Immunological Synapses Are Versatile Structures Enabling Selective T Cell Polarization

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Summary

Helper T cells discriminate among different antigen-presenting cells to provide their help in a selective fashion. The molecular mechanisms leading to this exquisite selectivity are still elusive. Here, we demonstrate that immunological synapses are dynamic and adaptable structures allowing T cells to communicate with multiple cells. We show that T cells can form simultaneous immunological synapses with cells presenting different levels of antigenic ligands but eventually polarize toward the strongest stimulus. Remarkably, living T cells form discrete foci of signal transduction of different intensities during the interaction with different antigen-presenting cells and rapidly relocate TCR and Golgi apparatus toward the cell providing the strongest stimulus. Our results illustrate that, although T cell activation requires sustained signaling, T cells are capable of rapid synapse remodeling and swift polarization responses. The combination of sustained signaling with preferential and rapid polarization provides a mechanism for the high sensitivity and selectivity of T cell responses.

Introduction

T lymphocytes are activated by the engagement of their antigen receptors (TCR) with peptide-MHC complexes displayed on the antigen-presenting cell (APC) surface (Germain, 1994). It is well established that antigen recognition in T lymphocytes involves the formation of an immunological synapse (IS) (Grakoui et al., 1999; Monks et al., 1998; reviewed in Gascoigne and Zal, 2004). In the original definition, the mature IS was described as a specialized signaling domain formed at the contact site between T cells and APC, characterized by large-scale molecular segregation of surface receptors and signaling components. Ongoing research led to a modification of this view, where the IS comprises a multitude of structures, all of them having in common the fact that

they are mediators of intercellular communication (Trautmann and Valitutti, 2003).

Even though it has been shown that signaling components are dynamic at the IS (Bunnell et al., 2002; Favier et al., 2001) and that T cell hybridomas can form more that one synapse with APC displaying different antigenic ligands (Zal et al., 2002), mature IS are traditionally studied as single stable structures at the cell-cell contact site.

Recent reports have contributed to a dynamic picture of T cell activation by showing that T cells can form multiple contacts with APC both in vitro and in vivo and become activated by summation of signals gathered on APC surfaces (Faroudi et al., 2003b; Gunzer et al., 2000; Huppa et al., 2003; Mempel et al., 2004).

While these reports introduced the new concept of sequential encounters in T cell activation, they did not address another challenging question: how do T cells respond when they encounter different stimulatory cells simultaneously? This is a situation that both resting and effector T cells are likely to face during the development of an immune response (Mempel et al., 2004).

In the present work, we investigate the possibility that IS may be dynamic structures allowing T cells to communicate with multiple APC simultaneously and give rapid responses to stimulation.

An important morphological parameter of T cell activation that can be studied in parallel with IS structure is the polarization of T cell secretory machinery toward the APC. In resting conditions, T lymphocytes are nonpolarized cells. TCR engagement by specific peptide-MHC complexes displayed on the surface of APC triggers the reorganization of intracellular structures, resulting in polarized cytokine secretion toward APC (Kupfer and Singer, 1989). This event is vital for efficient T cell functioning. Indeed, on the one hand, it allows cytotoxic T cells (CTL) to eliminate target cells in a selective way, and on the other hand, it allows helper T cells to dedicate their help to specific cellular partners (Kupfer and Singer, 1989). The mechanisms of T cell polarization are still not well understood; in particular it is not known how selective polarization is achieved in T cells interacting with multiple cells.

We studied immunological synapse formation in parallel with polarization of the secretory machinery in T cells interacting simultaneously with antigen-presenting cells offering different stimuli.

We show that even though T cells can form several IS with different adjacent cells, they selectively polarize their secretory machinery toward the APC offering the strongest stimulus. The intensity of molecular translocation at the different synapses reflects the strength of signals received via TCR and accessory molecules and drives polarization.

Results

Selective T Cell Polarization toward APC Providing the Strongest Stimulus

A key feature of the adaptive immune response is that Th cells are able to discriminate among different APC

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to provide their help in a selective fashion (Kupfer et al., 1994; Lanzavecchia, 1990).

In a previous study, we showed that T cells that have been activated by a previous encounter with peptide-pulsed APC and have extinguished signaling by TCR downregulation can be reactivated if conjugated with APC offering a stronger stimulus, polarizing IFNγ secretion toward these cells (Valitutti et al., 1996). In that study, we proposed that the downregulation of a large fraction of TCR allows T cells to calibrate their response to the level of antigenic stimulation, while the residual TCR allows T cells to detect subsequent APC. This implied that T cells are not desensitized by a previous interaction with APC and therefore can be activated by further encounters. That work provided a first stepping stone to the understanding of T cell activation and polarization in sequential interactions. However, it did not address the question of whether and how T cells could immediately discriminate signals of different intensities incoming simultaneously from different directions (Valitutti et al., 1996).

To address this question, we first investigated the morphological aspects of selective polarization in T cells interacting simultaneously with different APC. Th1 and Th2 clones were conjugated with EBV-B cells offering different levels of antigenic stimulation. The cells were then stained with anti-cytokine or with anti-tubulin antibodies.

In T cells interacting with both high- and low-peptide-pulsed APC, IFNγ, IL4, and the MTOC (microtubule organizing center) polarized toward the APC pulsed with the highest amount of peptide (Figure 1 and Table 1).

The above results extend our previous observation by showing that helper T cells can readily discriminate APC contacted simultaneously and dedicate their help to the APC providing the strongest antigenic stimulus.

