

Detection of antigen-specific T cells on p/MHC microarrays

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The development of high-throughput protein microarrays for rapidly determining antigen-specific T-cell receptor repertoires of diverse T-cell populations can enable comprehensive, broad-based analyses of T-cell responses. Promising applications include medical diagnostics, vaccine development, treatment of autoimmune diseases and detection of potential agents of bioterrorism. In this study, we examined the feasibility of using peptide/major histocompatibility complex (p/MHC) microarrays to selectively capture and enumerate antigen-specific T cells. Results are presented for p/MHC microarrays consisting of a dimeric MHC-immunoglobulin complex, K^b-Ig, loaded with either a cognate or non-cognate peptide for binding CD8⁺ T cells. We quantified the sensitivity of these K^b-Ig microarrays by measuring a lower detection limit of 0.05% antigen-specific CD8⁺ T cells mixed with splenocytes from C57BL/6J mouse. A fivefold increase in this lower detection limit (0.01%) was achieved using a secondary capture anti-Ig antibody to coat the microarray surface. This higher sensitivity is comparable to that obtained using standard state-of-the-art fluorescence activated cell sorting (FACS) instruments. We also found that contacting the T-cell suspension with the K^b-Ig microarrays under mild shear flow conditions produced more uniform distributions of captured T cells on the individual spots and better spot-to-spot reproducibility across the entire microarray.
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INTRODUCTION

At the core of the molecular events comprising adaptive immune response is the interaction of the T-cell receptor (TCR) with a complementary major histocompatibility complex (MHC) mediated by a small peptide, typically 8–10 amino acids in length for class I MHC molecules. Immune response often appears to be restricted to a small fraction of all potential peptide antigens, which is referred to as immunodominance, with responses to influenza or other viruses, such as Epstein-Barr, as notable examples (Hill *et al.*, 1995; Callan *et al.*, 1998; Tan *et al.*, 1999; Chen *et al.*, 2000; Gianfrani *et al.*, 2000; Davenport *et al.*, 2002). In contrast, the response to HIV or hepatitis, among other pathogens, appears very broad, and the diversity of this response seems to correlate with its effectiveness. A quantitative assessment of this diversity can facilitate the discovery of new peptide antigens (Gianfrani *et al.*, 2000). Factors that impact the heterogeneity of the immune response and the immunodominance of certain peptides are clearly important for understanding the underlying molecular mechanisms of adaptive immune response, as well as for biotechnological applications (Chen *et al.*, 2000).

Since each T cell is defined by a unique TCR, an array of immobilized peptide/MHC (p/MHC) complexes that differ in the amino acid sequence of the peptide can serve to distinguish T cells by their antigen-specific TCR. These p/MHC arrays have the potential for high throughput, systematic screening of peptide antigen-specific T cells in diverse, heterogeneous populations. Although monovalent p/MHC complexes have low affinity for TCR ($\sim 10^5$ – 10^6 M⁻¹), the stable binding of soluble complexes to cognate T cells has been achieved for multivalent p/MHC constructs that bind such cells with increased avidity (Altman *et al.*, 1996; Murali-Krishna *et al.*, 1998, 1999; La Rosa *et al.*, 2001). Recent developments of both tetrameric (Altman *et al.*, 1996; Holmberg *et al.*, 2003; Wang and Altman, 2003) and dimeric (Hamad *et al.*, 1998; Lebowitz *et al.*, 1998) MHC constructs have led to their use in microarrays for detecting antigen-specific T cells. Soen *et al.* (2003), for example, fabricated microarrays using both class I and II MHC tetramers (and dimers) and demonstrated their ability to capture CD8⁺ and CD4⁺ T cells as a function of the loaded peptide antigen. Stone *et al.* (2005) fabricated microarrays of p/MHC tetramers co-immobilized with co-stimulatory antibodies and anti-cytokine antibodies to screen for antigen-specific T cells and to detect locally their functional responses, including adhesion, cytokine secretion and the modulation of surface markers. Their results demonstrated the ability of these microarrays to

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screen significant numbers of T-cell epitopes for both antigen-specific recognition and induced T-cell activation.

