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TCR-Like Human Antibodies Expressed on Human CTLs Mediate Antibody Affinity-Dependent Cytolytic Activity¹

Patrick Chames,²* Ralph A. Willemsen, † Gertrudis Rojas,‡ Detlef Dieckmann,§ Louise Rem,* Gerald Schuler,§ Reinder L. Bolhuis,‡ and Hennie R. Hoogenboom ³*†

The recent increase in understanding of the immune responses associated with cancer and autoimmune disorders can further be advanced by recognizing the importance of the role played by peptide/MHC complexes in these diseases, and such knowledge provides various strategies for immunotherapy (1, 2). Molecules that bind specifically to these peptide MHC (pMHC)³ complexes, which are involved in the molecular and cellular processes of Ag presentation, have a wide variety of applications, including direct visualization of the pMHC complexes (both intracellular and at the cell surface), specific masking of complexes involved in autoimmune disease, targeted delivery of toxins and drugs, and adoptive transfer of CTLs expressing pMHC-specific molecules involved in the immune response against cancer or viral infections (3). Two potent classes of agents have been developed to bind to such complexes. Specific TCR, engineered from cloned T cells of known pMHC specificity, have been used to visualize cell surface pMHC complexes (4, 5), and MHC-restricted, peptide-specific mAb, identified by various immunization and screening schemes, have been isolated and used similarly (5–12). However, it has recently been suggested that in vitro selection of phage libraries displaying Ab fragments could be one of the most efficient ways to select specific pMHC binders (3). We have recently taken advantage of this technique to select a human Fab capable of binding an Ag of strong clinical value (1), the tumor-related pMHC complex HLA-A1/melanoma-associated Ag (MAGE)-A1 (13). Despite a moderate affinity of 250 nM, the selected Ab fragment Fab-G8 is highly specific for the HLA-A1/MAGE-A1 complex and does not bind to HLA-A1/MAGE-A3, a complex with a peptide that differs in only three residues from MAGE-A1. Nevertheless, most therapeutic applications, including targeting of toxins or cytokines and adoptive immunotherapy, are likely to demand a higher affinity. Interestingly, TCRs, the molecules that have been selected by evolution to bind pMHC complexes, always display very low affinities for their targets (10 to 0.1 μM) (14). The activity of T cells seems to be dependent on the affinity of the TCR/MHC interaction and/or the dissociation rate of the TCR from the peptide/MHC complex (15, 16). It was suggested by Valitutti et al. (17) that a single peptide/MHC complex on target cells engages multiple TCR on T cells to activate the effector T cells. This process of serial triggering of multiple TCR, essential for optimal T cell triggering, favors the interaction of low-affinity TCR with peptide/MHC complexes, because the longer dissociation times of high-affinity TCR might prevent serial triggering and thus optimal T cell activities. In this work we investigated to what extent such affinity constraints apply when T cells are artificially equipped with an Ag receptor. Indeed, there is recent evidence suggesting that in vitro affinity-matured TCRs also mediate increased peptide activation (18). While lower affinities may be acceptable in natural TCR-MHC-mediated cellular interactions, many therapeutic applications may benefit from higher-affinity TCRs or surrogate TCRs such as appropriately membrane-anchored antibodies.

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⁴ Abbreviations used in this paper: pMHC, peptide MHC; DC, dendritic cell; MAGE, melanoma-associated Ag; LAC, limited Ag concentration; COM, competition for binding to the Ag; SW, stringent wash; CDR, complementarity-determining region; HS, H chain-CDR3 spiking; LS, L chain shuffling; INF, influenza; B-LCL, B cell lymphoblast.
Abs or other receptors. We decided to see whether it would be possible to increase the affinity of Fab-G8 without losing the peptide fine specificity, and to subsequently examine the effect of such an affinity increase on chimeric Fab receptor-mediated T cell functions.

In vitro affinity maturation of Abs can be conducted using phage display methods. Genetic diversity is introduced into the genes encoding the parental Ab and, from the resulting phage library; variants showing improved affinity for the specific target are selected (for review see Ref. 19). We did not know a priori the specific amino acid residues in the Ab that confer the peptide fine specificity to Fab-G8. Therefore, we used two broadly complementary affinity maturation methods for diversifying the Ab genes, including 1) chain shuffling of the intact L chain, and 2) site-directed mutagenesis of the complementarity-determining region (CDR)3 of the H chain, which for most Abs forms the heart of the Ag binding site. Variants were selected under a variety of conditions, and those with highest affinity were tested for pMHC binding specificity and for binding to the pMHC complex on the surface of APCs. Finally, we expressed Fab-G8 and higher-affinity variants as chimeric Fabs fused to the FcεRI γ-chain signaling molecule on the surface of primary human T lymphocytes, and we examined the tumor cell killing capacity of these T cells expressing these low- or high-affinity pMHC binding molecules. The increased affinity of the chimeric receptor is clearly associated with faster cytotoxic responses, increased sensitivity, and an enhanced tumor cell killing capacity. We anticipate that this higher-affinity molecule will be more effective than Fab-G8 in the eradication of cancer cells in vivo.

