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Staphylokinase-Specific Cell-Mediated Immunity in Humans

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Staphylokinase is a highly fibrin-specific clot-dissolving agent that constitutes a promising drug for clinical development. It is of bacterial origin, and the majority of patients develop neutralizing Ab after its administration. Several antigenic regions, recognized by these Ab, have been identified, but the underlying immunogenic features of staphylokinase remain unknown. In this study, we show that staphylokinase is a T cell-dependent Ag, and that an immunological memory may be acquired, even without administration of staphylokinase. Thrombolyis with staphylokinase provokes the proliferation of staphylokinase-specific T lymphocytes, which remain elevated over 10 mo posttreatment. Interestingly, analysis of a large number of staphylokinase-specific T cell clones isolated from 10 unrelated donors revealed only six distinct immunogenic regions in the molecule. Moreover, five of the six regions are recognized by T lymphocytes from several individuals, indicating that these regions are not restricted to a single HLA-DR allele. Therefore, these new insights can guide the design of variants with a lower immunogenic profile in humans. The Journal of Immunology, 2002, 168: 155–161.

Although B lymphocytes are directly responsible for the production of Ab, they can only do so with the help of specific T lymphocytes. Therefore, in a humoral response in which B and T cells meet, the T cells provide the restricting elements (14). In fact, if circulating Ab recognize an Ag (i.e., antigenic), the binding does not predict whether that particular Ag will stimulate subsequent immune reactions (i.e., immunogenic) (15). If T cell help is insufficient or absent, the formation of Ab is greatly impaired, and the development of an immunological memory is hampered (16, 17).

In this study, the underlying cellular aspects of the SakSTAR-specific humoral response were studied. SakSTAR-specific T lymphocytes were detected both in patients and in healthy individuals who never received staphylokinase treatment. Detailed analysis of SakSTAR-specific T lymphocytes, cloned from 10 unrelated individuals, revealed the presence of six distinct immunogenic regions. These regions were not restricted to a single HLA-DR molecule, but were more widely recognized. The limited number of functional T cell regions combined with their potential promiscuous behavior with respect to HLA-DR binding may allow the design of SakSTAR variants with a reduced immunogenic profile in humans.

Materials and Methods

Blood donors and PBMC isolation

PBMC from healthy individuals were isolated from either blood or buffy coats (Red Cross Bloodbank, Leuven, Belgium), and from thrombembolic patients before and after (3–44 wk) SakSTAR treatment. The PBMC were isolated according to standard procedures using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation, and stored under liquid nitrogen until usage.

Ag and peptides

Two natural variants of recombinant staphylokinase were used, Sak42D and SakSTAR. The first was isolated and purified in Jena, Germany (18), and SakSTAR was expressed and purified as previously described (19). The purified preparation was further subjected to size exclusion chromatography on Superdex G75 (Pharmacia, Uppsala, Sweden), aliquoted, and stored at −20°C. Streptokinase (SK)3 was purchased from Behring (Hoechst, Brussels, Belgium), aliquoted, and stored at −20°C. The following SakSTAR-derived peptides, 17 mers overlapping 12 residues (1-SSSFDKGGYKGGDASY-17, 6-KGKYKGDASFYEPTG-22, 11-K

3 Abbreviations used in this paper: SK, streptokinase; BrdU, 5-bromo-2′-deoxyuridine; IHW, International Histocompatibility Workshop; SI, stimulation index.
The SI in this assay system has a maximum value of 200,000 which was obtained from the plated dilutions, and in which the OD was at least twice that of the spontaneous proliferation. The given SI is the highest value obtained by dividing the obtained OD from the PBMC in response to an Ag by the PBMC in culture medium alone. The visible T cell clones were harvested under the microscope and individually restimulated in a 96-well flat-bottom plate with irradiated autologous PBMC as APC. These wells were supplemented on day 1 with 20 U/ml human rIL-2 (Boehringer Mannheim, Mannheim, Germany) and allowed to grow for 8–11 days. Restimulation was repeated every 8–11 days under the same conditions. After three to six passages, autologous PBMC were replaced by autologous EBV-transformed B lymphocytes, as APC.

