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MMTV Superantigens Coerce an Unconventional Topology between the TCR and MHC Class II

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Mouse mammary tumor virus superantigens (vSAGs) are notorious for defying structural characterization, and a consensus has yet to be reached regarding their ability to bridge the TCR to MHC class II (MHCII). In this study, we determined the topology of the T cell signaling complex by examining the respective relation of vSAG7 with the MHCII molecule, MHCII-associated peptide, and TCR. We used covalently linked peptide/MHCII complexes to demonstrate that vSAG presentation is tolerant to variation in the protruding side chains of the peptide, but can be sensitive to the nature of the protruding N-terminal extension. An original approach in which vSAG was covalently linked to either MHCII chain conﬁrmed that vSAG binds outside the peptide binding groove. Also, whereas the C-terminal vSAG segment binds to the MHCII α-chain in a conformation-sensitive manner, the membrane-proximal N-terminal domain binds the β-chain. Because both moieties of the mature vSAG remain noncovalently associated after processing, our results suggest that vSAG crosslinks MHCII molecules. Comparing different T cell hybridomas, we identiﬁed key residues on the MHCII α-chain that are differentially recognized by the CDR3β when engaged by vSAG. Finally, we show that the highly conserved tyrosine residue found in the vSAG TGXY motif is required for T cell activation. Our results reveal a novel SAG/MHCII/TCR architecture in which vSAGs coerce a near-canonical docking between MHCII and TCR that allows eschewing of traditional CDR3 binding with the associated peptide in favor of MHCII α-chain binding. Our ﬁndings highlight the plasticity of the TCR CDRs. The Journal of Immunology, 2014, 192: 000–000.

Superantigens (SAGs) are bacterial or viral proteins that have the ability to stimulate up to 20% of the total T cell population, bypassing conventional MHC class II (MHCII) Ag processing and presentation (1). SAG-activated T cells undergo a strong proliferation phase that is followed by either anergy or deletion (2). In contrast to canonical MHCII/TCR docking, which relies on the intimate binding of the CDRs to the MHCII α helices and associated peptide, TCR bridging to MHCII by SAGs relies on distinct strategies (3). On the one side, they commonly bind to the TCR CDR2, framework region 3 (FR3), and hypervariable region (HV4) that are within a speciﬁc V family (4). On the other side, they either bind 1) the MHCII α-chain, 2) a binding interface composed of the MHCII α-chain and associated peptide, 3) the MHCII βHis81 residue through coordination of a zinc ion, or 4) the entire MHCII membrane-distal surface spanning the α-chain, β-chain, and the associated peptide (4, 5). Moreover, a handful of SAGs have the ability to crosslink or oligomerize MHCII molecules by using both the α- and β-chain binding sites (6). SAGs of viral origin have yet to provide a clear picture in terms of their ability to compel such interactions.

The best characterized viral SAGs (vSAGs) are those encoded by the mouse mammary tumor viruses (MMTVs). Contrary to other SAGs, vSAGs are produced by the host cell machinery and must undergo posttranslational modiﬁcations to stimulate T cells (7). First, a precursor polypeptide forming a type II transmembrane protein of 37 kDa is modiﬁed by the addition of up to ﬁve N-linked glycans (8). Then, the 45-kDa glycoprotein transits to the Golgi where it is cleaved at two speciﬁc RXXR dibasic motifs, resulting in the luminal N-vSAG and C-vSAG (also called 18 kDa) moieties. After processing, both N- and C-terminal domains remain noncovalently associated and detached from the membrane (9). This maturation allows cells to shed vSAGs, even when devoid of MHCII, a feature called paracrine transfer (10). Finally, by binding both MHCII and TCR, the C-vSAG domain is responsible for cognate T cell stimulation (9, 11). Within this fragment is the most polymorphic region of the MMTV SAGs, the C-terminal 21–38 residues responsible for VB speciﬁcity (12). Extensive TCR mutagenesis has revealed that the TCR binding
site of c-vSAG includes the TCR Vβ FR3 and HV4 (13–17). Interaction with MHCIIIs, in contrast, is puzzling and has been reasoned from competition experiments in which vSAG and the staphylococcal enterotoxin A (SEA) compete for binding on both the α- and β-chains (10, 18).

The molecular basis for successful T cell stimulation by vSAG7 is an interesting conundrum. Our group and others have described vSAG presentation to be influenced by the peptide repertoire (19, 20). However, it is unknown whether this characteristic is linked to vSAG binding to MHCII or TCR recognition of the vSAG-bound MHCII complex. Conflicting data of vSAG binding to the MHCII α-chain, β-chain, or peptide-binding groove exist in the literature and none provided a consensual model on how they successfully generate the T cell signaling complex (10, 18, 21, 22).

In the present study, we sought to determine the topology of the vSAG/MHCII/TCR complex by examining the respective relationship of vSAG with the MHCII chains, associated peptide, and TCR. To this end, we used a panel of covalently linked peptide/MHCII molecules, point mutants, and MHCII/vSAG single chain (SC) constructs. Altogether, our results define a novel MHCII/vSAG/TCR architecture.