In both studies, the spot-to-spot reproducibility of the microarrays was not reported, although images frequently revealed non-uniform distributions of captured T cells across the individual microarray spots. In addition, the detection limit reported by Soen *et al.* (2003) was in the range of 1%, which is low considering that the endogenous antigen-specific T-cell repertoire is in the range of 0.01%–0.1%. Stone *et al.* (2005) reported a much higher detection limit of ~0.01% using co-stimulatory molecules in addition to p/MHC complexes to capture the T cells, and anti-cytokine antibodies to detect them. Although this approach appears to give a higher sensitivity, caution must be taken in interpreting the antigenic repertoire from the cytokine response. Since only a few cells could be producing cytokines upon capture, the repertoire of antigen-specific cells might be significantly different than that seen by functional assays, such as cytokine release. The development of high-throughput p/MHC microarrays with high spot-to-spot reproducibility in addition to high sensitivity is essential to the widespread use of this technology.

In this work, we quantified the sensitivity of these microarrays by measuring the lower detection limit for dilute solutions of 2C CD8⁺ T cells in splenocytes from C57BL/6J mouse. We further investigated the use of a secondary antibody to enhance detection by increasing the number of functional K^b-Ig complexes on the microarray surface. Finally, we studied the time dependence of T-cell capture from cell suspensions contacted with the K^b-Ig microarray under mild shear flow conditions.

RESULTS

The system we chose to study was the murine class I restricted CD8⁺ 2C T-cell response, which recognizes the peptide, SIY, presented by the syngeneic murine class I H-2K^b MHC. Soluble, dimeric K^b-Ig complexes (Dal Porto *et al.*, 1993; Schneek, 2000) formed using immunoglobulin as a molecular scaffold takes advantage of the high degree of flexibility of the Ig hinge region to promote simultaneous binding to two TCRs, thus increasing avidity. The soluble K^b-Ig dimer has been used extensively to study the immune response of T cells to specific antigens, both *in vivo* (O'Herrin *et al.*, 2001) and *in vitro* (Fahmy *et al.*, 2002).

Figure 1 shows the binding specificity of 2C CD8⁺ T cells on two representative spots derivatized with either the cognate ^{SIY}K^b-Ig or the non-cognate ^{SIIN}K^b-Ig complex. High binding specificity is observed on the ^{SIY}K^b-Ig spot, while no cell binding is seen on the ^{SIIN}K^b-Ig spot. The ability of ^{SIY}K^b-Ig microarrays to detect low levels of 2C CD8⁺ T cells in mixtures with splenocytes isolated from C57BL/6J mouse was also evaluated. These results are shown in Figure 2 for 2C CD8⁺ T cells at five different concentrations: 50%, 5%, 1%, 0.5%, and 0.05% by cell number. 2C CD8⁺ T cells were detected at the lowest concentration of 0.05%, while no binding was observed at each concentration on the ^{SIIN}K^b-Ig spots, as shown in Figure 2 at one representative concentration of 1% 2C CD8⁺ T cells.

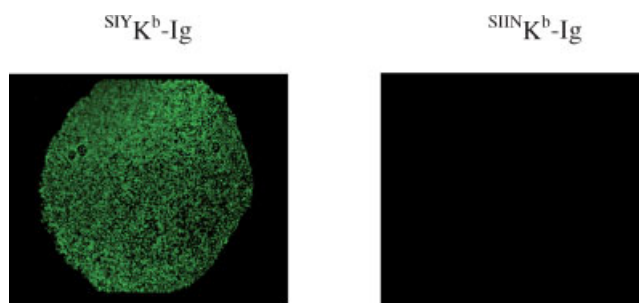


Figure 1. Binding of 2C CD8⁺ T cells labelled with CFSE (green dye) on two representative spots. One spot (left) consists of H-2K^b-Ig loaded with the peptide, SIYRYGL, the cognate peptide that binds to 2C T cells. The second spot (right) consists of H-2K^b-Ig loaded with the peptide, SIINFEKL, that is known to bind K^b, but does not bind to 2C T cells. Spots were printed manually by pipetting ~0.1 μl of ^{SIY}K^b-Ig or ^{SIIN}K^b-Ig (0.5 mg/ml) onto polyacrylamide film-coated glass slides. T-cell concentration: ~4 × 10⁶ T cells in 700 μl of phosphate buffer saline (PBS) solution.

