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Dynamic signaling by T follicular helper cells during germinal center B cell selection

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Abstract

T follicular helper (T_{FH}) cells select high-affinity, antibody-producing B cells for clonal expansion in germinal centers (GCs), but the nature of their interaction is not well defined. Using intravital imaging, we found that selection is mediated by large but transient contacts between T_{FH} and GC B cells presenting the highest levels of cognate peptide bound to major histocompatibility complex II. These interactions elicited transient and sustained increases in T_{FH} intracellular free calcium (Ca²⁺) that were associated with T_{FH} cell coexpression of the cytokines interleukin-4 and -21. However, increased intracellular Ca²⁺ did not arrest T_{FH} cell migration. Instead, T_{FH} cells remained motile and continually scanned the surface of many GC B cells, forming short-lived contacts that induced selection through further repeated transient elevations in intracellular Ca²⁺.

Germinal centers (GCs) are specialized microanatomical sites where B cells undergo clonal expansion, somatic hypermutation, and affinity maturation (1–3). Through iterative cycles of diversification and selection, the GC produces high-affinity memory B and plasma cells (2–4). Selection of high-affinity GC B cells requires their interaction with T follicular helper (T_{FH}) cells, which must discern among B cell clones according to their surface density of peptide–major histocompatibility complex II (pMHCII) (5). GC B cells are then programmed by T_{FH} cells to expand and hypermutate in direct proportion to the levels of cognate antigen presented (6). These events are controlled by T_{FH} cell–derived signals, including membrane-bound inducible costimulator (ICOS) and CD40L and the cytokines

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Supplementary Materials:

Materials and Methods

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interleukin-4 (IL-4) and IL-21 (7, 8), which are delivered in short-lived intercellular contacts (9).

To examine how the interactions between T_{FH} cells and GC B cells control selection, we imaged cells expressing genetically encoded fluorescent proteins in vivo by means of two-photon laser scanning microscopy (TPLSM). Ovalbumin (OVA)–specific, T cell receptor (TCR) transgenic OT-II T cells expressing DsRed were adoptively transferred into congenic mice before priming with OVA in alum. After 2 to 3 weeks, a 95:5 mixture of non fluorescent *Ly75*^{−/−} and GFP⁺ *Ly75*^{−/−} (*Ly75* encodes the cell-surface receptor DEC205) B cells, both specific for NP (4-hydroxy-3-nitrophenylacetyl; B1-8^{hi}) (10), was transferred before boosting with soluble OVA conjugated to NP (NP–OVA) (11, 12). To induce selection, we increased the levels of pMHCII on the surface of GC B cells 7 to 8 days later through injection of DEC205 antibody fused to the cognate antigen OVA (αDEC–OVA) (Fig. 1A). This chimeric antibody targets DEC205, an endocytic receptor that carries associated proteins into MHCII-processing compartments of GC B cells (5). As a result, targeted GC B cells are initially retained in the GC light zone (LZ) and thereafter proliferate in the dark zone (DZ) (5, 6). As a control, mice were injected with chimeric DEC205 antibody fused to an irrelevant antigen (*Plasmodium falciparum* circumsporozoite protein, αDEC–CS)(5). Popliteal lymph nodes were exposed after 4 to 10 hours, GCs were imaged by means of TPLSM (Fig. 1A), and the results were subjected to colocalization analysis (fig. S1).