**EBV B cell lines**

EBV was isolated from Marmoset B95-8 cells (ATCC CRL1612), according to standard centrifugation and filtration procedures (22). PBMC of the HLA-DR4 individual were cultured with autologous supernatant RPMI 1640 supplemented with 10% FBS, 10 μg/ml gentamycin, and 1 μg/ml cyclosporin A. Established EBV cell lines were then expanded conservatively, and after 3–4 wk of culture, aliquots were stored under liquid nitrogen. The cell lines could be maintained in culture for at least 1 year. These cell lines were tested for the expression of CD19, CD80, CD86, CD54, HLA-DR, and HLA-ABC. All EBV B cells expressed the indicated cell surface markers with minor intensity differences as determined by FACS analysis, and stained negative for CD3 and CD4. They were also tested for their Ag-presenting capacities, and showed to be functional for expanding T cell clones as well as in T cell epitope analysis. Heterologous EBV B cell lines were obtained from the European Collection of Animal Cell Cultures, for their homozygous HLA-DR typing by the International Histocompatibility Workshop (IHW). Ten unrelated donors were selected, and their PBMC were thawed, washed, and cultured in RPMI 1640 containing 5% FBS, 10 μg/ml gentamycin, and 1 μg/ml cyclosporin A. Established EBV B cell lines were then expanded for SakSTAR-specific T cell clones.

### Results

**Staphylokinase-specific cellular immune response in normal individuals**

Some patients develop SakSTAR-specific IgGs after treatment; therefore, the presence of circulating SakSTAR-specific T lymphocytes was studied. Classical proliferation assays in 96-well plates did not give reproducible data, because the frequency of SakSTAR-specific T lymphocytes in the periphery was found to be less than 1 in 200,000 PBMC. However, upscaling of the assay system, as described in Materials and Methods, resulted in the detection of a SakSTAR-specific cellular response. Fig. 1 shows the proliferation responses of PBMC from 80 healthy normal individuals to SakSTAR, Sak42D, and a classical recall Ag SK. A positive response to SK was found for 97% of the donors, indicating that all assay conditions for proliferation were present. In contrast, a correlation was found between a positive staphylokinase-specific cellular response (SI > 2) and age. In the oldest age groups (over 40 years of age), >70% tested positive, whereas in the youngest age groups (under 30 years old), less than 30% of the donors showed a staphylokinase-specific cellular response.

**Staphylokinase-specific cellular immune response in patients**

The vast majority of peripheral arterial occlusion patients, selected for SakSTAR treatment, exceed the age of 55 years. Therefore, it was expected that many of the eligible patients had established an immunological memory against staphylokinase. For a single SakSTAR-treated patient, who developed a high neutralizing Ab titer, the cellular response could be monitored up to 10 mo after treatment. Four weeks after treatment, the specific SakSTAR response reached the limits of our assay system (SI ≥ 30–40), indicating that the frequency of SakSTAR-specific T lymphocytes had largely increased. The SakSTAR-specific cellular response remained high for 10 mo after treatment. Subsequently, several patients were tested for the presence of SakSTAR-specific T lymphocytes before and 4 wk after treatment. In one patient who did not develop a humoral response, no SakSTAR-specific proliferation could be measured before nor 4 wk after treatment (Fig. 3A). Six patients who developed neutralizing Ab inactivating between 5 and 20 μg/ml (Fig. 3B) or >150 μg/ml (Fig. 3C) were tested twice for their specificity using the above 25 SakSTAR-derived peptides. Briefly, two specific T cell clones were expanded with mitomycin C-treated autologous EBV B cells (ratio 1:5) combined with Ag (no Ag, SK, negative controls; and SakSTAR, Sak42D, positive controls) or the SakSTAR-derived peptides were cultured in duplicate in a 96-well round-bottom plate for 4 days. Subsequently, they were pulsed for 20–24 h with BrdU, harvested, and analyzed for their BrdU content. Proliferation of the T cell clones was found positive, if the SI was at least three in the independent experiments. Similar assays were performed, but with the IHW cell lines as APC, to study the HLADR restriction of a particular T cell clone. Blocking assays were done by preincubating the autologous APC with anti-HLA-DR Ab (clone G46-6 (L243, mlgG2a,α); BD Biosciences, Heidelberg, Germany), and subsequently cultured as described above.

