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Local Cooperation Dominates Over Competition Between CD4⁺ T Cells of Different Antigen/MHC Specificity

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Interactions between CD4⁺ T cells in vivo are controlled by a balance between cooperation and competition. In this study the interaction between two populations of CD4⁺ T cells of different MHC/peptide specificity was probed at different precursor frequencies, delivering one or both Ags to APC using particle-mediated DNA delivery. Expansion of clonal populations of Ag (OVA and pigeon cytochrome c-specific) CD4⁺ T cells was limited at higher precursor frequencies, presumably reflecting intraclonal competition. In contrast, a strong enhancement of the number of cells expressing IFN-γ, IL-4, and IL-2 was observed in populations of cells at low precursor frequency in the presence of a high frequency of activated cells of a different Ag specificity. The helper effect was most potent when both Ags were delivered to the same dendritic cell (i.e., linked). This reflects the requirement of epitope or paracrine help for optimal activation of T cell clones at low frequency. A measure of help was also delivered in an endocrine manner (unlinked), especially for Th1 responses, suggesting that there is also limited diffusion of cytokines between dendritic cell clusters. The dominant effects of cooperation over competition between CD4⁺ T cells responding to different Ags may have important implications in terms of the efficacy of multivalent vaccines.

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Upon infection, many clones of CD4⁺ and CD8⁺ T cells are activated by Ags from the pathogen that constitute their common target. These two subsets of T cells are known to collaborate, since CD8⁺ T cell-mediated CTL responses are often enhanced by a contribution from CD4⁺ Th cells. Dendritic cells (DCs)³ have two major roles in the activation of these T cells. Firstly, DCs form Ag-specific synapses with individual T cell clones, leading to T cell activation. T cells then transiently up-regulate CD40 ligand (1), which, upon ligation with CD40 on the DC, enhances the expression of costimulatory molecules (2, 3), further boosting T cell activation. Secondly, by presenting a panel of different Ags derived from the same pathogen, DCs can recruit diverse T cell clones that share a common goal, allowing them to interact within the microenvironment of the DC cluster. Interactions between these T cells may be by paracrine (cytokine cross-talk between T cells) or epiprise signals that are transmitted via DCs. CD40/CD40 ligand ligation plays a central role in the epiprise pathway for CD4-CD8 T cell cooperation, but other signals may be delivered via DC (3–6). Less is known about the cooperation between CD4⁺ T cells, which involves a combination of CD40, OX40, and other unidentified mechanisms (7). Cytokines produced by Th1, Th2, and CTL also enhance and/or modulate the responses of other CD4⁺ clones in vitro (8, 9).

Although DCs promote intra- and interclonal cooperation in the ways outlined above, intraclonal competition, which limits the extent of T cells activation and division, can also occur at a high frequency of CD4⁺ (10, 11) or CD8⁺ T cells (12). CD8⁺ clones can compete for access to MHC/peptide complexes on DC (12), and those of the highest affinity are preferentially selected and expanded (13). Competition between CD8⁺ T cells of different Ag specificity is more limited (13) or insignificant (14). Competition between CD4⁺ T cells of different specificity has yet to be characterized.

In this study we investigated competition and cooperation in vivo between two transgenic (Tg) CD4⁺ T cell populations with different Ag/MHC specificity (OVA expressed on I-A⁺, and pigeon cytochrome c (PCC) expressed on I-E⁺). We varied the relative clonal precursor frequency of the two populations and examined their responses using particle-mediated DNA delivery (PMDD). The OVA-specific CD4⁺ T cells were activated either alone in the presence of nonactivated PCC-specific CD4⁺ T cells or concomitantly with PCC-specific T cells. In the latter case, both Ags were expressed by either the same DC or different DCs.

