) specific reactivity with the tested superantigens was not dependent on the HLA alleles expressed. MAM\(^+\) clones remained reactive regardless of the APCs used and MAM\(^-\) clones remained nonreactive.

![Figure 3](image)

**Figure 3.** Reactivity to MAM is not MHC-restricted. Four lymphoblastoid cell lines, 2P68 (HLA DR4), LS (HLA DR5), RF (HLA DR2,6), and 8866P (HLA DR15), were used for presentation of MAM, SEC2, and SEE to MAM\(^+\) and MAM\(^-\) T cell clones. In each group of T cell clones (V\(\beta17\), V\(\beta8\), V\(\beta12\), and V\(\beta5\)) specific reactivity with the tested superantigens was not dependent on the HLA alleles expressed. MAM\(^+\) clones remained reactive regardless of the APCs used and MAM\(^-\) clones remained nonreactive.

<table>
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<th>J17</th>
<th>Recipient cell line</th>
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<td></td>
<td>10 ng/ml</td>
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<td>0.1 ng/ml</td>
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<tr>
<td>MAM</td>
<td>362</td>
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Numbers indicate IL-2 U/ml produced after stimulation of transfectants with superantigens. Two TCR-\(\beta\) chains derived from clones N17 (MAM\(^+\)) and J17 (MAM\(^-\)) were expressed in mouse T hybridomas deficient for TCR-\(\beta\) chain (DS 23.27, with endogenous mouse V\(\alpha2\); or YL\(\beta\)-, with endogenous mouse V\(\alpha3.1\)). Resulting transfectants expressed functional TCRs formed by coupling of their endogenous TCR-\(\alpha\) chains with V\(\beta\) chains derived from clones N17 and J17 into two mouse T hybridoma cell lines, DS 23.27 and YL\(\beta\)-, that are \(\beta\) chain–deficient (Table 2). Surface expression of the endogenous mouse TCR-\(\alpha\) chain, either V\(\alpha2\) or V\(\alpha3.1\), respectively, is rescued by TCR complex formation with the transfected \(\beta\) chain. \(\beta\) chains N17 and J17 retained their differential MAM reactivity regardless of which \(\alpha\) chain they were paired with (Table 2).
one or two residues between I94 and Y106 (JIY-SY, JIY-NE, JIY-E). These mutants would be expected to differ in their peptide/MHC reactivity, because recognition of conventional peptide antigen is extremely sensitive to changes in CDR3 (34, 35). All three deletion mutants retained MAM reactivity (Fig. 4). Together with the data on MAM presentation by different MHC class II alleles (Fig. 3) these experiments exclude a role for MHC II-bound peptides in these MAM–TCR interactions. A direct contact between TCR CDR3-\(\beta\) and MAM is the most likely explanation for dependence on residues I94 and Y106. Based on recent crystallographic data, these two amino acids are located at the base of the CDR3 loop lying in close proximity to one another (36). The side chain of Y106 extends towards the exposed surface of the TCR (Y107 in reference 2). It appears that residues I94 and Y106 are accessible for direct interaction with MAM. These interactions are specific for MAM since none of the tested mutations affected reactivity with staphylococcal superantigens (Fig. 4).

**Discussion**

CDR3-dependent Superantigen. In this study we show that the CDR3 region of TCR-\(\beta\) chain mediates specific recognition of the MAM superantigen. Complementarity-determining regions (CDR loops) (2, 37) represent hyper-variable loops of the TCR that face the MHC during antigen recognition. On the \(\beta\) chain, both CDR1 and CDR2 as well as the HVR 4 loops can be involved in interactions with superantigens. TCR-\(\beta\) point mutations that affect specific interaction with a particular superantigen are shown in Fig. 5. The crystal structures of TCR-\(\beta\) chain with staphylococcal enterotoxins SEC2 and SEC3 revealed that these staphylococcal superantigens interact with CDR1, CDR2, FR3, and HVR 4 but not with the CDR3 loop (38). Most contacts (47%) are formed between the superantigen and the CDR2 loop. Since Y 106 of CDR3 (2) and exposed CDR2 residues are located at opposing faces of the TCR-\(\beta\) chain (1), our data suggest that recognition of MAM and staphylococcal superantigens is substantially different. That CDR2 Figure 4. Mapping of the TCR residues required for reactivity with MAM. Mutations based on alignment between N17 and J17 map the positions involved in interaction with MAM (I and Y). Deletions in the middle of the CDR3 loop (\(\Delta\)) did not affect reactivity, as well as several mutations in the HVR4 region. Constructs marked with * were transfected into both DS23.27 (mouse V\(\alpha2\)) and YL\(\alpha\)-(mouse V\(\alpha3\)) cells, and had identical reactivity regardless of the \(\alpha\) chain used. 10-fold dilutions of superantigens were tested in IL-2 assays as described in reference 26, (see also Table 2). Reactivity was scored as the minimal concentration of the tested superantigens required for an IL-2 response and the key to the symbols is: +++, 0.1 ng/ml, ++, 1 ng/ml, +, 10 ng/ml.