**T cell clone analysis**

All isolated T cell clones were tested twice for their specificity using the above 25 SakSTAR-derived peptides. Briefly, two specific T cell clones were expanded with mitomycin C-treated autologous EBV B cells (ratio 1:5) combined with Ag (no Ag, SK, negative controls; and SakSTAR, Sak42D, positive controls) or the SakSTAR-derived peptides were cultured in duplicate in a 96-well round-bottom plate for 4 days. Subsequently, they were pulsed for 20–24 h with BrdU, harvested, and analyzed for their BrdU content. Proliferation of the T cell clones was found positive, if the SI was at least three in the independent experiments. Similar assays were performed, but with the IHW cell lines as APC, to study the HLADR restriction of a particular T cell clone. Blocking assays were done by preincubating the autologous APC with anti-HLA-DR Ab (clone G46-6 (L243, mlgG2a,α); BD Biosciences, Heidelberg, Germany), and subsequently cultured as described above.
g/ml (Fig. 3C) SakSTAR were also tested for a SakSTAR-specific cellular immune response. Before treatment, one of these patients tested negative for SakSTAR-specific proliferation, four patients were found with a low but detectable SakSTAR-specific cellular response, and one patient showed a relative high specific proliferation. The SakSTAR-specific cellular immune response may be increased 4 wk after treatment in all of these patients, and seemed to be higher for the group of patients who developed high titers of SakSTAR-neutralizing IgG (Fig. 3C) compared with those with intermediate levels of IgG (Fig. 3B).

Staphylokinase-specific T cell epitopes

Most T lymphocytes, supporting a humoral response, recognize a peptide in the context of a particular HLA-DR molecule. Therefore, 10 unrelated donors, covering approximately 95% of the HLA-DR haplotypes occurring in Europe and the U.S., were selected for the cloning of their staphylokinase-specific T cells. Table I summarizes the results. Of 283 isolated T cell clones, 109 could be maintained and were found to be SakSTAR specific and of the Th phenotype (CD3⁺CD4⁺). Their specificity was identified in proliferation assays using overlapping SakSTAR-derived peptides. The majority of the isolated clones proliferated on one or two overlapping peptides, revealing the localization of a T cell epitope. It could have been expected that such an extensive T cell screening resulted in different epitopes for each HLA-DR heterozygous donor. Interestingly, this was not the case, as is clearly depicted in Fig. 4. T cell clones from the unrelated donors recognized similar areas in the SakSTAR molecule, revealing six distinct immunogenic regions.

Two independent T cell clones isolated from a single donor recognize the A1 immunogenic region, located at the amino terminal. Interestingly, one of these clones did not only proliferate on SakSTAR, Sak42D, or a peptide including this particular region of the SakSTAR molecule, but also when SK is given as an Ag.
The first immunogenic cluster, named D1, is recognized by T cell clones isolated from four different donors (40, 41, 57, and 58), and is located within aa 16–32 of SakSTAR. Since these donors do not share a common HLA-DR molecule (see Table I), region D1-specific T cell clones were tested in proliferation assays using heterologous EBV B cells as APC (IHW cell lines). Specific proliferation could be measured for individual T cell clones, if an HLA-DR2, DR3, DR7, or DR8 homozygous IHW cell line presented SakSTAR or the peptide including the D1 region of the SakSTAR molecule (Table II).

The immunogenic region F2 was identified by T cell clones isolated from donors 33, 59, and 66, and is located within the aa 56–72 of SakSTAR molecule. The HLA-DR restriction for this region is also not clear, because the donors do not share a common HLA-DR gene. HLA-DR1 and DR4 homozygous IHW cell lines could support the proliferation of region F2-specific T cell clones (Table II).