**Materials and Methods**

**Library construction**

*Chain-shuffling library construction.* To build the L chain-shuffling (LS) library, the G8 V H gene was cloned into a vector containing a library of human Ab κ and λ L chains. The latter libraries were generated during the construction of the large nonimmune Fab library (20). Briefly, the pCES1 vector containing Fab-G8 was digested with SfiI and BstEI, and the fragment corresponding to G8 V H was gel purified and extracted using the Qiagen extractor (Qiagen, Valencia, CA). The κ and λ libraries were similarly digested and gel purified. Large-scale ligation (using 20 μg of insert and 5 μg of vector) was performed overnight at 16°C. The mixture was ethanol precipitated and introduced into E. coli competent cells (E. coli DH5α) by electroporation. Cells were plated on 2× YT agar plates containing 100 μg/ml ampicillin and 2% glucose. After overnight incubation at 30°C, colonies were scraped from the plates and stored at −80°C in 2× YT containing 15% glycerol.

*H chain CDR3 mutagenesis for H chain-CDR3 spiking (HS) library construction.* To create the HS library in a one-step PCR amplification of the V H gene, we introduced diversity in the 13 amino acid residues of the H chain CDR3 by using a primer hybridizing on the CDR3 plus FR4 region. The primer used was 5′-GCTTGAACGTGCTGGGTAAGTGCTTT-3′, with the underlined residues using 90% of the wild-type nucleotide and 10% of an equimolar mixture of A, T, C, and G (purchased from Eurogentec, Liege, Belgium). The V H fragment was amplified by PCR using the pCES1-Fab-G8 as template. This fragment was digested with SfiI and BstEI and cloned into the pCES1 vector containing the G8 L chain. A library was made as before. Fingerprinting analysis was performed as previously described (21) using the primers pUC reverse (5′-AGCGGATAAATAATCACACAGC-3′) and Id-tet-seq24 (5′-TTT-GTCTGCTTTCAGAGTTAGT-3′); DNA sequencing was performed by Eurogentec, and a sequence reverse primer for V H and CH1-fw (5′-GAAG TAGCTTGCAGCCGAC-3′) for V L.

**Selection and screening procedures**

Except when mentioned in Results, all selections were conducted as described (13). The Ag-binding specificity of individual Fabs was assessed by phage or Fab ELISA using indirectly coated complexes, as described (22). Fab were purified by immobilized metal affinity chromatography as described (13).

**Surface plasmon resonance measurements**

Kinetic measurements were performed by surface plasmon resonance on a BIAcore 2000 (Pharmacia Biotech, Uppsala, Sweden). PBS (pH 8) plus 0.1% Tween 20 was chosen as running buffer. A nitrotriacetic acid chip (Pharmacia Biotech) was activated with 500 μM NiCl₂ for 1 min at 10 μl/min. Approximately 800 resonance units of hexahistidine-tagged Fab (20 μg/ml) was immobilized and different concentrations of pMHC complexes were subsequently injected at a flow rate of 20 μl/min to minimize rebinding effects. A blank (injection of the Ab only) was subtracted from each curve to take into account the dissociation of the Ab from the chip. The channels were regenerated by injection of 250 mM EDTA over a period of 2 min. Kinetic analysis was performed using BIAevaluation 2.0 software (Biacore, Uppsala, Sweden).

**Flow cytometry**

Dendritic cells (DC) were generated as described (23). In brief, BMCs were isolated from leukapheresis by Ficol1 (Amersham Biosciences, Piscataway, NJ) density gradient centrifugation. Monocytes were isolated by plastic adherence and cultured in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 1% plasma, 20 μg/ml gentamicin (Merck, West Point, PA), 2 mM glutamine (BioWhittaker), 800 U/ml IL-4 (Novartis Pharmaceuticals, East Hanover, NJ), and 1000 U/ml GM-CSF (Leukomax; Novartis Pharmaceuticals). At days 5–6, 2 ng/ml IL-1β (Sigma-Aldrich, St. Louis, MO), 1000 U/ml IL-6 (Novartis Pharmaceuticals), 1 μg/ml PGE₂ (Sigma-Aldrich), and 10 ng/ml TNF-α (Bender Medsystems, Vienna, Austria) were added. After an additional day of culture nonadherent cells were harvested. To demonstrate maturation of DC, FACS analysis was performed on CD80, CD83, and CD86 (all from BD Pharmingen, San Diego, CA). Mature DC were ≥90% double positive for costimulatory molecules and CD83.