Multiple IS in T Cells Interacting Simultaneously with Different APC

To understand how T cells discriminate between APC displaying various antigen levels, we studied IS formation in T cells conjugated with EBV-B cells offering different levels of antigenic stimulation. EBV-B cells pulsed with either a high or a low peptide concentration were conjugated with T cells (Th1 and Th2 clones) and incubated for 15 min at 37°C. Cells were stained with anti-CD2 and anti-phosphotyrosine (PTyr) antibodies to visualize IS formation (Leupin et al., 2000). In T cells interacting with one APC only, IS formation was detected at both antigenic concentrations, although it was more pronounced and frequent in conjugates containing APC pulsed with high antigen concentration (data not shown). In T cells interacting simultaneously with high- and low-peptide-pulsed APC, CD2 and PTyr accumulated preferentially at the contact site with the APC pulsed with the higher amount of peptide (Figure 2 and Table 1). Interestingly, in 39% of the scored conjugates (n = 64), a rudimentary IS was also observed at the contact site with the low-peptide-pulsed APC as detected by a minor accumulation of CD2 and PTyr staining (Figure 2). It should be noted that the measurement of polarization indexes in T cells was performed on unprocessed images and without the application of hand-drawn regions (see Experimental Procedures). The re-
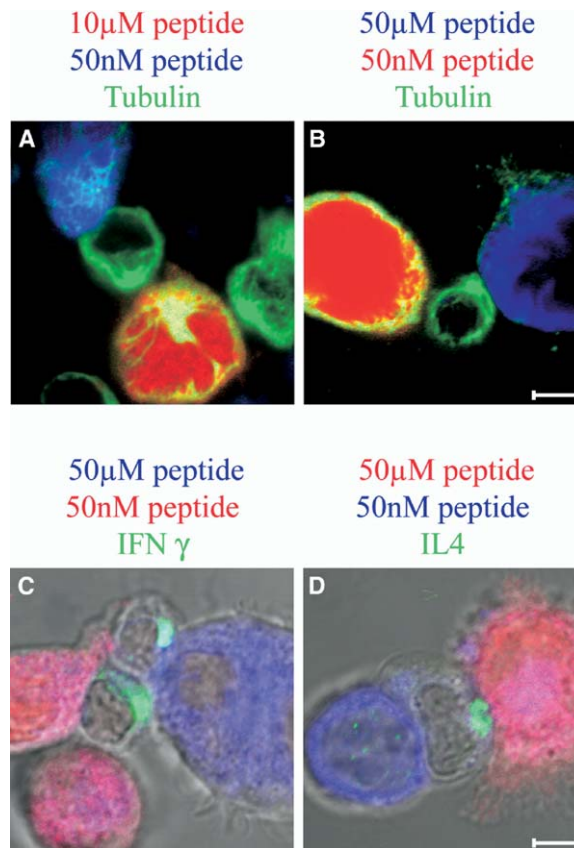

Figure 1. Selective T Cell Polarization toward the APC Offering the Strongest Stimulus

T cells were simultaneously conjugated with APC pulsed with 50 nM and either 10 μM or 50 μM peptide and loaded with CMTMR-Orange (red) or BODIPY 630 (blue) as indicated. After 15 min (A and B) or 150 min (C and D) incubation at 37°C, cells were stained with anti-tubulin (A and B) anti-IFNγ (C) and anti-IL4 (D) mAbs followed by FITC-labeled goat anti-mouse antibodies (green). (A), clone 6396 p5.1.2; (B and C), 6396 p5.1.5; (D), SDM 3.5. Similar results were obtained with another T cell clone (KS 140). Data are from one representative experiment out of at least three for each panel. Bar, 5 μm.

sults obtained are statistically relevant, as shown in Figure 4.

Similar results were obtained when the localization of the ζ chain of TCR/CD3 was studied in T cells interacting simultaneously with high- and low-peptide-pulsed APC (data not shown).

All quantifications of polarization in T cells were performed on randomly selected triplicates in which one T cell was contacting two APC simultaneously. Thus, data presented in Figure 4 and Table 1 were all obtained by measuring double contacts of T cells simultaneously conjugated with two different APC.

Taken together, the above results indicate that T cells can simultaneously scan different APC and form different immunological synapses reflecting the strength of antigenic stimulation.

CD2/CD58 Interaction Fine Tunes IS Formation and T Cell Polarization

The above results indicate that important differences in the strength of antigenic stimulation can shape the

Table 1. Measurement of the Distribution of PTyr, CD2, Tubulin, and IFNγ at the Contact Sites between T Cells and Two Different APC

Polarization Toward	High-Peptide-Concentration-Pulsed APC (%)	Low-Peptide-Concentration-Pulsed APC (%)	Undetermined
PY (n = 38)	87	10	3
CD2 (n = 38)	87	13	0
Tubulin (n = 31)	88	12	0
IFN γ (n = 29)	97	3	0
Polarization Toward	Anti-MHC-Class I-Treated APC (%)	Anti-CD58-Treated APC (%)	Undetermined
PY (n = 29)	90	10	0
CD2 (n = 36)	95	5	0
Tubulin (n = 42)	78	16	6
IFN γ (n = 31)	75	22	3

For each antibody, n T cell/APC conjugates (formed by one T cell simultaneously conjugated with two APC pulsed with low and high peptide concentration) were randomly selected from three independent experiments and scored for CD2 and PTyr enrichment and tubulin cytoskeleton and IFNγ polarization at the cell-cell contact site. Tubulin and IFNγ polarization were scored by visual inspection in a blind study. Enrichment of PTyr and CD2 were measured using the linescan function of the MetaMorph software as described in Experimental Procedures. Fluorescence intensity measurement was performed on unprocessed images. The polarization indexes for CD2 and PTyr staining were the following: 2.1 for CD2 high versus low peptide concentration; 2.2 for CD2 anti-CD58 versus anti-class I blocking Abs; 3 for PTyr high versus low peptide concentration; 1.9 for PTyr anti-CD58 versus anti-class I blocking Abs. %: percent of conjugates exhibiting a given pattern of staining for n = 100%. Similar results were obtained when one APC population was untreated instead of being treated with anti-class I blocking antibodies (not shown).

