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T Cell Epitope Mimicry between Sjögren's Syndrome Antigen A (SSA)/Ro60 and Oral, Gut, Skin and Vaginal Bacteria.

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Abstract

This study was undertaken to test the hypothesis that Sjogren's syndrome Antigen A (SSA)/Ro60-reactive T cells are activated by peptides originating from oral and gut bacteria. T cell hybridomas generated from HLA-DR3 transgenic mice recognized 3 regions on Ro60, with core epitopes mapped to amino acids 228-238, 246-256 and 371-381. BLAST analysis identified several mimicry peptides, originating from human oral, intestinal, skin and vaginal bacteria, as well as environmental bacteria. Amongst these, a peptide from the von Willebrand factor type A domain protein (vWFA) from the oral microbe *Capnocytophaga ochracea* was the most potent activator. Further, Ro60-reactive T cells were activated by recombinant vWFA protein and whole *E. coli* expressing this protein. These results demonstrate that peptides derived from normal human microbiota can activate Ro60-reactive T cells. Thus, immune responses to commensal microbiota and opportunistic pathogens should be explored as potential triggers for initiating autoimmunity in SLE and Sjögren's syndrome.

Keywords

Sjögren's syndrome; SLE; Ro60/SSA; Molecular Mimicry; T Epitopes; Microbiota

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Conflict of interest statement

None of the authors have any conflict of interest.

1. Introduction

Ro60/SSA, is one of the major autoantigens targeted in Sjögren's syndrome and SLE [1]. Anti-Ro60 autoantibodies are the most common autoantibody specificity found in these disorders, and in antinuclear antibody (ANA) positive normal individuals [2]. In Sjögren's syndrome, presence of anti-Ro60/SSA antibodies is used as one of the classification criteria for the diagnosis of the disease [3]. Clinically, Ro60 antibodies have been associated with severity of lymphocytic foci within the salivary glands, and extra glandular manifestations such as vasculitis in Sjögren's syndrome [4], and with photosensitivity in SLE [5], thereby suggesting a pathogenic role of these autoantibodies. Moreover, in some SLE patients, Ro60 reactive antibodies are the first autoantibody specificity detected in the serum [6]. Despite intense investigation, the initiating event(s) leading to the production of these autoantibodies remains much debated.

Cross-reactivity between Ro60 and EBV protein EBNA-1 has been proposed as a mechanism for the initiation of anti-Ro60 antibody response in lupus patients and in experimental rabbit model system [7]. Similarly, B cell cross-reactivity between Coxsackie virus 2B protein and Ro60 has been suggested to play a role in the initiation and perpetuation of anti-Ro60 autoantibody response in Sjögren's syndrome [8]. However, cross-reactivity at the B cell level alone does not provide a plausible explanation for the strong association of certain HLA-D regions with the pathogenesis of SLE and SS [9]. Our previous work has demonstrated that for sustaining anti-Ro60 antibody response, breaking tolerance at T cell level is highly critical [10]. We have also demonstrated that activation of HLA-DR3 restricted T cell responses against lupus-associated autoantigen SmD, by a T cell epitope mimicry peptide, was sufficient to induce autoantibody responses to SmD [11]. The pivotal role of T cells in induction of autoantibody responses in SLE is highlighted by the work of Putterman et al [12]. Immunization of BALB/c mice with a peptide mimotope of dsDNA on a branched poly lysine backbone induced anti-dsDNA antibodies. While initially this was attributed to B cell cross-reactivity, later studies have demonstrated the critical requirement of T cells in this process [13]. These studies underscore the importance of T cells in the generation of lupus related autoantibodies and can explain their strong association with certain HLA-D regions.