DC were then pulsed with 20 μM MAGE-A1 (EADPTGHSY) or MAGE-A3 (EVDPIGHLY), tyrosinase (SFKDICTEIY), and influenza (INF) nucleoprotein (CTELKLSDY) as negative controls for 3 h. DC were washed twice in PBS (BioWhittaker) and resuspended at 10⁶ cells/ml. All staining procedures were performed at 4°C. DC were incubated for 30 min with fd-Fab-Hyb3, G8, or H₂, washed again, and incubated with anti-M13 mAb (Zytomed, Berlin, Germany) for additional 30 min. After two rounds of washing in PBS, DC were incubated with goat anti-mouse PE Fab (Caltag Laboratories, Burlingame, CA) for 15 min. Cells were washed again and analyzed by flow cytometry (FACScan and CellQuest software; BD Biosciences, San Jose, CA). Cultured EBV-transformed B cell lymphoblasts (B-LCL) were pulsed with MAGE-A1 peptide or irrelevant INF virus peptide (10 μg/ml final concentration) for 1 h at 37°C. Unbound peptides were removed from the B-LCL by two washes with PBS. The peptide pulsed B-LCL were then incubated with fd-Fab-Hyb3, G8, or H2 essentially as described for the DC staining.

Primary human T lymphocytes (5 × 10⁵) were stained with soluble peptide/HLA-A1/streptavidin PE complexes as described (24). Briefly, T lymphocytes (5 × 10⁵) were incubated for 30 min on ice with fd-Fab-Hyb3, G8, or H2, washed again, and incubated with anti-M13 mAb (Zytomed, Berlin, Germany) for additional 30 min. After two rounds of washing in PBS, DC were incubated with goat anti-mouse PE Fab (Caltag Laboratories, Burlingame, CA) for 15 min. Cells were washed again and analyzed by flow cytometry (FACScan and CellQuest software; BD Biosciences). Human T lymphocytes were stained with soluble peptide/HLA-A1/streptavidin PE complexes and enriched via anti-PE mAb-coated magnetic beads and mini-MACS columns according to the manufacturer’s instructions (Miltenyi Biotec, Bergisch Gladbach, Germany).

**Construction of the chimeric Fab-CD4/8 receptors and retroviral gene transfer to human T lymphocytes**

The chimeric Fab-G8/γ and Fab-Hyb3/γ receptors were made as recently described (24). The chimeric Fab-G8/γ and Fab-Hyb3/γ receptors were independently cloned into the pBullet retroviral vector (24) and introduced into OKT-3 mAb-activated primary human T lymphocytes using retroen- tin-enhanced supernatant transduction (25). The retroviral supernatants were obtained from a mixture of Phoenix packaging cells (Phoenix Pharmaceu-ticals, Belmont, CA) (26) and 293T cells that had been transfected 2 days before harvest of the supernatants, with the following constructs: the GAG-POL construct pH8-60 (27), the pColt-GaLV vector, the pBullet vector with the Vr/CD4/γ chain, and the pBullet vector with the VC/CD8/γ chain. After retroviral transduction the human T lymphocytes were expanded as described elsewhere (28).

**Cytotoxicity assays**

Cytolytic activity of transduced human T lymphocytes was measured in 5¹Cr release assays as described previously (29). Peptide loading of target cells was performed by adding MAGE-A1 nonapeptide (EADPTGHSY;
Leiden University Medical Center, Leiden, The Netherlands) or irrelevant HLA-A1-binding INF peptide derived from INF virus A nucleoprotein (CTELKLSDY; Leiden University Medical Center) at indicated concentrations to the target cells 5–15 min before incubation with effector T lymphocytes at indicated E:T cell ratios. The incubation period of effector and target cells is indicated in the figures. The percentage of specific 51Cr release was calculated as follows: ([test counts – spontaneous counts]/(maximum counts – spontaneous counts)) × 100%.

**TNF-α ELISA**

To quantify the secreted amount of TNF-α, transduced human T lymphocytes (6 × 10^6) were cultured for 24 h in either the presence or absence of 2 × 10^6 adherent tumor cells in culture medium supplemented with 360 IU/ml rIL-2. Supernatants were harvested and levels of TNF-α were measured by standard ELISA according to the manufacturer’s instructions (CLB, Amsterdam, The Netherlands).