Detection of the 2C CD8⁺ T cells was enhanced by first coating the microarray surface with a goat anti-mouse IgG antibody that binds to the Fc domain of the K^b-Ig dimer prior to printing both ^{SIY}K^b-Ig and ^{SIIN}K^b-Ig spots on the microarray. Figure 3 compares representative spots on the pre-treated and untreated microarrays for a T-cell concentration of 0.05% (5000 2C CD8⁺ T cells in 10 million splenocytes). Fluorescent intensities corresponding to the number of captured T cells were found to be uniformly higher for the ^{SIY}K^b-Ig spots on the pre-treated microarray over the entire concentration range studied. The higher sensitivities with the anti-mouse Ig antibody suggests a higher efficiency for capturing the 2C CD8⁺ T cells, presumably due to the enhanced orientation and accessibility of ^{SIY}K^b-Ig on these surfaces. No T cells were detected on the spots printed with ^{SIIN}K^b-Ig for either the pre-treated and untreated microarrays. Using the secondary antibody, the lower detection limit of antigen-specific cells was enhanced fivefold to 0.01%.

The linear relationship between the logarithm of fluorescent intensity—roughly corresponding to the number of captured T cells—and the logarithm of T-cell concentration in the suspension is shown in Figure 4 for 2C T cells bound to spots containing anti-Ig+^{SIY}K^b-Ig and only ^{SIY}K^b-Ig. The slope is essentially the same in both cases, suggesting that the pre-treatment with secondary antibody improves the absolute sensitivity, but not the relative sensitivity of the two microarrays.

High binding specificity and virtually no non-specific binding of 2C T cells were also observed for K^b-Ig microarrays fabricated using the automated printer. However, the ^{SIY}K^b-Ig spot-to-spot variability across these microarrays in terms of the number of captured T cells was significant, presumably due to the smaller surface area of these spots and the much smaller volumes of p/MHC solution (2–3 nl) that were printed on them. To circumvent this problem, we examined the use of mild shear flow conditions to contact the cell suspension with the microarray surface. The effect of washing the microarray at higher flow rates after capture was also examined. Frames from the movies generated during this experiment are shown in Figure 5. Frames 3 and 4 show initial detectable, rapid binding of T cells on the ^{SIY}K^b-Ig spots as early as 7 min, and