In this investigation, the role of molecular mimicry for activation of Ro60 reactive T cells was explored by generating Ro60 reactive T cell hybridomas from HLA-DR3 transgenic mice. HLA-DR3 was selected, as this haplotype shows one of the strongest genetic associations for Sjögren's syndrome and SLE [14, 15]. The T cell epitopes recognized by these hybridomas were mapped and used to perform homology searches. Considering the important role of commensal microbiota in shaping of immune system [16], we focused more on the ability of mimicry peptides from oral, skin and gut bacteria for activation of a Ro60 reactive T cell hybridomas. The present study shows that several peptides derived from these bacteria were able to activate Ro60 reactive T cell hybridomas. Moreover, bacterial vWFA protein activated Ro60 reactive T cells. Our findings suggest that immune responses to commensal microbiota might play a critical role in the initiation of autoimmune responses to Ro60.

2. Material and methods

2.1. Mice, immunization, and hybridoma generation

A mouse strain that lacks murine MHC class II and expresses the HLA DR3 (DRB1*0301 and DRA1*0101 transgenes) was used in this study [17]. All mouse experiments were conducted as per the guidelines laid down by the National Institutes of Health, USA and were approved by the Institutional Animal Care and Use Committee at the University of Virginia. The mice were bred in the University of Virginia vivarium and given food and water *ad libitum*. T cell epitope mapping on Ro60 was done as previously described with a few modifications [11]. Briefly, mice were immunized in one foot pad and at the base of the tail with 100 µg of recombinant human Ro60 emulsified in Freund's incomplete adjuvant (Pierce Chemical Company, Rockford, IL, USA). On day 10 after immunization, the draining lymph node cells were harvested and stimulated for 3 days with recombinant Ro60. Cells were fused with BW5147 TCR^{-/-} fusion partner and T cell hybrids were selected using standard HAT medium [11]. Antigen reactive hybrids were cloned by limiting dilution method.

2.2. Synthetic peptides and recombinant proteins

The panel of Ro60 synthetic peptides covering the entire span of Ro60 (538 amino acids) has been described earlier [10,17]. For fine epitope mapping, overlapping peptides (15mers with 14 amino acid overlap) or alanine substituted peptides were obtained from JPT Peptide Technologies (Acton, MA, USA) in a library format. All mimicry peptides (15mers) were obtained in a library format from Bio-Synthesis INC (Lewisville, TX, USA). Recombinant Ro60-6X His tag protein was purified as described before [10]. Ro60 and La cDNA were cloned in to the pMAL-c5E vector (New England Biolabs, Ipswich, MA) to generate fusion proteins with Maltose Binding Protein (MBP). The recombinant proteins were purified using amylose resin (NEB, Ipswich, MA) following manufacturer's instructions. The entire coding region for vWFA protein (GenBank: ACU93083.1) from *Capnocytophaga (C.) ochracea* DSM 7271 was chemically synthesized and cloned in to the pIDTBlue vector by Integrated DNA Technologies (Coralville, IA, USA). This coding region was sub-cloned into the pQE30 expression vector (Qiagen INC, Valencia, CA) to generate a vWFA-6XHis fusion protein or in the pMAL-c5E vector to generate a vWFA-MBP fusion protein. The respective recombinant vWFA proteins were purified using Ni-NTA affinity chromatography (Qiagen INC, Valencia, CA) under native conditions and by using amylose resin affinity chromatography, following manufacturer's instructions. Purified proteins were dialyzed extensively against PBS and stored frozen in aliquots until use.

2.3. T cell hybridoma activation

T cell hybridomas were activated with antigen presenting cells (APC) loaded with proteins and peptides. Either spleen cells from HLA-DR3 transgenic mice or the lymphoblastoid B cell line, QBL, that expresses HLA-DR3, were used as APC. T hybridoma cells (10^5) were added to APCs ($2-5 \times 10^5$) with or without antigen, and cultured for 16h at 37°C in 5% CO₂. IL-2 in culture supernatants was analyzed by ELISA (BD Biosciences, San Jose, CA, USA).