Figure 5. Linear model of TCR-\(\beta\) indicating residues implicated as superantigen (SAG) interaction sites by mutation studies. Typically, superantigens interact with solvent-exposed residues of CDR1, CDR2, and HVR4 encoded by V\(\beta\) genes (24, 64-67). The interaction of MAM with TCR-\(\beta\) is clearly distinct, as it interacts both with the V\(\beta\) region because of its V\(\beta\) specificity (the exact site is not known), and with two residues located at the base of the CDR3-\(\beta\) loop.
regions are not involved in MAM recognition is supported by the observation that the primary sequence of the homologous human Vβ17 and mouse Vβ6 chains, both MAM-reactive, differs in the CDR 2 region.

Mutational and structural studies have shown that the CDR 3 loops of the α and β chains are central in peptide recognition (1, 2, 34, 35, 39, 40). CDR 3 regions are the most diverse parts of the TCR produced by extensive processing of end joints during antigen receptor rearrangement (3). Junctional diversity is increased by N-additions contributed by the lymphoid-specific enzyme terminal deoxynucleotidyltransferase (TDT; references 41–43). Although an N-region-deleted TCR repertoire from TDT-knockout mice was shown to be as efficient as normal TCR repertoire (44), the N-region-deleted TCRs were more promiscuous in peptide recognition. These TCRs recognized a significantly larger number of different peptides than did the wild-type TCRs when tested for reactivity against a library of random peptides (45).

The structural basis for the role of N-encoded TCR residues was revealed in a recently solved structure of the TCR-MHC interface (1). In this crystal, three interactions are formed between residue p5 of the peptide and the TCR. Two of these are contacts of p5 with N-encoded residues of the Vα-Dα and Vβ-Dβ junctional regions.

The specific interaction of MAM with the TCR appears to imitate the natural process of peptide MHC recognition. During peptide recognition, CDR 3 regions of α and β chains form contacts with MHC. The complex is stabilized by CDR 3–peptide interactions (46, 47). Recognition of MAM is clearly Vβ-dependent, as is the case with other superantigens, but it appears to be stabilized by additional contacts with the CDR 3-β region.

In all MAM-reactive Vβ17+ clones that we have sequenced, position 94 was represented by isoleucine. I94 is adjacent to the conserved end of Vβ region CASS and corresponds to position 2 of the CDR 3 region (48, 49). In random CDR 3 sequences, position 2 is rarely represented by isoleucine (49) but in Vβ17+ sequences I94 is more common, occurring in ~20% of the sequences (50). This residue is often assumed to be part of the N-region, but in the case of isoleucine it may be encoded by the 3' end of the germline Vβ17 sequence, codon ATA of the genomic sequence (51). However, in ~80% of the cases this codon is removed during Vβ-Dβ recombination and replaced by N-additions.

The second CDR 3-β position critical for interaction with MAM is tyrosine within the Q(Y/F)FG motif of Jβ2 (T cell clones using Jβ1 are rare and they were not analyzed in this study). At this position Y is encoded by Jβ2.3, Jβ2.4, Jβ2.5, and Jβ2.7. F, which does not allow MAM reactivity (Fig. 4), is encoded by Jβ2.1 and Jβ2.2. The Jβ2.1 segment, which was used by the MAM–T cell clones studied here (Fig. 2), is used by ~40% of all T cells from peripheral blood (52).