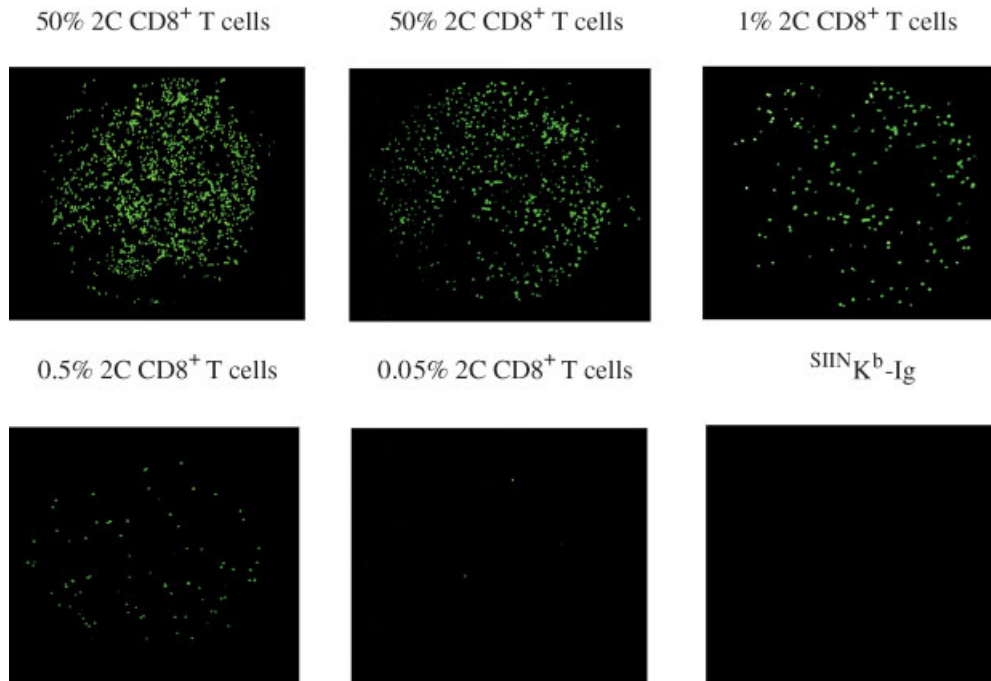


Figure 2. Binding of 2C CD8⁺ T cells labelled with CFSE (green dye) was measured to spots containing SIYK^b-Ig in a mixture of CD8⁺ T cells from 2C transgenic mouse and splenocytes from C57BL/6J mouse (labelled with Dil, a red dye) as a function of T-cell concentration in per cent by number of the 2C CD8⁺ T cells. The negative control consisted of contacting these solutions with spots containing the non-cognate SIIN^K^b-Ig for each T-cell concentration studied. The negative control is shown for 1% 2C CD8⁺ T cells. The spots were printed manually by pipeting $\sim 0.1 \mu\text{l}$ of SIYK^b-Ig or SIIN^K^b-Ig (0.5 mg/ml) onto polyacrylamide film-coated glass slides. Total cell concentration: $\sim 4 \times 10^6$ cells in $700 \mu\text{l}$ of PBS solution.

more clearly by the first 15–20 min at a flow rate of 0.01 ml/min. After 1 h (frames 8 and 9), the accumulation of T cells was nearly uniform on all SIYK^b-Ig spots. Significant non-specific binding is also noticeable in the interstitial area between these spots. After washing with PBS at a much higher flow rate of 5 ml/min, very few T cells disengaged from the SIYK^b-Ig spots (frame 10). However, virtually all the T cells that bound to the non-cognate SIIN^K^b-Ig spots (frame 11) were removed by washing (frame 12). The negligible loss of captured cells on the SIYK^b-Ig spots at this high flow rate indicates strong binding between the T cells and the SIYK^b-Ig complexes on the microarray surface.

DISCUSSION

Our results demonstrate the feasibility of using p/MHC microarrays to selectively capture antigen-specific T cells from suspensions at concentrations as low as 0.01%. The high sensitivity of p/MHC microarrays compares well with high-end state-of-the-art fluorescence activated cell sorting (FACS) instruments. In light of the fact that the microarrays fabricated here and the operating conditions used for selective T-cell capture have yet to be optimized, this high sensitivity represents a significant potential advantage of p/MHC microarray technology over FACS. Although the results