E. coli expressing recombinant proteins were used for T cell hybridoma activation. *E. coli* were grown in LB medium and log phase cultures (OD_{600nm} : 0.5 to 0.7) were induced with IPTG at a final concentration of 1mM. Cells were harvested after 4h and washed twice with ice cold PBS. Cells were then treated for 10 min with 70% Ethanol and washed 3 times with PBS. The number of bacteria were estimated by spectrophotometry ($O.D_{600nm} = 1$ corresponds to 10^9 bacteria/ml). Cells were resuspended in PBS, and equal numbers of bacterial cells from each group were fed to spleen cells from HLA-DR3 transgenic mice in the T cell hybridoma activation assay.

3 Results

3.1. T cell hybridomas reactive with Ro60 recognize 3 epitope regions on Ro60

A panel of T cell hybrids was generated from HLA-DR3 transgenic mice immunized with Ro60 and epitope mapping was performed using 52 synthetic peptides. The initial screening was done by employing 13 pools of peptides (4 peptides/pool) followed by screening with individual peptides within the respective positive pool. The results from mapping the reactivities for 4 hybrids (Hyb-9, Hyb-24, Hyb-34, and Hyb-26) showed that the T cell epitopes localized to 3 regions on Ro60 (figure 1). Hyb-9 recognized peptides within the pool covering amino acids 361-420. When individual peptides were used, 2 overlapping peptides within this pool Ro60₃₆₁₋₃₈₀ and Ro60₃₈₁₋₄₂₀ were able to stimulate the hybridoma thereby localizing the epitope to Ro60₃₆₁₋₃₉₀. Similarly, the epitope for Hyb-24 and Hyb-34 was localized within Ro60₂₂₁₋₂₅₀, while the epitope for Hyb-26 localized to Ro60₂₄₁₋₂₆₀. Although there is a significant overlap between the regions recognized by these hybrids, the T epitopes recognized are distinct. Peptide Ro60₂₄₁₋₂₆₀ only activated hybrid 26 but not hybrids 24 and 34. All hybrids were cloned by limiting dilution and the respective clones were used for further experiments.

3.2. Mapping core amino acid residues within Ro60 T cell epitopes

Fine epitope mapping was done by using overlapping synthetic peptides covering each epitope region to stimulate the respective T cell hybridoma (figure 2). Hyb-9.5 was activated with peptides within Ro60₃₆₁₋₃₉₀, only if they had the amino acid sequence of FLLAVDVSASM within them. Removal of phenylalanine at the N-terminal or methionine at the C-terminal abrogated the ability of the peptides to activate T cells. Similarly the core epitopes for other hybridomas were identified and the results are summarized in table 1. It is interesting to note that although hyb-24.1 and hyb-34.14 recognize the same epitope region on Ro60, their core epitopes are different. For hyb-24.1 it is Ro60₂₂₈₋₂₃₇ (YLEAVEKVKR), whereas for hyb-34.14 it is Ro60₂₂₉₋₂₃₈ (LEAVEKVKRT).

To further determine which amino acids are critical for activation of the hybridomas, peptides (15mers) which included the core T epitope region for each hybrid were synthesized, and each amino acid within the peptide epitopes were either replaced with alanine or with glycine (if the native residue was alanine). The ability of substituted peptides to activate the respective T cell hybridoma was tested. The results are shown in figure 3. If replacement of an amino acid residue resulted in reduction of T cell activation by more than 50%, then that amino acid residue was considered critical. For example, each amino acid

within peptide KRFLAVDVSASMNQ was either replaced with alanine or glycine and the substituted peptides were used to activate hyb-9.5. Substitution of amino acids within the core T epitope at positions 374 (A), 375(V), 376(D), 377(V), 378(S), 379(A), and 380(S), considerably affected the activation of hyb-9.5, indicating that these are the critical residues. A similar analysis was carried out for the other 3 hybridomas and is summarized in table 1.