The most noticeable immunogenic region is located within aa 71–87 (C3), as the vast majority of the isolated T cell clones recognized it. Moreover, region C3-specific T cell clones could be isolated from 9 of the 10 donors, and therefore the HLA-DR restriction, if any, is not obvious. Two region C3-specific T cell clones were tested for proliferation in the presence of peptide and anti-HLA-DR-preincubated APC. The proliferation of both clones could be inhibited in a dose-dependent manner (Fig. 5). Several clones from different donors were then tested for proliferation when using the IHW cell lines as APC. It was found that HLA-DR2, DR3, DR4, DR6, DR7, or DR8 homozygous IHW cell lines could support the proliferation of individual region C3-specific T cell clones (Table II).

T cell clones from the donors 38, 40, 44, 52, and 66, who all share HLA-DR3, revealed the immunogenic region D4, which is located within the aa 106–122 of the SakSTAR molecule. Several region D4-specific T cell clones seemed to be HLA-DR3 restricted, but others could proliferate on the SakSTAR peptide, including the D4 region presented by an HLA-DR6 homozygous IHW cell line (Table II).

The final immunogenic region, named A5, is found at the carboxyl terminus of the SakSTAR molecule. Again the HLA-DR restriction of this region is not obvious, because of the different HLA-DR backgrounds of the five donors. HLA-DR restriction analysis of the region A5-specific T cell clones revealed that at least HLA-DR2, DR3, DR6, and DR8 homozygous IHW cell lines could present this region (Table II).

**Immunogenic region-specific cellular immune response in normal individuals**

Human T cell clones identified the described immunogenic regions in SakSTAR. To relate their immunogenic importance for SakSTAR in humans, 50 new individuals over 45 years of age were tested for an immunogenic region-specific cellular response. Therefore, PBMC were primed with SakSTAR, followed by a restimulation with either SakSTAR or each of the immunogenic peptides. The results of the proliferation responses are summarized in Fig. 6. Of the 50 donors, 37 tested positive for SakSTAR. Only a single donor recognized the A1 region, whereas 7, 8, and 7 donors proliferated on the peptides, including the D1, F2, and A5 regions, respectively, which corresponds to approximately 20% of the

![Image](https://example.com/image.png)

**FIGURE 4.** Alignment of the identified immunogenic regions recognized by T cell clones isolated from 10 unrelated healthy individuals with the SakSTAR sequence. Several T cell clones were isolated from each donor by repeated SakSTAR stimulation. The SakSTAR-specific T cell clones were then tested for their specificity using 25 17-mer peptides overlapping 12 residues, spanning the entire SakSTAR molecule. None of the individual T cell clones responded to more than two overlapping peptides. The regions of the SakSTAR molecule that induced proliferation of T cell clones from a particular donor are given, in which the amino acids from the corresponding peptides are depicted in lower case letters and the overlapping residues are depicted in upper case letters. The identified immunogenic areas are named, as indicated at the bottom line.

<table>
<thead>
<tr>
<th>Donor</th>
<th>HLA-DR Typing</th>
<th>No. of SakSTAR-SPECIFIC T Cell Clones Isolated</th>
<th>No. of Immunogenic Regions Recognized</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>DR1, DR6(14)</td>
<td>13</td>
<td>2 (F2 and A5)</td>
</tr>
<tr>
<td>38</td>
<td>DR3, DR6(13)</td>
<td>4</td>
<td>2 (C3 and D4)</td>
</tr>
<tr>
<td>40</td>
<td>DR3, DR5(11)</td>
<td>16</td>
<td>4 (A1, D1, C3, and D4)</td>
</tr>
<tr>
<td>41</td>
<td>DR5(1), DR7</td>
<td>10</td>
<td>3 (D1, C3, and A5)</td>
</tr>
<tr>
<td>44</td>
<td>DR3, DR8</td>
<td>4</td>
<td>2 (C3 and D4)</td>
</tr>
<tr>
<td>52</td>
<td>DR3, DR6(13)</td>
<td>6</td>
<td>2 (C3 and D4)</td>
</tr>
<tr>
<td>57</td>
<td>DR2/15, DR7</td>
<td>21</td>
<td>3 (D1, C3, and A5)</td>
</tr>
<tr>
<td>58</td>
<td>DR7, DR8</td>
<td>14</td>
<td>3 (D1, C3, and A5)</td>
</tr>
<tr>
<td>59</td>
<td>DR4, DR5</td>
<td>4</td>
<td>3 (F2, C3, and D4)</td>
</tr>
<tr>
<td>66</td>
<td>DR2(16), DR3</td>
<td>17</td>
<td>3 (F2, C3, and D4)</td>
</tr>
</tbody>
</table>