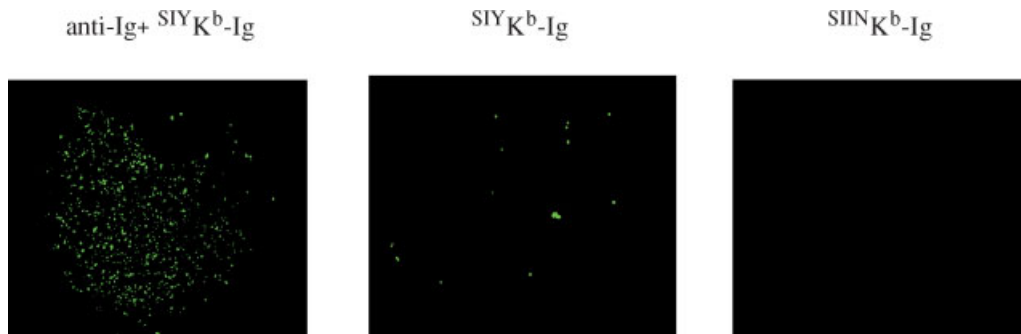


Figure 3. Measured binding of 2C CD8⁺ T cells labelled with CFSE (green dye) on a representative spot containing SIYK^b-Ig anchored to an anti-Ig antibody in a mixture of 5000 CD8⁺ T cells from 2C transgenic mouse and 10 million splenocytes from C57BL/6J mouse (labelled with Dil, a red dye). For comparison, a representative spot containing SIYK^b-Ig without the secondary anti-Ig antibody is also shown. The negative control consisted of contacting this solution with spots containing SIIN^K^b-Ig. The entire surface of the polyacrylamide film-coated glass slide was first pre-treated with secondary anti-Ig antibody before manually pipeting $\sim 0.1 \mu\text{l}$ of SIYK^b-Ig or SIIN^K^b-Ig (0.5 mg/ml) to make the individual spots.

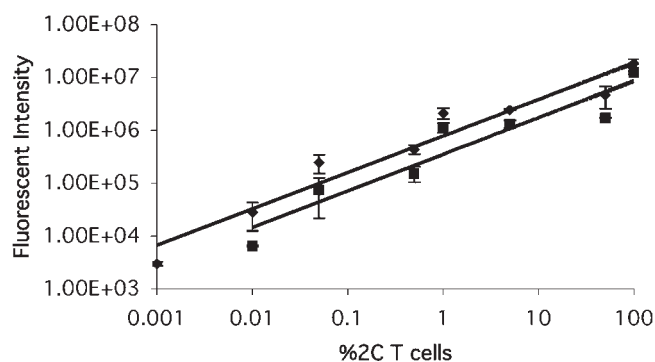


Figure 4. Fluorescent intensities as a function of T-cell concentration for 2C CD8⁺ T cells labelled with CFSE (green dye) bound to spots containing anti-Ig+^{SIY}K^b-Ig (upper) and only ^{SIY}K^b-Ig (lower). Buffer solutions and surface preparations are given in Figures 1 and 2. Linear fits of log(intensity) versus log(concentration) yield slopes of 0.690 and 0.693, and identical intercepts of 7.71×10^5 for the anti-Ig+^{SIY}K^b-Ig spots and the ^{SIY}K^b-Ig spots, respectively. Error bars represent standard deviations for multiple data points at each concentration.

are encouraging, spot-to-spot reproducibility was found to be poor at low CD8⁺ T-cell concentrations when the cell suspension was contacted with the microarray under static conditions. For example, few CD8⁺ T cells were observed on some ^{SIY}K^b-Ig spots adjacent to other spots containing many CD8⁺ T cells at the lowest T-cell concentration of as 0.01%.

Contacting the cell suspension with the microarray under mild shear flow conditions produced a more uniform distribution of captured T cells on the individual ^{SIY}K^b-Ig spots and improved the spot-to-spot reproducibility across the entire microarray, as shown in Figure 5 (frames 9 and 10). In contrast, incubation under static conditions produced erratic distributions of captured T cells on the individual ^{SIY}K^b-Ig spots, and spot-to-spot reproducibilities that were much worse. A possible explanation for these observations is an increased number of encounters between the TCRs and p/MHC complexes under flow conditions which enhances the binding of 2C CD8⁺ T cells on the ^{SIY}K^b-Ig spots (Chang and Hammer, 1999). It is interesting to note that Pierres *et al.* (1994) captured lymphoid cells bearing CD8 molecules on an anti-CD8-coated surface under shear flow and found that the rate constant for adhesion increases with relative velocity. The Peclet number calculated for our flow experiment is 16.5, which indicates that the mild shear flow conditions we used corresponds to a high adhesion rate (Pierres *et al.*, 1994; Chang and Hammer, 1999).