Materials and Methods

Plasmids and mutagenesis

The vSAG7 sequence was codon optimized using GeneOptimizer (Invitrogen). pBUD-DRα and −DRβ have been described elsewhere (23). Lumenal portions of DRα (aa 1–191) and DRβ (aa 1–199) were linked to the luminal part of vSAG7 (aa 67–322) or II (aa 72–297) by the PCR overlap extension method (24). The chains were joined by a small glycine-serine linker (G3-S-G3) introduced in DR chains, li and vSAG7 with complementary overlapping primers. DRα mutants, peptide-linked DRβ (25) hemagglutinin SC trimer (HASCT) and SC dimer (SCD)ACI were generated by the PCR overlap extension method using the Phusion polymerase (New England BioLabs). The resulting cDNAs were cloned into pCDNA3 vector (Invitrogen) and sequenced. The IHA, DRβ, and GSPS-HADR peptide-linked DRβ coding sequences were obtained by chemical synthesis (Life Technologies) and cloned in pCDNA3.

Abs and reagents

The VS7 (IgG1) mouse mAb is specific to the C-terminal end of MMTV7 SAG (26). L243 (mouse IgG2a mAb) and ISCR3 (mouse IgG2b mAb) bind to the β1 domain of all HLA-DR molecules (28). BU45 (mouse IgG1 mAb) binds the C-terminal portion of human li (29), and CetCLIP1 (mouse IgG1 mAb) recognizes the N-terminal portion of CLIP (30). The anti-DRα (antiserum against the DRα-chain) and anti-DRβ (antiserum against the DRβ*0101 chain) were obtained following the immunization of rabbits with proteins purified from SDS-PAGE (R.P.S., unpublished). The anti-SEA mAb was a gift of Dr. W. Mourad (University of Montreal) (31). SEA was purchased from Sigma-Aldrich (St. Louis, MO).

Cell lines and transfections

Human epithelial HEK 293T, HeLa DM.5 and HeLa CITA cells (20), and murine DAP cells were culture in DMEM (Wisent) with 5% FBS (Wisent), whereas Knls 13.11 and Knls 12.6 T cell hybridomas (32) were cultured in DMEM with 10% FBS. CTLL-2 (TIB-214; American Type Culture Collection) and HA1.7 TCR+ Jurkat cell line, CH7C17 (33), were cultured in RPMI 1640 (Wisent) with 10% FBS and 4 μM 2-ME. HeLa and DAP cells were transfected using Lipofectamine LTX reagent (Invitrogen) in RPMI 1640 (Wisent) with 10% FBS and 4 μM 2-ME. HeLa and DAP cells were transfected using Lipofectamine LTX reagent (Invitrogen) according to the manufacturer’s instructions. The cells were selected for resistance against gentamicin (500 μg/ml; Wisent), and MHCII+ cells were sorted by flow cytometry on a FACSVerse SE (BD Biosciences). HEK 293T cells were transfected using the calcium phosphate method as described previously (34) using 8 μg plasmid DNA. Cells were harvested and analyzed 48 h after transfection.

Flow cytometry

Cells were incubated in PBS with the primary Ab for 30 min on ice. Cells were washed twice with PBS and incubated a further 30 min with the Alexa Fluor 488–coupled goat anti-mouse IgG Ab (Invitrogen) for 30 min on ice.
transfection efficiencies and plotted mean fluorescence intensity (MFI) in bar graphs.

First, we tested whether the PFRs of CLIP were affecting vSAG stimulation. We hypothesized that the hallmark N-terminal extension of CLIP would affect vSAG7 presentation based on analyses of CLIP peptides found in T2-DR3 cells, which all bear four to six PFRs (35, 45). We generated cell lines expressing MHCII charged with either long CLIP81–101 (cCLIP) or core CLIP87–101 (cCLIP) peptides (Fig. 1B). To ensure that the quaternary structure of the different complexes was not disturbed by the peptide, we used two different mAbs: L243, which binds a conformational epitope on the DRα-chain, and XD5.117, which binds a linear epitope on the DRβ-chain (28, 46). Fig. 1C shows for each cell line the MHCII cell surface expression and the MFI of the reporter GFP (lower panels). Both cell lines expressed similar amount of MHCIIIs and were similarly receptive to DNA upon the subsequent transient transfection of vSAG7 and GFP.

After vSAG7 transfection, cells were incubated with the Vβ6+ T cell hybridomas Kmls 13.11 or 12.6. As shown in Fig. 1D, vSAG7 presentation by ICLIP-DR was inefficient compared with cCLIP-DR, the latter giving rise to strong T cell response. Interestingly, we also observed a decrease in L243 mAb reactivity with the ICLIP-DR1 transfectant (Fig. 1C), better portrayed by the graph in Fig. 1E showing normalized MFI for L243 over XD5. The reduced L243 reactivity for ICLIP/MHCII complexes can either stem from an MHCII conformation alteration or steric hindrance between the CLIP81–86 extension and L243. This result suggests that vSAG7 presentation is sensitive to variations in pMHCII that are also sensed by the mAb L243. However, it is hard to pinpoint whether it is one of these effects, or a combination of both, that accounts for the reduction in stimulatory capacity of vSAG7. On the one hand, it was reported that the L243-like 14-4-4S mAb, specific for mouse MHCII, competes against vSAG7 on I-Ea (47). Likewise, staphylococcal enterotoxin B (SEB), which binds to the MHCII α-chain and competes with L243, is also affected by ICLIP (48, 49). On the other hand, superimposition of the crystal structures of DR1 bound to either CLIP86–101 or CLIP90–101 showed conformational alteration in the loop connecting the third and fourth strands of the MHCII β-sheet platform that includes lysine α39 (50). Mutation of K39 abolishes L243 and SEB binding but does not affect vSAG presentation (51). Therefore, although there is a known conformational change between CLIP-MHCII variants, it is unlikely to affect vSAG7 presentation. Taken together, these results suggest that the reduced vSAG7 stimulation observed when presented by ICLIP-DR1 is due to steric hindrance of vSAG7 by terminal PFRs of CLIP. This conclusion is strengthened by the fact that a HA peptide bearing the N-terminal extension of CLIP (CLIP81–87) was much less permissive in allowing vSAG7 stimulation than the wild-type (wt) HA sequence of the corresponding length (Supplemental Fig. 1). Also, the above-described data corroborate the notion that vSAG7 binds to the MHCII α-chain (10).