Materials and Methods

Plasmids

The pVAC1 (empty vector) and pVAC1.OVA (expressing full-length OVA) were supplied by Drs. M. Young and I. Catchpole (GlaxoSmithKline, Stevenage, U.K.). The full-length PCC gene preceded by an IgG secretion signal was synthesized by PCR using overlapping oligonucleotides and was cloned into pVAC1. The pVAC1 vectors contain a CMV promoter, an internal ribosome entry site, and an SV40 poly(A) sequence. All plasmids were prepared for immunization using Endofree Maxiprep kits (Qiagen, Valencia, CA), resulting in <0.1 endotoxin units/μg of DNA.

Cartridge preparation for PMDD

Cartridges were prepared as previously described (15) from five different preparations of gold particles coated with various combinations of plasmids as illustrated in Fig. 2A. All gold particles were coated with a total amount of 2 μg of DNA/mg of gold. An “all beads linked” preparation was obtained by mixing pVAC1.PCC and pVAC1.OVA in a ratio of 1/1 for the DNA number of copies (i.e., 43.5 and 56.5% in amount, respectively) and coprecipitating on gold particles. A “half the beads linked” preparation was achieved by mixing in a 1/1 volume ratio an all beads linked preparation with one made exclusively with pVAC1. For unlinked preparations, first
pVAC1.PCC and pVAC1 (43.5 and 56.5%, respectively) were mixed and cocoated as described above. In parallel, pVAC1.OVA and pVAC1 (56.5 and 43.5%, respectively) were likewise cocoated. After washes for non-bound DNA removal, the two preparations were mixed in a 1/1 volume ratio. All beads OVA and half beads OVA preparations were made in exactly the same manner as the all beads and half beads linked preparations, except that pVAC1.PCC was replaced by pVAC1.

Micromanipulation of gold particles and PCR
A small aliquot of gold/DNA suspension was spread onto a glass slide, and after ethanol evaporation, single beads were micromanipulated with a fine micropipette into PCR tubes. Short fragments contained in the PCC and OVA inserts were amplified by PCR using two sets of specific primers (Invitrogen, San Diego, CA), dNTPs (Roche, Indianapolis, IN), and Advantage cDNA polymerase (Clontech, Palo Alto, CA), in 30–35 cycles comprising 30-s denaturation at 94°C and 3-min annealing/extension at 68°C. PCR products were separated in 3% agarose gels.

Mice
All transfers were between fully histocompatible strains of B10 back-ground. The OVA-specific TCR-Tg mice (OVA-B10D2) were obtained by backcrossing D0.11.10 onto B10D2 mice for nine generations by Dr. T. Kamradt (Deutsches RheumaForschungs Zentrum, Berlin, Germany) and an additional three to five generations at University College London. 5CC7 rag2+/− mice (B10A background) carrying the PCC-specific TCR transgene were obtained from Dr. W. Paul (National Institutes of Health, Bethesda, MD). Since the two TCRs recognize Ag on distinct MHC molecules (I-A^d and I-E^k), F1 hybrids (bred at University College London) were used as both donors and recipients. Donors were 8–12 wk F1 (OVA-B10D2 × B10A) or (5CC7 rag2+/− × B10D2). Offspring were typed for transgene expression by flow cytometry. Recipients were 6- to 10-wk-old B10D2 × B10A. All Tg strains were bred in University College London facilities. B10A, B10D2 and B10D2 × B10A F1 mice were purchased from Harlan U.K. All experiments were conducted in accordance with U.K. animal experiment legislation.

FACS analysis and adoptive transfer of splenocytes
Splenocytes from donor mice were analyzed for TCR expression by flow cytometry using either anti-β2-PE (0.15 μg; BD PharmMingen, San Diego, CA) with anti-va11-biotin (0.4 μg; BD PharmMingen)/streptavidin-Quantum Red (3 μl; Sigma-Aldrich, St. Louis, MO) for PCC-specific Tg T cells or KJ1.26-PE (TCR clonotype, 0.15 μg; Caltag Laboratories, Burlingame, CA) with anti-CD4-TriColor (0.15 μg; Caltag Laboratories) for OVA-specific Tg T cells. The frequency of β3^+ va11+ or KJ1.26^+ CD4^+ was used to calculate the number of donor splenocytes required to transfer the desired number of Tg T cells. These cells were injected in 0.2 ml into the lateral tail vein of recipients.