The flow experiments also established that washing the microarrays had no deleterious effect on the captured T cells. In fact, washing was found to reduce significantly the background from non-specific T-cell binding, and thus, increase the sensitivity to specific binding. It is important to emphasize that flow rates of 2 and 5 ml/min for washing the microarrays correspond to shear stresses of 8×10^{-2} Pa and of 2×10^{-1} Pa, respectively, which are high for routine flow assays. Even at these high rates, T-cell disengagement from the ^{SIY}K^b-Ig spots was negligible, which suggests a mechanism for T-cell capture that is enhanced by flow, consistent with the earlier studies of Pierres *et al.* (1994) and Chang and Hammer (1999).

CONCLUSIONS

We established that microarrays functionalized with the MHC-Ig dimer, K^b-Ig, can selectively capture T cells based on their peptide antigen specificity, and can detect T cells at concentrations that are comparable to those for state-of-the-art fluorescence activated cell sorting (FACS) instruments. Microarrays fabricated by either manually printing the individual MHC-Ig spots or automated printing of a large number of smaller spots exhibited similar characteristics of specificity and sensitivity for the capture and detection of antigen-specific CD8⁺ T cells. Contacting T cells with these microarrays under mild shear flow conditions improved performance in terms of promoting a uniform distribution of captured T cells on the individual spots and a much higher spot-to-spot reproducibility across the entire microarray. Although these flow experiments using p/MHC microarrays fabricated by automated printing must be considered preliminary, the results warrant a systematic study of the influence of flow on p/MHC microarray performance in applications that involve the selective capture and enumeration of diverse T-cell populations.

EXPERIMENTAL SECTION

Preparation of soluble K^b-Ig dimer

The J558L murine cell line transfected with plasmid containing genetically fused class I MHC to the amino termini of the immunoglobulin heavy chain (IgG1) (Dal Porto *et al.*, 1993) was used to produce soluble, dimeric K^b-Ig. The cells were grown in complete RPMI media (GIBCO Invitrogen, Carlsbad, CA) and were expanded in Hybridoma-SFM (GIBCO Invitrogen) spinner flasks. After a week of culture in spinner flasks, cell supernatant was harvested by centrifugation. The supernatant was filtered and pH adjusted to 7.0. The K^b-Ig protein was purified by affinity chromatography using a 4-hydroxy-3-nitrophenyl (NP)-sepharose column. The fusion constructs were eluted from the column with excess NP without the need for harsh acidic or basic elution conditions. The excess NP was then separated from the fusion proteins by multiple steps of washing and centrifugation in phosphate buffered saline (PBS).

The purified, soluble K^b-Ig was loaded using the protocol described previously (Schneck *et al.*, 2000b). Briefly, K^b-Ig protein was stripped of endogenous peptide by denaturation in an alkaline buffer (150 mM NaCl + 15 mM Na₂CO₃, pH: 11.5) for 15 min. The protein was then refolded in the presence of 100-fold excess peptide (SIY or SIIN) at neutral pH for 48 h. This protocol has been shown to efficiently load 95% of the protein with the peptide of interest.

Isolation and labelling of CD8⁺ T cells

Single cell splenocyte suspension was prepared from harvested mouse spleens, and red blood cells were removed by lysing them in ACK buffer (BIOSOURCE, Camarillo, CA). CD8 T cells were purified from the splenocytes by