Secondly, we sought to evaluate the contribution of TCR-contacting peptide residues by comparing the capacity of peptides with distinct protruding side chains to present vSAG7. To this end, we employed a covalently linked HA307–319 peptide (HADR1). Transfected cell lines from (C), (F), and (H) were used as APCs to stimulate Kmls 13.11, Kmls 12.6 hybridsomas, or Jurkat T cells (D, G, I, J). All data are representative of at least three independent experiments.
Our rationale was to be able to distinguish both the conventional and vSAG presentation in the same setting, using different T cells. Additionally, to minimize adverse effects linked to the bound peptide’s primary sequence, we performed the same assays using another peptide supporting vSAG presentation, the tetanus toxin peptide (TT829–842). Similar to HA-DR1, TT-DR1 has great kinetic stability and also fills the P1 pocket with a tyrosine, thus providing a system to test the exclusive contribution of peptide-protruding side chains (Fig. 1B) (53, 54). The TCR contacting side chains of these two peptides were interchanged, resulting in the backbone (b) HAα and TTβ variants, and involved a charge modification at P-1 (K-Q), a change of a small residue (V) to a bulkier one (I) at P2, and finally a change from a positively charged residue to a highly hydrophobic one (K-F) at P8. When any of the peptide-protruding side chains were implicated in specific vSAG7 contacts, one should expect to see differential stimulation between these peptides, independently of the T cell Vβ specificity. The stably transfected HAβ and TTβ constructs (Fig. 1F, 1H) were then tested for their capacity to stimulate T cells (Fig. 1G, 1I). Every variant successfully presented vSAG7, suggesting that a peptide’s protruding side chains are not critical for vSAG7-mediated response (Fig. 1G, 1I). Conversely, presentation of HAα failed to stimulate Jurkat T cells (Fig. 1J), as specific TCR contact residues have been modified (52). Many covalently-linked peptides supported strong vSAG7 presentation, ruling out a role for the peptide’s C-terminal linkers and thus PFR at this end. Altogether, these results suggest a model in which vSAG7 presentation is tolerant of the nature of the peptide’s protruding side chains, but can be sensitive to the conformation adopted by the N-terminal extension of some peptides.

**N-vSAG7 has an overlapping binding site with SEA on the DRβ-chain**

The above-described data support the notion that C-vSAG7 binds to the MHCII α-chain. However, it has been proposed that the vSAG7 polypeptide has two binding sites on MHCII, one of which shares the same interface as SEA on the β-chain or binds the peptide-binding groove (18, 21, 22). Maturing vSAGs are composed of two domains that remain noncovalently associated upon processing. C-vSAG participates in MHCII/TCR bridging but the role of N-vSAG is less clear. To gain insight on the role of the latter, we designed two SC polypeptides in which the luminal domain of either DR chain is linked to that of vSAG7. These straightforward SCs were inspired by a similar fusion between MHCII and Ii (55). Such SCs were made possible because HLA-DR and vSAG7 are type I and type II proteins, respectively, allowing fusion of the C terminus of DR chains to the N terminus of vSAG7. The resulting DRα- and DRβ-based fusion molecules were named αSCD and βSCD, respectively, and are depicted in Fig. 2A. For brevity, when an SC is mentioned in an experimental setting, it is always cotransfected with its matching complementary wt DR chain partner; for example, DRα plus βSCD is referred to as βSCD.

First, we characterized the interaction between the SCs and their complementary MHCII chain partner. For these experiments, as Jurkat) (33, 52). Our rationale was to be able to distinguish the peptide’s protruding side chains, but can be sensitive to the conformation adopted by the N-terminal extension of some peptides.