Immunizations
Recipient mice (three per group) were immunized by PMDD (typically 1–2 μg of Ag in two shots on the shaved abdomen) at 500 psi of helium pressure using the Helios gene gun (Bio-Rad, Hercules, CA) provided by Dr. J. Brookes (University College London).

FACS analysis of lymph node cells
On day 5 postimmunization, inguinal and periaortic lymph nodes were collected, pooled, and made into a single-cell suspension. PCC-specific and OVA-specific T cells were analyzed for transgene expression and for CD69 and CD62L by flow cytometry as described above.

Cytokine-producing cells (ELISPOT)
Multiscreen 96-well filtration plates (Millipore, Bedford, MA) were coated with capture Abs for IFN-γ, IL-2, or IL-4 (750 ng/well; BD PharmMingen or eBioscience, San Diego, CA) overnight. Plates were blocked with complete RPMI medium (10% FCS). Lymph node cells were collected from three mice for each experimental group and pooled. Lymph node cells (1–8 × 10^6 cells/well) were cultured in medium alone or medium with either 1 μM PCC or OVA for 24 h. After 24-h incubation at 37°C in 5% CO_2, plates were incubated for 2 h with biotinylated detection Abs (50 ng/well; BD PharmMingen), for 2 h with streptavidin-conjugated alkaline phosphophatase (Caltag Laboratories), and ~10 min with substrate (alkaline phosphophatase conjugate substrate kit; Bio-Rad). At least three washes with PBS were performed between each step. Spots were counted using plate reader and Eli 2.9 software from Autoimmun Diagnostika (Starzberg, Germany). The number of spots in duplicate cultures was averaged (replicate cultures usually gave values within 10% of each other) and normalized per million cells. Background ELISPOT values from cultures in the absence of peptide were always low and were subtracted from the corresponding cultures in the presence of peptide. Each experiment was repeated a minimum of three times on different groups of mice, and the mean from all the experiments is shown in the figures. Significance between experimental groups was calculated using Student’s t test or ANOVA as appropriate and as detailed in the figures.

Results
Characterization of the DNA on beads prepared for linked and unlinked conditions
To investigate the interaction between OVA-specific and PCC-specific CD4^+ T cells, five sets of preparations were made for PMDD (Fig. 1A). Preparations 1 and 2 carried only OVA-coding DNA (and empty vector) to measure the response of OVA-specific T cells at different precursor frequencies. In preparations 4 and 5, OVA- and PCC-coding DNA were cocoated on the same beads so as to favor copresentation of both Ags to the two populations of T cells by the same DC (linked). In preparation 3 the DNA for OVA and PCC were coated on different beads, and the beads were mixed. This arrangement should favor the segregation of the two Ags on different DC (unlinked). The half beads OVA, unlinked, and half beads linked preparations all contained approximately the same number of beads coated with OVA. The all beads OVA, unlinked, and all beads linked preparations all carried the same total number of Ag-coated beads. The dose of DNA per bead for each Ag was equalized in all five preparations, as described in Materials and Methods.
Single beads from the half beads linked and unlinked preparations were isolated, and the presence of the OVA and/or the PCC insert was detected by PCR. Of 23 randomly isolated beads from the former preparation (Fig. 1B), 11 tested positive for both OVA and PCC, while the remaining 12 were negative for both. This confirms the expected ratio of 1/1. Of 28 beads from the unlinked preparation (Fig. 1C), 11 tested positive for OVA only, and 12 tested positive for PCC only. Five beads, however, were positive for both OVA and PCC. It is unlikely that plasmid transfers between beads after coating, because if this were the case, the 1/1 ratio in Fig. 1B would be altered (double-negative beads would become positive). The results probably reflect occasions when aggregates of two or more small beads have been picked up as single beads. Such clumps are likely to disaggregate during bombardment. In view of the small number of DC transfected in vivo (16), it is extremely unlikely that one DC in the skin would be transfected with two separate beads.

