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A SMALL NUMBER OF ANTI-CD3 MOLECULES ON DENDRITIC CELLS STIMULATE DNA SYNTHESIS IN MOUSE T LYMPHOCYTES

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T lymphocytes proliferate if challenged with a mAb to the CD3 portion of the TCR complex and with an additional stimulus that is usually provided by phorbol esters or by accessory cells (1–3). In the latter instance, proliferation requires an intact anti-CD3 antibody (4) as well as Fc receptors (FcR) on the accessory cells (5–7). The presentation of anti-CD3 mAb by FcR on accessory cells is thought to mimic presentation of antigens on MHC molecules to the clonally specific, α/β heterodimer of the TCR complex. There is little information on the number of anti-CD3 mAb on the accessory cell that is necessary to induce growth. For the presentation of specific antigens, it likewise is not clear how many MHC molecules can be associated with any one type of peptide fragment to which individual T cell clones are specifically precommitted (8–10).

We provide some information on this matter in the studies we describe here on the anti-CD3 response with epidermal Langerhans cells (LC)[1] as the accessory cell. Even though LC express FcR (11–14), we could not predict whether LC would act as accessory cells since these receptors become undetectable after a day of culture (14, 15). Concomitant with this decrease in FcR, there is a 10–30-fold increase in sensitizing function for T cell proliferative responses to a variety of stimuli (15–17). We wondered if both freshly isolated and cultured LC would be weak accessory cells for anti-CD3 responses. Fresh FcR^+ LC would lack needed sensitizing functions, and cultured FcR^- LC would lack the FcR needed to present anti-CD3 to the T cell.

Surprisingly, cultured LC proved to be unusually active. This finding was analyzed quantitatively by determining the number of lymphocytes that were synthesizing DNA while in contact with individual LC, and by measuring the number of requisite FcR molecules on the LC surface. The data indicate that T cell growth was being induced by a very small number of anti-CD3 on the LC surface, ~200–300 molecules.

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[1] *Abbreviations used in this paper:* LC, Langerhans cells.

Materials and Methods

Mice. H-2d [BALB/c × DBA/2]F_1 [CxD2], H-2b [C57BL/6] [B6], H-2b × d [B6 × DBA/2]F_1 [B6xD2] mice were obtained from the Trudeau Institute (Saranac Lake, NY) and H-2k CBA/J from The Jackson Laboratories (Bar Harbor, ME). Mice of all strains and of both sexes, 6–12 wk old, gave similar results.

Epidermal LC. Single cell suspensions were prepared by trypsinization of mouse ear epidermal sheets (15, 16). The LC were enriched either immediately (fresh LC) or after 2–3 d in culture (cultured LC). 15–20 × 10^6 epidermal cells were cultured per 100-mm tissue culture dish (No. 3003, Falcon Labware, Oxnard, CA) in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 5% FCS (Hazelton Systems, Inc., Aberdeen, MD), 5 × 10^{-5} M 2-ME (Sigma Chemical Co., St. Louis, MO) and 20 μg/ml gentamicin (Gibco Laboratories). Fresh and cultured LC were enriched from the suspension as described (16). Briefly, epidermal cells were treated with mAb anti-Thy-1 plus complement to kill most keratinocytes, followed by 0.1% trypsin–160 μg/ml DNase to remove dead cells. After coding with rat mAb to the mouse common leukocyte antigen (CD45; clone M1/9, TIB122 from the American Type Culture Collection [ATCC], Rockville, MD), the cells were panned on petri dishes coated with goat-anti-rat Ig. The nonattached cells were washed off, and the adherent LC were eluted by pipetting in the presence of rat Ig. The populations thus obtained consisted of 65–90% fresh LC and 90–98% cultured LC as determined by immunofluorescence with FITC-conjugated mAb anti-I-$A^{b,d}$ (B21-2, TIB229 from ATCC) and by phase-contrast microscopy in the case of cultured LC. Previously it was shown that the panning method did not significantly alter the accessory function of LC during MLR stimulation (16). To show that coating with mAb did not affect function in anti-CD3 responses, the final panning step was omitted in some experiments; the resulting fresh and cultured LC were only 6–12% and 30–50% LC but the accessory function per LC was similar (data not shown). In addition, cultured LC were also enriched by flotation on bovine plasma albumin columns (15). This was the most convenient method, providing populations that were 30–60% LC and that functioned comparably to panned, highly enriched LC.

Other Accessory Cells. Peritoneal washout cells were used as the source of macrophages (~30% macrophages) in most experiments. Dendritic cells were prepared from the low density, adherent cell fraction of collagenase-digested spleens and thymi. After overnight culture, the cells that had eluted from the culture dish were depleted of macrophages and B cells by rosetting with EA to provide an EA^- dendritic cell fraction (18, 19). Spleen and thymus macrophages were the cells that remained adherent after overnight culture. They were released from the dishes by treatment with 5 mM EDTA in PBS for 30 min at 37°C. B cells included the A20 B lymphoma cell line (TIB208, ATCC), as well as LPS-induced B lymphoblasts that were prepared from spleen.

Responder T Cells. Spleen and/or mesenteric lymph nodes were passed over nylon wool columns and the nonadherent cells treated with anti-Ia mAb (B21-2, TIB229 and M5/114, TIB120 for H-2 d,b, and bxd strains; 10.216, TIB93 for H-2k strains) plus complement. Where indicated, $CD4^+$ and $CD8^+$ T cell subsets were enriched by adding mAb anti-Lyt-2 (CD8, 3.155, TIB211) or GK 1.5 (CD4, TIB207) during the complement treatment above. Purity of the subsets was verified to be >95% by flow cytometry with FITC-anti-CD8 (clone 53–6.72) and PE-anti-CD4 (clone GK 1.5) purchased from Becton Dickinson & Co. (Mountain View, CA). Except when we used lymph node as the source of T cells, the populations were enriched by an additional step involving sedimentation in discontinuous Percoll gradients (45–54–63% Percoll) in which dead cells and remaining accessory cells floated.