**FIGURE 2.** The N-terminal domain of vSAG7 overlaps the SEA binding site on the MHCII β-chain. (A) Schematic representations of the constructs used throughout this study. Different peptides were appended to DRβ (pepDRβ) C terminus based on the strategy by Kozono et al. (25). The C terminus of DR1 β-chains was covalently attached to the N terminus of Ii (iSCD), vSAG7 (SCD), or N-vSAG (SCDΔCt) by a flexible GLY-SER linker. Similarly, the C terminus of DR1 α-chain was covalently attached to the N terminus of vSAG7 (αSCD). In the SCT, a peptide was also linked to the N terminus of the SCD. The linkers between DR and either the peptide, vSAG7 or Ii, are represented as light gray boxes. The arrowheads represent the three vSAG7 cleavage sites. (B) L243 and VS7 cell surface staining of HEK 293T cells transfected with the αSCD or βSCD and the appropriate complementary DR chain or a wt DR and vSAG7 control. L243 and VS7 mAbs recognize a conformational epitope on DRα and the C-terminal end of vSAG7, respectively. (C) HEK 293T cells were transiently transfected with wt DR and vSAG7, DRα and the βSCDs, or βiSCDs. Forty-eight hours after transfection, cells were harvested, split, and incubated on ice with L243 or SEA for 3h. After washing, bound SEA was detected by flow cytometry using an anti-SEA mAb. These staining profiles are representative of at least five independent experiments. (D) The MFIs were plotted as a ratio where the error bars represent the SD to the mean of three stainings on independent populations of transfected cells. (E) Same as (D), but using DRα K39A mutant, which prevents SEA binding to the α-chain. All data are representative of at least three independent experiments.
we used HEK 293T cells given their high transfection efficiency. First, the α-SCD apparently failed to form a functional complex with DRβ, as judged by the lack of L243 surface staining and the fact that DRβ remained entirely EndoH-sensitive (Fig. 2B, Supplemental Fig. 2, left panel). Also, the α-SCD migrates as two major bands, which are smaller than β-SCD and appear to be devoid of sugars (Supplemental Fig. 2, middle and right panels). In contrast, the β-SCD properly associated into heterodimers with DRα, trafficked to the plasma membrane, and was recognized by a conformational mAb (Fig. 2B, left panel). Using the anti-C-vSAG7 mAb VS7, we also observed MHCII-bound vSAGs at the cell surface (Fig. 2B, right panel). Altogether, these results indicate that only the β-SCD can generate ordered complexes and allow vSAG surface expression.

Next, to confirm that vSAG7 behaved similar to the wt vSAG when part of the β-SCD, we verified whether it still competed the binding of SEA. We transfected HEK 293T cells with either wt DR or β-SCD and performed SEA binding assays monitored by flow cytometry. Fig. 2C shows the DR (left panel) and SEA (right panel) staining profiles, which reveal weak SEA binding to cells expressing β-SCD. Once normalized for MHCII surface expression, we noted a marked reduction in the binding of SEA for β-SCD compared with the wt or a control βII-SCD controls (Fig. 2A, 2D). These results indicated that the vSAG moiety on DRβ interferes with SEA binding. However, because SEA has a minor low-affinity binding site on the MHCII α-chain, the possibility remained that the observed competition was due to the presence of vSAG7 on DRα (56, 57). To test this, we performed the same experiment using a DRα mutant that is unable to bind SEA (DRαK39A) (56). As shown in Fig. 2E, binding of SEA is the same whether DRβ is associated with wt MHCII α-chain or K39A mutant. Moreover, truncating C-vSAG from β-SCD, generating the βSCDΔCt, still reduced SEA binding, confirming that N-vSAG7 and SEA have an overlapping binding interface (Fig. 2D). The high SEA concentration and affinity for βHis81 are factors likely to favor displacement of some of the vSAG7 molecules and could explain why competition was not total. Likewise, processing of the vSAG7 moiety is observed and could favor its dissociation from the β-chain (Supplemental Fig. 2). These data confirmed that N-vSAG has an overlapping binding site with SEA on the MHCII β-chain.

vSAG7 does not bind the MHCII peptide groove

Our results described so far define the binding site of C-vSAG7 and N-vSAG to the MHCII α-chain and β-chain, respectively. Thus, it was difficult to envision a model where vSAG7 interacted with both DR chains at once. Huber and colleagues (22) proposed that a segment of MMTV SAGs (called the MHCII peptide-binding motif) interacts with the MHCII peptide-binding groove upon synthesis in the endoplasmic reticulum. The MHCII peptide-binding motif is part of N-vSAG and could bridge both vSAG segments through the MHCII cleft. To verify whether vSAG7 occupies the MHCII groove, we designed a new construct, HASCT, in which the HA peptide was appended to the β-SCD N terminus (Fig. 2A). Based on the Hsu et al. (22) premise, our rationale was that N-vSAG7 would prevent HA binding to the groove. Fig. 3A shows the surface DR expression for both β-SCD and HASCT in comparison with wt DR and HADR. Interestingly, DR surface staining was increased in both transfectants expressing the linked HA peptide. These data corroborated well with the increased stability of MHCII when optimal peptide occupies the groove (25). Soluble versions of DRβ and HAβ (sβ and sHAβ) chains were used as controls and ruled out transmembrane domain–mediated bias between the transmembrane-devoid β-SCD and controls. L243 MFI ratios of sβ and β-SCD against sHAβ and HASCT, respectively, showed a 4-fold increase in expression when HA was fused to DRβ (Fig. 3B). That the tethered HA peptide increased expression of DR is incompatible with a model in which vSAG would occupy the binding groove.

To confirm that the HA peptide was lodged inside the groove and not displaced by the immature vSAG7 polypeptide, we tested the complex for SDS sensitivity. Indeed, HA/DR1 complexes resist SDS denaturation and migrate as compact peptide-loaded heterodimers.