Response of OVA-specific TCR-Tg cells at different precursor frequencies in the presence or the absence of concomitant PCC activation

In a first set of experiments we examined whether the response of OVA-specific TCR-Tg cells, present at a range of different precursor frequencies, was influenced by concomitant activation of PCC-specific cells (protocol illustrated in Fig. 2). Recipient B10D2xB10A F1 recipients received 1 x 10^6 naive PCC-specific CD4^+ T cells (vβ3^+ vα11^+ ) and a variable number of naive OVA-specific T cells (KJ1^+ CD4^+ ): 1 x 10^6, 1 x 10^5, 1 x 10^4, or none. The next day recipient mice were immunized with either pVAC1.OVA alone or pVAC1.PCC and pVAC1.OVA cocoted on the same gold particles (groups 2 and 5; Fig. 1A). Five days later draining lymph nodes were collected for analysis of both PCC-specific (Fig. 3, A–D) and OVA-specific (Fig. 3, E–H) responses by flow cytometry and ELISPOT.

In the absence of PCC DNA, PCC-specific T cells remained at the same baseline frequency (~0.5%; Fig. 3A) as in nonimmunized mice (0.5%; not shown) regardless of the presence or the absence of OVA-specific TCR-Tg cells. The PCC-specific cells were CD69^{low} and CD62L^{high} (not shown) and failed to express IFN-γ (Fig. 3B) or IL-4 (Fig. 3C). A few of them produced IL-2 upon subsequent peptide stimulation in the ex vivo assay (Fig. 3D). In contrast, when mice were immunized with PCC as well as OVA, a strong PCC-specific response (net clonal expansion and cytokine production) was evident (Fig. 3, left panels, circles). In addition, CD69 expression was strongly up-regulated (data not shown). Neither the proportion of PCC TCR-Tg cells, nor their cytokine responses were significantly affected by the presence of different numbers of transferred OVA-specific T cells, whether naive (not shown) or activated (as shown by the flatness of the lines in all left panels of Fig. 3).

The net clonal expansion and cytokine responses of OVA-specific cells are shown in Fig. 3, right, E–H. We will consider first the OVA-specific response in the absence of concomitant PCC activation (Fig. 3, E–H, squares). The OVA-specific response increases with the number of TCR-Tg cells transferred, with no detectable TCR expression or cytokine response in the absence of OVA TCR-Tg transferred cells, presumably because the number of OVA-specific precursors in the recipients is too small to give a detectable response following a primary 5-day immunization. However, neither clonal frequency (Fig. 3E) nor cytokine response (Fig. 3, F–H) rose in proportion to the number of cells transferred. The inverse relationship between the extent of the immune response and precursor frequency is clearly demonstrated in Fig. 3, I–L (squares), where the OVA-specific clonal frequency and cytokine production after transfer of 10^4, 10^5, and 10^6 OVA-specific TCR-Tg T cells have been divided by 1, 10, and 100, respectively, to normalize for the number of cells transferred and then plotted against the initial number of cells transferred (relative output in relation to cell input).

The analysis of the OVA response at different precursor frequencies shown in Fig. 3 suggests that intraclonal competition is limiting the OVA response as frequency increases. In contrast, there is no evidence that the presence of a concomitant PCC-specific response in the presence of a high frequency of PCC TCR-Tg transferred cells competes with the OVA response (compare circles and squares in Fig. 3, E–H). Clonal expansion is, in fact, the same in the presence or the absence of a PCC-specific response (Fig. 3E). In contrast, the cytokine secretion of all three cytokines measured is strongly enhanced upon concomitant activation of PCC-specific T cells (Fig. 3, F–H). The enhancement was most dramatic at the lowest numbers of OVA-specific T cells. Even in the absence of any OVA-specific TCR-Tg transfer, where the OVA-specific response relies on the endogenous population of non-Tg T cells in the recipients that recognize the OVA_{323–339} peptide, concomitant activation with PCC-specific responses resulted in activation of detectable OVA-specific IFN-γ, IL-4, and IL-2 responses (Fig. 3, F–H). The presence of PCC-specific activated T cells did not, however, override the decrease in relative OVA-specific responses as OVA-specific precursor frequency increased (Fig. 3, I–L, circles), which was presumed to reflect intraclonal competition.