**Results**

**Construction of LS and HS libraries**

In the absence of any information regarding the specificity of the antibody, we decided to use two complementary library generation methods in parallel. The Fab-G8 V_H gene has 18 mutations compared with the nearest germline sequence (subgroup 01.3.4, annotated JH538 by the V base index at www.mrc-cpe.cam.ac.uk/mlt-doc/public/INTRO.html), whereas the V_L gene has only one mutation. We decided to use V_L chain shuffling to identify germline mutations that are important for binding and to possibly generate variants with a higher affinity for the HLA-A1/MAGE-A1 complex. However, the H chain often dominates the interaction with Ag, and its CDR3 region is usually responsible for crucial interactions with the Ag. We surmised that the H chain CDR3 might provide direct contact in the G8-Ag interaction; therefore, we created a library of G8-V_H-CDR3 variants. The long length of the Fab-G8 V_H-CDR3 (13 residues) prevents a representative sampling of the CDR3 when made by complete randomization of each residue. Therefore, we decided to introduce a low percentage of mutation into each residue position using a “spiked” oligonucleotide (see Materials and Methods). This should result in the introduction of one to three mutations per clone, spread along all the CDR3.

Both libraries were built using standard cloning procedures (20) (see Materials and Methods). We obtained a library of 2 × 10^6 clones for the V_L chain shuffling (LS) library and 2 × 10^7 for the HS library. The quality of these unselected libraries was checked by fingerprint analysis using the restriction enzyme BstNI, by DNA sequencing, and by ELISA. Both libraries showed a high diversity (>88%); >95% of the LS clones and 65% of the HS clones displayed a correct open reading frame (Table I).

**Selection against HLA-A1/MAGE-A1**

Both repertoires were independently selected against the biotinylated HLA-A1/MAGE-1 complex as previously described (13). We initially chose to carry out several rounds of selection using decreasing Ag concentration to favor selection of high-affinity binders. For both repertoires, after the first round of selection, 92 of 92 clones bound the Ag in phage ELISA. However, after three rounds of selection, most of the clones (82%) showed a high cross-reactivity with HLA-A1/MAGE-A3 complexes by phage ELISA (data not shown). Because we wanted to use the frequency of Ag positives as a readout of the stringency of the selection procedure, we also wanted to avoid the selection of these cross-reactive clones, we decided to optimize the selection procedure by using only one round of selection. Different conditions for selection were tested, and clones were screened to find affinity variants with higher affinity while maintaining peptide fine specificity.

We compared three selection strategies based on different principles, including 1) limited Ag concentrations (LAC), 2) stringent washes (SW), favoring low dissociation rate (k_off), or 3) competition for binding to the Ag (COM). After selection, 40 clones derived from each selection method and each library were tested for binding to HLA/MAGE-A1 in ELISA. Clones showing the best signals were produced as Fab and screened for affinity by BLAcore. As indicated in Tables II–IV, the first two methods did not yield any significant increase of affinity and, surprisingly, the average K_d of the selected clones was above that of the parental clone value of 250 nM (see Fig. 1). The third selection process (COM) was based on competition for binding and involved adding soluble Fab-G8 directed against the same epitope. To choose a relevant concentration range for the competitor molecule, we first performed a phage-Ab ELISA mimicking the selection conditions and chose different concentrations of competitor yielding from 90% (for 40 nM) to 10% (for 5 μM) of the control experiment without competition. During the selections, increasing the competitor concentration resulted in a decrease of the output titer as well as in the number of positive clones in ELISA (see Table IV), and at 5 μM competitor none of the output phages was positive for binding to HLA-A1/MAGE-A1. Contrasting with results obtained from the other selection procedures, the affinities of all the ELISA-positive clones were better than the affinity of the parental clone, with the best affinities being 4.5-fold stronger. The best results were obtained with the HS library, with clones Com6 and Com7 showing an affinity of 55 and 60 nM, respectively (Fig. 1). In all cases, the affinity improvement was essentially due to a decrease of the k_off.

**Construction of V_H/V_L hybrids**

We next investigated whether the effects of the mutations selected in the L chain could be additive with those in the H chain. The best L chain available (from clone Lac3, 83 nM, 3-fold improvement) was combined with the H chain of clone Lac7 (85 nM, 3-fold improvement), Com7 (60 nM, 4-fold improvement), and Com6 (55 nM, <5-fold improvement) to give three new clones called Hyb1, Hyb2, and Hyb3, respectively. These clones have an additional mutation (G8: V_H RdR) that is important for binding and maintained a similar affinity.