Interestingly, alanine substitution at some amino acids outside the core T epitopes also influenced T cell activation. In Hyb-9.5, alanine substitution at position 382 (N) led to loss of activity while 226(L), 227(K) and 228(Y) for Hyb-34.14, substantially increased IL-2 production, making the substituted peptides super agonists. This is not surprising since amino acid side chains near T epitopes can influence the manner in which the peptide fits into the HLA binding peptide groove and interacts with the T cell receptor. However, whether the reduced IL-2 production is due to lowered or abolished binding to HLA-DR3 or is due to failure to contact the TCR is not known. Regardless the data shows that these amino acid residues are important within the mapped epitope region.

3.3. Several peptides from bacterial proteins activate Ro60 reactive hybridomas

To identify putative cross reactive peptides, Basic Local Alignment Search Tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) analysis was performed using the information on core sequences and critical amino acid residues. For hyb9.5, twenty two peptide sequences were selected for synthesis based on the criterion that they came from bacteria included in the Human Oral Microbiome Database (<http://www.homd.org/>). Two additional peptides, one from a fungal protein and one from a primate protein were also synthesized. Similarly 16 putative mimicry peptides were synthesized for hyb24.1 and hyb34.14, and 13 mimicry peptides for hyb26.14.

Of the 22 mimicry peptides tested, 11 peptides were able to stimulate hyb-9.5 (figure 4 left panel). The other 12 peptides did not induce any IL-2 production over the background. The positive peptides mainly belonged to 3 proteins, vWFA, BatA and Trove domain containing protein (table 2). Hyb34.14 was activated by a single mimicry peptide P121 from aldehyde dehydrogenase from *Acinetobacter johnsonii* (right panel of figure 4). Of the 13 peptides tested, only one peptide from the beta lactamase protein from *Listeria grayi* activated hyb-26.4 (right panel of figure 4). The mimicry peptides for hyb34.14 and hyb26.4 were less potent than the corresponding Ro60 peptides.

In the 13 peptide mimics that activated Ro60 reactive T cells, 3 are found in oral flora (*Prevotella disiens*, *Capnocytophaga sputigena* and *Capnocytophaga ochracea*); 4 are in the gut flora (*Bacterioides finegoldii*, *Bacteroides intestinalis*, *Bacteroides fragilis* and *Alistipes finegoldii*) and 2 are found on the skin (*Corynebacterium amycolatum* and *Acinetobacter johnsonii*). While *Prevotella disiens* is also found in the vagina and is associated with vaginosis, *Pseudomonas mendocina* is an opportunistic pathogen linked with endocarditis. Two mimics (*Flavobacterium bacterium* and *Listeria grayi*) are found in marine flora and in contaminated food respectively. While table 2 only summarizes few bacteria harboring these 13 peptides, a more extensive list of bacteria from human microbiome is provided in Supplemental table 1. This table provides testimony to the presence of multiple putative mimics of Ro60/SSA in the human microbiome and the environment.

Amongst all positive peptides, peptide p106 was the strongest activator of hyb-9.5. This peptide is from the vWFA protein from an oral commensal bacteria *C. ochracea*. Figure 5 shows dose response curves for all mimicry peptides for hyb-9.5, as well as a comparison between Ro60 peptide p38 and vWFA peptide p106. The data (figure 5C) show that the vWFA peptide was at least 60 times more potent than the native Ro60 peptide.

3.4. vWFA protein from *C. ochracea* can activate hyb-9.5

Since P106 was the most potent peptide for activating hyb-9.5, we focused further on the vWFA protein from *C. ochracea*. Purified recombinant vWFA protein was generated and its ability to activate hyb-9.5 investigated. Figure 6 shows that vWFA protein induced IL-2 production in hyb-9.5 in a dose dependent fashion. MBP and La-MBP used as negative controls did not induce any IL-2 production, whereas Ro60-MBP used as positive control, induced robust IL-2 production.