FIGURE 3. vSAG7 binding to MHCII α- and β-chains occurs on distinct MHCII molecules. (A) Cell surface expression of MHCII on HEK 293T cells transfected with DRβ or HAβ (left panel) and β-SCD or HASCT (right panel) stained with the L243 mAb. (B) The MFIs of the transfectants bearing the HA peptide were plotted as a ratio against the ones without HA. The error bars represent the SD to the mean of three stainings on independent populations of transfected cells. (C) Compact SDS-resistant and -sensitive forms of DR were assessed by immunoblot (IB) analysis of total protein extracts (boiled or heated to 65˚C) from the transfectants in (A). β-SCD (βI) was used as control. The IB was revealed with the anti-DRβ mAb XDS. The two compact complexes are marked with I or II (lanes 2 and 8) and schematized next to the gel. (D) Comparison of HA-specific T cell stimulation between endogenous MHCII* or MHCII* HeLa cells transfected with different MHCII β-chains. (E) Same as (D) but comparing vSAG7-specific T cell stimulation. All data are representative of at least three independent experiments.
under nonboiled conditions (58). Cell lysates obtained from the transfectants presented in Fig. 3A as well as a β2mSCD control were analyzed by SDS-PAGE. XD5 mAb was used to probe the immunoblots. As expected, in absence of HLA-DM, neither 1i nor vsAG7 SCDS formed complex mutants with DRα (Fig. 3C, lanes 4 and 6). In contrast, when HA was linked to either DRβ or βSCD (lanes 2 and 8), complex, SDS-resistant heterodimers were observed, confirming that the HA peptide was present in the groove.

To unequivocally prove that vsAG7 did not bind to the MHCII cleft, we tested whether the HASCT could induce both Jurkat and vsAG-specific T cell responses. To this end, we moved from the HEK 293T to HeLa cells, the former being unable to present vsAG (20). Of note, although the molecular basis of this defect remains obscure, HeLa cells are unable to present vsAGs unless transfected with CIITA or treated with IFN-γ (20). This provided a useful system to evaluate the presentation of HA independently of vsAG in the absence of CIITA and of both HA and vsAG7 in the presence of CIITA. We transiently transfected our various β-chains into either HeLa or HeLa CIITA α cells and assessed the response of Jurkat or Kmls 13.11 cells. As expected, vsAG7 presentation only occurred in the presence of CIITA, whether from the SCD or SCT (Fig. 3E). Surprisingly, HeLa cells transfected with HASCT failed to stimulate Jurkat cells in contrast to the control shAB (Fig. 3D).

The lack of HA presentation can be attributed to either an MHCII conformation flaw or to the vsAG moiety of the HASCT hampering proper TCR docking. We argue for the latter alternative because the tested mAbs did not distinguished the HASCT from the sHA β control (Fig. 3A), and in the presence of CIITA, HA presentation was restored (Fig. 3D). However, that Jurkat failed to respond to the HASCT in CIITA α cells was puzzling. This is not due to degradation of the SCT-HA peptide and its subsequent association to endogenously expressed MHCII, as the DRβ0102 allele in the homozygous HeLa CIITA α cells does not present HA307–318 to Jurkat T cells (59). vSAG presentation only occurred in the absence of vSAG presentation, thus revealing HA to the HA-DR1 moieties of the SCT. One explanation is that in presence of a pool of MHCII, C-vSAG is transferred from the processed SCT onto the endogenous pMHCII, thus contributing to the presentation of HA independently of vSAG.

vSAG7 presentation relies on MHCII/TCR interactions

To better pinpoint the C-vSAG7 binding site on DRβ and investigate the TCR binding topology, we generated an array of mutants from DQ2 and IAq, which present vSAG poorly and span aa 63–68, the interface associated with SEB binding (12). Fig. 4A shows the point mutants on the HA-DR1 model (Protein Data Bank no. 1FY7) as well as DRβ63–71. vSAG-specific T cells were transfected with vSAG7 or a mock control before monitoring Jurkat and lvekit proliferation. All data are representative of at least three independent experiments. (G) Model of vSAG7 binding to MHCII and TCR in which the multiple binding regions between vSAG7, both MHCII chains, and the TCRs are highlighted. The N-vSAG and C-vSAG moieties remain noncovalently associated after crosslinking of MHCII molecules. Binding of C-vSAG7 to the MHCII α-chain is conformation-dependent and abrogated by a peptide N-terminal extension. The TCRβ6 CDR3 binding site on the MHCII-TCR interactions.
DR expression in DAP cells is given in Fig. 4B as assessed by L243 and XD5 staining. As above, stable cell lines were transiently cotransfected with vSAG7 and the reporter GFP. Then, cells were cocultured with Kmls 13.11 and Kmls 12.6 T cells and IL-2 production was measured. In an effort to minimize the effect of the cells’ intrinsic associated peptides, we coexpressed each DRα variant with the HADFRβ-chain. Additionally, this allowed us to monitor the stimulation of HA-specific Jurkat cells, which could be impeded by the α-chain mutations (Fig. 4D). As predicted from the HA/DR1/HA1.7 TCR cocystal (52), only mutation of TCR contact residues α64, α65, and α67 abrogated presentation of HA to Jurkat cells.

Of note, the reactivity of L243 was strongly decreased toward DR mutants α63 and α67 (Fig. 4B). This is best portrayed by the graph in Fig. 4C showing L243 MFI over the MFI of the conformation-insensitive XD5 mAb. Surprisingly, those same DR mutants had a dramatically reduced ability to present vSAG7 (Fig. 4E, 4F), suggesting again overlapping binding regions between L243 and vSAG. Because the side-chain of α63 points laterally, the weakened stimulation observed with both hybridomas by the α163E MHCIIs mutant cell line suggests that C-vSAG7 binding is somehow affected by this mutation. Indeed, a less obstructive mutation, αI63A, restored vSAG7 stimulation (Supplemental Fig. 3A, 3B).