structure of the synapse and guide the direction of T cell help. However, they do not address the question of how T cells can discriminate among APC offering similar amounts of antigen. To address this question, we investigated the possibility that signals derived from
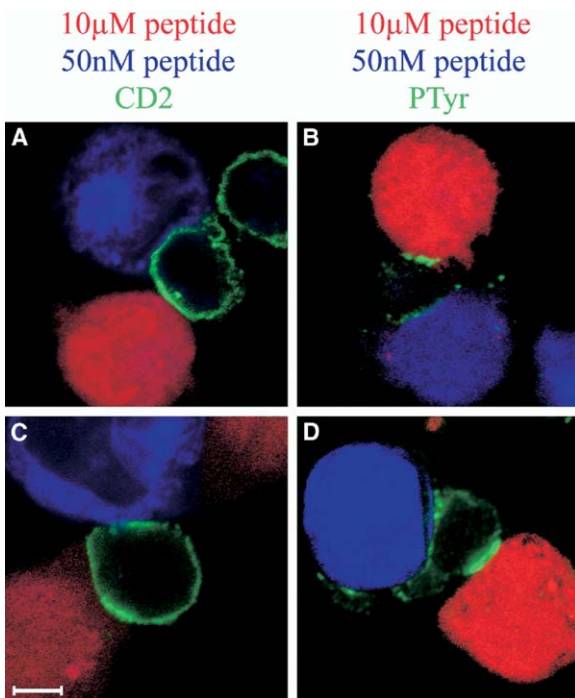

Figure 2. IS Formation in Th Cells Interacting Simultaneously with Different APC

T cells were conjugated for 15 min with APC pulsed with either 50 nM (blue) or 10 μM (red) peptide. Cells were stained with anti-CD2 or anti-PTyr mAbs followed by FITC-labeled goat anti-mouse antibodies (green) as indicated. (A and B), clone KS 140; (C), 6396 p5.1.2; (D), clone SDM 3.5. Data are from one representative experiment out of six. Bar, 5 μm.

accessory molecules at the IS may provide additional information for APC discrimination.

We have previously shown that blocking the interaction between CD2 and CD58 (by treating the APC with anti-CD58 antibodies) alters the structure of the IS and affects several T cell responses (Zaru et al., 2002). To test whether the binding of CD2 with CD58 may contribute to selective polarization of helper T cells, Th1 or Th2 cells were conjugated with EBV-B cells pulsed with the same amount of antigenic peptide (10 μM) and treated with either anti-CD58 or anti-MHC class I control antibodies. Cells were stained for markers of the IS and for the "help machinery."

T cells simultaneously conjugated with two peptide-pulsed APC (one treated with anti-CD58 and the other treated with anti-class I abs) exhibited a reduced tyrosine phosphorylation and no CD2 enrichment at the contact site with the APC where the CD2/CD58 binding was impeded. Conversely, IS formation was not affected at the contact site with the APC treated with control antibodies (Figures 3A, 3B, and 4 and Table 1).

These results indicate that the CD2/CD58 interaction contributes to the reorganization of the signaling cascade in T cells, resulting in the formation of a well-structured IS only at the contact site with the APC offering an optimal level of CD2 ligands. While CD2 enrichment was absent at the contact site with the APC treated with anti-CD58 antibodies (Figure 3A; in agreement with previously reported data [Zaru et al., 2002]), in 51% of scored conjugates (n = 45), a minor enrichment of PTyr staining was detected at this contact site (Figure 3B). Thus, also in these experimental conditions, T cells tend to form more than one IS; the intensity of signaling at each IS reflects the strength of signals gathered from the APC surface.

Interestingly, in the above-described conditions, cytokines and microtubule cytoskeleton selectively polarized toward the APC not treated with anti-CD58 blocking antibodies (Figures 3C and 3D and Table 1).

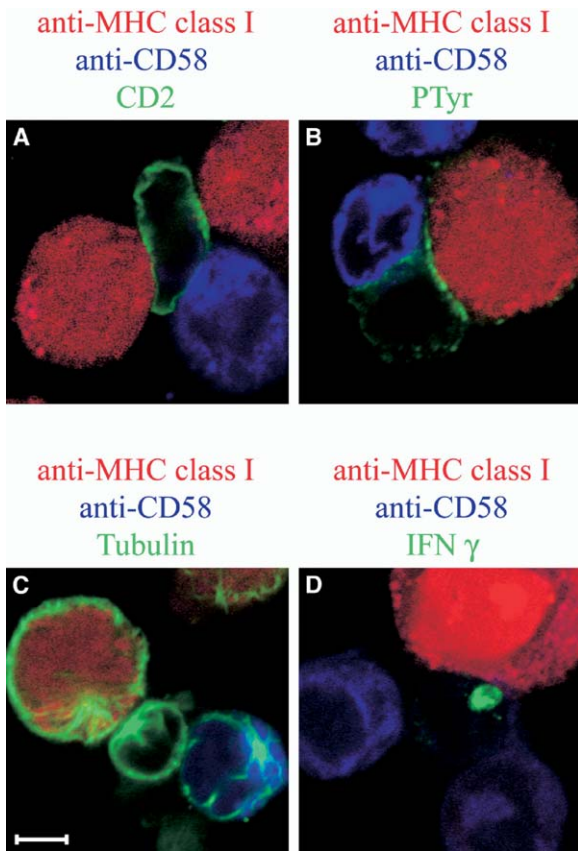Also in this case, all quantifications of polarization in

Figure 3. CD2/CD58 Interaction Fine Tunes the Orientation of T Cell Help Delivery

T cells were conjugated with peptide-pulsed APC either treated with anti-MHC class I mAb (red) or treated with anti-CD58 mAb (blue). After 15 min (A–C) or 150 min (D) incubation at 37°C, cells were stained with anti-CD2, anti-PTyr, anti-tubulin, or anti-IFNγ mAb (green) as indicated. (A and B), clone KS 140; (C and D), 6396 p5.1.2. Data are from one representative experiment out of three for each panel. Bar, 5 μm.