Repeated attempts to show that *C. ochracea* can activate hyb-9.5 were not successful. This failure might be due to the presence of a suppressor factor produced by *C. ochracea* that has been shown to suppress mitogen stimulated lymphocyte proliferation [32]. In order to determine whether APCs can present the P106 epitope from whole bacteria, we used *E. coli* expressing vWFA protein as a surrogate to stimulate hyb-9.5. *E. coli* induced to express Ro60-MBP were used as positive control, and *E. coli* expressing La-MBP and MBP were used as negative controls. Figure 7 shows that vWFA and Ro60 expressing *E. coli* induced IL-2 production in hyb-9.5, whereas those expressing La and MBP did not activate the hybridoma. Another hybridoma, hyb-341.14 was activated only by *E. coli* expressing Ro60 and not vWFA demonstrating that the stimulation by vWFA was specific to hyb-9.5. In summary these results demonstrate that the cross-reactive peptide from vWFA protein can activate Ro60 reactive T cells, when presented as a synthetic peptide or as a naturally processed and presented from the whole protein.

4. Discussion

This study was undertaken to investigate the role of molecular mimicry in activation of Ro60 reactive T cells. Molecular mimicry has been proposed to play an important role in the activation of Ro60 reactive B cells [7, 8]. Cross-reactive B cells recognizing Epstein bar virus nuclear antigen 1 (EBNA1) peptide 58-72 and Ro60 peptide 169-180 have been suggested to initiate the anti-Ro60 antibody responses in lupus patients [7]. Anti-Ro60₁₆₉₋₁₈₀ affinity purified antibodies reacted with EBNA-1 and recognized the EBNA-1 peptide 58-72. Moreover, immunization of rabbits with the EBNA-1 peptide 58-72, led to the generation of anti-Ro60 antibodies. However, the role of T cells in this process was not explored. In another study, Stathopoulou *et al* have demonstrated that anti-Ro60₂₁₆₋₂₃₂ antibodies from Sjögren's syndrome patients were cross-reactive with Coxsackie virus 2B protein peptide 31-47 [8]. They suggest that B cells cross-reactive with Coxsackie virus 2B protein and Ro60 might be involved in the initiation of anti-Ro60 antibody response. Our study demonstrates for the first time that cross-reactivity at T cell epitope level exists for Ro60 and might play a critical role in the immune response to Ro60. In addition, the HLA-

DR3 restriction of these cross-reactive T cell epitopes might provide an explanation for the importance of this region in autoimmune responses to Ro60.

In this study we generated a panel of HLA-DR3 restricted T cell hybrids that recognized 3 epitope regions, Ro60₂₂₁₋₂₅₀, Ro60₂₄₁₋₂₆₀ and Ro60₃₆₁₋₃₉₀. While the latter 2 regions were reported as T cell epitopes in our previous study [17], Ro60₂₂₁₋₂₅₀ is a newly defined T cell epitope. For Ro60₃₆₁₋₃₉₀ reactive hyb-9.5, of the 22 homologous bacterial peptides tested, 11 peptides were able to activate this hybridoma. These peptides came from proteins within the vWFA domain superfamily. The vWFA domain was originally described in the blood coagulation protein von Willebrand factor and is present in a large number of prokaryotic and eukaryotic proteins [33, 34]. Ro60, which is also known as TROVE2 has 2 distinct conserved domains, the TROVE domain and the vWFA domain [35]. The core epitope recognized by hyb-9.5, Ro60₃₇₁₋₃₈₁, resides within the vWFA domain of Ro60 (amino acids 372-489). We focused our study on the vWFA protein from *C. ochracea*, as the peptide derived from this protein was the strongest activator of hyb-9.5 (figure 5). *C. ochracea* is a gram negative, facultative anaerobic bacterium that was originally isolated from dental plaque obtained from both healthy and diseased gingival sites [36]. It is now established that *C. ochracea* is a commensal bacterium present in the oral cavity and is also an opportunistic pathogen with the potential to cause severe infections [23]. To determine, whether the whole vWFA protein can activate hyb-9.5, the coding sequence was artificially synthesized and cloned to generate recombinant vWFA protein. This protein was able to activate the Ro60 reactive T cells, either when fed to antigen presenting cells as purified recombinant protein or in whole *E. coli* expressing the protein. These data demonstrate that the cross-reactive peptide from the microbial protein can be naturally processed and presented by antigen presenting cells in the context of HLA-DR3. Unfortunately, our attempts to activate these T cells with intact *C. ochracea* were not successful. One of the reasons for this might be the suppressive factor made by *C. ochracea* [32]. This suppressive factor has been shown to suppress *in vitro* murine spleen cell proliferation. In our experiments we also observed that *C. ochracea* readily inhibited T cell activation of hybridomas by synthetic peptides (data not shown). However, it should be noted that antibodies to *C. ochracea* are present in periodontitis patients [37], suggesting that the *in vivo* immune response is distinct from the *in vitro* suppression. Thus, whether *C. ochracea* and other bacteria listed in table 2 can activate Ro60 reactive T cells *in vivo* will be an important question to address.