Response of PCC-specific TCR-Tg cells at different precursor frequencies and their influence on the endogenous OVA-specific response

To determine whether cooperation can be observed in the presence of lower (and perhaps more physiological) numbers of helper cells, variable numbers of PCC-specific naive TCR-Tg T cells (3 x 10^4, 3 x 10^5, and 3 x 10^6) were injected into B10D2 x B10A recipient mice (experimental protocol outlined in Fig. 4A). On the following day these mice were immunized by particle-mediated delivery of linked pVAC1.PCC and pVAC1.OVA (cocoted on the same gold particles). On day 5 postimmunization, the in vivo expansion of the transfected PCC-specific CD4^+ T cells was assessed by flow cytometry (Fig. 4B), and the cytokine response was analyzed on...
FIGURE 3. Enhancement of OVA-specific cytokine responses by concomitant activation with PCC-specific T cells. The adoptive transfer protocol is described fully in Fig. 2. Graphs show the mean ± SEM from three independent experiments, except for nonimmunized mice, where the mean ± SD from five mice in two separate experiments was used. The OVA cytokine responses in the presence and the absence of concomitant PCC activation (i.e., squares and circles in F–H) were compared using Student’s t test (unpaired), and all points at which concomitant activation resulted in significant enhancement (p < 0.05) are marked by an asterisk. Overall comparison of the response in the presence and the absence of PCC, using two-way ANOVA was highly significant for all cytokines (p < 0.001). I–L, Clonal expansion, IFN-γ, IL-4, and IL-2 responses, respectively, to OVA in the presence (○) or absence (□) of PCC activation (data from E–H) normalized to the number of OVA-specific TCR-Tg cells injected. The data was calculated by taking the OVA-specific clonal frequency and cytokine production after transfer of 10^4, 10^5, and 10^6 OVA-Tg cells (E–H), dividing by 1, 10, and 100, respectively. This figure was then plotted against initial number of cells transferred (relative output in relation to cell input). The number of endogenous precursors was too small to measure, so this point was not included.

To explore whether the cooperative interaction between the two T cell populations shown in Figs. 3 and 4 required linkage, the experimental protocol shown in Fig. 2 was repeated, but mice were immunized with OVA alone, OVA/PCC unlinked, or OVA/PCC linked (groups 1, 3, and 5; Fig. 1A). The number of OVA-coated beads was half that used previously (Fig. 3) to allow better comparison with the unlinked preparation. Linked delivery of PCC and OVA strongly enhanced the OVA-specific IFN-γ, IL-4, and IL-2 responses (Fig. 5, right panels), as shown in Fig. 3. Delivery of the two Ags on separate beads (unlinked) showed an intermediate degree of enhancement. At high precursor frequencies, linked delivery of the two Ags was not significantly better than unlinked (p > 0.05). In contrast, at the lower precursor frequency of OVA-specific T cells, linked help was significantly better than unlinked (p < 0.05) for all cytokines tested.

Linked delivery of PCC and OVA strongly enhanced the OVA-specific IFN-γ, IL-4, and IL-2 responses (Fig. 5, right panels), as shown in Fig. 3. Delivery of the two Ags on separate beads (unlinked) showed an intermediate degree of enhancement. At high precursor frequencies, linked delivery of the two Ags was not significantly better than unlinked (p > 0.05). In contrast, at the lower precursor frequency of OVA-specific T cells, linked help was significantly better than unlinked (p < 0.05) for all cytokines tested.

