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Cutting Edge

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Cutting Edge: Distinct Waves of BCL6 Expression during T Follicular Helper Cell Development

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T follicular helper (T_{FH}) cells are central to the development and regulation of T cell-dependent humoral immune responses. The transcriptional repressor BCL6 is required for T_{FH} responses, but the kinetics of BCL6 protein expression in activated $CD4^+$ T cells have not been established. We measured BCL6 expression during T_{FH} cell development at the single-cell level using intracellular staining and YFP-BCL6 fusion protein reporter mice. BCL6 was immediately upregulated in all dividing T cells during dendritic cell–T cell interactions. A second wave of early BCL6 expression coincided with the induction of CXCR5, resulting in a distinct and stable T_{FH} cell population. Cognate B cells were not required for the induction of BCL6, but supported the expansion of T_{FH} cells. These data suggest that BCL6 participates in very early events in T_{FH} cell development, and that repeated encounters with APCs reinforce BCL6 expression, thereby establishing the T_{FH} cell phenotype. ***The Journal of Immunology*****, 2011, 187: 2089–2092.**

T follicular helper (T_{FH}) cells are the prototypical Th cells involved in the initiation and maintenance of germinal center (GC) responses that generate memory B cells and long-lived plasma cells (1–4). T_{FH} cells express molecules that facilitate their functional interaction with Ag-specific B cells (1), including the chemokine receptor CXCR5 (5); the costimulatory molecules PD-1, ICOS, and BTLA; and the cytokine IL-21 (6, 7). In addition, T_{FH} cells shape the humoral immune response by producing CD40L and cytokines that influence the Ig isotype produced by B cells (8–10). The transcriptional repressor BCL6 is essential for T_{FH} cell development (11–13). *Bcl6* mRNA is induced in activated T cells (14) and is abundant in T_{FH} cells (15). However, BCL6 expression is subject to several modes of post-translational regulation (16, 17), and single-cell analysis of BCL6 protein expression has been hampered by a lack of reliable detection reagents. In this study, we define the precise kinetics of BCL6 protein expression during T_{FH} cell development in vivo. Early BCL6 upregulation occurs at the first cell division and can be induced by Ag-presenting dendritic cells (DCs). A second wave of BCL6 expression coincides with the induction of CXCR5 expression and results in the development of a distinct $BCL6^+CXCR5^+$ T_{FH} cell population, even in the absence of cognate B cell interactions.

Materials and Methods

Mice

TCR-transgenic (tg) OT-II (004194) mice were purchased from The Jackson Laboratory (JAX) and crossed with B6.SJL-$Ptprc^a$ $Pepc^b$/BoyJ (002014) mice to obtain congenic CD45.1/2 offspring for adoptive transfers. TCR-tg SMARTA/BoyJ mice were provided by M. Matloubian and BCR-tg MD4/BoyJ mice by A. DeFranco (both from University of California, San Francisco). TCR-tg Do11.10/$Rag2^{-/-}$ mice were purchased from Taconic Farms. C57BL/6 (Charles River) or congenic B6-LY5.2/Cr (National Cancer Institute) mice were used as recipients. BALB/c (JAX) mice were recipients for Do11.10 cells. YFP-BCL6 knock-in reporter mice (18), in which a *yfp* construct was inserted into exon 1 of the *Bcl6* gene resulting in the expression of a chimeric YFP-BCL6 protein, were crossed with OT-II and SMARTA mice to obtain heterozygous $Bcl6^{YFP/+}$ offspring for adoptive transfers. All experiments were done according to the Institutional Animal Care and Use Committee guidelines of the University of California, San Francisco.