This study provides a snap shot of cross-reactive microbial peptides capable of activating Ro60 reactive T cells. In view of our previous mapping studies [17], showing 2 epitopes in the C-terminal region, Ro60₄₂₁₋₄₄₅ and Ro60₄₆₆₋₄₈₅, it was surprising that hybrids reactive with these T cell epitopes were not generated. Moreover, T cell Epitope prediction tools (<http://www.iedb.org/>) suggest that in addition to the epitopes reported by us, additional regions on Ro60 also harbor HLA-DR3 restricted T cell epitopes (data not shown). Clearly these additional T cell epitopes are likely to be cross-reactive with additional microbial peptides and they will be identified in the future.

The findings from this study can provide valuable clues towards understanding the possible influence of oral and gut microbiota in autoimmunity. It is now well accepted that oral, gut and skin microbiota plays a critical role in the development of the human immune system

[16]. In our study, hyb-9.5 was activated by peptides originating from oral and gut microbes; hyb-26.4 by a peptide from gut pathogen *Listeria grayi*; and hyb-34.14 by a peptide from skin commensal *Acinetobacter johnsonii*. The multiple T cell epitopes of Ro60, with many of its mimics in the environment provides an ample opportunity for the immune system to encounter them. It is quite possible that this might be one of the reasons for the observation that anti-Ro60 antibodies are the most prevalent autoantibody in normal population [2]. In Sjögren's syndrome and SLE prone individuals, the progression of this preclinical autoimmune response to autoimmunity would be then dictated by multiple susceptibility genes and pathways [9].

In conclusion, considering recent reports implicating just a single gut bacteria *Prevotella copri*, in the pathogenesis of rheumatoid arthritis [38], it is quite possible that the bacteria described in this study, as well as other yet unidentified commensal bacteria, might be involved in the initiation or/and perpetuation of anti-Ro60 immune responses. If proven experimentally, one can speculate that rather than a specific infection, a dysregulated immune response to normal microbiota might be involved in the pathogenesis of Sjögren's syndrome and SLE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ANA	Anti-nuclear antibody
APC	Antigen presenting cells
BLAST	Basic local alignment search tool
<i>C. ochracea</i>	<i>Capnocytophaga ochracea</i>
HAT	Hypoxanthine Aminopterin Thymidine
O.D.	Optical density
SLE	Systemic lupus erythematosus
SSA	Sjögren's syndrome Antigen A
vWFA	von Willebrand factor type A domain protein

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Highlights

HLA-DR3 restricted T cell epitopes on Sjogren's syndrome Antigen A (SSA)/Ro60 defined.

Peptides originating from multiple bacteria activate Ro60-reactive T cells.

vWFA bacterial protein can activate Ro60-reactive T cells.

Need to consider immune response to normal microbiota in autoimmunity.