T cells were performed on randomly selected triplicates in which one T cell was contacting two APC simultaneously.

We next investigated whether the block of CD2/CD58 interaction could compensate for the level of antigenic stimulation in directing T cell polarization.

T cells were simultaneously conjugated with APC pulsed with high antigen concentration and treated with anti-CD58 antibodies and with APC pulsed with low antigen concentration and treated with anti-MHC class I antibodies. Our results show that, in these experimental conditions, the block of CD2/CD58 interaction has a moderate effect on T cell polarization. Indeed, both microtubule cytoskeleton (74% of 31 triplicates scored) and IFNγ (60% of 35 triplicates scored) still preferentially polarized toward the APC displaying the highest antigenic stimulus.

Taken together, the above results indicate that CD2/CD58 interaction fine tunes the formation of IS at the T cell/APC contact site and contributes to direct T cell polarization.
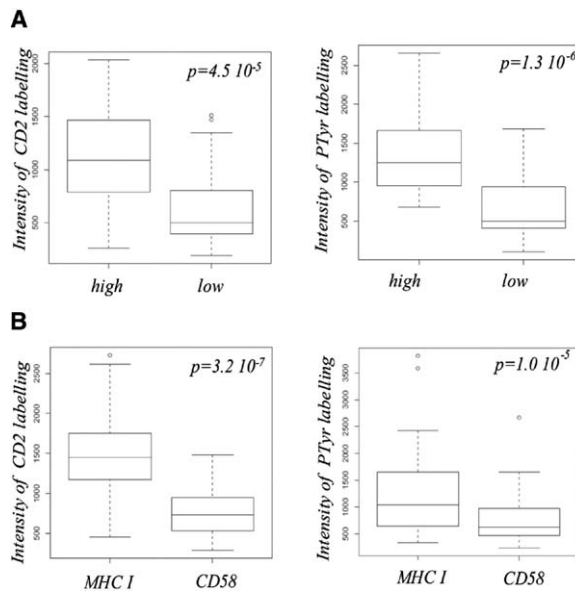

Figure 4. Statistical Analysis of Morphological Data

(A) Statistical analysis of CD2 and PTyr polarization toward T cells simultaneously conjugated with APC pulsed with high or low peptide concentration.

(B) Statistical analysis of CD2 and PTyr polarization toward T cells simultaneously conjugated with pulsed APC either treated with anti-MHC class I mAb or with anti-CD58 mAb. Enrichment of PTyr and CD2 were measured using the linescan function of the Meta-Morph software as described in the Experimental Procedures. The values correspond to measurements of PTyr and CD2 fluorescence intensity in the T cell/APC conjugates presented in Table 1. Florescence intensity measurement was performed on unprocessed images. The panels give a graphical summary of the data distribution in the form of box plots (further details in the Experimental Procedures). The median (and mean) values of polarization between corresponding images are significantly different (p values < 0.0001); exact p values inside each graph.

Engagement of Extrasynaptic TCR Triggers Rapid T Cell Repolarization

Taken together, the above results sketch a novel "dynamic" aspect of T cell activation: T cells may contact different APC at the same time and form multiple synapses, reflecting the amount of cell-cell information transfer. This process allows T cells to quickly respond to changes in signaling intensity via rapid repolarization toward an APC offering a stronger stimulus. To examine real-time T cell responses to signaling intensity, we studied TCR dynamics in living T cells. Cells were stained with Cy5-labeled anti-CD3 Fab and conjugated with APC pulsed with either 100 nM or 100 μM specific peptide. In T cells interacting with a single APC (pulsed with either antigen concentration), TCR enrichment toward the APC was observed (although it was more pronounced at high antigen concentration; data not shown). Interestingly, when a T cell interacting with an APC pulsed with the low antigen concentration (already exhibiting an enrichment of TCR/CD3 at the IS) entered in contact with an APC offering a higher antigenic stimulus, a clear TCR relocalization was observed. As shown in Figures 5A and 5B and Supplemental Movies S1 and S2, a large fraction of TCR/CD3 complexes moved to-
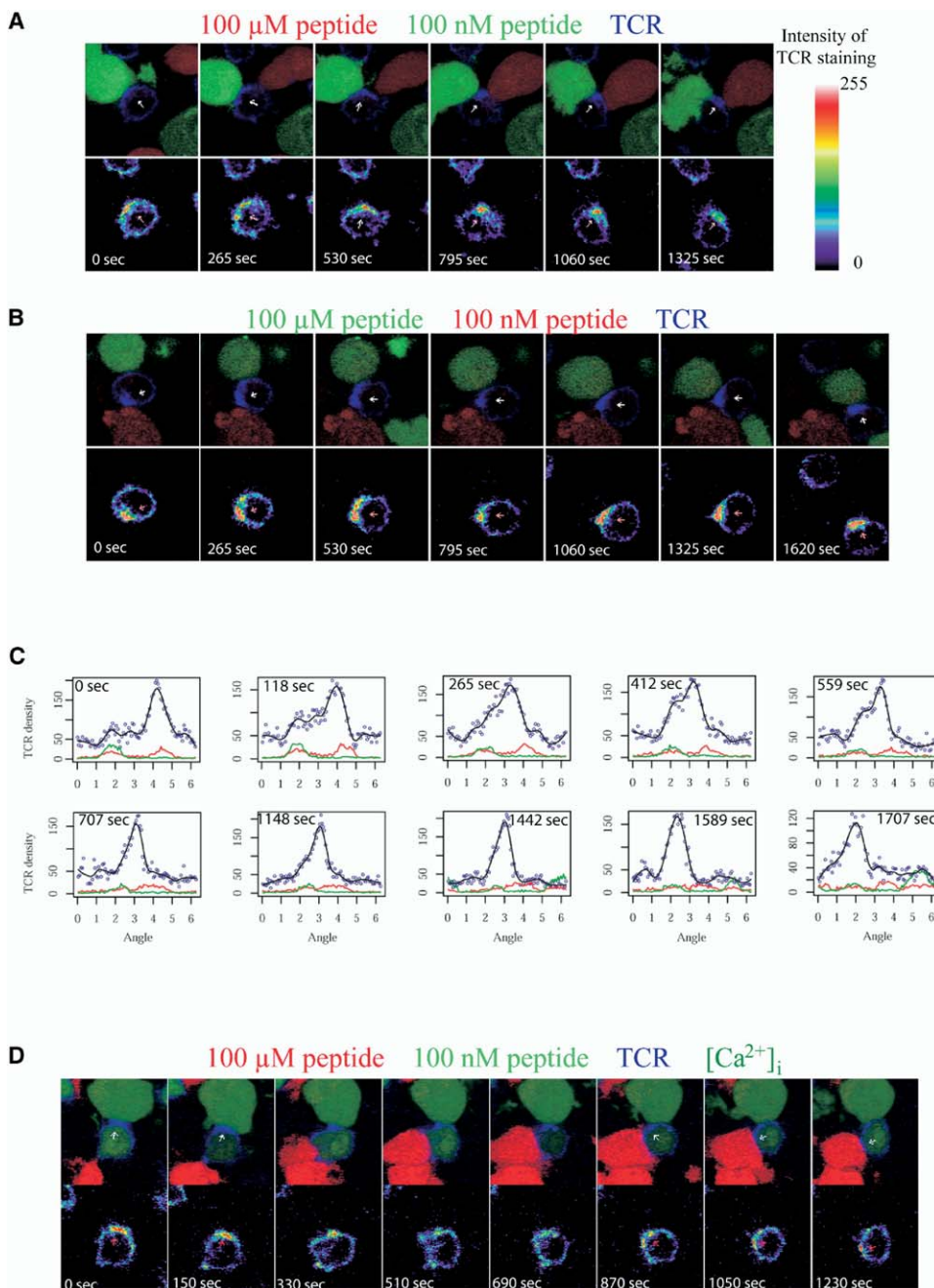
Figure 5. TCR Dynamics in Living T Cells Simultaneously Conjugated with Two APC