Proliferation Assays. Graded doses of accessory cells (irradiated with 1,000 rad ^{137}Cs; A20 cells received 9,000 rad) were added to 2–3 × 10^5 syngeneic T cells in flat-bottomed, 96-well plates (No. 25860; Corning Glass Works, Corning, NY). mAb 2C11, hamster anti-murine CD3 (20), was a generous gift of Dr. J. Bluestone (University of Chicago, Chicago, IL) and was added either as ascites (1:2,000 final dilution) or as culture supernatant (10% final concentration). Cells were cultured in RPMI 1640 with 1–5% FCS, gentamicin, and 2-ME. [^{3}H]Thymidine incorporation was measured at 28–44 h (4 μCi/ml; 6 Ci/mmol). The response to Con A (1 μg/ml; Miles Laboratories, Naperville, IL) was measured identically. The allogeneic MLR was run in medium containing 5% FCS and was routinely pulsed 72–90 h.

To check for blocking activity, mAbs (see Results) were used as culture supernatants (5–25% vol/vol) except for 2.4G2 anti-FcR mAb, which was also used as a purified Ig and Fab fragment (21).

Single Cell Studies to Monitor T Cell Proliferation. To measure the number of T lymphoblasts that were induced by a given number of added LC, the size of the T cells stimulated as above was evaluated at 24–52 h by forward light scatter in a FACScan instrument (Becton Dickinson & Co., Mountain View, CA). Before analysis, a viable (trypan blue–negative) cell count was taken. Then the cells were exposed to 2 μg/ml ethidium bromide, to label and gate out dead cells, and vigorously resuspended to dissociate clusters of LC and T lymphoblasts. The latter showed at least twice the forward light scattering of small T cells (see Results). To enumerate the number of T cells in the S phase of the cell cycle while in contact with LC, autoradiography and anti-Ia immunolabeling were combined on cytospin samples of the cultures. Two to three cytospins could be prepared from each microtest culture which had been pulsed at 30–36 h with 4 μCi/ml [^{3}H]TdR. Most of the medium was replaced with fresh medium to dilute out unincorporated radiolabel before sedimentation onto glass slides (Shandon Cytospin 2, Southern Instruments Inc., Pittsburgh, PA). The slides were air dried, fixed in acetone (5 min, room temperature), and stained for Ia antigens using biotin–anti-mouse Ia (2 μg/ml each of B21-2 and 14-4-5S, HB32), avidin-biotin-peroxidase complex (Vectastain; Vector Laboratories, Burlingame, CA), and diaminobenzidine/H_2O_2. After dehydration in ethanol the air-dried cytospins were dipped in Nuclear Track Emulsion (Eastman Kodak Co., Rochester, NY) and exposed at 4°C for 36 h. The slides were developed (Kodak D-19), fixed (Kodak Rapid-Fix), stained with hematoxylin, and mounted in Permount. [^{3}H]TdR-labeled cells that were clustered with Ia$^+$ LC were counted on most microscopic fields at ×500. >500 T cells on duplicate or triplicate specimens were examined.

Expression of FcR. For flow cytometry, cell suspensions were double-labeled with mAb, one of which was an anti-Ia to identify the Ia$^+$ LC and the other a rat anti–mouse mAb of the IgG2b isotype (14, 19), including: 2.4G2 anti-FcR; M1/70 anti-C3biR or CD11b; B5-3 anti-Thy-1; GK1.5 anti-CD4; 33D1 anti–dendritic cell; F441.8 anti-LFA-1 or CD11a; and B21-2 anti–I-A. These other mAbs served as positive and negative controls, 33D1 and B5-3 being nonreactive with mouse LC. The cells were incubated in sequence with rat mAb (culture supernatants), FITC–mouse anti-rat Ig (2 μg/ml; Boehringer Mannheim Biochemicals, Indianapolis, IN), biotin mouse anti-I-E (2 μg/ml; clone 14-4-5S), and PE-streptavidin (B-D). The suspensions were fixed in 3.7% formaldehyde and analyzed on a FACScan (B-D). Dead cells and debris were gated out as events with a lower forward light scatter than lymphocytes.

For the detection of intracellular FcR, fresh and cultured LC were cytospun onto glass slides, fixed with acetone (10 min/−20°C), air dried, and incubated with culture supernatants of the 6B7C rat IgG2a anti-FcR mAb (22), with 53-7.3 and 53-6.7 anti-CD5 and CD8 as nonreactive controls. Alternatively, the panel consisted of rat IgG2b hybridomas: 2.4G2 anti-FcR, M1/70 anti-C3biR, and B21-2 anti-I-A with 33D1 anti-DC as nonreactive control. The secondary mAb was FITC–mouse anti-rat (as above).

Cell surface FcR were quantitated with ^{125}I-2.4G2 binding assays done in suspension in 96-well round-bottomed plates (Flow Laboratories, McLean, VA) in a total volume of 100 μl. 2 × 10^5 cells were incubated for 60 min at 4°C on a nutator with the ^{125}I-2.4G2 at 1 μg/ml, 2–5 × 10^6 cpm/μg, in PBS containing 1% BSA and 0.02% sodium azide. After three washes the cells were transferred to plastic Eppendorf tubes for gamma scintillation counting. Specificity was calculated by blocking with a 50-fold excess of cold 2.4G2 added before the radiolabeled mAb for 30 min at 4°C. The binding was saturable as described (21, 23).

Results

Cultured LC Are Potent Accessory Cells in Anti-CD3 Responses. To study accessory cells for murine T cell responses to anti-CD3, it was necessary to extend standard methods for depleting accessory function in T cell preparations. Under our culture conditions, nylon wool–nonadherent, Ia$^-$ spleen cells did not proliferate to 1 μg/ml Con A but did give sizable backgrounds to anti-CD3, up to 20,000 cpm per culture. This

could be reduced to <1,000 cpm by using lymph node rather than spleen cells, or more conveniently, by adding a step to the spleen T cell purification involving sedimentation in dense Percoll (see Materials and Methods). Such T cells responded vigorously in the MLR and to the lectin Con A (Fig. 1) when low doses of cultured LC, or spleen or thymic dendritic cells, were added. Freshly isolated LC were much less active as accessory cells (Fig. 1) as noted previously (15, 16, 24).

When anti-CD3 was the mitogen, cultured LC were again much more potent than fresh LC (Fig. 1). This activity was unexpected since anti-CD3 responses require FcR (4–7), and cultured LC had shown little rosetting of erythrocytes maximally coated with antibody (EA) nor reactivity with the 2.4G2 anti-FcR mAb (14, 15). Yet the LC were active as FcR-rich peritoneal macrophages [Fig. 1].