**Table I. T cell cloning overview from 10 unrelated healthy individuals**
SakSTAR\(^+\) donors. PBMC from 18 donors proliferated upon re-stimulation with the D4 region containing peptide, and 25 recognized the C3 region containing peptide, corresponding to approximately 50% and 70% of the SakSTAR\(^+\) donors, respectively.

**Discussion**

Thrombolytic treatment with nonhuman protein drugs, such as SK and SakSTAR, has been shown to result in the generation of neutralizing IgG. For SK, neutralizing Ab are generally found in humans before treatment (8, 23, 24). Infusion triggers a secondary immune response, as demonstrated by an increase of SK-specific IgG within 3–5 days, peaking within 2 wk (8, 23), and remains in the circulation for years (24). In contrast, SakSTAR-neutralizing Ab are virtually undetectable in healthy individuals (8). Administration of SakSTAR results in a humoral response in the majority of patients, which is characterized by intermediate and incidentally high titers of IgG, which start to increase 3 wk after treatment (6, 8, 25). Although these latter characteristics do not fit with a classical secondary immune response, staphylokinase is of bacterial origin and, therefore, the human immune system may have encountered this protein Ag before treatment.

Streptokinase is associated with group A streptococci (26), which explains its characteristics as a classical recall Ag (Fig. 1) (27). It has been reported that the presence of staphylokinase in a *Staphylococcus* strain is related to its lysogenic status (28), but reports on the percentage of staphylokinase-positive strains remain

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Table II. *Proliferation of SakSTAR-specific T cell clones on HLA-DR-homozygous IHW cell lines as APC*\(^+\)

<table>
<thead>
<tr>
<th>Donors</th>
<th>A1</th>
<th>D1</th>
<th>F2</th>
<th>C3</th>
<th>D4</th>
<th>A5</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>ND</td>
<td>DR3: IHW9022</td>
<td>DR1: IHW9004</td>
<td>DR3: IHW9022</td>
<td>DR3: IHW9022</td>
<td>DR6: IHW9057</td>
</tr>
<tr>
<td>40</td>
<td>ND</td>
<td>DR7: IHW9050</td>
<td>DR3: IHW9022</td>
<td>DR5: IHW9036/9041</td>
<td></td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>DR2: IHW9014/9010</td>
<td>DR7: IHW9050</td>
<td>DR3: IHW9022</td>
<td>DR6: IHW9057/9063</td>
<td></td>
<td></td>
</tr>
<tr>
<td>52</td>
<td>DR4: IHW9031</td>
<td>DR6: IHW9057/9063</td>
<td>DR3: IHW9022</td>
<td>DR6: IHW9057/9063</td>
<td></td>
<td></td>
</tr>
<tr>
<td>57</td>
<td>DR7: IHW9050</td>
<td>DR8: IHW9068</td>
<td>DR2: IHW9010/9014</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>58</td>
<td>DR5: IHW9036/9041</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>59</td>
<td>DR5: IHW9036/9041</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>66</td>
<td>ND</td>
<td>DR2: IHW9009/9014</td>
<td>DR3: IHW9022</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The SakSTAR-specific T cell clones were cocultured with mitomycin C-treated IHW cell lines and Ag (negative controls; no Ag, SK, and positive controls; SakSTAR, Sak42D, and the relevant peptide) for 4 days. The cultures were pulsed for 20–24 h with BrdU and subsequently harvested and analyzed for their BrdU content. The specific proliferation on a particular IHW cell line is indicated.*