<table>
<thead>
<tr>
<th>Library</th>
<th>Size</th>
<th>Insert^a</th>
<th>Diversity^b</th>
<th>Open Reading Frame^c</th>
<th>ELISA^d</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS</td>
<td>2 × 10^6</td>
<td>40/40</td>
<td>35 different clones/40 (fingerprint)</td>
<td>15/15</td>
<td>0/92</td>
</tr>
<tr>
<td>HS</td>
<td>3 × 10^7</td>
<td>37/40</td>
<td>20 different clones/20 (sequencing)</td>
<td>13/20</td>
<td>3/92</td>
</tr>
</tbody>
</table>

^a Number of clones presenting a full-length insert (checked by PCR).

^b Number of different clones identified/total number of clones sequenced.

^c Number of clones without any stop codons, deletions, insertions, or frame shifts/total number of clones examined.

^d Number of clones positive against HLA-A1/MAGE-A1 in indirect ELISA/total number of clones examined.
Hyb2, and Hyb3, respectively (see Fig. 2). The Fab encoded by these genes were produced and purified for BIACore measurements. All three clones have $K_d$ s well below the $K_d$ of the parental clone (Fig. 2). At 14 nM, the $K_d$ of the best clone, Hyb3, was 18-fold lower than that of the parental clone, thereby demonstrating a synergistic effect among the selected mutations.

Analyzing the Ag recognition fine specificity
The binding specificity of G8 and its derivatives for the HLA-A1/MAGE-A1 complex was confirmed by BIACore analysis and by comparing binding to the HLA-A1/MAGE-A3 complex. No binding to the latter Ag could be detected for the selected clones (Lac3, Lac7, Com6, Com7, Hyb1, Hyb2, and Hyb3; data not shown). We further compared the specificity of G8 (250 nM), Lac7 (85 nM), and Hyb3 (14 nM) by sandwich ELISA using indirect coating of nine different biotinylated HLA-A1/peptide complexes with streptavidin. To choose the peptides for this study, we investigated the homology between the two MAGE peptides: MAGE-A1 differs at three positions from MAGE-A3 (V2A, I5T, and L8S). To investigate which residue is critical for G8 and Hyb3 binding, we synthesized hybrid peptides corresponding to MAGE-A3 or INF with residues from MAGE-A3 at positions 2, 5, and 8 and used them to make HLA-A1/peptide complexes. Surprisingly, the absence of a threonine in position 5 is thus necessary to allow G8 binding. None of the other complexes were properly refolded. As shown in Fig. 3, Fab-G8 gave a strong signal against MAGE-A1 and no signal for MAGE-A3 or INF. Interestingly, M3T was strongly recognized by G8, implying that the threonine at position 5 is crucial for binding; however, INF with this central threonine was not recognized. The presence of a threonine in position 5 is thus necessary but not sufficient to allow G8 binding. None of the other complexes was recognized by the Abs if the threshold for binding was set at a signal lower than three times the background; when set at twice the background, some binding was seen for the peptide M3S. Clones Lac7 and Hyb3 gave an identical binding pattern, but, as expected for MAGE-A1, with higher intensities (Fig. 3). This result confirms that the peptide fine specificity of Fab-G8 was maintained during the affinity maturation process.

Cell binding of G8 wild-type and affinity variants
Clone Fab-G8 was used in our previous work to detect the complex HLA-A1/MAGE-A1 in flow cytometry, after recloning the Ab genes for display on fd particles (to increase the number of Fab per particle) (13). Fab-G8 expression on fd particles yielded a difference between control cells and MAGE-A1-pulsed cells of less than one log shift in the mean fluorescence index. To evaluate the effect of the 18-fold affinity increase on diagnostic assays, e.g., flow cytometry, Hyb3 was also recloned for multivalent display and compared with fd-Fab-G8 in staining of peptide-loaded HLA-A1-expressing human DC or EBV-transformed B cell blasts (APD). After loading mature DC or APD with the MAGE-A1 peptide, a strong positive staining was obtained with fd-Fab-Hyb3 (Fig. 4). In accordance with our previous results, staining with fd-Fab-G8 was also positive on DC but almost two logs lower than fd-Fab-Hyb3 (Fig. 4A) and not detectable on APD (Fig. 4B). As expected, binding with control fd-Fab-H2 as well as binding of fd-Fab-Hyb3 to DC and APD loaded with a control peptide (derived from the cancer-related protein tyrosinase or derived from the INF virus, respectively) (Fig. 4), to DC loaded with MAGE-A3 (data not shown), or to cells with an irrelevant haplotype (data not shown) were all negative. Thus, fd-Fab-Hyb3 reliably visualizes HLA-A1/MAGE-A1 complexes on cells, showing its exquisite specificity and improved sensitivity over the lower-affinity variant G8.