When cultured LC were compared with other dendritic cells, EA^- thymic dendritic cells were as active, but spleen dendritic cells were weak (Fig. 1). The function of spleen dendritic cells could be due to small numbers (<5%) of contaminating EA^+ macrophages and B cells and was not pursued further. Instead, the potent accessory function of cultured LC and thymic dendritic cells was evaluated.

Surface Molecules Involved in the Anti-CD3 Response. 2C11 anti-CD3 was the only anti-T cell reagent that could stimulate proliferation in the presence of LC. Inactive mAb were: anti-Thy-1, clones HO-13.4 and B5-3; anti-Lyt-1/CD5, clone 53-7.3; anti-CD4, GK1.5; anti-CD8, clones 53-6.7 and 3.155; anti-TCR, clone KJ16; anti-CD45, clone M1/9; and F441.8 anti-LFA-1 (not shown).

The 2.4G2 mAb to the murine, trypsin-resistant, immune complex receptor (FcR II) inhibited the proliferative response to LC plus anti-CD3. The inhibition was >90% irrespective of the form of 2.4G2 used: purified Ig (2–10 μg/ml), Fab fragments (10 μg/ml), or hybridoma culture supernatant (25% vol/vol) (Fig. 2, *top*). In contrast to anti-CD3 responses, 2.4G2 did not block proliferation to the mitogen Con A (Fig. 2, *top*) or in the MLR (not shown). We were unable to block the anti-CD3 response if either the LC or the T cells, or both, were treated with 2.4G2 and washed before coculture (not shown).

The anti-CD3 response was resistant to anti-CD4 and anti-Ia mAb. These mAb did inhibit the MLR induced by LC, as expected for an antigen-specific response (Fig. 2, *middle*). The F441.8 anti-LFA-1 mAb markedly blocked the 2C11 response (Fig. 2, *bottom*). Both CD4 and CD8 T cell subsets were stimulated by LC plus anti-CD3 (Fig. 2, *bottom*). Isotype-matched control mAb did not block, such as M1/70 anti-CD 11b; F4/80 anti-macrophage; B5-3 anti-Thy-1. Nor was blocking observed with high doses of mouse myelomas (LPC-1, IgG2a; J606, IgG3) or human Ig that interact with other FcR (not shown). Therefore proliferative responses to anti-CD3 and cultured LC require FcR II and CD3, but do not seem to require MHC class I/II or CD4/CD8 molecules.

Detection of Small Numbers of FcR on Cultured LC but not T Cells. We used a FACScan, a new sensitive cytofluorograph, and two-color immunolabeling to reinvestigate the expression of FcR on Ia^+ LC with 2.4G2 mAb. This rat IgG2b mAb stained *fresh* LC at 10–20 times the background observed with no primary mAb or isotype-matched nonreactive controls (GK 1.5 anti-CD4; B5-3 anti-Thy-1; RA3-6A anti-B220; 33D1 anti-dendritic cell) (Fig. 3 *A*). After 1–3 d of culture, the LC still stained with 2.4G2, but the staining was very much weaker than on fresh LC and

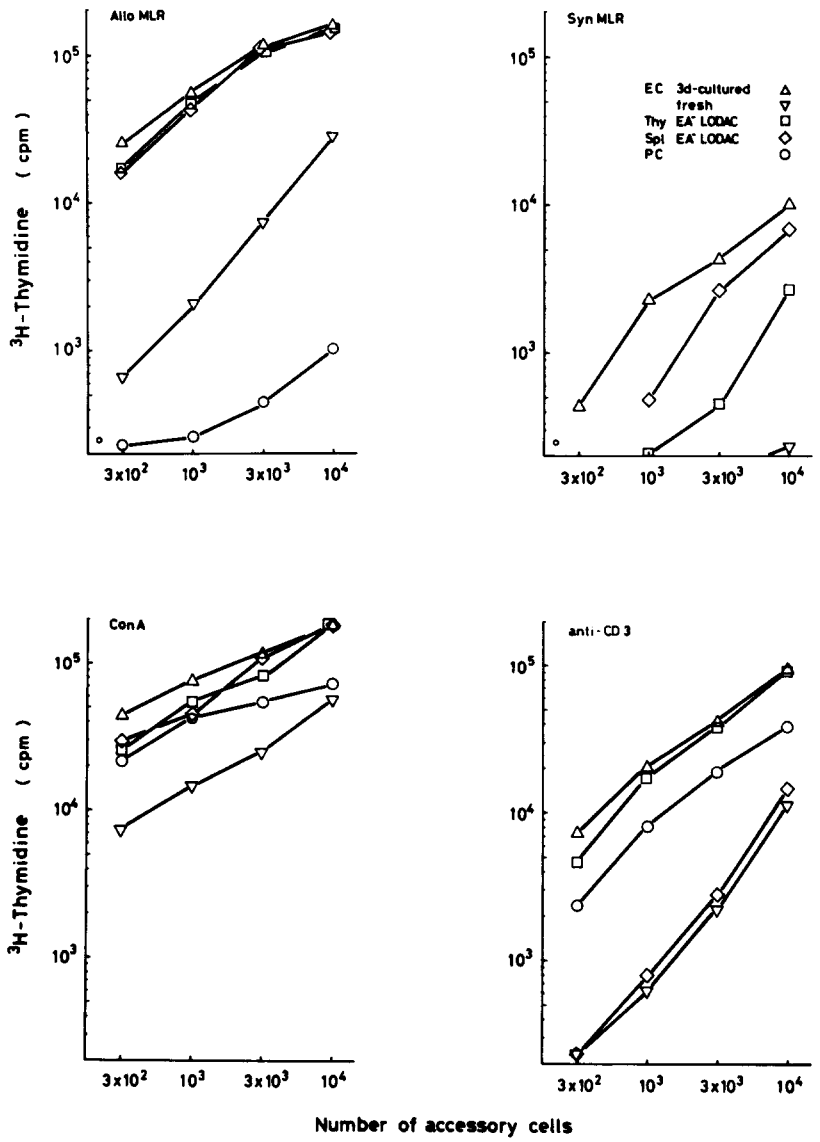


FIGURE 1. The function of five different populations, including fresh and cultured LC, during T cell mitogenesis. Graded doses of fresh and 3-d cultured LC (here labeled EC), spleen and thymic dendritic cells (Fc receptor weak dendritic cells were isolated from low density adherent cells, LODAC, by depleting cells that rosetted with antibody-coated erythrocytes EA), and peritoneal macrophages were added to 3 × 10^5 T cells in microtest cultures. All accessory cells were from B6xD2 H-2 bxd mice and were irradiated with 1,000 rad. The T cells were from the same mice, except for the allogeneic MLR, where CxD2 H-2d T cells were used. Proliferative responses were measured at 72–90 h in the allogeneic and syngeneic MLRs, and at 28–44 h in the Con A and anti-CD3 responses.

