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CD30 Is Required for CCL21 Expression and CD4 T Cell Recruitment in the Absence of Lymphotoxin Signals

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Lymphoid tissue inducer cells express a diverse array of tumor necrosis family ligands, including those that bind CD30 and the lymphotoxin β receptor. Both of these signaling pathways have been linked with B/T segregation in the spleen. In this study, we have dissected a lymphotoxin-independent CD30-dependent signal for the induction of expression of the T zone chemokine, CCL21. Reduced expression of CCL21 due to CD30 deficiency was functionally significant: mice deficient in both lymphokine and CD30 (dKO) signals had significantly smaller accumulations of lymphocytes in their splenic white pulp areas, with no evidence of focal aggregation of T cells. Furthermore, recruitment of wild-type CD4 T cells was poor in dKO mice compared with both wild-type or lymphotoxin-deficient mice. Phylogeny suggests that CD30 signals predated those through the lymphotoxin β receptor. We suggest that CD30 signals from lymphoid tissue inducer cells were a primitive mechanism to recruit and prime CD4 T cells. This would have been a stepping stone in the evolution of the highly organized lymphokine dependent B and T white pulp areas within which CD4-dependent memory Ab responses now develop. The Journal of Immunology, 2009, 182: 4771–4775.

The white pulp areas of the spleen are organized into B and T cell areas by the expression of homeostatic chemokines within defined fixed stromal cells in B (CXCL13) and T (CCL19, CCL21) cell areas (1). Although required for the formation of B follicles, B/T segregation is not dependent on CXCL13, as B and T cells segregate normally in mice deficient in CXCR5 (2). Of the two CCR7 ligands, CCL21 is produced in greater abundance (100-fold greater protein levels than CCL19) (3). Furthermore, ectopic expression of CCL21 induces larger and more organized infiltrates than CCL19, so it is functionally and quantitatively more important than CCL19.

The main structural difference between CCL19 and CCL21 is that the latter has an extra 32 amino acid C terminus-containing basic amino acids (4, 5). Through this basic motif, it binds negatively charged structures, including the highly glycosylated mucin-type protein podoplanin (6) expressed on T zone stroma (7). As a consequence of its immobilization, CCL21 is less chemotactic than soluble CCL19 (4, 5). The result of this immobilization is that unlike CCL19, CCL21 does not desensitize its receptor (8). Furthermore, immobilization of CCL21 in vitro (simulating immobilization on podoplanin-expressing T zone stroma) stimulates integrin-independent T cell chemokinesis (9, 10), a process fundamental to the promotion of interactions between T cells migrating on T zone stromal cells (11) and dendritic cells (DCs) in the T zone.

The relationship between CCL21 and podoplanin implies that the coexpression of the two molecules by T zone stromal cells is fundamental to their function, and they require lymphotoxin β receptor (LTβR) signals for the expression of both (12, 13). Recently, we identified a distinct role for another TNF receptor, CD30, in splenic B/T segregation (14). Despite normal levels of homeostatic chemokines including CCL21, CD30-deficient (CD30−/−) mice demonstrate impaired B/T segregation, with blurring of the normally crisp boundary between the B follicle and T zone. This defect maps to lack of expression of podoplanin, supporting the view that podoplanin is functionally important for B/T segregation.

The work described above suggests that LTβR and CD30-signal coordinate T zone stromal differentiation. Although lymphokine signals can be provided by lymphocytes, lymphoid tissue inducer cells (LTi) can provide both LTα and CD30L (15). During splenic ontogeny, lymphoxygen-expressing LTi are the first cells to associate with embryonic T zone stroma and activate the expression of VCAM-1 (16). At this stage of their development, LTi fail to express CD30L (17), which appears around 1 wk of age, coincident with the up-regulation of podoplanin and B/T segregation (14). It was therefore possible that LTi CD30 signals were dependent on upstream lymphokine signals also provided by lymphocytes (14). Phylogeny, however, indicates otherwise: CD30 and its ligand are present in mammalian and avian genomes, whereas the LTβR and the lymphokine genes are exclusive to mammals (18). So the effects of CD30 on chemokine expression are just as likely to be independent of lymphokine. This suggested that CD30 might have effects on chemokine expression independent of lymphokine. To decide between the two scenarios, we generated mice deficient in both CD30 and LTα and compared their phenotype to single deficient and wild-type (WT) mice. In this study, we...
demonstrate that in the absence of lymphotoxin signals, CD30 has a clear effect on CCL21 expression. Mice deficient in both CD30 and lymphotoxin signals, have white pulp areas that are smaller and even less organized than those of lymphotoxin deficient mice, and exhibit impaired recruitment of CD4 T cells.