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**FIGURE 5.** Proliferation response of region C3-specific T cell clone in the presence or absence of anti-HLA-DR Ab. Two region C3-specific T cell clones (○ and ●) were cocultured with the C3 peptide; irradiated autologous APC prebound with different concentrations of anti-HLA-DR Ab. After 4 days of culture, BrdU was added, and after 24 h harvested. The BrdU incorporation was analyzed, and the SI was calculated. The maximal proliferation was determined by the proliferation of the C3 peptide alone and was set at 100%. The proliferation found in the presence of different concentrations of anti-HLA-DR Ab is plotted relative to that of the C3 peptide alone.

**FIGURE 6.** Proliferation responses of PBMC to SakSTAR or a SakSTAR-derived immunogenic peptide. PBMC from 50 new individuals older than 45 years of age were isolated and primed with SakSTAR. After 10 days of culture, the cells were restimulated with irradiated autologous CD3-depleted PBMC and Ag/peptides, as indicated. After 4 days of culture, the cells were pulsed with BrdU and harvested after 20–24 h. Their BrdU content was analyzed, and the SI was calculated. The number of donors with a positive cellular response is plotted, and the percentage given in the bars is the relative frequency of the region-specific response among the SakSTAR-positive donors.
unclear. Of 100 consecutive cultures from a bacteriology laboratory, only four strains were reported to produce plasminogen activator activity (29), while significantly increased staphylokinase-specific Ab were reported in 3 of 17 patients with *S. aureus* bacteremia (8). Other studies with pathogenic strains have reported as many as 80% of their *S. aureus* isolates to produce staphylokinase (30, 31). Furthermore, it has been suggested that bacteria with a plasminogen-activating capacity are more invasive and virulent (26). We found an age-related SakSTAR-specific cellular response in healthy individuals, probably reflecting that not all *S. aureus* strains do express staphylokinase, and suggesting an increased probability for an individual to have encountered a positive strain over time.

Although the frequency of SakSTAR-specific T lymphocytes was determined to be low, the human immune system can acquire a cellular memory for staphylokinase without ever receiving SakSTAR treatment. The absence of a staphylokinase-specific cellular immune response may indicate that an individual never encountered staphylokinase, or that the amount of specific T cells dropped below detection limits, or that the individual is a nonresponder. Therefore, no predictions can be made on the outcome of the humoral response if such a person is treated with SakSTAR. Two patients tested negative for the presence of SakSTAR-specific T lymphocytes, before treatment. One patient was found to be a nonresponder, whereas the other generated high levels of neutralizing Ab and specific T cells were detected after treatment. In contrast, if an individual tested positive for SakSTAR-specific T lymphocytes, a secondary immune response may be expected upon thrombolytic therapy. This is observed in five patients showing increased levels of neutralizing IgG and a possible increased SakSTAR-specific cellular response after treatment. Furthermore, 70% of the older healthy donors were positive for a staphylokinase-specific cellular response. Consequently, at least as many of the SakSTAR-treated patients (the vast majority exceeds the age of 55) would be expected to develop a secondary immune response. This is in agreement with studies showing that ≥80% of the SakSTAR-treated patients develop neutralizing Ab titers (6, 25). In conclusion, SakSTAR classifies as a T cell-dependent Ag, and thrombolytic treatment probably triggers a secondary immune response in most patients. However, the observed late onset of IgG production after treatment remains unexplained.