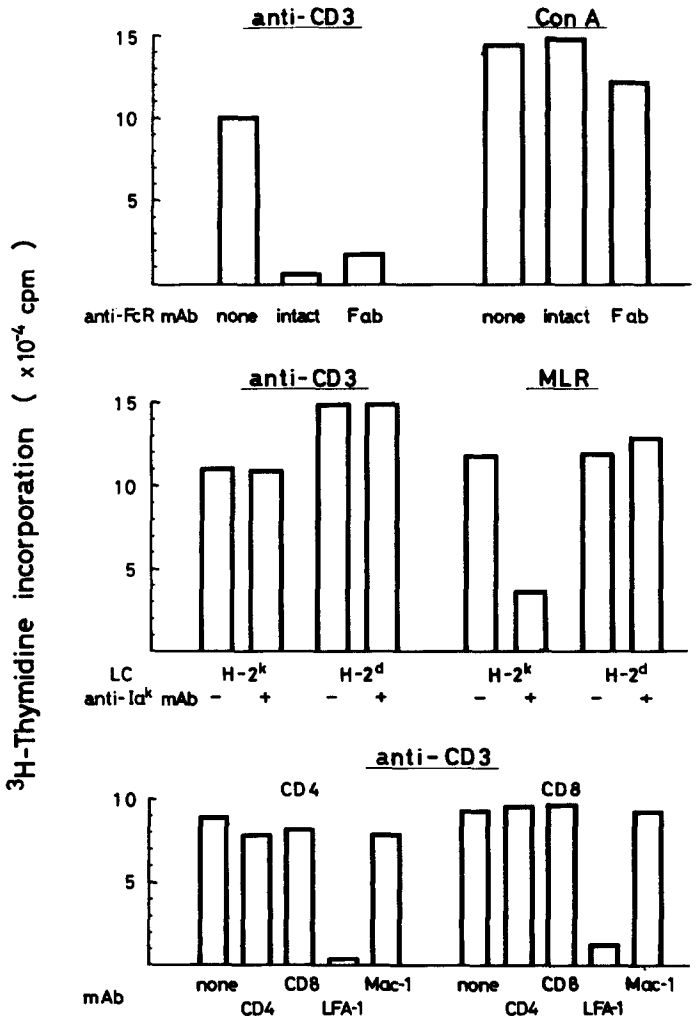


FIGURE 2. Blocking of the anti-CD3 response with mAbs. Proliferative responses were generated with 3×10^5 T cells and 3×10^3 (*top*) or 10^3 (*middle, bottom*) cultured LC. (*Top*) Blocking of anti-CD3 but not Con A responses with 2.4G2 anti-FcR mAb. Cultures were set up with no mAb, 10 μg/ml intact Ig, and 10 μg/ml Fab fragment of 2.4G2 Ig. (*Middle*) Anti-Ia blocks the MLR induced by LC, but not the anti-CD3 response. The LC were from CBA/J H-2k mice or from CxD2 H-2d mice. Syngeneic cells were used for the anti-CD3 response, and the reciprocal allogeneic cells for the MLR. The anti-Ia^k mAb was 10-2.16 anti-I-A^k. Hybridoma culture supernatants were used at a dose of 25% vol/vol. (*Bottom*) Anti-LFA-1 but not other mAb blocks the anti-CD3 response of both CD4 and CD8 T cell subsets.

about twice background (Fig. 3 *B*). Reactivity with anti-Ia increased fivefold, as described (14).

In contrast to LC, T cells gave strong signals with anti-LFA-1 and anti-Thy-1, but no staining at all with 2.4G2 and anti-Ia (not shown).

We then did two-color FACS analyses on the mitogenesis cultures, using anti-Ia to distinguish the LC from T cells (activated mouse T cells do not express Ia). T

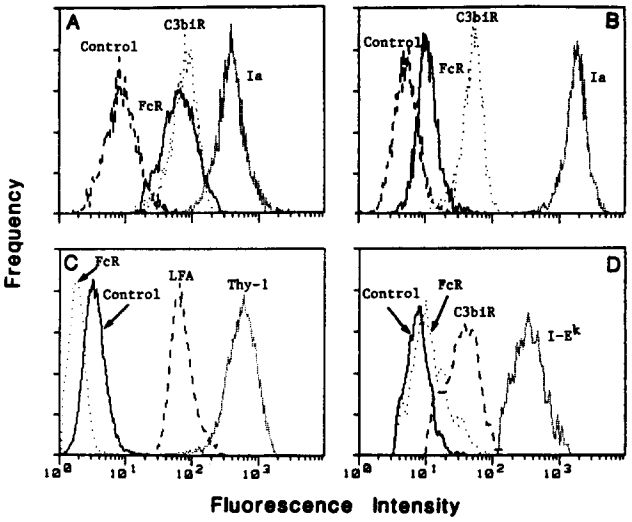


FIGURE 3. Detection of Fc receptors by flow cytometry. Fresh (*A*) and cultured (*B*) epidermal cell suspensions were exposed to culture supernatants from rat anti-mouse hybridomas all of the IgG2b isotype. These were: 2.4G2 anti-Fc receptor, M1/70 anti-C3bi receptor, B21-2 anti-I-A, and 33D1 anti-dendritic cell. After washing, the suspensions were treated with FITC-mouse anti-rat Ig, biotin mouse anti-I-E, and PE-streptavidin. The suspensions were then analyzed for PE and FITC fluorescence, the former identifying the LC component. Shown here are one-dimensional FITC distributions for the different mAb on fresh (*A*) and cultured (*B*) LC. The background stain is that exhibited by 33D1, which gave the same staining as the no primary control, as did other nonreactive IgG2b mAb GK 1.5 anti-CD4 and B5-3 anti-Thy-1 (not shown).