Sequences of snapshots depicting TCR repositioning toward the APC offering the strongest stimulus are shown.

(A) T cells (clone 6396 p5.1.2) stained with anti-CD3 Fab (blue) are interacting with APC loaded with 100 nM (green) and 100 μM (red) peptide concentration.

(B) T cells stained with anti-CD3 Fab (blue) are interacting with APC loaded with 100 nM (red) and 100 μM (green) peptide. Lower panels depict the TCR staining intensity using a pseudocolor scale. Data are from two representative experiments out of five. Snapshot sequence (A) corresponds to Supplemental Movie S1, and snapshot sequence (B) corresponds to Supplemental Movie S2. Arrows indicate the bulk of TCR.

(C) Analysis of TCR distribution over the T cell surface in T cells interacting with APC pulsed with low and high antigen concentration. Data are from Supplemental Movie S2. Florescence intensity measurement was performed on unprocessed images. The depicted data are one representative of a total of three data sets.

(D) T cells (clone 6396 p5.1.2) previously loaded with Fluo-4 AM and stained with anti-CD3 Fab (blue) are interacting with APC loaded with 100 nM (green) and 100 μM (red) peptide concentration. Snapshot sequence (D) corresponds to Supplemental Movie S4. The green intracellular staining of the T cell depicts $[Ca^{2+}]_i$ increase as compared to T cells alone (see Supplemental Movies S4 and S5). Lower panels depict the TCR staining intensity using a pseudocolor scale. Data are from one representative experiment out of three.

ward the APC offering the stronger stimulus. While TCR repositioning was completed in different times for different T cell/APC conjugates, the initiation of this process was always detected within 200–300 s after conjugation with the second APC. In some T cell/APC conjugates, repolarized TCR appeared to accumulate in between the two APC. However, the cluster of receptors always pointed toward the APC offering the stronger stimulus (Supplemental Movie S1). When T cells conjugated with APC offering a high antigen concentration encountered APC offering a lower antigen concentration, no repositioning of TCR was observed (Supplemental Movie S3).

To better visualize remodeling of the IS in living cells, a region (the linescan option of the MetaMorph software) was drawn at the T cell surface to quantify TCR staining. As shown in Figure 5C, TCR staining is initially accumulated at the contact site with the first APC in a symmetric distribution curve. During the time of repolarization, the TCR distribution tends to lose symmetry with TCR being mainly dispersed between the two contact sites with the APC; this probably reflects the flux of TCR to the new contact site. At the end of repolarization, a symmetric distribution is reacquired (Figure 5C).

We next asked whether a T cell must have ceased signal transduction at the contact site with the first APC in order to be able to polarize toward a new APC or whether it can chose among different APC while signaling is still going on.

We visualized TCR repositioning in parallel with detection of $[Ca^{2+}]_i$ increase in T cell/APC conjugates. As shown in Figure 5D and Supplemental Movie S4, a T cell in the process of undergoing $[Ca^{2+}]_i$ increase repolarizes toward an incoming APC offering a stronger stimulus without having ceased signal transduction.

Taken together, the above results indicate that a relatively small number of extrasynaptic TCR can outcompete the signals derived from a large number of TCR already enriched at the IS and drive their relocation.

To understand whether the T cell secretory machinery may exhibit similar dynamics, we stained T cells with BODIPY FL C5 Ceramide to track the T cell Golgi apparatus. Most of the T cells conjugated with APC pulsed with low antigen concentration exhibited Golgi polarization toward the APC (data not shown). When APC pulsed with the high antigen concentration entered into contact with already polarized T cells, a rapid relocation of the Golgi apparatus toward the incoming cell occurred within a few minutes, suggesting that reorientation of the secretory machinery is achieved before complete TCR repositioning (Figure 6A and Supplemental Movies S6 and S7). Interestingly, not only the Golgi apparatus but also the mitochondria of T cells interacting with APC offering different stimuli rapidly relocated toward the APC offering the stronger stimulus (Supplemental Movie S8). This indicates that early after detection of a stronger stimulus coming from a new direction both the secretory and the metabolic machineries of the T cell consistently adjust their location.