Figure 1. T cell hybrids reactive with Ro60 recognize 4 epitope regions within Ro60

T cell hybrids were screened for reactivity to 13 peptide pools, followed by screening with individual peptides within the positive pool. T cell hybrids (10^5) were incubated overnight with syngeneic spleen cells (2×10^5) fed with synthetic peptides ($10\mu\text{g/ml}$). IL-2 in the culture supernatants was detected by ELISA and data are presented as mean O.D. at 450nm. Hyb-9 was activated by Ro60₃₆₁₋₃₈₀ and Ro60₃₇₁₋₃₉₀. Hybs-24 and -34 were activated by Ro60₂₂₁₋₂₄₀ and Ro60₂₃₁₋₂₅₀. Hyb-26 was activated by Ro60₂₄₁₋₂₆₀

Figure 2. Mapping of core T cell epitopes for Ro60 reactive hybridomas

Overlapping synthetic peptides (15 mers with 14 amino acid overlap) spanning the broader epitope region for each hybridoma were used to activate the T cell hybridomas. HLA-DR3+ human B cell lymphoblastoid line QBL was used as APC. IL-2 in culture supernatant was estimated by ELISA and data are presented as mean IL-2 *pg/ml* $\pm$ SEM from 2 independent experiments. The core peptide sequence for each hybridoma is marked by a rectangle and denotes that amino acids within this rectangle have to be present for activation of the T cell hybridomas.

Figure 3. Identification of amino acids critical for the activation of T cell hybridomas by alanine scanning

T cell hybridomas were stimulated with a sequential panel of synthetic peptides substituted with either alanine or glycine at consecutive amino acid residues. Data are presented as mean percent IL-2 produced relative to the unsubstituted peptide (■) and are from at least 2 independent experiments.

Figure 4. Bacterial peptides activate Ro60 reactive T cell hybridomas

Multiple mimicry peptides were used to stimulate T cell hybridomas with QBL cell line as APC. The peptide corresponding to the native Ro60 sequence is shown in solid bar (■). Hyb-9.5 (left panel) was activated by 11 mimicry peptides (P87, P91, P93, P94, P99, P100, P103, P104, P106, P107 and P108). P38 is the corresponding Ro60 peptide. Hyb-34.14 (right panel) was activated by a single mimicry peptide P121 and P23 represents the parental Ro60 peptide. Hyb-26.4 was also activated by a single mimicry peptide P135. P25 represents the corresponding Ro60 peptide. The data are presented as mean IL-2 pg/ml $\pm$ SEM from 2 independent experiments.

Figure 5. Dose response curves for activation of hyb-9.5 with mimicry peptides

P106 from vWFA protein from *C. Ochracea* is the most potent stimulatory peptide. Mimicry peptides at different concentrations were used to activate hyb-9.5. Panel A shows activation by peptides P87, P91, P93, P94, and panel B shows peptides P99, P100, P103, P104, P106, P107 and P108. P38 is the corresponding Ro60 peptide. Panel C shows IL2 production following stimulation with a wider range of peptide concentrations with P38 (the Ro60 peptide) and the mimicry peptide P106 (the vWFA peptide from *C. ochracea*). P106 is at least 60 fold more potent than the Ro60 peptide. The data are presented as mean IL-2 pg/ml $\pm$ SEM from 2 independent experiments.

Figure 6. Activation of Ro60 reactive T cells by vWFA protein from *C. ochracea*

Hyb-9.5 was incubated overnight with syngeneic spleen cells and purified recombinant proteins. IL-2 in culture supernatants was estimated by ELISA and data are presented as mean IL-2 (pg/ml) $\pm$ SEM from 2 independent experiments. Both Ro60 and vWFA proteins induce IL-2 production, whereas MBP and La-MBP failed to do so.

Figure 7. Activation of Ro60 reactive hyb-9.5 by E coli expressing recombinant proteins

E coli expressing different proteins were induced with IPTG for 2h and killed with alcohol. Equal numbers (2.5×10^7 /ml) were fed to spleen cells from HLA-DR3 transgenic mice. T cell hybridomas were incubated with these spleen cells overnight and IL-2 in culture supernatants was estimated by ELISA. The data are presented as mean IL-2 (pg/ml) $\pm$ SEM from 2 independent experiments. Hyb-9.5 was activated by E coli expressing Ro60 and vWFA, while hyb-34.14 was only activated by Ro60 expressing E coli.