Genetic retargeting of human T lymphocytes with affinity-matured Fab-CD4/γ receptors
To determine whether an increased affinity for the HLA-A1/MAGE-A1 complex results in enhanced chimeric Fab-based receptor-mediated T cell functions, we constructed chimeric receptors comprising either Fab-G8 or Hyb3. Retroviral transduction of

| Table II. Selection results: LAC$^a$ |
|-----------------|-----------------|-----------------|
|                  | LS              | HS              |
|                  | Output (10$^6$ CFU) | Positive (%) | Output (10$^6$ CFU) | Positive (%) |
| Ag (pM)$^b$      | 10,000          | 11              | 55              | 79             | 85          |
|                  | 1,000           | 1.3             | 7               | 5.8            | 67          |
|                  | 100             | 0.83            | 0               | 0.34           | 7           |
|                  | 10              | 3               | 0               | 0.58           | 0           |
|                  | 1               | 5.6             | 5               | 0.86           | 0           |
|                  | 0.1             | 1.4             | 0               | 1.6            | 0           |
|                  | 0.01            | 1.3             | 0               | 1.1            | 2           |

<table>
<thead>
<tr>
<th>Output (10$^6$ CFU)</th>
<th>Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>55</td>
</tr>
<tr>
<td>1.3</td>
<td>7</td>
</tr>
<tr>
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<td>0</td>
</tr>
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</tr>
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</tr>
<tr>
<td>1.4</td>
<td>0</td>
</tr>
<tr>
<td>1.3</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$ Input for LS library, 1.1 × 10$^5$ CFU; input for HS library, 1.6 × 10$^5$ CFU.

$^b$ Time of incubation with the Ag, 6 h.

| Table III. Selection results: SW favoring low $k_{d}$$^a$ |
|-----------------|-----------------|-----------------|
|                  | LS              | HS              |
|                  | Output (CFU) | Positive (%) | Output (CFU) | Positive (%) |
| Washes          | Positive (%) | Output (CFU) | Positive (%) | Output (CFU) | Positive (%) |
| 3h$^e$          | 510            | 80             | 140           | 2              |
| Overnight$^e$   | 2              | 0              | 2             | 100$^e$        |
| 10              | 6,500         | 87             | 260           | 50             |

$^a$ Input for LS library, 1.3 × 10$^{10}$ CFU; input for HS library, 1.5 × 10$^{10}$.

$^b$ Depletion on immunotubes coated with HLA-A1/MAGE-A3 prior to selection.

$^e$ Ten times for 15 min.

$^e$ Three-hour washes plus a last wash overnight.

$^e$ Only two clones tested.
the G8-CD4/γ receptor gene and the Hyb3-CD4/γ receptor gene into primary human T lymphocytes resulted in stable expression of the receptors on the T cell surface after enrichment using HLA-A1/MAGE-A1 tetramers and anti-PE-coated magnetic beads (Fig. 5). Enriched G8-CD4/γ+ T lymphocytes and Hyb3-CD4/γ+ T lymphocytes were expanded and analyzed for receptor-mediated tumor cell killing by incubation with 51Cr-labeled target cells. Fig. 6A shows that human T lymphocytes, derived from the same donor and transduced with the G8-CD4/γ receptor or Hyb3-CD4/γ receptor, were capable of lysing native HLA-A1/MAGE-A1+ melanoma cells. However, at identical E/T cell ratios the Hyb3-CD4/γ+ T lymphocytes demonstrated considerably higher cytolytic activity than the G8-CD4/γ+ T lymphocytes. The kinetics of Fab receptor-mediated tumor cell lysis were analyzed in cytotoxicity experiments with a duration of 1, 2, and 4 h. As shown in Fig. 6B, the Hyb3-CD4/γ+ T lymphocytes demonstrated faster kinetics with respect to their capacity to kill both peptide-loaded and MAGE-A1+ tumor cells. Furthermore, we investigated the sensitivity of G8-CD4/γ+ and Hyb3-CD4/γ+ T lymphocytes by incubating them with target cells that had been pulsed with increasing amounts of MAGE-A1 peptide. As demonstrated in Fig. 6C, Hyb3-CD4/γ+ T lymphocytes required much less peptide to be activated and kill target cells than G8-CD4/γ+ lymphocytes. In addition, Hyb3-CD4/γ+ T lymphocytes produced more TNF-α than G8-CD4/γ+ T lymphocytes when incubated with HLA-A1/MAGE-A1+ melanoma cells (Fig. 7).

**Discussion**

In this study, we investigated whether the Ab fragment G8 directed against the pMHC complex HLA-A1/MAGE-A1 could be affinity matured without loss of peptide fine specificity, and whether such genetically grafted chimeric receptors with increased affinity for HLA-A1/MAGE-A1 would accordingly result in enhanced chimeric receptor-mediated and immune-specific T cell functions such as cytotoxicity and cytokine production. In addition, we investigated whether such affinity-matured Fab could be used to efficiently detect pMHC complexes on APCs.