Anti-CD3 induced mitogenesis cultures (LC and T cells) were also stained at 24 h with the same system, i.e., PE-anti-Ia to identify LC and a second FITC mAb. Cells from H-2k mice were used so that the B21-2 anti-I-A^d mAb served as nonreactive isotype-matched rat IgG2b control. The T cells were separated into small lymphocytes and large lymphoblasts (see Fig. 6 below) on the basis of forward light scattering. Shown here is the lack of 2.4G2 staining on the T blasts (*C*) and the LC (*D*).

blasts and small T cells were separated by forward light scattering (see below). Both T cell populations were Thy-1^+, LFA-1^+, FcR^- (Fig. 3 *C*). The Ia^+ cells had even less FcR than the LC added to the mitogenesis culture (Fig. 3 *D*), possibly because the FcR were occupied by the 2C11 anti-CD3 mAb.

We did additional studies looking for FcR since the above studies would fail to detect pools of intracellular and occupied FcR. Cytospun, permeabilized specimens of the cells were stained with 2.4G2 or with a second anti-FcR mAb 6B7C. The latter IgG2a mAb recognizes a membrane proximal epitope (25) that is found on all FcR molecules recognized by 2.4G2 but is normally obscured on live cells (22). The 6B7C epitope however is readily detected in B cells in tissue sections and cell lysates (22). When either the 6B7C or 2.4G2 mAb were used to stain the LC-T cell cultures, no staining was observed, in contrast to staining with anti-Ia, which was bright (Fig. 4). Clear cut staining with both anti-FcR mAbs was observed in peritoneal macrophages when the latter were used as APC for the anti-CD3 response (Fig. 4) and in A20 B lymphoma cells (not shown).

To quantitate FcR on cultured LC and other FcR^+ cells, binding assays were performed with ^{125}I-2.4G2. In all cases, the binding was specific since it was abrogated by a 50-fold excess of cold 2.4G2 but not by other Ig. Fresh LC had about 20,000 binding sites/cell, while cultured LC had 2,000 (Table I). Spleen dendritic cells had about 1,000 binding sites, but it was not determined if this was due to a few contaminating B cells or macrophages. The latter cells, exemplified by LPS-induced B blasts and peritoneal macrophages, expressed 20-60,000 binding sites/cell (Table I).

Two approaches (not shown) verified that the small amounts of ^{125}I-2.4G2 binding were due to LC, not keratinocytes. By autoradiography, the 2.4G2-labeled cells all had the cytologic features of LC. Second, we bound ^{125}I-2.4G2 to mixtures of LC and keratinocytes and then found that the label was carried by cells that could be panned with anti-Ia mAb (anti-LC) but not with anti-Thy-1 (anti-keratinocyte).

We conclude from this group of studies that a very small number of FcR are ex-

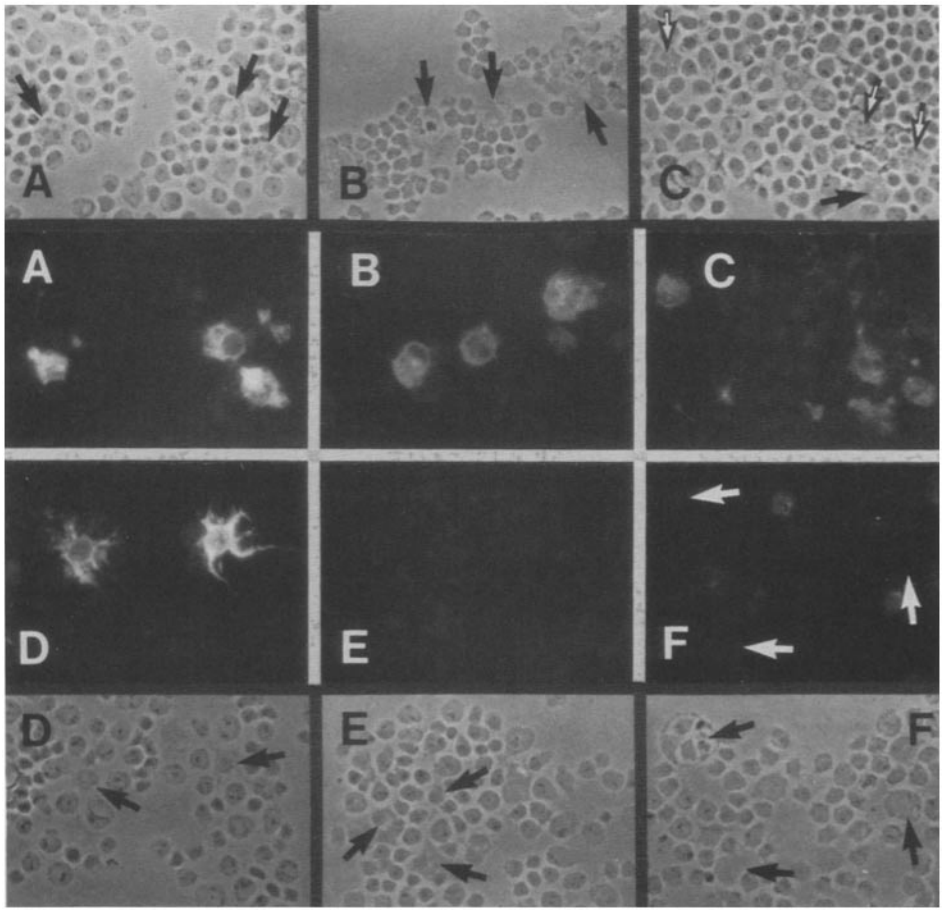


FIGURE 4. Failure to identify pools of intracellular FcR in LC or T cells during anti-CD3 mitogenesis responses. Mitogenesis was induced using FcR-rich peritoneal macrophages [A-C, F] as accessory cells or FcR-poor cultured LC [D, E]. At 24 h the cells were cytospun onto slides, fixed in acetone, and stained with a panel of mAb followed by FITC-mouse anti-rat Ig. The IgG2b mAb were M1/70 anti-C3biR or CD11b [A], 2.4G2 anti-FcR [B, E], B21-2 anti-I-A [D] and 33D1 anti-DC [the non-reactive control, not shown]. The IgG2a mAb were 6B7C anti-FcR [C] and the isotype-matched control 53-6.7 anti-CD8 [F; reactive with T cells but not APC]. All micrographs were exposed and developed identically to allow for a comparison of fluorescence intensities. Phase contrast and fluorescence are shown for representative fields. The arrows mark the APC. 200×.

pressed on cultured LC and that these are essential for presenting anti-CD3 to FcR negative T cells.