Taken together, the above results suggest that the intensity of localized signal transduction is readily translated in T cell polarized responses. However, they do not prove that different foci of signal transduction can be simultaneously formed at different contact sites in living T cells undergoing multiple contacts.

To address this issue, we visualized the accumulation of the Pleckstrin homology (PH) domain of Akt fused to GFP in antigen-specific T cell hybridomas interacting with APC pulsed with different antigen concentrations. This cell system has been previously employed to show that, after conjugate formation with peptide-pulsed APC, PI3-kinase activation leads to preferential accumulation of PH-Akt/GFP within the IS (Harriague and Bismuth, 2002).

As shown in Figure 6B and Supplemental Movie S9, T cells form simultaneous foci of localized signal transduction when interacting with multiple APC. Interestingly, during sustained interaction, signal transduction at the contact site with the APC offering the stronger stimulus progressively dominates.

To obtain more quantitative information on PH-Akt/GFP accumulation at the two contact sites, we took several snapshots of the living cells by randomly changing the field at the end of the movie recording. A total of 33 snapshots were recorded in 3 independent sessions, and the polarization of PH-Akt/GFP accumulation was scored by visual inspection in a blind study. Only T cells in simultaneous contact with two APC offering different antigenic stimuli and exhibiting a clear PH-Akt/GFP accumulation were scored. In total, 40 triplicates were scored; 67% of T cells exhibited clear PH-Akt/GFP accumulation toward the APC pulsed with a high antigen concentration.

Taken together, the above results indicate that T cells rapidly translate the strength of antigenic stimulations in localized changes in signaling intensity and adjust polarization.

Discussion

In the present work, we show that both synapse formation and T cell polarization are dynamic and flexible processes that are modulated by the engagement of extrasynaptic T cell antigen receptors. Our data shed new light on the mechanisms of cell-cell cooperation during T cell activation by specific antigen.

It is well established that, upon conjugation with cognate APC, T cells undergo sustained signaling that is required for cytokine production and proliferation (Goldsmith and Weiss, 1988; Negulescu et al., 1996; Valitutti et al., 1995a). While CTL are known to form transient conjugates with target cells and recycle from one target to another (Rothstein et al., 1978), CD4^{+} T helper cells are known to form stable clusters with cognate APC (Kupfer and Singer, 1989) and are therefore considered to be rather "monogamous." Conversely, recent studies performed both in vitro and in vivo have shown that some Th cell biological functions can be activated by multiple sequential contacts with APC and by intermittent signaling, suggesting that in some circumstances Th lymphocytes can indeed exhibit "polygamous" behavior (Faroudi et al., 2003b; Gunzer et al., 2000; Mempel et al., 2004).

Our present results confirm and extend this concept, since we show that, in addition to the capacity for collecting activation signals on the surface of several APC
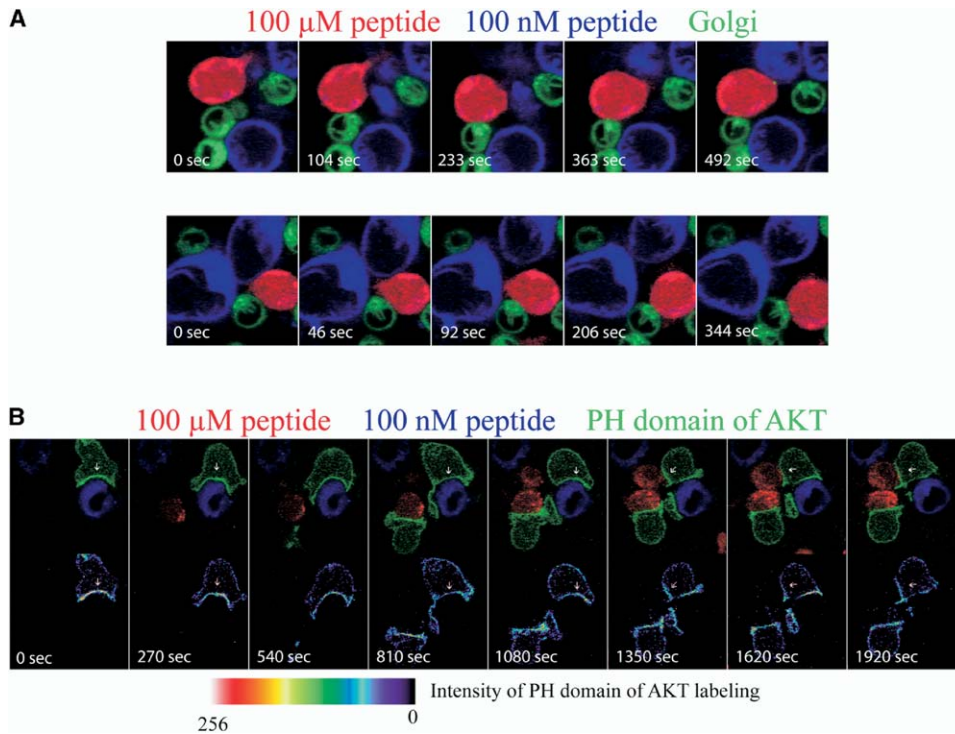
Figure 6. Dynamics of Golgi Apparatus and of PH-Akt/GFP in Living T Cells Simultaneously Conjugated with Two APC

(A) T cells swiftly repolarize Golgi apparatus toward incoming APC offering a stronger stimulus. Sequences of snapshots depicting T cells stained with BODIPY FL5-Ceramide (green) interacting with APC loaded with 100 nM (blue) and 100 µM (red) peptide concentration. Data are from two representative experiments out of four. Upper snapshot sequence corresponds to Supplemental Movie S6; lower snapshot sequence corresponds to Supplemental Movie S7.