Interestingly, whereas DRαA64R allowed a strong Kmls 13.11 stimulation, Kmls 12.6 failed to respond (Fig. 4E, 4F). This result indicates a TCR recognition effect unrelated to vSAG7 binding and highlights the importance of specific DRα/TCR contacts for efficient T cell stimulation. In line with this, it became difficult to evaluate whether the effect of DRα K67A on vSAG presentation is actually linked to a weakened association between vSAG and DRα or to a TCR recognition defect. Indeed, DRα K67 is characterized by a lack of both L243 reactivity and inefficient presentation of HA peptide to Jurkat cells, making both outcomes plausible (Fig. 2C, 2D). Although weak, the stimulatory response observed exclusively with the Kmls 13.11 hybridoma suggests that TCR recognition is a factor.

The solvent-exposed DRα-α helix is used by SEB to bridge TCRs (61). The fact that vSAG7 presentation implicates DRα/TCR interactions demonstrates that the topology of the ternary complex is different from the one involving SEB. Interestingly, only the DRα mutant A64R cell line was unable to support SEB presentation (Supplemental Fig. 3C). The highly conserved A64 is buried inside the SEB/DRα interface and the Arg substitute is likely to sterically hinder SEB association, probably reflecting the effect of I63E on vSAG7 presentation. Overall, it is apparent that the mechanism leading to TCR recognition of the vSAG/MHCIIs complexes differs from SEB and canonical peptide/MHCIIs recognition.

vSAG7 mediates TCR activation through a conserved TGXY motif

The specific interactions between the TCR and the MHCII α-chain suggest the existence of conserved vSAG/TCR contacts. Given the numerous responsive Vβs for all vSAGs, it is unlikely that the TCR interaction is solely based on the polymorphic vSAG C terminus, as it would severely impede the likelihood of a cognate partnership. Interestingly, an alignment of MMTV SAGs and MMTV SAG-related domain from other viruses (e.g., herpesvirus) revealed a conserved TGXY motif (Fig. 5A) located at residues 226–229 of C-vSAG. We mutated the Tyr229 to a Phe and assessed the ability of vSAG7-YF to stimulate different T cell hybridomas. Fig. 5B indicates that the Y229F mutation introduced either in full length or SC vSAG7 abrogated the activity.

The TGXY motif could impact vSAG activity in many ways. On the one hand, it could perturb C-vSAG binding to MHCIIIs or its overall structural integrity. Both of these hypotheses are refuted by the fact that the mutation did not affect surface expression of MHCIIIs or vSAG7 (Fig. 5C, 5D). On the other hand, the Tyr229 could be implicated in direct TCR contact. Supporting this mechanism is the presence of a similar motif, LGNY, on a SEB contacting loop that interacts with the HV4 region of the TCRβ. Accordingly, mutation of the Tyr on SEB prevents its presentation (63, 64). Furthermore, Digglemann and colleagues (65) described the region of C-vSAG bearing the TGXY motif as part of the TCR-interacting domain. These results suggest that in addition to highly polymorphic regions responsible for Vβ specificities, vSAG binding also relies on conserved vSAG-specific TCR interactions.

Discussion

The data presented in this study define how vSAG7 bridges MHCII to the TCR. C-vSAG binds the MHCII α-chain in a conformation-sensitive manner at the interface formed between the α helix and the β-sheet platform. We demonstrated that, when coerced by vSAG, the TCR recognizes the MHCII in a near canonical manner, which constitutes a unique topology among those previously described for SAGs. Next, our results suggest that maturing N- and C-vSAG7 bind the MHCII β- and α-chains, respectively, on distinct MHCIIIs. Finally, we identified a conserved motif in MMTV SAGs responsible for TCR binding independently of the TCR Vβ specificity. Based on our results and those of others, we propose a model in which the N- and C-terminal domains of a single vSAG crosslink two MHCIIIs and bridge only specific TCR-bearing Vβ elements that can be skewed to recognize the MHCII α-chain instead of the associated peptide (Fig. 4G).
C-vSAG7 binding to the MHCII α-chain is influenced by the associated peptide

Presentation of vSAG7 requires that MHCII be filled with a diverse peptide repertoire (11, 20, 38). In the present study, we demonstrated that a single peptide supports vSAG7 presentation. However, the MHCII associated peptide must meet certain criteria to be part of a permissive pMHCII. By comparing cell lines expressing either cCLIP-DR1, ICLIP-DR1, and CLIP-HADR1, we showed that the CLIP peptide PFRs inhibit vSAG7 presentation (Fig. 1D, Supplemental Fig. 1). Interestingly, it was previously described that CLIP N-terminal extensions also interfered with SEB binding to MHCII (49). The crystal structures of CLIP-DR3 and CLIP-I-αβ show that the CLIP N-terminal extension is disordered, suggesting an idiosyncratic effect of this CLIP region on vSAG function (66, 67). Future studies will address this issue by characterizing the impact of a larger panel of N-terminal extensions linked to various core peptides. Nevertheless, knowing the profound effect of PFRs on canonical and toxic shock syndrome toxin-1 T cell responses (39, 68, 69), and that DM favors the binding of peptides that tightly fit the groove, our findings point to N-terminal peptide trimming as a potentially important determinant for vSAG presentation (40, 70). These results explain why DM-deficient cells, either murine or human, are unable to present vSAG7, as they are predominantly charged with CLIP peptide bearing a 4- to 6-aa extension (34, 45, 71). Hsu et al. (22) reported that a DM-β cell line was able to present vSAG7. However, as opposed to T2 cells expressing a single MHCII (DR3) or our DP cell lines (DR1), the proportion of the various alleles of DR, DQ, or DP that are bound to CLIP at steady-state, or which form of CLIP is bound, was not determined in this DM-β model.