Materials and Methods

**Mice**

All experiments were performed in accordance with U.K. laws and with the approval of the University of Birmingham ethics committee. Mice were bred in our animal facility and were of the C57BL/6 background. We generated CD30 and LT$^{-/}$ double-deficient mice by crossing CD30$^{-/-}$ and LT$^{-/-}$ mice. Male or female mice were used when they were 6–10 wk old.

**Confocal microscopy and quantitative real-time PCR**

Tissue preparation, staining, and image acquisition and analysis were performed as previously described (14, 19). B cells were detected after staining with anti-mouse IgM conjugated to Rhodamine red (Jackson ImmunoResearch Laboratories) or anti-mouse B220 conjugated to Pacific blue (eBioscience). T cells were stained either with anti-mouse CD3e-FITC (eBioscience) or CD3-biotin (eBioscience) followed by streptavidin-Alexa Fluor-647 (Invitrogen). CD45.1$^{-/-}$ transferred cells were detected in situ after staining with anti-mouse CD45.1-FITC (eBioscience). Sample preparation for and analysis of quantitative real-time PCR was as previously described (14, 19). Primer/probe sequences (ccl19, ccl21, cxcl13, β-actin) were published previously (14).

**Cell transfers and immunizations**

OT2 (20) spleen cells were adoptively transferred i.v. in WT, LTα$^{-/-}$, or dKO mice at a density of 5 × 10$^6$ cells per mouse. One day later, mice were immunized i.p. with 200 μg OVA (Sigma-Aldrich), which was precipitated with a commercial alum gel, Alu-Gel-S (SERVA).

**Statistical analysis**

All statistical analyses were performed with the nonparametrical Mann-Whitney $U$ test using StatView 5.0 ($p < 0.05$ is considered significant).

**Results**

Mice deficient in both CD30 and LTα have smaller splenic white pulp areas and impaired T cell retention relative to mice deficient in LTα alone

To test the hypothesis that CD30 and LTα might have independent effects on B/T segregation, we made mice deficient in both. We
first compared the size of white pulp areas in WT, LTα−/−, and CD30−/−-deficient mice (Fig. 1A). WT mice as expected demonstrate substantial white pulp areas with crisp segregation into B and T cell areas. In contrast, CD30−/− mice present with impaired B/T segregation and small T zones although B follicles and total white pulp areas are normal (14). In LTα−/− mice, B follicles are lost and the size of the white pulp areas is reduced. Although B/T segregation is disrupted, T cells are concentrated in the center of the white pulp areas whereas B cells are located peripherally. Spleens that lack both CD30 and LTα (dKO) have smaller white pulp areas (Fig. 1A), and there is little evidence that T cells are concentrated in the center of white pulp areas.

To assess this defect quantitatively, we defined white pulp areas by staining for CD3 and B220, which demarcates the white pulp more clearly than IgM (Fig. 1B). No differences in white pulp areas were identified between WT and CD30−/− mice using this method (data not shown and Ref. 14). To identify whether there were specific differences between LTα−/− and dKO white pulp areas, we evaluated the size of splenic white pulp areas (Fig. 1C). We found that the white pulp size of dKO (median = 113000 μm²) was significantly reduced compared with WT (median = 175000 μm²) mice (p < 0.0001) (Fig. 1C).

The decreased white pulp areas observed in dKO mice were also correlated with decreased frequency of CD3+ pixels/μm² of total spleen (1.0 pixels/μm² dKO vs 1.22 pixels/μm² LTα−/−; p = 0.04) (Fig. 2B). When we calculated the percentage of CD3+ pixels in red and white pulp areas, we found that a higher percentage of CD3+ pixels were located in the red pulp of dKO mice (Fig. 2A). However, this abnormal ratio was attributed solely to decreased CD3+ pixel densities in white pulp areas (1.7 pixels/μm² dKO vs 2.32 pixels/μm² LTα−/−; p = 0.0006) (Fig. 2B). Red pulp CD3+ frequency was not significantly different (0.64 pixels/μm² dKO vs 0.69 pixels/μm² LTα−/−; p = 0.8). When the total white pulp was taken into account, the numbers of CD3+ pixels/white pulp was ~2-fold greater in LTα−/− vs dKO mice (median 4.1 × 10⁵ vs 2 × 10⁵ pixels/white pulp). However, the total numbers of CD3+ pixels/red pulp was not significantly different (p = 0.6). In contrast to CD3 cells, there were no significant differences in either the total number of IgM+ pixels/μm² or in their distribution between red and white pulp areas (data not shown). Furthermore, when we examined localization of CD11c+ DCs, there were no obvious differences in either total DC numbers, or their relative distribution between red and white pulp areas (data not shown).