Adoptive cell transfers and immunizations

Naive $CD4^+$ T cells ($CD4^+CD8^-CD25^-CD44^{low}CD62L^{high}$) were enriched from spleens and lymph nodes (LNs) with the $CD4^+$ negative isolation kit (Invitrogen) and further purified on a FACS Aria II cell sorter (BD Biosciences). T cells were labeled with 5 μM CellTrace Violet (Invitrogen) and injected i.v. into recipient mice. NP_{16}-OVA (Biosearch Technologies) was mixed with Imject Alum (Pierce), and 5 μg NP_{16}-OVA was injected s.c. into each footpad or 50 μg s.c. in the base of tail and flank. Bone marrow-derived DCs were generated, as previously described (19), using rGM-CSF (PeproTech). Maturation was induced with 0.5 μg/ml LPS (Sigma-Aldrich) overnight. Mature DCs were loaded with 3 μM $OVA_{323–339}$ peptide (Anaspec) and washed extensively to remove free peptide. OVA-DCs or 5 μg OVA protein plus 2 μg LPS were injected s.c. into each footpad. For viral infections, 5×10^5 PFU lymphocytic choriomeningitis virus Armstrong were injected s.c. into each footpad of SMARTA cell recipients.

Flow cytometry

Single-cell suspensions were prepared by mincing and filtering LN cells through fine mesh. Abs were purchased from eBioscience, BD Biosciences, or Biolegend. Biotinylated anti-CXCR5 (BD Biosciences) was visualized with streptavidin-allophycocyanin. The anti-BCL6 Ab (clone K112-91) was from BD Biosciences. Unspecific binding was blocked with anti-CD16/CD32 plus 2% normal mouse/rat serum. Intracellular BCL6 staining was performed with the Foxp3 Staining Set (eBioscience). Samples were acquired on a LSR II cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star), gating out doublets and B cells. Dead cells were excluded with 7-amino-actinomycin D or Fixable Viability Dye eFluor780 (eBioscience).

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Abbreviations used in this article: DC, dendritic cell; GC, germinal center; LN, lymph node; T_{FH}, T follicular helper; tg, transgenic; wt, wild-type.

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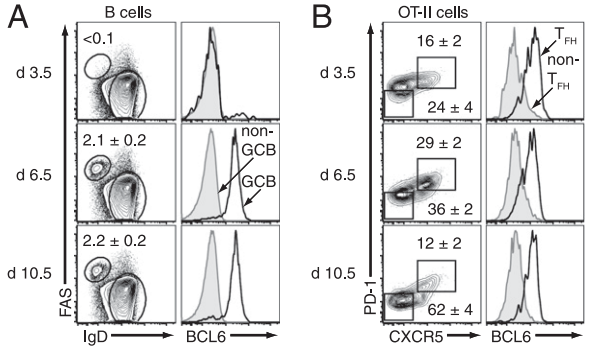

FIGURE 1. BCL6 protein expression in T_{FH} cells. Low numbers of OVA-specific OT-II cells (5×10^4) were adoptively transferred into wt recipients, followed by s.c. immunization with NP_{16}-OVA/alum in the footpads. Draining (popliteal) LNs were dissected on days 3.5, 6.5, and 10.5 after immunization and analyzed by FACS. *A*, *Left column*, Identification of GC B cells ($IgD^{low}FAS^{+}$) versus non-GC B cells ($IgD^{high}FAS^{low}$) among total B cells ($B220^{+}CD19^{+}$). Gate frequency ± SEM is shown for GC B cells ($n = 5$). Representative histograms of BCL6 expression in GC B cells versus non-GC B cells are depicted in the *right column*. *B*, *Left column*, Frequency of T_{FH} ($CXCR5^{+}PD\text{-}1^{+}$) versus non-T_{FH} ($CXCR5^{low}PD\text{-}1^{low}$) cells among transferred OT-II cells ($B220^{-}CD4^{+}CD45.1^{+}$). Gate frequencies ± SEM are shown ($n = 5$). *Right column*, Representative histograms of BCL6 expression in T_{FH} versus non-T_{FH} cells. Data are representative of three independent experiments.