In the absence of any structural information about this Ab-Ag interaction, several libraries were built to generate G8 variants, and different selection techniques were compared. The first two approaches devised to select higher-affinity binders used either low

### Table IV. Selection results: COM

<table>
<thead>
<tr>
<th>Fab (nM)</th>
<th>LS Library</th>
<th>Positive (%)</th>
<th>Output (10^3 CFU)</th>
<th>Positive (%)</th>
<th>HS Library</th>
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<tr>
<td>5000</td>
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<td>62</td>
<td>40</td>
<td>690</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>120</td>
<td>70</td>
<td>1600</td>
<td>82</td>
<td></td>
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</tbody>
</table>

*Concentration of the Ag: 10 nM (6-h incubation with Ag and competitor). Input: 1 × 10^12 CFU.

![FIGURE 1. Affinity distribution of the selected clones: LAC, nine clones; SW, 15 clones; COM, eight clones. A, Kd ranking. B, kcat ranking. Measurements were done by surface plasmon resonance using a nitrilotriacetic acid chip (BIAcore) coated with 800 resonance units of each Fab via nickel. Different concentrations of pMHC complexes were injected at 20 μl/min. In both cases, the line shows the value corresponding to parental clone G8. The given values are the mean from three experiments.](http://www.jimmunol.org/)

![FIGURE 2. BIAcore sensogram (A) and kinetic values (B) of selected and hybrid clones. BIAcore measurements were performed as described in Fig. 1. The given values are the mean from three experiments.](http://www.jimmunol.org/)
Ag concentration or long washes. Unfortunately, both of these methods yielded poor binders, with some clones even showing affinities weaker than that of the parent clone G8.

Why such clones could compete with higher-affinity binders in such stringent conditions is unclear, but the phenomenon might be explained by a dominant avidity effect of those phage that are most tightly bound to the beads, the phage particles that display multiple Fabs on their surface (30). In a third approach, we competed off low-affinity binders during the selection with a high concentration of soluble Fab directed against the same epitope. Such competition would in principle also lower the frequency of avid interactions and may therefore also favor selection on the basis of affinity.

Indeed, in contrast to the previous two methods used, this competitive selection led to binders all having higher affinities than the original Fab-G8 clone (see Fig. 1).

After sampling both libraries using this selection method, the best improvements in affinities found were only in the range of 3- to 5-fold. Sampling of the full repertoire was made difficult by the presence in the library of variants with higher affinity but altered peptide specificity. For this reason the selection was limited to one round only and screening of a limited set of clones from this repertoire. Instead of further selecting the separate libraries, we decided to combine the best-selected H chains with the best-selected L chain. In the best hybrid clone, Hyb3, we obtained an improvement in affinity up to 18-fold compared with Fab-G8, suggesting that the effect of the mutations was synergistic. This synergistic effect is somewhat surprising because mutations selected in parallel are usually difficult to combine in a single protein (31).

Strikingly, all selected L chains originated from the same germline gene. However, it is difficult to pinpoint the crucial residues involved in binding, because most L chains have several mutations that differ from the germline.

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FIGURE 3. Fine specificity of the affinity-matured clones by phage-ELISA. pMHC complexes were refolded using each peptide and coated via streptavidin on an ELISA plate. Equal concentrations of phage-Abs were incubated for 1 h at room temperature. After extensive washing, bound phages were detected with an anti-M13 mAb coupled to HRP. mAb TÜ155 binds only to HLA complexes presenting a peptide and was used to demonstrate a proper conformation of the refolded complexes. H2 is an irrelevant phage-Ab (anti-Ras; Ref. 41).

FIGURE 4. Staining of externally loaded human DC and EBV-transformed B cell blasts using fd-Fab-G8 and fd-Fab-Hyb3. A, DC were pulsed with 20 μM peptides, washed, incubated with phage-Abs, washed again, and incubated with anti-M13 Fab followed by anti-Fab coupled to PE. fd-Fab-H2 is an irrelevant phage-Ab. Thin line, Control peptide tyrosinase; thick line, MAGE-A1 peptide. B, B-LCL APD was pulsed with 10 μM peptide, washed, incubated with phage-Abs, washed again, and incubated with anti-M13 Fab followed by anti-Fab coupled to PE. fd-Fab-H2 is an irrelevant phage-Ab. Thin line, Control peptide INF peptide; thick line, MAGE-A1 peptide.