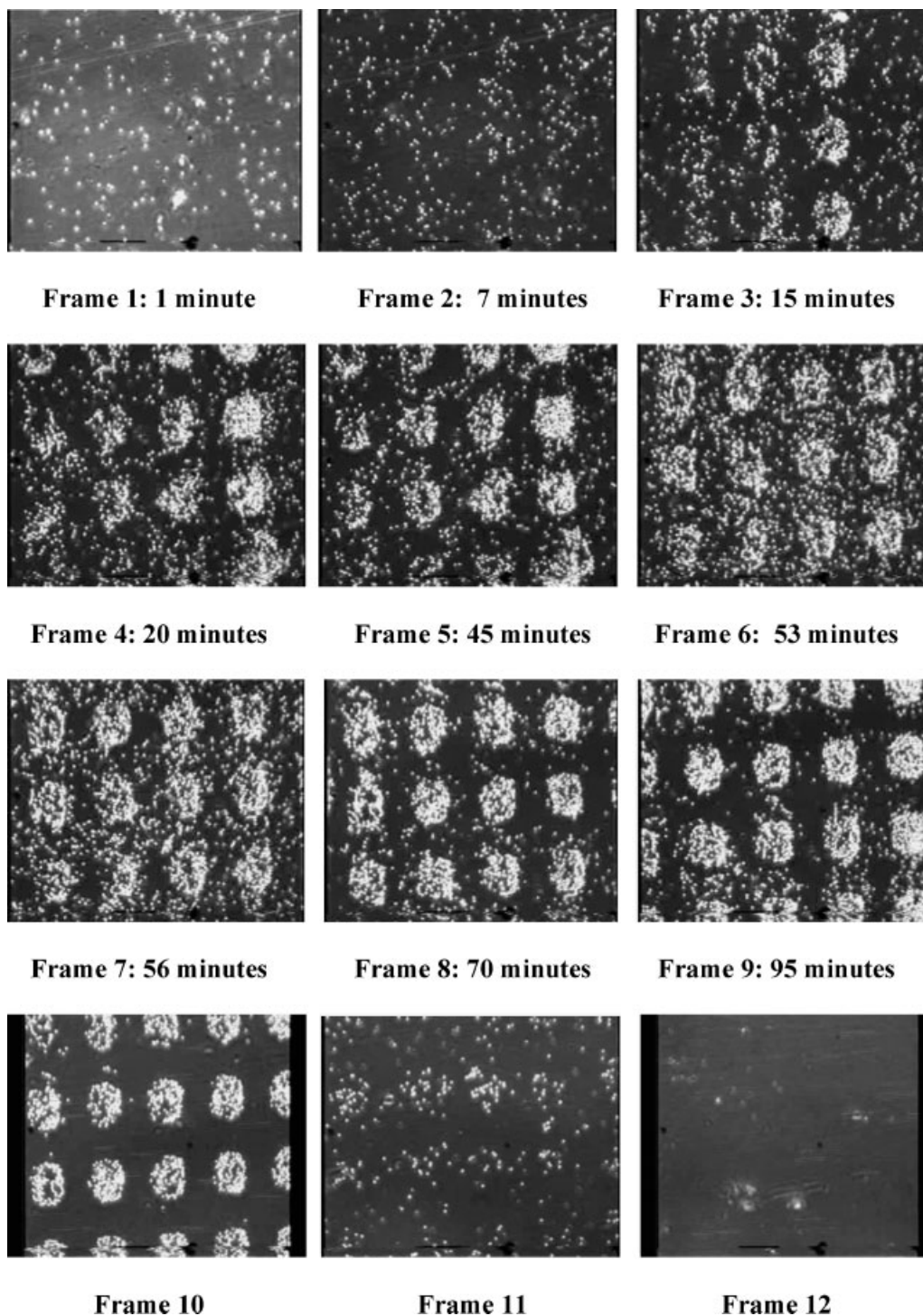


Figure 5. Time dependence of binding 2C CD8⁺ T cells labelled with CFSE (green dye) under flow conditions to spots containing the ^{S¹YK^b}-Ig and ^{S¹INK^b}-Ig complexes. Flow is from right to left. Spots were printed using an automated printer as described in the text. The individual spots consist of 2–3 nl of p/MHC solution (0.5 mg/ml) and are ~100 microns in diameter. T-cell concentration: ~3 × 10⁶ T cells in 1 ml of PBS solution. Flow rate = 0.01 ml/min. Frames 1–9 show T cells binding on ^{S¹YK^b}-Ig spots with time up to 1 h and 35 min. Frame 10 shows T cells bound to the ^{S¹YK^b}-Ig spots after washing with cell-free PBS solution at a flow rate of 5 ml/min for 5 min. Frames 11 and 12 compare T-cell binding to the ^{S¹INK^b}-Ig spots before and after washing at the same flow rate.

negative selection columns (R&D Systems, Minneapolis, MN). The purity of isolation was greater than 95% as assessed by 1B2 mAb (specific for 2C TCR) and anti-CD8 staining (data not shown). In some experiments, splenocytes

from C57BL/6J were prepared and used without further isolation.