Results and Discussion

BCL6 protein expression in T_{FH} cells

We investigated the expression of BCL6 protein in T_{FH} cells at the single-cell level using an optimized protocol for intracellular BCL6 staining. Wild-type (wt) mice were seeded with naive OVA-specific TCR-tg $CD4^{+}$ (OT-II) T cells, and then immunized with OVA/alum in the hind footpads. By day 6.5, immunization provoked the generation of IgD^{low} FAS^{+} GC B cells that also stained brightly for BCL6. Because BCL6 is strongly upregulated in GC B cells, these data validate the specificity of the anti-BCL6 mAb (Fig. 1*A*). OT-II cells with a T_{FH} phenotype ($CXCR5^{+}PD\text{-}1^{+}$) were already detectable at day 3.5, peaked around day 6.5, and declined by day 10.5 (Fig. 1*B*). T_{FH} cells expressed higher levels of BCL6 than $CXCR5^{low}PD\text{-}1^{low}$ non-T_{FH} cells at all time points analyzed (Fig. 1*B*), consistent with previous findings on the mRNA level (15). Furthermore, BCL6 expression positively correlated with the expression level of the T_{FH} cell markers CXCR5 and PD-1, as shown by intracellular staining and visualization of a transgenic YFP-BCL6 fusion protein in OT-II and SMARTA T cells after protein immunization and viral infection, respectively (Supplemental Fig. 1). In summary, these data clearly showed that T_{FH} cells expressed significant amounts of BCL6 protein and that T_{FH} cells developed before the appearance of GC B cells.

Two waves of BCL6 expression during naive $CD4^{+}$ T cell activation

Next, we combined adoptive transfer of CellTrace-labeled naive OT-II cells with FACS-based analysis of intracellular BCL6 protein expression to visualize the expression kinetics of BCL6 during early stages of T_{FH} cell development. By 48 h after immunization, OT-II cells had started to divide in the draining, but not in the distal LNs (Fig. 2*A*). We observed a strong increase in BCL6 expression in dividing OT-II cells after the first cell division (Fig. 2*A*). Because CXCR5 was not yet induced at this early time point (Fig. 2*B*), BCL6 expression preceded the emergence of the T_{FH} cell surface phenotype.

A strong increase in BCL6 expression at the first cell division stage was also detectable on day 3.5 (Fig. 2*C*). BCL6 expression waned with successive cell divisions in most of the dividing cells. After approximately five cell divisions, BCL6 was further upregulated in a subset of cells, although a clear boundary