(B) Accumulation of PH-Akt/GFP reflects the strength of antigenic stimulation in T cells interacting simultaneously with two APC. Sequence of snapshots depicting hybridoma T cell T8.1 transfected with PH-Akt/GFP interacting with APC loaded with 100 nM (blue) and 100 µM (red) peptide concentration. Data are from one representative experiment out of four. Snapshot sequence corresponds to Supplemental Movie S9.

encountered in sequence, Th cells can also integrate signals from multiple APC at the same time. In other words, they would not only be polygamous in time but also in space. It is likely that this high degree of flexibility of T cell contacts plays an important role in allowing T cells to scan and compare a large number of adjacent APC within a short time.

A second key feature of T lymphocyte activation by specific antigen is the extraordinary sensitivity of T cell responses to antigenic stimulation (Demotz et al., 1990; Harding and Unanue, 1990; Valitutti et al., 1995b). We have previously shown that one mechanism contributing to the high sensitivity of T cells is the serial triggering of a large number of TCR by a small number of peptide/MHC complexes displayed on the APC surface, resulting in a sustained and amplified signal (Valitutti et al., 1995b). This implies that T cells can collect and add up signals during a sustained time period and achieve corresponding levels of activation, which has indeed been recently demonstrated (Faroudi et al., 2003b; Huppa et al., 2003). While signal summation is instrumental in favoring T cell sensitivity, it may be detrimental for selective T cell polarization. Since T cell responses are saturated by a small number of specific peptide/MHC complexes, it is not clear how T cells can selectively polarize their help toward the best APC.

In the present work, we answer this question by showing that, in addition to the accumulation of sustained signals, T cells can also rapidly translate changes in the level of antigenic stimulation in different localized signal transduction (Figures 5 and 6B; Supplemental Movies S1–S4 and S9) and therefore efficiently polarize toward the stronger stimulus (Figure 6A and Supplemental Movies S6 and S7). This creates actual competition among APC for T cell help. Our results provide direct evidence for this competition by showing that already "focused" TCR rapidly relocate toward an incoming APC offering a stronger stimulus, neglecting the previous cell (Figure 5 and Supplemental Movies S1–S4).

A recent study on CTL/target cell interaction came to a rather different conclusion by showing that, in the case of CTL conjugated simultaneously with two targets offering similar densities of antigenic ligands, the tubulin cytoskeleton oscillates between the two targets. This suggests that in CTL polarization and serial killing are stochastic events not guided by specific TCR signaling (Kuhn and Poenie, 2002). However, in this study, the dynamics of polarization in T cells conjugated with APC offering different densities of antigenic ligands was not investigated. Therefore, our results extend these described findings.

We also studied the role of accessory molecules in T cell polarization. It is known that the outcome of T cell/APC cognate interaction depends on the integration of signals from TCR with signals from accessory molecules (Anton van der Merwe et al., 2000; Zaru et al., 2002). Here, we show that accessory molecules also contribute to T cell polarization (Figure 3). The additional signals derived from accessory molecules may help to discriminate among APC offering similar densities of antigenic ligands. Since CD58 expression is upregulated by a variety of stimuli, such as BCR triggering in B cells (R.Z. and D.D., unpublished observations) and Toll-like receptor triggering in other cells (Hertz et al., 2001), the regulated expression of CD58 may favor T cell polarization toward cells that have been strongly activated. This may favor T cell polarization toward B cells triggered via high-affinity BCR or toward APC activated by pathogen-derived products. It is tempting to speculate that this mechanism of fine tuning of T cell polarization is not exclusive to the CD2/CD58 interaction and could be a common regulatory feature of other accessory molecules at the IS.

As a whole, our results give insight into the activation dialog between T cells and APC during cognate interactions. It is well established that T cell activation is a slow process resulting from sustained TCR engagement and triggering (Goldsmith and Weiss, 1988; Huppa et al., 2003; Valitutti et al., 1995a). Here, we show that in addition to this, T cells quickly translate intensities of stimulation derived from different directions in discrete foci of signal transduction and consequently remodel synapses and adjust polarization.

The combination of sustained signaling with preferential and rapid polarization provides a mechanism for the high sensitivity and selectivity of T cell responses.

Experimental Procedures

T Cells and APCs
Three DRB1*0101-restricted T cell clones (6396p5.1.2, 6396p5.1.5, and SDM 3.5) specific for the measles virus fusion protein peptide F254-268 and a DRBI*1104-restricted T cell clone (KS140) specific for the tetanus toxin peptide TT830-843 were used. 6396p5.1.2, 6396p5.1.5, and KS140 are Th1 clones, and SDM 3.5 is a Th2 clone. DR matched Epstein-Barr virus (EBV)-transformed B cells were used as APC. T cell clones and EBV-B cell lines were generated and maintained as described (Valitutti et al., 1995a).

T cell hybridoma (T8.1) expressing a human-mouse chimeric TCR specific for the tetanus toxin TT830-843 restricted to HLA-DRB1*1102 and the PH domain of AKT fused with GFP was used to visualize PI3K activation at the IS. Mouse fibroblasts (L625.7) expressing DR-matched MHC class II molecules were used as APCs (Harriague and Bismuth, 2002).

Intracellular Staining
EBV-B cells were pulsed for 2 hr at 37°C with either low (50 nM) or high (either 10 or 50 μM) concentration of the indicated peptide as previously described (Leupin et al., 2000). During the last 10 min of pulsing, cells were loaded at 37°C with either 0.5 μM CMTMR-Orange (Molecular Probes, Leiden, The Netherlands) or 0.5 μM BODIPY 630 (Molecular Probes).