It is unclear whether the previously reported need for peptide diversity reflects a direct contribution of the peptide’s sequence to part of the vSAG binding site or is an indirect negative effect echoed in the MHCII conformation. The findings presented in this study support the latter hypothesis. It is unlikely that vSAG7 interacts with the MHCII-bound peptide’s protruding side chains because severe changes in charge or size between those of HA, TT, and CLIP did not affect their ability to activate Vβ6- and Vβ8.1-bearing T cells (Fig. 1, Supplemental Fig. 3D). Although we did not conduct a thorough saturation mutagenesis of the peptides, our observation of DRα/TCR contacts portray a setting in which vSAG7/peptide interactions are improbable (see below).

Our results suggest that C-vSAG7 binds to the lateral interface formed by the solvent-exposed DRα α-helix/β-sheet junction (Fig. 4A), an area highly susceptible to peptide-induced conformational changes (72). That HA-DR1αI63E, but not HA-DR1αI63A, reduced both L243 mAb reactivity and vSAG7 presentation capabilities points to an MHCII conformation defect (Fig. 4C, 4D, Supplemental Fig. 3A, 3B). It was previously reported that mice expressing Eo-bound I-αβ as the sole pMHCII were unable to mount vSAG7-specific responses, supporting the conclusion that a diverse MHCII peptide repertoire was required (38). In light of our results, we can speculate as to why this pMHCII failed to present vSAG. The Eo(52–68) peptide bears no N-terminal PFRs, and the C-terminal linker between the peptide and the MHCII β-chain does not influence vSAG presentation. Thus, as previously presented by the authors, the inability of Eo-I-αβ to successfully present vSAG must be due to its intrinsic conformation (38). Indeed, whereas the 25-9-17 mAb binds a panel of peptide-bound I-αβ, Eo-I-αβ was not recognized (73, 74). One possibility is that vSAG7 binding is affected by the αβ24 residue, reported to be pushed outward by the bulky p1-filling Phe of Eo at the C-vSAG7 binding site (75). Although we did not observe an effect of a Y-A substitution at the p1 HA residue (Supplemental Fig. 3E), it would be interesting to determine whether a bulkier amino acid (e.g., Trp) substitution would reduce vSAG7 presentation accordingly. Collectively, these results indicate that the interface formed on the DRα α-helix/β-sheet junction is paramount for vSAG7 binding and that its presentation is vulnerable to both peptide-mediated conformational changes and N-terminal PFRs.

TCR engagement by vSAG7 is unique among SAGs

Most of the SAG-mediated T cell signaling complexes outline a typical interaction between the TCR Vβ and the SAG, where the Vβ sometimes participates in MHCII β-chain binding (4, 76, 77). Consequently, Vβ/MHCII α-chain contacts are sterically precluded by the bound bacterial SAGs. Given the lack of an MMTV SAG crystal structure, it is challenging to predict how vSAGs force MHCII/TCR association. Previous analyses of the response of mature peripheral T cells against SAGs have clearly established that non-Vβ components of the TCR are involved in the pMHCII/vSAG complex recognition (78–81). The Vβ-chain was shown to be an important part of the complex, and a skewed repertoire in responding cells has been identified (82, 83). Within a given Vβ family, reactivity was seen with only certain Vβ subfamily members (83, 84). Thus, the variations in the CDR3 regions, especially CDR3, are likely to affect the capacity of these TCRs to bind the MHCII and might be at the origin of many of the reported cases of MHCII allelic/isotype restriction (32, 47, 83, 85).

Based on these facts and our results demonstrating that DRα A64R and K67A mutant cell lines differentially stimulated distinct hybridomas bearing the same Vβ, it is clear that the TCR/MHCII contacts are imperative to vSAG7 signal transduction (Fig. 4E, 4F). Moreover, these findings highlight direct MHCII α-chain/TCRβ contacts and would imply that vSAG7-mediated TCR engagement resembles that of conventional peptides, as hypothesized by the Marrack and Kappler group following their discovery that the TCR βT24Y mutant abrogated both conventional Ag and vSAG presentation (13, 86). Along the same lines, our MHCII α64 and α67 mutants were also unable to trigger HA-specific activation of Jurkat cells (Fig. 4D). Based on the crystal structure of HA/DR1/HA1.7 TCR, DRα A64 and K67 are bound to the CDR2β D51 (52). However, one must be cautious in unifying these results. Indeed, as we have shown with our many different peptide/MHCII combinations, the peptide’s protruding side chain did not affect vSAG response by the tested hybridomas, as would be expected in a traditional pMHCII/TCR setting (Fig. 1). Furthermore, mutations at residues α65 and α68, which are also implicated in TCR binding (52), had no effect on the ability of MHCII to present vSAG7 (Fig. 4D, 4E). Thus, it is fitting to picture a near canonical docking between MHCII and TCR that is coerced by vSAG. Such architecture allows eschewing of traditional CDR1/3 binding with the associated peptide in favor of MHCII chain binding as proposed by Nguyen et al. (87). These Vβ/MHCIIβ and Vβ/MHCIIα contacts remain to be verified biochemically (88).