Isolated 2C CD8⁺ T cells were labelled with carboxy-fluorescein diacetate succinimidyl ester using the manu-

facturer's protocol (CFDA-SE, Molecular Probes, Invitrogen, Carlsbad, CA). Splenocytes from C57BL/6J were labelled with DiI using the manufacturer's protocol (Molecular Probes, Invitrogen).

Printing antibody and p/MHC microarrays

The $^{SIY}K^b$ -Ig and $^{SIIN}K^b$ -Ig complexes were printed manually on polyacrylamide film-coated glass slides (Schott Nexterion[®], Elmsford, NY) using a hand pipette to dispense the samples in the volume range, 0.1–2 μ l. Nearly spherical spots 1.0–1.5 mm in diameter were produced from 0.1 μ l of 0.5 mg/ml p/MHC solution.

A plastic 2-pad incubation chamber (Schleicher & Schuell, Elmsford, NY) was placed on the glass slides and then a custom-made polystyrene template (2.5 \times 7.5 \times 3 mm) was placed on this incubation chamber. Proper alignment was achieved by holding this assembly in a FAST frame (Schleicher & Schuell). Spots were printed onto the slides through 1 mm diameter holes in the template to produce two 2 \times 2 cm microarrays each containing 16 spots. The printed slides were incubated for 1 h at room temperature, followed by rinsing three times with PBS, then blocked for 1 h in blocking buffer (50 mM ethanolamine + 50 mM borate at pH 8.0) and rinsed again three times with PBS.

For some experiments, goat anti-mouse IgG secondary antibody (BD Pharmingen, San Diego, CA) was used to orient the K^b -Ig complexes for more efficient binding to the TCRs. In these experiments, the slides were first coated completely with the secondary antibody and then spots were manually printed with $^{SIY}K^b$ -Ig and $^{SIIN}K^b$ -Ig (0.5 mg/ml). After 1-h incubation, slides were blocked and rinsed three times in PBS before use.

Automated printing with the VersArray Chip Writer system produced 25, 50 or 64 spots containing 2–3 nl of antibodies or p/MHC on the same polyacrylamide film-

coated glass slides. The spot size was \sim 100 μ m in diameter, producing a 100-fold smaller surface area compared to the manually printed spots. The same incubation and blocking protocols were used as those described above for the experiments with manually printed microarrays.

Flow-based adhesion assay

A parallel-plate flow chamber with a 500 μ m channel depth and 1 cm channel width (Ahn *et al.*, 2005) was used to follow the time dependence of CD8⁺ T-cell capture on microarrays printed with $^{SIY}K^b$ -Ig and with $^{SIIN}K^b$ -Ig as the negative control. The flow chamber was configured with a mounting stage above an inverted microscope (Nikon TE300, Melville, NY) equipped with 10 \times phase objectives, a 0.55 \times projection lens and a video feed camera (CCD100 Dage-MTI, Michigan City, IN) connected to a VCR and monitor.

Microarrays with 64 (8 \times 8) spots of $^{SIY}K^b$ -Ig and 64 (8 \times 8) spots of $^{SIIN}K^b$ -Ig were printed with the automated printer. A CD8⁺ T-cell suspension was perfused over these microarrays at a flow rate of 0.01 ml/min, corresponding to a shear stress of 4×10^{-4} Pa (Bird *et al.*, 1960), for 2 h and T-cell capture visualized at locations corresponding to both the $^{SIY}K^b$ -Ig and $^{SIIN}K^b$ -Ig spots. A cell-free PBS solution was then perfused over the microarrays at a flow rate of 2 ml/min, corresponding to a shear stress of 8×10^{-2} Pa, to wash the microarrays and the extent to which captured T cells removed by washing was monitored.

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