Comparison of Cultured LC with other FcR-bearing APC. Dose-response curves were obtained to compare the function of cultured LC with many other types of accessory cell. For each population, we measured the average number of specific 2.4G2 binding sites. In all cases, the cultured LC were as or more active than other, FcR-rich APC. Cultured LC were more active than fresh LC, as active as peritoneal macrophages and the A20 lymphoma, and more active than LPS-induced B blasts (Fig. 5). The activity of every population was blocked >90% by 2.4G2 mAb (Fig. 5, *bottom*).

The Frequency of T Cells that Enter Cell Cycle per LC. To detect responding cells that developed upon coculture with cultured LC plus anti-CD3, the mitogenesis cultures were analyzed for forward light scattering as a measure of cell size. In two experiments after 38 h of coculture with 2×10^3 LC and anti-CD3 mAb, ~50% of all viable cells were blasts (Fig. 6). This corresponds to 50 blasts per LC in 38 h. Since T cells could go through two rounds of division in this time, we estimated that at least 25 blasts are induced by one LC in a day. The response, whether measured using [^{3}H]TdR uptake or numbers of lymphoblasts, was directly related to the dose of LC (Fig. 6, *bottom*).

Since T cells could be binding to and then disengaging from LC during the anti-CD3 response, we made cytospin preparations of the [^{3}H]TdR-labeled cultures to identify those T blasts that were in contact with LC at any one time. Giemsa staining roughly distinguished the cell types by their tinctorial properties, and showed large numbers of lymphoblasts in contact with the LC (Fig. 7, *left*). If the preparations were stained with anti-Ia and then processed for autoradiography, T cells that were in S phase while in contact with Ia$^+$ LC could be counted (Fig. 7, *right*). On average, 7.5 (two experiments) proliferating cells per LC were found in the clusters, regardless of the dose of LC used to initiate the response (Fig. 6, *legend*).

TABLE I

The Number of FcR (2.4G2 Binding Sites) per Cell

Cell type	Binding sites ± SD	Number of experiments
Langerhans cells, fresh	19,000 ± 11,000	3
Langerhans cells, cultured	2,000 ± 1,000	11
Resident peritoneal macrophages	67,000 ± 50,000	6
Spleen macrophages	63,000	2
Thymus macrophages	119,000	1
Spleen dendritic cells	1,000 ± 300	3
Thymic dendritic cells	10,000	2
Lymph node dendritic cells	2,000	1
Spleen EA$^+$ adherent cells	9,000 ± 5,000	3
Thymic EA$^+$ adherent cells	12,000	2
A20 B cell lymphoma	57,000 ± 24,000	6
LPS-induced B blasts	31,000	1

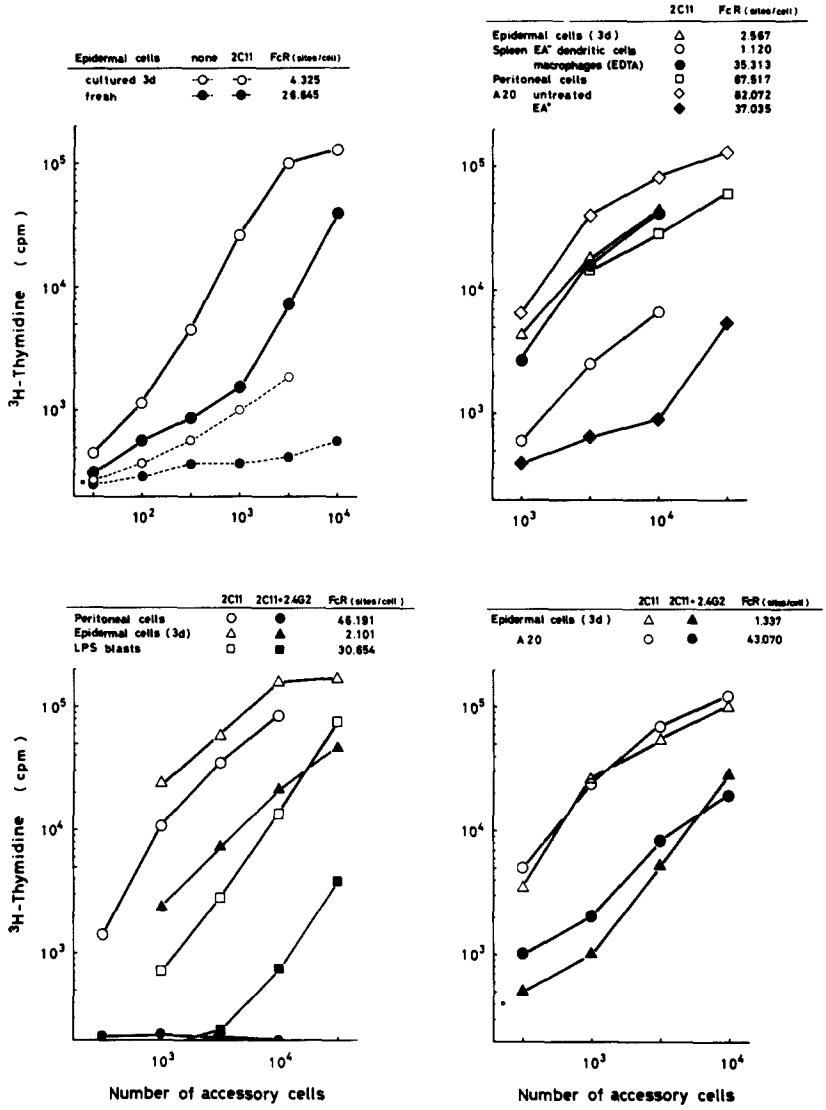
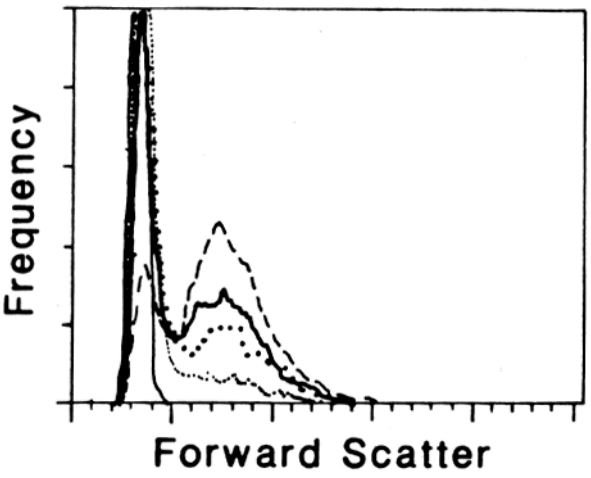