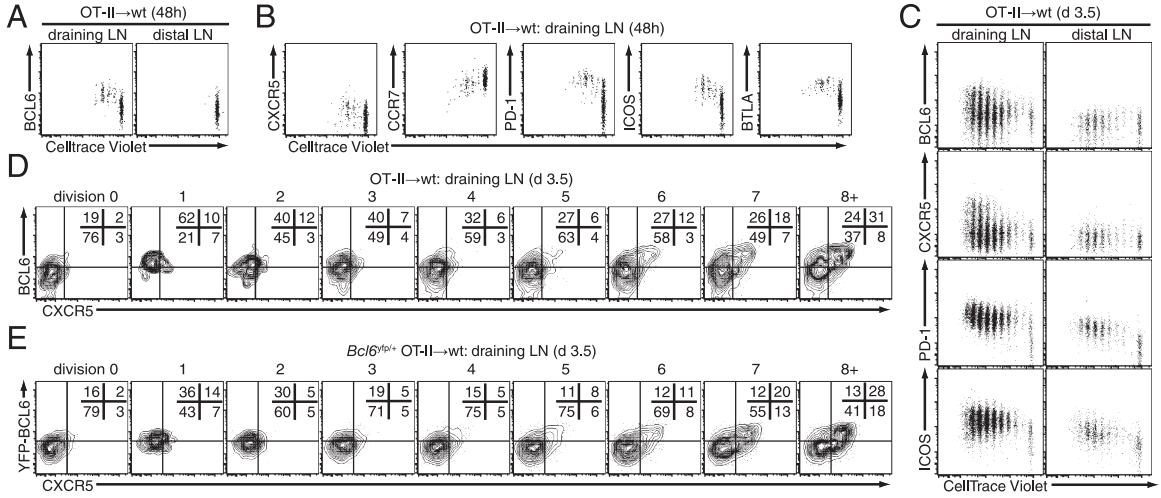

FIGURE 2. Two waves of BCL6 expression during $CD4^{+}$ T cell activation. *A*, CellTrace-labeled OT-II cells (5×10^5) were adoptively transferred into wt recipients, followed by s.c. immunization with NP_{16}-OVA/alum in the footpads. Draining (popliteal) and distal (axillary + brachial) LNs were dissected 48 h later and analyzed by FACS. Dot plots display BCL6 expression on OT-II cells in relation to CellTrace dilution. Data are pooled from five individual mice analyzed. *B*, Dot plots display CXCR5, CCR7, PD-1, ICOS, and BTLA expression on dividing OT-II cells in the draining LN, as described in *A*. *C*, CellTrace-labeled OT-II cells (4×10^5) were adoptively transferred into wt recipients, followed by immunization, as described in *A*. LNs were dissected on day 3.5 and analyzed by FACS. Dot plots display BCL6, CXCR5, PD-1, and ICOS expression on OT-II cells in relation to CellTrace dilution. Data are pooled from three individual mice analyzed. *D*, CellTrace dilution was used to identify divided cell populations in the data from *C*. Contour plots display BCL6 and CXCR5 coexpression for each division. Gates were drawn according to the expression levels in undivided OT-II cells from distal LNs, and quadrant frequencies are indicated in each plot. *E*, CellTrace-labeled OT-II cells (1×10^6) from $Bcl6^{yfp/+}$ reporter mice were adoptively transferred into wt recipients, followed by immunization, as described in *A*. Transferred OT-II cells were analyzed by FACS on day 3.5, as described in *D*. Data shown are pooled from two individual mice analyzed. Data are representative of two (*A*, *B*), more than five (*C*, *D*), and three (*E*) independent experiments with two to five mice each.

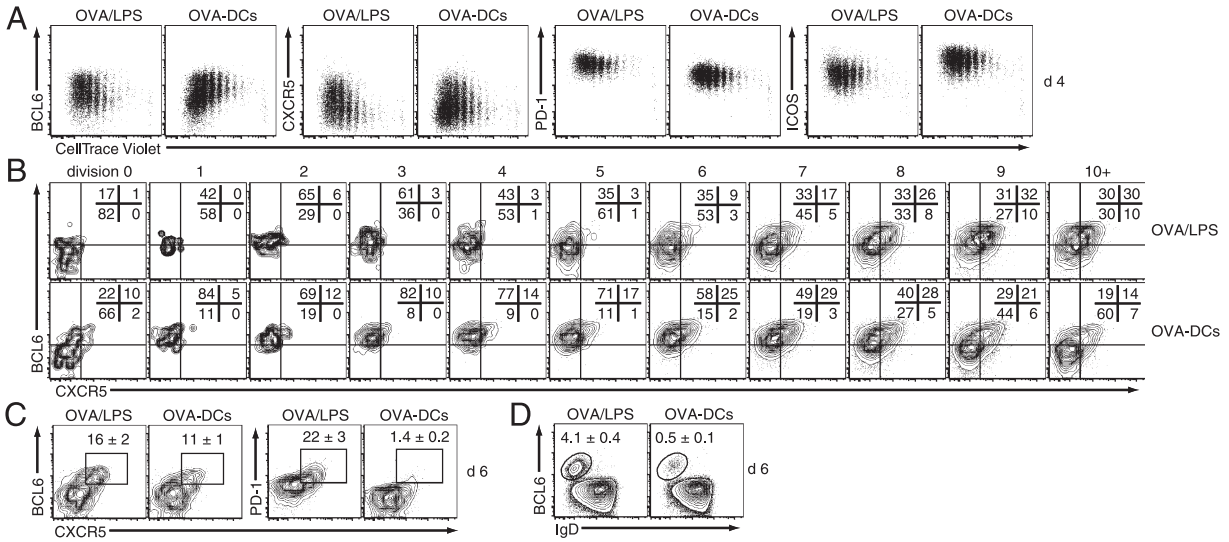

FIGURE 3. DCs are potent inducers of BCL6 expression in naive CD4$^+$ T cells. CellTrace-labeled OVA-specific Do11.10/Rag2$^{-/-}$ cells (2.5×10^5) were adoptively transferred into wt recipients. The next day, mice were immunized in the footpads with either NP_{16}-OVA/LPS or $OVA_{323–339}$-loaded LPS-matured DCs (5×10^5). *A*, KJ1-26$^+$ Do11.10 cells from draining LNs were analyzed by FACS for BCL6, CXCR5, PD-1, and ICOS expression on day 4. Dot plots shown are representative of four LNs analyzed per condition. *B*, Kinetics of CXCR5 versus BCL6 expression in Do11.10 cells in relation to the number of cell divisions, as determined by CellTrace dilution. Data shown are pooled from four LNs analyzed per condition. Quadrant frequencies are indicated in each plot. *C*, Do11.10 cells (2.8×10^5) were adoptively transferred into wt recipients, followed by immunization, as described in *A*. Generation of CXCR5$^+$BCL6$^+$ and CXCR5$^+$PD-1$^+$ T_{FH} cells (*C*) and IgD^{low}BCL6$^+$ GC B cells (*D*) was analyzed on day 6 after immunization ($n = 5$). Gate frequencies ± SEM are shown. Data are representative of four independent experiments.