A large body of literature suggests that the β-chain junctional region could influence TCR recognition of SAGs (79, 89–91). Especially relevant was the demonstration by many groups that Vβ6 thymic deletion is incomplete in vSAG7 mice and that Vβ junctional diversity regulates vSAG reactivity (91, 92). At first glance, the need for a diverse peptide repertoire and the importance of Vβ junctional diversity may seem paradoxical. Because many peptides support vSAG binding, we do not expect skewing in CDR3. However, our results point to the existence of nonpermissive peptides regulating vSAG binding and to unconventional
interactions between MHCII and TCR. In order words, there may be extra pressure on CDR3s to have more intimate contacts with the MHCII chains than during canonical peptide recognition. Indeed, thymic positive selection does not directly influence the fine specificity of T cells toward MHCII, and it has been suggested that TCR/MHCII interactions taking place during sSAG presentation are unconventional and linked to TCR recognition of haplotype-specific MHCII residues (79, 87). This is in line with the recent demonstration by Kilgannon et al. (92) that three of eight Vβ6+ T cells, although all specific for the same pMHCII (K5-I-Aβ), did not respond to vSAG7.

A recent report by Stadinski et al. (93) stressed the fact that TCR specificity for pMHC ligands is not driven by germline-encoded pairwise interactions. Notably, they described a single TCR Vβ using alternate strategies to bind pMHC when paired with different Vα. The authors argued that because CDR1 and CDR2 loops have the flexibility to bind their ligands in many ways, this could not fit a pairwise coevolution model. Accordingly, it demonstrated that the CDR3 loops can markedly alter those evolutionarily selected contacts (94). Canonical CDR/pMHCII contacts can differ under different pressures, supporting a model in which vSAG enforces such unconventional docking.

vSAG7 moieties bind distinct MHCII

Given that vSAG7 N- and C-terminal moieties bind respectively the MHCII β1 and α1 domains, one must ask how such binding is possible while remaining noncovalently associated. We propose a model where C-vSAG binds the α-chain of an adjacent MHCII, while still bound to N-vSAG, with the latter attached to the β-chain. In other words, unable to reach the α-chain of its MHCII partner in cis, C-vSAG interacts with another MHCII in trans. This model highly resembles the manner in which SEA crosslinks MHCII molecules via a low-affinity binding site to the α-chain and the Zα-dependent binding site on the β-chain (95, 96). In the context of the SCT, our results showing that HA presentation to Jurkat cells is null in endogenous MHCII as opposed to MHCII+ cell lines strongly support such a model (Fig. 3D). Indeed, we speculate that in the absence of surrounding endogenous pMHCII, both vSAG7 moieties remain associated with the parent MHCII, blocking the groove and preventing the TCR from recognizing the HA peptide. Also, because each MHCII is covalently linked to vSAG, it is possible the C-vSAG prevents any approaching vSAG from contacting the α-chain in trans. In contrast, in HeLa CITIA cells, C-vSAG7 will find numerous vSAG-free endogenous MHCII α-chains to associate with, freeing the MHCII membrane-distal region for the HA-1-7 TCR binding. Although our data best fit a model where vSAG crosslinks MHCII molecules, we cannot entirely rule out that vSAG binds across the groove on the α- and β-chains of a single molecule. Biochemical and crystallographic studies will be needed to decisively demonstrate the binding of a single vSAG to two different MHCII molecules.

Highly conserved TCR binding motif in sSAGs

Our results indicate a more intricate vSAG7 presentation than previously acknowledged. vSAG stimulatory activity is paired to the TCR interaction of DRα, a structural aspect that will likely modulate its potency in terms of the responding T cell repertoire and the strength of the signal. Additionally, vSAG recognition of specific TCR Vβ elements is mediated through its C-terminal 30 or so highly variable amino acids, complicating speculation as to how this family of sSAGs could mediate such a broad immune response without a conserved binding scheme.

In conclusion, our observation that conserved DRα residues are implicated in the recognition of the vSAG/peptide/MHCII by the TCR suggests that T cell activation is influenced by the polymorphic CDR3β region, linked to the recognition of the MHCII α-chain. To our knowledge, this represents a novel SAG-mediated MHCII/TCR architecture. According to a recent study by Nur-ur Rahman et al. suggesting that the TCR CDR2β is the critical determinant for the functional recognition of bacterial SAGs, it is clear that the topology of vSAG differs from those previously described (97). Because both the MHCII allele and associated peptide influence the potential interaction between MHCII and TCR, a given TCR could only recognize a fraction of the vSAG/pMHCII complexes (79). MMTV SAGs are expressed at very low density on the cell surface and still remain highly potent T cell activators. This expression pattern is of great importance, as it mimics the low density of conventional antigenic peptide/MHCII complexes, critical for T cell activation (98, 99).

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Disclosures

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