In the absence of LTα, CD30 signals induce modest expression of CCL21

Our data suggested that there was a specific defect in localization of CD3+ T cells in white pulp areas in dKO vs LTα−/− mice.
CD30 INDUCES CCL21 EXPRESSION IN THE ABSENCE OF LYMPHOXIN

76-fold lower (1.3%) in LT14. In contrast, there is clear evidence that LT
pulp areas and central localization of CD3
H11011) of CCL21, and that this correlates with the increased white
the absence of LT

Distribution of recruited CD4 T cells in red and white pulp areas of WT and gene deficient mice. Spleen sections from immunized WT ( ), LTα−/− ( ), and dKO ( ) mice that received OT2 cells were analyzed as described in Fig. 4. A, Numbers of OT2 cells in total (white + red pulp), white, or red pulp of WT, LTα−/− and dKO recipients. B, The percentage of OT2 cells in white and red pulp of WT, LTα−/− and dKO recipient mice. OT2 cells were counted in white and red pulp and were expressed as a percentage relative to total (white + red pulp) cells. Each symbol represents data from one micrograph. Results are representative of five mice per strain.

reflected also in the decreased size of white pulp areas. Expression of homeostatic chemokines is central to B/T segregation. Although DCs produce CCL19, CCL21 is produced exclusively by stroma. We have previously found no deficit in mRNA expression of any of these three chemokines in CD30−/− mice (Fig. 3, A–C and Ref. 14). In contrast, there is clear evidence that LTα drives mRNA expression of all three chemokines (Fig. 3, A–C and Ref. 21). A comparison between dKO and LTα−/− mice revealed no difference in expression of mRNA for CCL19 (p = 0.3) or CXCL13 (p = 0.6). In contrast, although median expression of CCL21 is 76-fold lower (~1.3%) in LTα−/− vs WT mice, CCL21 levels are ~56-fold lower again in dKO vs WT mice, i.e., below the limits of detection by quantitative PCR (Fig. 3C). This clearly shows that in the absence of LTα, CD30 signals induce modest expression (~1%) of CCL21, and that this correlates with the increased white pulp areas and central localization of CD3+ cells within these white pulp areas. Although LTα can interact with TNFR1, TNFR2, and HVEM, previous work has shown that its heterotrimmer form, LTαβ2, is the main switch for splenic CCL21 (12). LTαβ2 binds the LTβR and accordingly we found that in LTβR−/− mice reduction of CCL21 matched that in LTα−/− mice (Fig. 3C).

Impaired CD4 T cell recruitment in mice double deficient for CD30 and LTα compared with LTα−/− mice

Priming of CD4 T cells has been reported to be normal in LTα−/− vs WT mice (22). In contrast, CD30 has a clear effect on CD4 T cell survival independent of any effect on B/T segregation (23, 24). To distinguish between CD30 effects on forming the microenvironment and CD4 T cell responses, we injected WT CD45.1+ OVA-specific OT2 CD4 cells (20) into CD45.2+ dKO, LTα−/−, or WT hosts. All recipient mice were immunized with OVA and the numbers of OT2 cells in red and white pulp of the spleen were enumerated 5 days postimmunization.

Transferred CD45.1+ CD4+ cells were easily detectable in the spleens of recipient immunized mice (Fig. 4). OT2 cells were identified by their coexpression of CD4, CD3, and CD45.1 in spleen sections. In WT mice, OT2 cells were clearly demarcated within the T zone areas, with few transgenic cells found outside this location (Fig. 4A). In LTα−/− mice, OT2 cells were also concentrated in the T cell-rich central compartment of the white pulp areas (Fig. 4B). However, in dKO mice, OT2 cells were poorly localized to the small white pulp areas and could also be identified in the red pulp (Fig. 4C).