between BCL6^{high} and BCL6^{low} cells was not evident. Increased BCL6 expression coincided with the induction of a population of CXCR5$^+$ cells. Plotting CXCR5 versus BCL6 expression at each cell division confirmed that BCL6 expression preceded the induction of CXCR5 (Fig. 2*D*). Importantly, this culminated in the emergence of a distinct BCL6$^+$CXCR5$^+$ T_{FH} cell population among the most divided cells. The transgenic YFP-BCL6 fusion protein was expressed with similar kinetics (Fig. 2*E*, Supplemental Fig. 2). Divided OT-II cells recovered from distal LNs only expressed marginal levels of BCL6 and CXCR5, and most likely represented emigrants that were primed in the draining LNs (Fig. 2*C*, Supplemental Fig. 2).

Consistent with our previous reports, the chemokine receptors CXCR5 and CCR7 underwent reciprocal regulation during the first 3.5 d after immunization (Fig. 2*B*, Supplemental Fig. 2), reflecting their roles in positioning activated T cells in proximity to activated B cells (20, 21). CXCR5 was gradually upregulated on dividing OT-II cells, whereas CCR7 was rapidly lost upon T cell activation. The costimulatory molecules PD-1, ICOS, and BTLA were rapidly upregulated during OT-II cell priming (Fig. 2*B*). PD-1 levels further increased at later divisions, correlating with the increase in BCL6 and CXCR5 levels (Fig. 2*C*, Supplemental Fig. 2). ICOS expression was maintained at high levels during ensuing divisions. Taken together, these data demonstrate that BCL6 is induced very early during the priming of naive T cells, and that a subset of dividing Ag-specific T cells undergoes a second, stronger wave of BCL6 expression.

DCs are potent inducers of BCL6 expression in naive CD4$^+$ T cells

The kinetics of early BCL6 upregulation suggested that BCL6 might be induced by DCs during T cell priming. To restrict Ag presentation to DCs, we immunized mice with LPS-matured and $OVA_{323–339}$ peptide-loaded bone marrow-derived DCs. As a control, we immunized mice with OVA/LPS. Using LPS or alum as adjuvant yielded similar results (compare Figs. 3*A*, 2*C*). DC immunization induced significantly higher early BCL6 expression as compared with whole protein immunization (Fig. 3*A*, 3*B*). However, at later cell divisions, the percentage of BCL6$^+$ cells rapidly declined in OVA-DC–

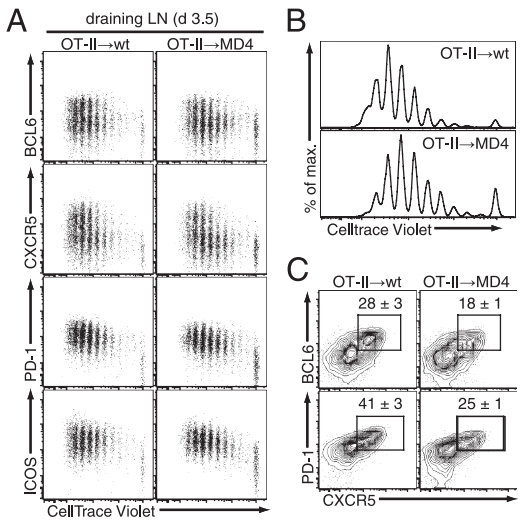

FIGURE 4. Cognate interactions with B cells are dispensible for early BCL6 expression. *A*, CellTrace-labeled OT-II cells (5×10^5) were adoptively transferred into wt ($n = 4$) or MD4 ($n = 4$) mice, which were then s.c. immunized with NP_{16}-OVA/alum in the base of tail and in the flank. Transferred OT-II cells recovered from draining inguinal, axillary, and brachial LNs were analyzed by FACS for BCL6, CXCR5, PD-1, and ICOS expression on day 3.5 after immunization. Data shown are pooled from four individually analyzed mice per condition. *B*, Histograms display CellTrace dilution by OT-II cells. *C*, Identification of OT-II CXCR5$^+$BCL6$^+$ and CXCR5$^+$PD-1$^+$ T_{FH} cells on day 3.5. Representative contour plots are depicted, and gate frequencies ± SEM are indicated ($n = 4$). Data are representative of two independent experiments.