Table 1

Summary of T cell epitopes on R06O mapped with hybridoma panel.

Hybridoma	R06O epitope region	Core Epitope	Amino Acid Sequence	Critical Residues
24.1	221-250	228-237	YLEAVEKVKR	^I <u>Y</u> <u>LEAVEK</u> VKR
34.14	221-250	229-238	LEAVEKVKRT	<u>LEAVEK</u> VKR
26.14	241-260	246-255	HLIEEHQLVR	<u>HLIEEH</u> <u>QLV</u> RE
9.5	361-390	371-381	FLLAVDVSASM	FLL <u>AVDVS</u> ASM

^I Amino acids shown in bold and underlined are critical residues within the core epitope. Replacement of these amino acids either with alanine or in some cases glycine, abolished the ability of the corresponding peptide to activate the respective T cell hybridomas.

Table 2

List of mimicry peptides capable of activating Ro60-reactive T cell hybridomas

Hybridoma	Peptide	Peptide Sequence	Protein	Organism	Comments (Ref)
Hyb-9.5	P87	INIMLAVDISASMLS	vWFA ₈₈₋₁₀₂	<i>Prevotella disiens</i>	Oral and Vaginal. Associated with bacterial vaginosis [18]
	P93	RDLLAVDVSGSMAY	vWFA ₉₀₋₁₀₄	<i>Pseudomonas mendocina</i>	Opportunistic pathogen, can cause endocarditis [19, 20]
	P103	IDIMLAVDVSTSLA	vWFA ₈₈₋₁₀₂	<i>Bacteroides finegoldii</i>	Normal gut flora, isolated from human faeces [21]
	P104	IDIMMAIDVSTSLA	vWFA ₈₈₋₁₀₂	<i>Bacteroides intestinalis</i>	Normal gut flora, isolated from human faeces [22]
	P106	IDIVMAIDVSASMLS	vWFA ₉₂₋₁₀₆	<i>Capnocytophaga ochracea</i>	Normal oral flora, opportunistic periodontitis, sepsis [23]
	P107	IDIVMAIDVSSSMLA	vWFA ₈₉₋₁₀₃	<i>Flavobacterium bacterium</i>	Environmental, marine flora [24]
	P108	IDIVMAIDVSSSMLS	vWFA ₉₂₋₁₀₆	<i>Capnocytophaga sputigena</i>	Normal oral flora, opportunistic pathogen [25]
	P100	IDIMLAIDVSTSLA	BatA ₈₈₋₁₀₂	<i>Bacteroides fragilis</i>	Normal gut flora, opportunistic pathogen causes diarrhea [26]
	P94	IDIVLAVDVSASMLA	BatA ₉₅₋₁₀₉	<i>Flavobacterium bacterium</i>	Environmental, marine flora [24]
	P99	IDIMLAIDVSGSMLA	Mg-chelatase subunit ChlD ₈₈₋₁₀₂	<i>Alistipes finegoldii</i>	Gut microbiota, acute appendicitis [27]
	P91	KRTLLSLDVASAMHW	TROVE domain protein ₃₆₆₋₃₈₀	<i>Corynebacterium amycolatum</i>	Skin flora, can cause endocarditis, sepsis [28, 29]
Hyb-34. 14	P121	DKFLEMAVERVKRIK	Aldeide dehydrogenase ₃₁₅₋₃₂₉	<i>Acinetobacter johnsonii</i>	Skin flora [30]
Hyb-26.4	P135	VIVGQLIEEHRLRYD	Beta lactamase ₁₂₁₋₁₃₅	<i>Listeria grayi</i>	Food borne, opportunistic pathogen [31]