The numbers of transferred OT2 cells in white and red pulp areas were quantified (an example of how the analysis was performed is shown in Fig. 4D). We found that the total number of OT2 cells per section area (Fig. 5A), in both dKO (median = 32 OT2 cells/section) and LTα−/− hosts (median = 41 OT2 cells/section), was significantly reduced compared with cells in WT hosts (median = 81 OT2 cells/section) (p < 0.0001). However, OT2 numbers in dKO recipient mice were also significantly reduced compared with LTα−/− mice (p = 0.04) (Fig. 5A). When this was analyzed further, we found that numbers of OT2 cells/white pulp were significantly reduced in dKO mice (median = 22 OT2 cells/white pulp) compared with LTα−/− (median = 35 OT2 cells/white pulp) (p = 0.002) and WT mice (median = 75 OT2 cells/white pulp) (p < 0.0001). In addition, a smaller percentage of OT2 cells were contained in the white pulp of dKO compared with either LTα−/− or WT (p < 0.0001) mice (Fig. 5B).

Discussion

Phylogeny suggests that the development of the capacity to make high affinity memory Ab responses was linked closely with the development of organized lymphoid structures (25), and we have highlighted the central role of LTi in the development of both functions (15). LTi are found tightly associated with fixed B and T zone stromal cells in secondary lymphoid organs (26, 27). They constitutively express a diverse set of TNF ligands (TRANCE, TNF-α and lymphotixins, α and β, OX40, and CD30L). All of these genes with the exception of OX40L have been linked with the development and organization of secondary lymphoid structures (14, 28, 29); in addition we have shown that OX40L and CD30L together are critical for the generation and maintenance of CD4 memory (24, 26).

The crucial point about CD30 signals is that they alone are linked with both functions: they contribute to organization in the presence of lymphotixin signals (14) but also to CD4 memory (23). Because a comparison of mammalian genomes with those of lower vertebrates suggested that CD30 signaling predated LTβR signals, we speculated that a function for CD30 in organization might be revealed in LTα−/− mice. To test this, we generated mice deficient in both CD30 and lymphotixin (dKO), and compared their phenotype to LTα−/− mice. Our studies show that white pulp areas in dKO mice are significantly smaller and less organized than in LTα−/− mice and that this is correlated with decreased expression of CCL21. Furthermore, we demonstrated a functional role for this decreased organization by showing that recruitment and localization of WT CD4 T cells is impaired in dKO compared with LTα−/− mice.

Although LTβR and CD30 are both members of the TNF receptor family, they are located on different chromosomes (in both man and mouse), in different TNF receptor clusters. The TNF receptor cluster containing CD30 (TNFR2, CD30, 4–1BB, DR3, 4–1RR, OX40).
HVEM, OX40, GITR) is principally linked with T cell memory (15, 30, 31) whereas the LTβR cluster (TNFR1, LTβR, and CD27) is primarily associated with organization. The two gene clusters are likely to be derived from tandem gene duplication (32), with subsequent separation and relocation to different chromosomes. Evidence for this (besides the fact that both locations contain TNF receptor family members) is that both gene clusters encode structurally and functionally related serine proteases that catalytically activate complement component C4. On human chromosome 1, MASp2 (activates C4 via the mannann binding lectin pathway) is ~1MB away from human CD30. On human chromosome 12, the C1rs complex (activates C4 via the classical C1q pathway) is 0.7 MB from LTβR. We think that the residual CD30-dependent induction of CCL21 expression in lymphophotinin deficient animals could be the modern remainder of the CCL21 induction elicited by signals from the ancestral TNF receptor before its duplication.

The main advantage conferred by gene duplication is the creation of redundancy, which allows diversification of function among duplicated genes. Chromosomal translocation is equally important, as it creates opportunities for associations with unrelated genes to provide new function. In this respect, the flanking genes of the LTβR are instructive: CD4, which controls the development of CD4 T cells, is only 0.4MB from LTβR, and the activation induced cytidine deaminase, required for somatic mutation and Ig class switching, 2.2 MB away. Is this a chance association? We suspect not. Inactivation of any of these three genes results in failure to generate high affinity surface switched Abs. Examination of the syntenic fish locus shows activation induced cytidine deaminase and CD4 are linked with TNFR1 but there is no true LTβR homologue. This suggests to us that LTβR dependent organization (generation of B follicles) evolved in the context of genes also essential for the generation of high affinity Ab responses. We suspect that such linkage confers a minor advantage for the selection of new function, which when iterated over millions of generations translates into a substantial advantage in performance. In this regard, mammalian CD30 is also linked with the gene, podoplaxin (1.8 MB distant), whose expression on splenic T zone stroma is CD30 dependent (14).

The data presented in this study therefore are consistent with the proposal that CD30 and LTβR are the descendants, by way of a gene duplication event, of an ancestral TNF receptor which organized the signals from the ancestral TNF receptor before its duplication.

Disclosures

The authors have no financial conflict of interest.

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