Consistent with previous observations (9, 11, 13–16), GC lymphocytes were highly motile (T_{FH} cells, 9 μm/min and B cells, 6.6 μm/min) (Fig. 1, B and C, and movie S1). Under steady-state conditions, in which an unknown fraction of B cells are being positively selected, the majority of T–B contacts were short-lived (Fig. 1, B and D, and movie S1). Positive selection through αDEC–OVA injection was associated with a reduction in both GC B and T_{FH} cell velocities, along with an increase in the duration of the T–B contacts ($P < 0.0001$) (Fig. 1, B to D, and movies S2 and S3). In particular, the fraction of conjugates lasting 5 min or longer increased from 2.7 to 20.7% of the total interactions (Fig. 1D), and these occasionally moved at the B cell velocity (4.16 μm/min on average) (Fig. 1E). Although most of the conjugates moved short distances, and it was not possible to determine which one of the partners drags the other (movie S2), in those cases that could be interpreted the conjugates were led by the B cell and rarely, if at all, by the T cell (movie S3). Thus, even under conditions of enforced selection, most T–B interactions resembled those found under physiologic conditions in that they remained transient, with T cells forming and breaking contacts with multiple B cells (Fig. 1D and movie S3). Volume analysis of the T–B colocalized area revealed that the average contact size of the stable T–B conjugates (>5 min) was enlarged threefold during positive selection compared with control (Fig. 1F). As expected, polyclonal follicular B cells within the mantle zone did not slow down or form contacts with T_{FH} cells after αDEC–OVA injection (fig. S2 and movie S4). We conclude that positive selection through increased pMHCII on GC B cells is associated with longer but dynamic T–B contacts involving a larger surface area between the two interacting cells.

To examine whether positive selection interferes with the interactions between T_{FH} cells and GC B cells presenting low levels of pMHCII, we directly imaged selected and nonselected B

cells within the same GC. GCs containing OT-II DsRed⁺ T cells and a mixture of B1-8^{hi} GFP⁺ *Ly75*^{-/-}, CFP⁺ *Ly75*^{+/+}, and nonfluorescent *Ly75*^{-/-} B cells at a ~5:5:90 ratio were generated and imaged as in Fig. 1A. Positive selection of the B1-8^{hi} CFP⁺ *Ly75*^{+/+} B cells was induced by injecting αDEC-OVA. When compared directly with *Ly75*^{-/-} B cells presenting lower levels of pMHCII in the same GC, positively selected *Ly75*^{+/+} B cells interacted for a longer time with T_{FH} cells ($P < 0.0001$) (Fig. 2, A and B, and movie S5) and formed a greater number of stable contacts (> 5 min) (Fig. 2C). However, selection of *Ly75*^{+/+} GC B cells did not alter the behavior of non selected *Ly75*^{-/-} cells, which showed no significant change in contact duration or in the total number of interactions (> 0.5 min) with T_{FH} cells (Figs. 1D and 2, B and C, and movie S5). The majority of T_{FH} cells interacting with selected cells concurrently formed transient contacts with nonselected B cells (Fig. 2A and movie S5). We conclude that the increase in contact duration of T_{FH} cells with selected B cells does not substantially affect their interactions with nonselected B cells.

Our experiments indicate that increased contact size and duration correlates with the amount of pMHCII presented by GC B cells and their subsequent clonal expansion in the DZ (6), yet how these interactions affect T_{FH} T cell receptor (TCR) signaling is unknown. To examine the relationship between T-B contacts, TCR signaling, and B cell selection in the GC, we sought to measure intracellular Ca²⁺ levels in T_{FH} cells. Traditional Ca²⁺ dyes cannot be used for this purpose because T cells divide extensively and dilute such tracers before reaching the GC (17). To circumvent this issue, we used mice expressing a genetically encoded Ca²⁺ indicator (GCaMP3), which changes its fluorescence intensity according to intracellular Ca²⁺ levels (18, 19). Lymphocytes from these mice showed an increase in intracellular fluorescence when stimulated with a Ca²⁺ ionophore or when stimulated through their antigen receptor (fig. S3 and movie S6).