The major subset of T cells targeted by MAM is the Vβ17 subset (21). Yet not all Vβ17+ T cells respond to MAM, and the response is limited to cells expressing two appropriate residues at the base of the CDR 3-β loop. This suggests that T cell activation by MAM is dependent on TCR junctional diversity, thus limiting the number of potential MAM-reactive T cell clones. The frequency of T cells responding to this superantigen could be significantly lower than to other superantigens (for example SEB, SEC1, SEC2, and SEC3). Among the minor MAM-responsive TCR phenotypes (Vβ5, Vβ8, and Vβ12), the frequency is even lower than within the Vβ17 subset, probably because Vα usage is an additional requirement as suggested by the data in Fig. 2. As previously shown, MAM-responsive T cells expressing Vβ5, Vβ8, and Vβ12 can only be detected by testing individual clones and these Vβ phenotypes do not expand in polyclonal cultures exposed to MAM (18). However, TCRs of the minor phenotype also appear to use the Jβ-encoded tyrosine for MAM recognition (Fig. 2).

It is possible that Jβ usage can affect low affinity interactions of TCR with other superantigens. Incomplete deletion of Vβ8+ or Vβ6+ cells in mice carrying Mls-1a (Mtv-7) revealed that TCRs that escaped deletion used distinct Jβ regions (53, 54). In this experimental system, Jβ1.2 seemed to protect Vβ6+ TCR from interaction with a retroviral superantigen, Mls-1a. However, these studies did not find any conserved motifs in the CDR 3 region that could be implicated in unresponsiveness to Mls-1a (54). Staphylococcal superantigens seem to be CDR 3-independent (Fig. 4 and reference 55). However, in the case of SEB and Ürtia dioica superantigens, it was suggested that the Jβ segment, but not the CDR 3 region, can affect superantigen binding by influencing the quaternary structure of the TCR-β chain (55).

The major MAM-responsive Vβ phenotype is strikingly dependent on discrete residues of CDR 3. This fact clearly distinguishes MAM from other superantigens. Thus, recognition of MAM is dependent on junctional diversity of the TCR-β chain.

Autoimmunity. Mycoplasma arthritides is a microorganism that causes a disease in rodents that is remarkably similar to human rheumatoid arthritis (RA). It is also the only mycoplasma that produces MAM. It should be emphasized that Mycoplasma arthritides is not a human pathogen. Surprisingly, preferentially expanded T cell clonotypes found in human RA often belong to the MAM-reactive TCR phenotype. Dominant clones from RA patients, characterized in three independent studies, were found to use Vβ17 followed by isoleucine at position 94 (50, 56, 57). In one of the studies, these T cells were shown to be autoreactive against a lymphoblastoid cell line expressing HLA-DR 4 (50). Similar autoreactivity was reported in a TCR-transgenic mouse model of RA (58). Inflammatory synovitis in mice was triggered by the transgenic TCR recognition of host MHC class II (A*β). This transgenic TCR-β chain used a mVβ6 followed by isoleucine (CASSI) (D. Mathis, personal communication). Murine Vβ6 is the closest homologue of human Vβ17, and mouse Vβ6+ T cells are also MAM-reactive (15). Thus, both murine and human TCRs associated with RA appear to express a conserved isoleu-
cine at the COOH-terminal end of the Vβ region. The role of the conserved isoleucine in the CDR3-β region of arthritogenic TCRs remains to be determined. Is it necessary for interaction with MHC class II or a putative joint-specific peptide? Is it possible that tolerized self-reactive clones can be reactivated by MAM in mice infected by Mycoplasma arthritides?

There is a strong genetic link between certain DR alleles (class II MHC) and RA in humans (59, 60). Position 71 of the particular DR4 or DR1 β chains, a lysine residue, is associated with susceptibility for RA (61). This residue determines the nature of the peptides in the class II groove by interacting with P4/P5 of the peptide (for review see reference 62). Conserved CDR3-β motifs that include isoleucine 94 have been proposed as evidence for antigen-driven immune response in RA (50). Whether superantigens like MAM have a role in RA pathogenesis is still an open question.

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