In a parallel set of experiments, EBV-B cells were all pulsed with 10 μM peptide; one half of the cells were loaded with CMTMR-Orange, the other half were loaded with BODIPY 630. Cells loaded with the two different probes were treated with either anti-CD58 mAbs (BD Pharmingen, San Diego, CA) or anti-MHC class I mAbs (W6/32, ATCC) as described (Zaru et al., 2002).

T cells were conjugated with EBV-B cells as described (Leupin et al., 2000). In preparative experiments, we tested different T cell/APC ratios in order to define the ratio allowing relatively frequent detection of three cells conjugates (one T cell with two different APC). Best results were obtained conjugating either 10^5 or 2×10^5 EBV-B cells of each color with 2×10^5 T cells. After conjugation, cells were fixed and permeabilized with 0.1% Triton X-100 as described (Zaru et al., 2002) and stained with anti-phosphotyrosine mAbs (Santa Cruz Biotechnology, Santa Cruz, CA), anti-CD2 (BD Pharmingen), or anti-tubulin mAbs (Sigma, Saint-Louis, MO) followed by FITC-labeled goat anti-mouse Abs (Southern Biotechnology Associates, Birmingham, AL) as described (Zaru et al., 2002). For staining with anti-IFNγ and anti-IL4 (ImmunoKontact, Oxon, UK), cells were permeabilized with 0.1% saponin as described (Zaru et al., 2002).

The samples were mounted and examined using a Carl Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena, Germany) as described (Faroudi et al., 2003b).

Image Quantification
To evaluate polarization of T cell secretory machinery toward APC, T cell/APC conjugates formed by three cells (triplicates) were scored visually. To score IS formation at the two different cell-cell contact sites, unprocessed images showing three-cell conjugates were analyzed using the linescan function of the MetaMorph software (Universal Imaging Corporation). Briefly, two reference lines are drawn at the center of each contact site. The software calculates the mean green fluorescence intensity all along the reference lines for 25 pixels of width (12 and 12 laterally to each reference lines) and plots the measurements. The integral of the curves obtained are ratioed to define the polarization index.

Statistical Analysis
The statistics of the measurement of the fluorescence intensity of all the conjugates scored (see number of conjugates scored in Table 1) are depicted by means of boxplots in Figure 4.

Boxplots are a standard way of giving a graphical summary of a distribution. The box in the middle indicates the quartiles of the distribution, which means that the middle line of each box represents the median of the distribution, and the upper and lower boundaries of the box depict the 25% and 75% quartile (in other words, the central 50% of the data are contained within the box). The lines show the largest/smallest observation falling within in a distance of 1.5 times the box size; if additional single points are depicted, they are extreme values falling outside this range.

The Wilcoxon test (same as Mann-Whitney test) as implemented in the R software package is used for statistical testing. Since the data were recorded in pairs—each T cell has two contact sites with differing APC—a paired test can be used, i.e., we can test whether the differences in labeling intensity between each pair of contact sites show a significant tendency. Florescence intensity measurement was performed on unprocessed images.

Dynamics of TCR, Golgi Apparatus, and PH-Akt/GFP in Living Cells
Anti-CD3ε (TR66, IgG1 [Lanzavecchia and Scheidegger, 1987]) were digested by using the IgG1 Fab and F(ab)$'_2$ kit (Pierce Biotechnology, Rockford, IL). Fab fragments (5 mg/ml in 0.1 M sodium carbonate [pH 9.3]) were labeled with Cy5 monoreactive dye pack (Amersham Bioscience, Piscataway, NJ). The labeled Fab fragments were separated from nonconjugated dye by gel chromatography by the use of Nap-10 Columns (Amersham Bioscience). This step was followed by an overnight dialysis in 1× PBS by using a Lyser Dialysis cassettes (Pierce Biotechnology). T lymphocytes were labeled with Cy5 TR66 Fab at 20 μg/ml in RPMI at 4°C for 30 min and conjugated with APC pulsed with either 100 nM or 100 μM of the indicated peptide for 2 hr at 37°C. Ten minutes before the end of pulsing, APC were labeled with either CMTMR-Orange or CFDA SE (often called CFSE, Molecular Probes).

In control experiments, T cells stained with Cy5 TR66 Fab or untreated T cells exhibited similar calcium mobilization responses when conjugated with peptide-pulsed APC as detected by FACS analysis in Indo-1 loaded T cells (data not shown).

APC pulsed with the low peptide concentration were seeded into microchambers (Lab-tek Chamber coverglass, Nalge Nunc International, Naperville, IL) previously coated with poly-D-lysine (Sigma). Measurements were started after the addition of the T cells into the chamber followed by the addition of APC pulsed with high peptide concentration. Fluorescence measurements were done on a Zeiss LSM-510 confocal microscope at 37°C, 5% CO2. Image sequences of the time-lapse recording were processed using MetaMorph software.

In parallel experiments, T cells were loaded with 1 µM Fluo-4 AM for 45 min at 37°C before labeling with Cy5 TR66 Fab. The green fluorescence emission of Fluo-4 increases with the increase of $[Ca^{2+}]_i$ (Faroudi et al., 2003a).

Additional experiments were performed labeling T cell Golgi apparatus by pretreating T cells for 30 min at 37°C with 5 µM BODIPY FL C5-Ceramide (Molecular Probes) followed by 30 min at room temperature. Image acquisition and processing were performed as described above.

Additional experiments were performed labeling T cell mitochondria by pretreating T cells for 30 min at 37°C with 20 nM GreenMitoTracker-AM (Molecular Probes). Image acquisition and processing were performed as described above.

PI3-kinase activation was measured using T8.1 T cells and L625.7 cells as APC. L625.7 cells were pulsed overnight with 100 nM or 100 µM peptide. T8.1 cells and L625.7 were conjugated and image acquisition and processing were performed as above.

Supplemental Data
Ten movies are available as Supplemental Data at http://www.immunity.com/cgi/content/full/22/2/185/DC1/.

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