Changes in Ca²⁺ fluorescence are best measured by comparison with a second dye that is insensitive to changes in intracellular Ca²⁺ levels. We therefore induced and imaged GCs, as described in Fig. 1A, using OT-II GCaMP3⁺ DsRed⁺ T cells and measured the ratio of GCaMP3:DsRed fluorescence intensity (fig. S4). To determine whether T_{FH} cell Ca²⁺ content is associated with changes in cellular dynamics, velocities at successive time points were measured (instantaneous velocity) and correlated with intracellular Ca²⁺ content (17). Under steady-state conditions, spikes in Ca²⁺ content in GC T_{FH} cells were rare (Fig. 3, A to C; fig. S5A; and movie S7). In contrast, αDEC-OVA injection increased the proportion of GC T_{FH} cells with GCaMP3:DsRed ratios above 0.05 from 9 to 68% (Fig. 3, A to C, and movie S7) and the average ratio by 8.3-fold (fig. S5A). Single T_{FH} cells that were actively engaged in B cell selection showed sustained increases in intracellular Ca²⁺ over time, which did not drop to control levels during the observation period (Fig. 3B). In addition, single T_{FH} cells also displayed frequent Ca²⁺ spike transients (Fig. 3, B and D). Both sustained and transient increases in T_{FH} intracellular Ca²⁺ levels were a result of TCR:pMHCII interactions (Fig. 3, B to D, and fig. S5A). We conclude that selection is associated with TCR:pMHCII-dependent increases in T_{FH} cell Ca²⁺ content that were both transient and long-term.

Increases in intracellular Ca²⁺ content in naïve T cells result in motility arrest and enhanced effector functions (17, 20, 21). By correlating intracellular Ca²⁺ content and instantaneous

velocity in unperturbed GCs, we found that T_{FH} cells moved at an average speed of 8.76 $\mu\text{m}/\text{min}$ regardless of their Ca^{2+} content (Fig. 3C and fig. S5B). Consistently, in αDEC -OVA-targeted GCs the velocity of T_{FH} cells with high- and low- Ca^{2+} content was decreased to an average of 6.24 $\mu\text{m}/\text{min}$, and few, if any, of the actively engaged T_{FH} cells stopped moving or lost their morphological polarity while signaling (Fig. 3C, fig. S5B, and movies S7 to S9). However, there was no clear correlation between the level of Ca^{2+} increase and cell motility because both T_{FH} cells with high- and low- Ca^{2+} content had the same average velocity (Fig. 3C, fig. S5B, and movie S7). Nevertheless, by synchronizing transient small Ca^{2+} peaks of several T_{FH} cells, we found that these were precisely associated with a reduction in instantaneous velocity of $\sim 2.5 \mu\text{m}/\text{min}$ (Fig. 3E, fig. S6, and movie S10).

Given these broad changes in Ca^{2+} content and the regulated T-B interactions, we sought to examine T_{FH} intracellular Ca^{2+} levels during the formation of T-B contacts. To this end, we imaged selection in GCs containing a 95:5 mixture of B1-8hi nonfluorescent $\text{Ly}75^{-/-}$ B cells, tdTomato⁺ $\text{Ly}75^{+/+}$ B cells, and OT-II GCaMP3⁺ T cells. Injection of αDEC -OVA induced the formation of T cell contacts with $\text{Ly}75^{+/+}$ B cells that were associated with transient spikes in T_{FH} intracellular Ca^{2+} content (Fig. 3, F and G, and movies S11 and S12). To determine whether these Ca^{2+} transients take place preferentially during T-B conjugate formation, we synchronized clearly isolated contact events and measured the average GCaMP3 fluorescence intensity in T_{FH} cells before and during these interactions. Although the intracellular Ca^{2+} levels in T_{FH} cells were high in αDEC -OVA-targeted GCs (Fig. 3B), an additional small increase at the onset of contact with selected B cells was detected (Fig. 3H and movie S10).