primed Do11.10 cells, whereas BCL6 expression stabilized in the cells that had divided the most following protein immunization. Again, BCL6 expression preceded expression of CXCR5 in both immunization regimens. The inability of OVA-DCs to stabilize the $CXCR5^+BCL6^+$ T_{FH} phenotype at this early time point correlated with a decrease in T_{FH} cells on day 6 after immunization (Fig. 3*C*). As expected from the DC-restricted peptide immunization design, which omitted both specific Ag presentation by and BCR triggering of cognate B cells, DC immunization failed to induce potent GCs as compared with protein immunization (Fig. 3*D*). Interestingly, DC immunization led to elevated ICOS levels, but reduced PD-1 levels on proliferating T cells at all time points analyzed (Fig. 3*A*, 3*C*). It was recently reported that peptide-based booster immunization can induce a T_{FH} phenotype regardless of cognate interactions with B cells, possibly due to sustained interaction with Ag-presenting DCs (22). Our data indicate that DCs are indeed able to induce a T_{FH} phenotype, including early expression of BCL6, but that other mechanisms are required to sustain BCL6 expression in developing T_{FH} cells following protein immunization.

Cognate interactions with B cells are dispensible for early BCL6 expression

Because B cells are required for the development of T_{FH} cells (11, 20), we tested whether they were also responsible for inducing BCL6 expression in activated T cells early in the immune response. We transferred CellTrace-labeled naive OT-II cells into wt mice with a normal B cell population or MD4 BCR-tg mice that harbor a nearly monoclonal B cell repertoire that cannot respond to OVA. We observed similar kinetics of BCL6, CXCR5, PD-1, and ICOS expression in dividing cells in both groups of recipients (Fig. 4*A*). However, OT-II cells divided slightly less in MD4 recipients (Fig. 4*B*). Because the most divided cells are enriched for $BCL6^+CXCR5^+$ cells, MD4 recipients produced less T_{FH} cells (Fig. 4*C*). These data clearly demonstrate that cognate interactions with B cells are not required for BCL6 expression at the initial priming stage, but that they begin to play an important role in sustaining and reinforcing T_{FH} cell expansion and phenotype as early as 3.5 d into the primary response, prior to the formation of GCs. At later time points, B cells become the major APCs for follicle-homing T cells, and are then required to maintain T_{FH} cells (9, 11, 20, 22).

In summary, we have shown that BCL6 is induced at the protein level in $CD4^+$ T cells very early in primary immune responses. An initial transient wave of BCL6 expression occurs during the priming of naive $CD4^+$ T cells by DCs. Although BCL6 expression subsequently wanes in most of the dividing cells, a second wave of BCL6 expression in a subset of cells coincides with upregulation of CXCR5, resulting in the emergence of a distinct $BCL6^+CXCR5^+$ T_{FH} cell population. Early cognate interactions with B cells enhanced the development of T_{FH} cells by supporting increased T cell division, but were not directly required for the upregulation of BCL6 or CXCR5 in responding cells. Thus, B cells apparently function primarily as amplifiers of early T_{FH} development, although they may play a more direct role in maintaining BCL6 expression and the T_{FH} gene expression program after GCs are established. These data provide evidence for tightly regulated BCL6 expression starting very early in the development of T_{FH} cells in vivo and clarify the potential for BCL6 to induce the T_{FH} cell gene expression program even prior to interaction with Ag-specific B cells and the development of GCs. A better understanding of T_{FH} cell development may facilitate the development of more effective vaccines, or novel therapies that target this $CD4^+$ T cell subset in the context of autoimmune diseases and allergies.

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Disclosures

The authors have no financial conflicts of interest.

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