T cell cooperation requires linkage at low cell numbers

To determine whether cooperation between OVA and PCC can be reciprocal and whether cooperation can be detected at the precursor frequencies of naive T cells (i.e., in the complete absence of Tg T cells), the experiment shown in Fig. 6 was conducted. B10D2×B10A mice received 3 × 10^6 PCC-specific or 3 × 10^6 OVA-specific TCR-Tg cells, or received no Tg cells (left panel). Mice were immunized the next day with both PCC and OVA beads delivered either unlinked (separate beads) or linked (same beads). Mice receiving PCC-specific TCR-Tg cells mounted a...
strong PCC-specific response to both linked and unlinked deliveries. In addition, mice receiving linked Ags, but not unlinked, mounted a significant endogenous OVA-specific response, confirming the cooperative effect of previous determinations. In the reverse experiment mice receiving OVA-specific TCR-Tg cells mounted a strong OVA-specific response to both linked and unlinked deliveries. Mice receiving linked, but not unlinked, Ags also mounted a significant endogenous PCC-specific response, confirming that the OVA-Tg cells can provide help for the low frequency of endogenous PCC-specific cells present in the recipients.

Discussion

This study explored the interactions between CD4 T cells of different Ag specificities, at different precursor frequencies. Titration of precursor frequencies over a 100-fold range for both OVA- and PCC-specific T cells showed strong evidence of regulation, presumably mediated by intraclonal competition, as demonstrated previously for both CD4 and CD8 T cells (10, 12) or other mechanisms (11). Clonal expansion, for example, was ~20 times
Cytokine responses (IFN-γ, IL-4, and IL-2) from a low frequency OVA-specific population are greatly enhanced, for example, in the presence of activated PCC-specific T cells. Neither clonal expansion nor CD69 expression was affected. The signal delivered by the Th cells, therefore, appeared to drive an increased differentiation of target cells toward an effector or memory/effector phenotype. Although, most experiments presented in this study were conducted using populations of transferred TCR Tg cells, Fig. 6 demonstrates that a similar phenomenon can also be observed with endogenous T cells, at least for IL-2 production. Similar cooperative effects have been observed using other dual Ag combinations delivered by PMDD in both primary and prime-boost models (our manuscript in preparation). The delivery of DNA on either the same or different beads was used to probe the nature of the signals mediating T cell cooperation. Although cotransfection of one DC with two beads or ectopic expression of Ag in skin, followed by reprocessing and presentation, could theoretically abrogate linkage, at low precursor frequencies an absolute requirement for linkage was observed. As T cells numbers increased, linkage became less important. The reduced requirement for linkage may reflect the increasing importance of endocrine cytokine activity, diffusing between DC clusters within the lymph nodes. At low T cell numbers, paracrine IFN-γ or IL-4 production within autonomous DC clusters may be the dominant force driving independent Th1 or Th2 differentiation (19, 20).

The mechanisms that impose intraclonal competition, but at the same time allow interclonal cooperation, remain to be worked out. It is tempting to speculate that the answer may lie in the properties of the immunological synapse (21) and the recently described directional export of MHC/peptide complexes (22). Thus, I-Ek/PCC complexes will be concentrated increasingly into one area of the DC surface, where PCC-specific T cells will compete for interaction, while I-Aβ/OVA will be progressively excluded from this area, but become available to T cells specific for OVA on a different area of cell membrane. The OVA-specific T cells will, however, still benefit from epiricnic activation (e.g., via increased CD40 ligation) or paracrine activation under the influence of T cell-derived cytokines. Indeed, it is likely that multiple molecular interactions will drive T-T cooperation in the context of a single DC cluster.

In conclusion, two opposing forces shape the emergence of the T cell repertoire following immunization. Competition, acting at the level of Ag/MHC, limits the response to each epitope specifically. Cooperation, acting between all T cells activated within an individual DC cluster, whether specific for the same epitope or not, enhances T cell differentiation and can permit the detection of responses even when the T cells are present at very low precursor frequencies (as in a naive animal). Crucially, our study shows that cooperation at low T cell numbers is predominantly confined to a single DC cluster, thus permitting different DC/T cell clusters to act autonomously. Cytokine bias within one cluster, for example, will not necessarily influence cytokine bias in another (19, 23). Although one cannot extrapolate directly from mouse to man, these results may contribute to the observed enhanced efficacy of multivalent vs monovalent vaccines (24–26). Reassuringly, multivalency may particularly benefit individuals with reduced responsiveness.

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