Changes in intracellular Ca^{2+} concentration control T cell effector functions, including the expression of IL-4 and IL-21 (20, 22), both of which are required for effective B cell immune responses (23). T effector cells can express either or both of these cytokines, and this multifunctionality is associated with enhanced immunity and vaccine responses (24–28). To determine whether the long-term increase in T_{FH} intracellular Ca^{2+} levels alters the quality of GC T_{FH} cells, we examined T cells derived from IL4/IL21 double reporter mice expressing *Il4-IRES-GFP* (29) and *Il21-IRES-Katushka* (fig. S7). Polyclonal OVA primed CD4⁺ T cells isolated from these mice were adoptively transferred into TCR β -deficient mice along with B1-8hi $\text{Ly}75^{+/+}$ and $\text{Ly}75^{-/-}$ B cells at a 5:95 ratio. Recipients were boosted with NP-OVA and injected 7 days later with either αDEC -CS or αDEC -OVA so as to induce selection. T_{FH} cells were examined for cytokine expression 9 hours later by means of flow cytometry. αDEC -OVA injection resulted in a significant decrease in IL-4–IL-21–T_{FH} cells and a concomitant increase in IL-4⁺IL-21⁺ multifunctional T_{FH} cells in the absence of cell division or a significant change in the number of single-cytokine-producing cells (Fig. 4, A and B, and fig. S8). Moreover, there was also a small but significant increase in the amount of IL-21, but not IL-4, produced by the double-positive cells, as measured by an increase in the mean fluorescence intensity of the reporters (Fig. 4, C and D). Thus, increased T_{FH} Ca^{2+} signaling during B cell selection is associated with a rapid change in the quality of the GC T_{FH} response, with increased development of multifunctional T_{FH} cells producing Ca^{2+} -dependent cytokines.

Our results demonstrate that T_{FH} cells respond to pMHCII on GC B cells during selection differently than the prolonged interactions of T cells with dendritic cells (DCs) in the T zone or with B cells at the T-B border (30–36). The transient interactional dynamics of T_{FH} cells in GCs allow them to continuously seek and find B cells presenting high levels of pMHCII and provide them with preferential help while permitting competitive opportunities for other GC B cells. This mode of B cell scanning allows T_{FH} cells to interact with many cells presenting a range of pMHCII levels, rather than forming a prolonged contact with a single high-affinity B cell.

Our experiments show that the increased size and duration of contacts between GC T_{FH} and selected B cells prolongs Ca²⁺ signaling and modifies the quality of the GC T_{FH} response. These events induce coexpression of the Ca²⁺-dependent cytokines IL-4 and IL-21, which endow T_{FH} cells with effector capabilities that facilitate high affinity B cell selection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Dynamics of T_{FH} and B cell interactions in the GC

(A) Timeline of the experimental protocol. i.p., intraperitoneally; s.c., subcutaneously. (B) GCs containing a 5:95 mixture of B1-8^{hi} Ly75^{+/+} GFP⁺ B cells (cyan), B1-8^{hi} Ly75^{-/-} B cells (non-fluorescent) and OT-II DsRed⁺ T cells (red) were imaged by TPMSM after s.c. injection of αDEC-OVA or αDEC-CS as control. T-B contacts (green) were detected by red and green pixel co-localization. Collapsed z-stacks of 40 μm depth (in 5 μm steps) are shown. Bottom panels depict T and B cell dynamics over time. Images correspond to movies S1–3. (C) Velocity analysis of OT-II T cells and B1-8^{hi} B cells in GCs. (D) T-B contact duration as measured by the lifetime of the T-B co-localized areas. Percentages indicate events lasting > 5 min, marked by dashed red box. (E) T-B conjugate velocity was measured as the velocity of the T-B co-localized area. (F) Contact size analysis as measured by T-B co-localized area. Each data point represents a single cell and red lines represent mean values. Data in C–F were pooled from 2–4 mice imaged in 2–4 independent experiments. * p < 0.0001; two-tailed Student's *t*-test.