FIGURE 5. A comparison of several different types of accessory cells for anti-CD3 mitogenesis responses in which the populations were also monitored for the number of ^{125}I-2.4G2 binding sites. All the LC (here labeled epidermal cells) populations were purified by panning. The top left panel compares fresh and cultured LC in the absence or presence of 2C11 anti-CD3 mAb. The top right compares cultured LC with the dendritic and macrophage components of spleen adherent cells, as well as peritoneal cells and the A20 lymphoma. The latter were tested without or with prior exposure to the antibody coated erythrocytes (EA) that are used to deplete FcR bearing cells. The bottom panels are comparable experiments, but 2.4G2 anti-FcR mAb was added to show that the mitogenesis responses require functioning FcR.



Key	LC* plated	Blasts‡ at 38h	[^{3}H]TdR§ /LC	DNA‖ synthesis
				cpm × *10*$^{-3}$
----	3	76.4	ND	456
——	1	51.6	8.3	287
••••••	0.3	33.4	7.2	149
·····	0.1	12.1	8.2	25
NI	0.03	3.0	7.3	6
NI	0.01	1.3	ND	2
——	0	0.2	0	0.8

FIGURE 6. The proliferative response to anti-CD3 mAb and graded doses of cultured LC: single cell analyses. Anti-CD3 mitogenesis cultures were set up in standard microtest cultures with 2 × 10^5 T cells and graded doses of cultured LC. At 38 h, the T cell response was monitored with a number of different assays on replicate samples. The frequency vs. forward light scatter plots provide a measurement of cell size using samples in which dead cells were gated out by ethidium bromide staining. Below are the symbols for the different doses of LC (*) and the yields of blasts (‡; the cells in the high forward scatter peak). Also shown in the table is the level of DNA synthesis after a pulse with [^{3}H]TdR from 31 to 37 h (‖) and the ratio of the total number of [^{3}H]TdR-positive cells to the number of Ia$^+$ LC in discrete LC-T cell clusters (§; see Fig. 7). NI, Not included in the figure.

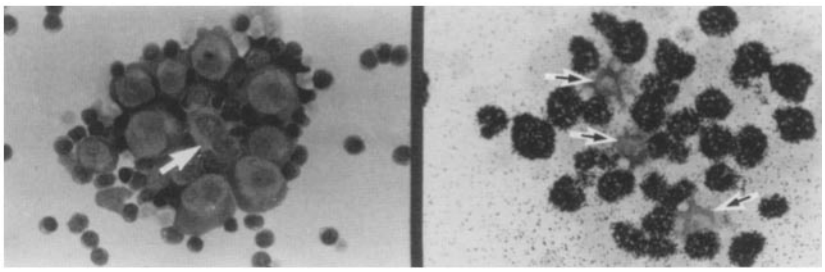


FIGURE 7. Cytologic approaches to the proliferative response to anti-CD3 mAb and cultured LC. Anti-CD3 mitogenesis responses were induced with graded doses of LC as in Fig. 6. Aliquots of the cultures were stained with Giemsa to distinguish enlarged lymphoblasts from small T cells (*left*). A presumptive LC, the cytoplasm of which does not take up the dyes in the Giemsa stain, is arrowed. To quantitate blastogenesis relative to the number of LC, the cultures were exposed to [^{3}H]TdR at 30–36 h. The cells were spun onto glass slides, stained with anti-Ia using an immunoperoxidase method (biotin anti-I-A and anti-I-E plus avidin-biotin-peroxidase complex), and then processed for autoradiography followed by hematoxylin staining (*right*). Note the large numbers of cells with silver grains in association with the peroxidase labeled LC (*arrows*). ×240.

Discussion

Detection of FcR on Some Types of Dendritic Cells. One of the characteristics features of most dendritic cells is a lack of FcR. This has been apparent by many criteria: weak reactivity with anti-FcR mAb (14, 26), little or no binding of immune complexes and antibody-coated particles in vitro and in situ (18, 27), and weak accessory function for anti-CD3 stimulation of T cells (28). The one exception has been the epidermal LC, which expresses FcR in situ and immediately upon isolation (11–14) but loses most of these FcR within a day of culture (15).

We now find that some dendritic cells, particularly those in thymus and in cultured epidermal suspensions, retain small but detectable levels of FcR. These low levels were detected using binding assays with ^{125}I-2.4G2 mAb and by flow cytometry using a polyclonal FITC–mouse anti–rat Ig reagent and a sensitive FACScan instrument (Table I, Fig. 3). In the accompanying paper, we will show that freshly isolated LC are very active in presenting exogenous protein antigens to chronically stimulated T cell clones, while cultured LC are inactive. We suspect that the physiologic role of the relatively high levels of FcR on fresh LC may be to help process immune complexes. The capacity to handle immune complexes, like the capacity to handle soluble proteins (accompanying paper) may be downregulated as LC acquire the additional functions required to sensitize T cells.

In discussing the findings of this paper, we are assuming that the capacity of LC to stimulate anti-CD3 responses may not relate directly to the physiologic role of LC FcR but instead provides a model for the efficacy of natural ligands for the TCR, i.e., complexes of antigen and MHC. At present, there is no way to quantitate specific MHC-peptide complexes directly, so the anti-CD3 system is essentially a working model in which one can obtain quantitative information. The model is a fascinating one when the quantitative findings are considered. Since LC only express ~2,000 surface FcR, and we are unable to detect a significant intracellular pool, then an average of 200–300 FcR are sufficient to stimulate any of the seven to eight T cells that a LC is triggering at any one time (Figs. 6, 7).