FIGURE 5. Cell surface expression of the Fab-CD4+ receptors on transduced primary human T lymphocytes. Cell surface expression of the Fab-G8-CD4+ and Fab-Hyb3-CD4+ receptors on the transduced T lymphocytes was analyzed by incubation with MAGE-A1/HLA-A1/streptavidin(Fab) complexes. Results are shown as histograms. Data acquisition was restricted to viable cells.
Sequences of clones selected from the HS library showed a small number of changes compared with G8, whereas the unselected clones showed an average of two to three mutations at the amino acid level, scattered all along the 13-residue CDR. These selected clones showed an average of two to three mutations at the amino acid level, leading to higher affinity without a major change in cross-reactivity. It was indeed postulated that these types of mutations are often selected during a natural in vivo affinity maturation process and may be responsible for major affinity increases (32).

As expected, the 18-fold improvement measured by BIAcore resulted in a much stronger binding for Hyb3 compared with G8 (almost two logs), as demonstrated by flow cytometry on externally loaded DC and EBV-transformed B cell blasts. These assays used fd particles, which allow multivalent display (up to five Fabs per phage particle) (33), thereby favoring avidity effects. This increased avidity is probably responsible in part for the large increase in cell binding by Hyb3 relative to G8. Staining was shown to be specific, because HLA-A1-positive DC loaded with irrelevant peptides did not show positive staining. Staining was also negative with a control phage Ab and with DC displaying an irrelevant haplotype (data not shown). The affinity-matured Ab Fab-Hyb3 allows for the first time the direct assessment of the level of class I pMHC Ag presentation at the single cell level in the human system. This work is similar to that of Krosggaard et al. (34), who showed class II pMHC staining. However, the quantity of peptide available for binding is thought to be much lower for class I complexes (for review see Ref. 35), making this task definitely a more challenging one.

We compared primary human T lymphocytes expressing chimeric receptors comprising Fab-G8 or Hyb3 fused to the FcεRI γ-chain signaling molecule, with respect to their tumor cell killing capacity. T cells expressing the high-affinity chimeric receptor displayed higher lytic activity and faster kinetics of cell lysis, required a much lower density of epitope to be activated, and produced more TNF-α upon incubation with target cells. These results are in full agreement with the work of Derby et al. (36), who recently demonstrated that high-avidity CTL provide better protection against viral infection for two reasons. First, they recognize lower Ag densities present earlier in the course of infection of each cell. Second, they initiate lysis more rapidly and thus more rapidly eliminate infected targets. Consequently, they prevent the accumulation of new virus particles much more efficiently than low-avidity CTL. In a cancer therapy perspective, high-affinity chimeric receptor T cells might be very advantageous. Indeed, tumor cells often express very low levels of HLA-A1, as is the case for the MZ2-MEL 2.2 cell line (data not shown), or low levels of MAGE-A1. In this case, only T cells harboring the high-affinity receptors will show a strong anti-cancer effect. Another clear advantage of chimeric receptor approach for cancer therapy is that...
receptors chimerized to alternative signaling molecules other than TCRs bypass TCR-mediated proximal signaling events, which are often defective in cancer patients (37). Our results allow a direct comparison of the effect of the receptor affinity without any possible interference due to different receptor expression levels or signal transduction efficiency. Our results also show improved sensitivity and faster kinetics for cells displaying the high-affinity receptor. The results obtained by us and Holler et al. (18) are in conflict with the serial triggering model, which proposes that high-affinity TCR, with longer interaction times between TCR and peptide/MHC, results in decreased numbers of TCR interacting with specific peptide/MHC complexes on target cells. A decrease in interaction of high-affinity TCR interacting with peptide/MHC complexes, according to this model, would result in decreased T cell functions such as cytotoxicity and cytokine production. On the contrary, our results clearly demonstrate that an increase in affinity of the TCR-like receptors results in enhanced T cell functions.

A further demonstration of the utility of high-affinity receptors has recently been described by Stanislawski et al. (38). The authors were able to circumvent self-tolerance of autologous T lymphocytes to universal tumor Ags by transfecting them with genes encoding a high-affinity TCR, thereby producing efficient and broad-spectrum tumor-directed CTLs.

As shown by these last works and our results, high-affinity TCRs are highly desirable. Using phage display, we have recently isolated Abs against 5 different HLA-A2-based complexes, most of them displaying affinity in the 10–50 nM range for their particular peptide complex (39, 40). This suggests that it is possible to select such molecules against any pMHC complexes in <3 wk. This efficient selection of high-affinity, specific pMHC binders, together with the versatility of Abs in terms of protein engineering, should make anti-pMHC Fab very attractive tools in a variety of virus-related and cancer applications, including diagnosis with the Fab or its engineered variants directly, or in therapy, as chimeric Fab-based TCRs.

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References