Fig. 2. T_{FH} cell interactions with selected and non-selected GC B cells

(A) GCs containing a ~5:5:90 mixture of B1-8^{hi} *Ly75*^{+/+} CFP⁺ B cells (blue), B1-8^{hi} *Ly75*^{-/-} GFP⁺ (green), *Ly75*^{-/-} non-fluorescent B cells and OT-II DsRed⁺ T cells (red) were imaged by TPLSM after s.c. injection of αDEC-OVA. Contacts between OT-II T cells and either B1-8^{hi} *Ly75*^{+/+} or *Ly75*^{-/-} B cells are shown in yellow and grey, respectively. 40 μm deep, collapsed z-stacks (5 μm steps) are shown. Arrowheads indicate B1-8^{hi} *Ly75*^{+/+} and *Ly75*^{-/-} GC B cells interacting simultaneously with one OT-II T cell. (B) Quantitation of contact duration between OT-II T cells and B1-8^{hi} *Ly75*^{+/+} or *Ly75*^{-/-} B cells. Each data point represents a single cell and red lines represent mean values. Percentages indicate events > 5 min, marked by dashed red box. (C) The number of B cell contacts (> 0.5 min, left and > 5 min, right) with OT-II T cells was normalized to the number of B cells in each group. Exp., experiment. Data in B-C were pooled from 4 independent experiments. * $p < 0.0001$; two-tailed Student's *t*-test.

Fig. 3. T_{FH} cell Ca²⁺ signaling during B cell selection

(A) GCs containing a 5:95 mixture of B1-8^{hi} *Ly75*^{+/+} CFP⁺ B cells (cyan), B1-8^{hi} *Ly75*^{-/-} B cells (non-fluorescent) and OT-II DsRed⁺ GCaMP3⁺ T cells (red, Ca²⁺ low; yellow, Ca²⁺ high) were imaged by TPLSM in untreated mice or after s.c. injection of αDEC-OVA. The bottom panels depict dynamic changes in GCaMP3 fluorescence in individual OT-II T cells over time. (B) Traces show changes in the GCaMP3/DsRed ratios over time for 6 single T cells in each condition. αI-A^b was injected intravenously after αDEC-OVA injection. (C) Scatter plots depict the GCaMP3/DsRed ratio versus instantaneous velocity as measured at successive 30 sec intervals. Each dot represents a single cell at a single time point. Average velocity (V) and GCaMP3/DsRed ratios (R) of 2-3 experiments are indicated in red and green, respectively. (D) GCaMP3/DsRed ratio fluctuations in single cells (expressed as variance) under control and αDEC-OVA conditions. (E) GCaMP3/DsRed spikes of 9 cells were synchronized. Traces of GCaMP3/DsRed ratios average and corresponding instantaneous velocities are shown. The synchronized spikes fall in the black rectangle. Error bars, SEM. (F) OT-II GCaMP3⁺ T cells and B1-8^{hi} *Ly75*^{+/+} tdTomato⁺ B cells were imaged in GCs over time as in A. Images correspond to movie S11. (G) Traces show GCaMP3 mean fluorescence intensities for 5 OT-II T cells in contact with B1-8^{hi} *Ly75*^{+/+}

tdTomato⁺ GC B cells. **(H)** Initiation of contacts were synchronized in 4 OT-II GCaMP3⁺ T cells and the corresponding average of GCaMP3 fluorescence intensity was traced before and during the contact (au, arbitrary units). Data is representative of 2–3 mice imaged in 2–3 independent experiments. * $p < 0.0001$; two-tailed Student's *t*-test.

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Fig. 4. Multifunctional T_{FH} cells during B cell selection

(A-B) $CD4^{+}$ T cells derived from OVA immunized *Il4-IRES-GFP* and *Il21-IRES-Katushka* double knock-in mice and a 5:95 ratio of $B1-8^{hi}$ $Ly75^{+/+}$ and $Ly75^{-/-}$ B cells were transferred into TCR β deficient mice, before boosting with NP-OVA. After 7 days, mice were injected with α DEC-CS or α DEC-OVA and lymph nodes were analyzed 9 hours later. The proportions of cytokine-expressing cells among T_{FH} ($CD8^{-}$, $B220^{-}$, $CD4^{+}$, $CD44^{+}$, $CD62L^{-}$, $CXCR5^{high}$ and $PD-1^{high}$) subsets are indicated. (C-D) gMFI of IL-4 (C) or IL-21 (D) reporter expression. Each data point represents a single mouse and lines represent mean values. Pooled data from 3 experiments each with 3–4 mice per condition. * $p = 0.021$; ** $p = 0.014$ ***; $p = 0.012$; two-tailed Student's t -test. NS, not significant.