Possible Roles of the FcR in the Anti-CD3 Response. Soluble anti-CD3 mAb, in the absence of accessory cells, does not induce human T cells to enlarge, secrete lymphokines, or become responsive to low doses of IL-2 (29). We have confirmed this in the 2C11 anti–murine CD3 system (Inaba, K., N. Romani, and R. M. Steinman, submitted for publication). Therefore a limiting feature of the anti-CD3 response is the small number of anti-CD3 bound to LC FcR molecules. One hypothesis for the role of FcR on the accessory cell is to allow aggregation of the anti-CD3 molecules, which then more effectively stimulate the T cell. This hypothesis stems from the enhanced stimulatory capacity of anti-CD3, for T cell clones, if coupled to solid supports like Sepharose (30). If FcR are needed to form aggregates of anti-CD3, it is of interest that these can assemble in spite of their low numbers on cultured LC.

FcR on the LC also may provide a required stimulus in addition to anti-CD3. We have not identified FcR-mediated second signals as yet. We asked whether the FcR-CD3 interaction might activate the physiologic system for antigen presentation in which MHC molecules on the APC and CD4 molecules on the T cell participate. However, mAb to Ia and to CD4, which block LC function in the MLR, did not significantly block the anti-CD3 response (Fig. 2). We have looked for soluble activating factors that might emerge from clusters of anti-CD3-stimulated LC and

T cells but have found none. Specifically, if in one chamber of a two-chamber culture system we place clusters of LC and responding T cells, and into the second chamber place anti-CD3 plus T cells, the latter do not enlarge, become IL-2 responsive, or synthesize DNA (Inaba, K., et al., submitted for publication).

A third possibility is that FcR do not directly trigger any change in the LC but simply approximate the anti-CD3 "opsonized" T cells to the LC. Then other properties of the LC induce mitogenesis. This too does not seem to be the case. We have found that cultured LC and T cells cluster with one another to comparable extents in the presence or absence of anti-CD3 when the cells are mixed for just 3 h. These LC-T cell clusters *then* proliferate vigorously if anti-CD3 is added. Therefore anti-CD3 is not simply an opsonin for LC FcR. Anti-CD3 molecules on LC FcR are providing a stimulus to the T cell that seems to be independent of another mechanism that brings dendritic and T cells together.

The Accessory Function of LC Develops in Culture. Prior studies have shown that cultured LC are much more active accessory cells for a variety of T-dependent responses (15, 24, 31), and that this development of accessory function can be mediated by granulocyte/macrophage CSF (GM-CSF) (16, 17). One difficulty in interpreting these prior experiments is that the ligand for the TCR for antigen was not able to be quantitated, so that the observed increase in LC accessory function could reflect an increase in ligands for the TCR. For example, in the MLR we quantitated MHC products (15, 16), but an MHC product in the absence of bound peptides (32) may not be recognized by the TCR. In oxidative mitogenesis (15, 17), one also does not know the ligand for the TCR.

However, in anti-CD3 responses, the ligand for the TCR complex is anti-CD3, there being no evidence for a role for other molecules like CD4 and MHC products (Fig. 2). What we have found is that LC function during anti-CD3 responses increases markedly in culture even though the level of FcR, the only known ligand for the TCR/CD3 complex in this model, decreases (Table I, Fig. 2). This suggests that some other aspect of LC function, other than presentation of ligand to the TCR, is developing in culture.

One such function, which as mentioned above is independent of FcR, is the capacity to form tight, stable aggregates with T lymphocytes (Inaba, K., et al., submitted for publication). We find that freshly isolated LC, with sizable numbers of FcR, can form clusters with T cells, but that these clusters are small and unstable even in the presence of anti-CD3. The aggregates are easily dissociated by handling, and disassemble spontaneously if placed in culture. The stability of clusters formed with LC that have been cultured one day is also very weak relative to those formed with day 3 cultured LC. Therefore, the capacity of cultured LC to stimulate a CD3 response may reflect, at least in part, the capacity to retain T cells for prolonged periods and/or to provide surface-dependent stimulatory signals.

Can a Small Number of Antigen-MHC Complexes on a Dendritic Cell Induce T Cell Mitogenesis? One of the features to emerge from recent studies of antigen presentation is that it may be very difficult to occupy a large proportion of an APC's complement of MHC molecules with one type of antigenic peptide. When peptides are generated by proteolysis, there may be many different peptides to which an APC is exposed at any one time, including those derived from self proteins in the environment of the APC.

Antigens likely interact with the α/β component of the TCR complex and then

the CD3 system is engaged. It is considered that anti-CD3 simply bypasses the signal delivered by antigen-MHC to the TCR complex (29). To the extent that anti-CD3 engagement of the TCR is analogous to physiologic engagement by antigens, very small numbers of antigen-MHC complexes may be effective when presented on dendritic cells. This would mean that the function of high levels of MHC molecules on dendritic cells is to carry different kinds of antigen rather than large amounts of just one peptide. Given the capacity of dendritic cells to fully activate at least 25 T cells per day, and 8 T cells at any one time, it is possible that many different lymphocyte clones can be selected by individual APC.

Summary

Resting T cells enter cell cycle when challenged with anti-CD3 mAb and accessory cells that bear required Fc receptors (FcR). Presentation of anti-CD3 is thought to be a model for antigens presented by accessory cells to the TCR complex. We have obtained evidence that the number of anti-CD3 molecules that are associated with the accessory cell can be very small. We first noticed that thymic dendritic cells and cultured, but not freshly isolated, epidermal Langerhans cells (LC) were active accessory cells for responses to anti-CD3 mAb. DNA synthesis was abrogated by a mAb to the FcR but not by mAb to other molecules used in clonally specific antigen recognition, i.e., class I and II MHC products or CD4 and CD8. The requisite FcR could be identified on the LC but in small numbers. Freshly isolated LC had 20,000 FcR per cell, while the more active cultured LC had only 2,000 sites, using ^{125}I-anti-FcR mAb in quantitative binding studies. Individual LC had similar levels of FcR, as evidenced with a sensitive FACS. FcR could not be detected on T cells or within the dendritic cell cytoplasm, at the start of or during the mitogenesis response. When the response was assessed at 30 h with single cell assays, at least 20 T cells became lymphoblasts per added LC, and at least 8 T cells were synthesizing DNA while in contact with the LC in discrete cell clusters. To the extent that anti-CD3 represents a polyclonal model for antigen presentation to specific T cell clones, these results suggest two conclusions. First, only 200–300 molecules of ligand on dendritic cells may be required to trigger a T cell. Second, the maturation of LC in culture entails "sensitizing" functions other than ligand presentation (anti-CD3 on FcR) to clonotypic T cell receptors.

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