When 10 healthy HLA-DR heterozygous individuals were chosen for T cell cloning experiments, two to three T cell immunoreactive regions were identified per donor, as was expected, since T cell epitopes are genetically restricted. However, alignment of these T cell regions from all donors revealed the localization of only six SakSTAR immunogenic regions. Anti-HLA-DR Ab blocked the proliferation of two independent T cell clones, indicating that at least some of the SakSTAR-specific clones are HLA-DR restricted. Several SakSTAR-specific T cell clones could specifically proliferate on autologous EBV B cells, as well as on IHW cell lines. Although the HLA-DR homozygous IHW cell lines were matched with the HLA-DR B1 alleles from our donors, they are transformed human B cells, and consequently, they express other HLA molecules such as HLA-A, HLA-B, HLA-C, HLA-DP, and HLA-DQ. Thus, if a SakSTAR-specific T cell clone was found to proliferate on a particular IHW cell line, it points to the HLA-DR restriction for that T cell clone, although the involvement of another HLA molecule cannot be ruled out. Five of the SakSTAR immunogenic regions were recognized by T lymphocytes in the context of genetically distinct EBV B cells. Therefore, it is likely that the identified regions can accommodate more than a single HLA molecule, or may even be promiscuous in its HLA-DR binding, as was implicated for the C3 region. Peptide cross-reactivity has been reported, and it is generally understood to be an essential feature of the TCR (32). Thus, a single TCR can recognize a number of peptides, but a cross-reactive Ag/peptide is often found by chance. Such a unique T cell clone was isolated and proliferated on the peptide comprising SakSTAR residues 1–17, SakSTAR, Sak42D, but also when SK was given as Ag. SakSTAR and SK do not share an overall sequence homology, and alignment of the SakSTAR amino-terminal part with SK reveals only a low sequence homology. However, TCR cross-reactivity is not necessarily due to a strong sequence homology. Moreover, a single TCR has been reported to recognize two different peptides in distinct MHC molecules (33). Although we did not confirm the proliferation with a SK peptide, the proliferation of the cross-reactive T cell clone in response to SakSTAR and SK implies that a cellular memory for SakSTAR may be acquired via a streptococcus infection in certain individuals.

A detailed analysis of the six T cell immunoreactive areas in SakSTAR is currently under study, to locate possible T cell epitopes and identify important residues for T cell reactivity. Recently, in the scope of developing peptide vaccines, several bioinformatic tools have emerged to discover good T cell epitope candidates (34, 35). These algorithms based on databases of candidate T cell epitopes and our own dead-end elimination computer program, evaluating the interaction energy between a HLA-DR peptide-binding groove and a peptide (36), will help to locate the T cell epitopes within the identified immunogenic regions. Studies combining computer models with functional assays using the SakSTAR-specific T cell clones may lead to the design of SakSTAR variants, aiming at the elimination of T cell reactivity, while retaining thrombolytic activity. Theoretically, the more widely recognized human T cell epitopes are eliminated from a protein, the lower the immunogenic potential for the human population.

In this study, the immunogenic regions of SakSTAR were identified by human T cell clones, and as they were obtained by selection and cloning procedures, they may not represent a normal distribution of their relative importance for the immunogenicity of SakSTAR. Therefore, 50 new individuals older than 45 years of age were evaluated for an immunogenic peptide-specific cellular immune response. Culturing PBMC with SakSTAR-derived peptides did not result in a specific proliferation, probably because the frequency of peptide-specific T lymphocytes is too low. However, this could be evaluated when PBMC were primed with SakSTAR, and subsequently restimulated with the immunogenic peptides. Similar as in the cellular assay for the age group over 45 years of age, 74% was found positive for SakSTAR. Only a single donor recognized the A1 region, whereas the D1, F2, and A5 regions were recognized by approximately 20% of the SakSTAR-positive donors, approximately 50% responded to the D4 region, and 70% to the C3 region. These data confirm the relative importance of five of the six identified immunogenic regions, and in particular the possible contribution of the C3 region to support a humoral response in humans when exposed to SakSTAR.

One earlier reported B cell epitope (19) overlaps with the most prominent C3 T cell immunoreactive region. Several SakSTAR variants, with multiple mutations in this area, were tried in patients with peripheral arterial occlusion. Interestingly, the Ab response was found significantly reduced compared with patients treated with wild-type SakSTAR (12, 13). The observed reduced humoral response may, however, be explained at least in part by the mutation of an important immunoreactive T cell region in these SakSTAR variants.

In conclusion, the identification of the clustered immunogenic regions in SakSTAR will guide rational studies to eliminate T cell reactivity of these areas, which may result in a SakSTAR variant
with a reduced immunogenic profile, but with intact thrombolytic properties.

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