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Commentary

Ectopic Lymphoid Organogenesis

A Fast Track for Autoimmunity

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In the current issue of *The American Journal of Pathology*, Armengol and colleagues[1] document the structural characteristics and functional competence of ectopic germinal centers (GCs) in autoimmune thyroid disease. An immunohistochemical dissection of the microarchitecture of intrathyroidal lymphoid follicles identified them as analogs of follicular structures found in secondary lymphoid tissues. Morphological studies were complemented by carefully chosen molecular studies that probed the competence of the GCs. Cell proliferation and apoptosis in the ectopic lymphoid microstructures resembled those of ongoing GC reactions. Homing chemokines and high endothelial venules were identified as necessary components of cell recruitment and compartmentalization into functional zones.

Probably the most important result of the study is the finding that two of the major autoantigens implicated in autoimmune thyroid disease, thyroglobulin and thyroidal peroxidase, were selectively bound in the ectopic GCs, suggesting that the GC reactions were committed to these self antigens. GCs are critical in the development of normal B cell immune responses because they provide an infrastructure to capture and store antigen, which drives B-cell division and maturation, selection of B cells with high-affinity immunoglobulin receptors, and differentiation of memory B cells and plasma cells. The current study extends the biological function of GC reactions to a pathogenic role in autoimmune responses. If intrathyroidal lymphoid follicles are committed to the recognition of the major autoantigens, pathways leading to their establishment gain relevance in the disease process and need to be considered as fundamental pathogenic factors. The study by Armengol and colleagues[1] establishes that the immunogenicity of self-antigens is ultimately determined by their access to highly specialized lymphoid structures. The host's ability to create lymphoid microstructures in nonlymphoid organs may, therefore, be a key determinant in the pathogenesis of autoimmune diseases.

Lymphoid Organogenesis—The Cellular and Molecular Ingredients

The task of the immune system, to protect the host from all possible invading pathogens and from malignancies, is enormous. To meet this challenge, it is equipped with an array of T cells and B cells, each expressing a unique receptor for a particular antigen. The diversity of these receptors is so gigantic that antigenic invasion may not elicit a sustained response unless the immune system optimizes recognition and response. To generate protective antibody responses, the infrequent T- and B-cell clones specific for the same antigen need to gather at a site where the antigen is also available. The purpose of secondary lymphoid tissue is exactly that of bringing together antigen, T cells, and B cells, thereby establishing a microenvironment that, by virtue of its three-dimensional structure, optimizes communication between T cells and B cells and the presentation of antigen to facilitate antigen-driven selection of expanding clones.[2,3] Naive T cells and B cells percolate through secondary lymphoid organs where antigens are being delivered by sophisticated transport systems. When T cells and B cells enter secondary lymphoid tissues, they home to distinct compartments, the T-cell areas and the B-cell follicles. They stay for several hours and then move on unless they are held back by their specific antigen. Initial antigen-specific interaction occurs at the interface of the T- and B-cell zones. If appropriate conditions are met, GC reactions are initiated. After migrating into the network of follicular dendritic cells, B cells proliferate and mutate their immunoglobulin genes and high-affinity mutants are selected for survival. B-cell follicles with GC reactions

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exemplify the optimal relationship between microarchitecture and function.

Of particular interest, GCs can be formed *de novo* in nonlymphoid tissues by the process of lymphoid neogenesis.[4] Follicular hyperplasia is typical for Hashimoto's thyroiditis,[5] is found in salivary glands of patients with Sjögren's syndrome,[6–8] in the thymus of patients with Myasthenia gravis,[9,10] and in the synovial membrane of patients with rheumatoid arthritis.[11–18] In addition to autoimmune syndromes, chronic infections with *Helicobacter pylori*,[19,20] hepatitis C,[21,22] and *Borrelia burgdorferi*[23] can also be associated with the formation of ectopic GCs.

A series of chemokines and cytokines has been implicated in providing the cues for the cellular homing and interaction in lymphoid organogenesis. The study by Armengol and colleagues[1] confirms that the identical molecular mediators are also involved in inflammation-associated lymphoid neogenesis, suggesting, not unexpectedly, that biological principles are shared in secondary and tertiary lymphoid structures. Among the chemokines that regulate the compartmentalization of T cells and B cells, CXCL13 (formerly BLC or BCA-1) and CCL21 (formerly 6Ckine or SLC), have received particular attention. CCL21, which binds to CCR7, is expressed by high endothelial venules and by cells in the T-cell areas.[24] Insufficient expression of CCL21 (characteristic for mice with the spontaneous *plt* mutation)[25] or disruption of CCL21-CCR7 interaction (generated by knocking out the CCR7 gene)[26] produces a defect in the entry of naive T cells into lymphoid organs across high endothelial venules and a disorganization of T-cell areas in lymph nodes. Defects in lymphocyte compartmentalization in mice deficient in RelB[27] have also been attributed to defective production of CCL21. Naive T cells can be attracted by a second ligand of CCR7, CCL19 (formerly MIP-3β or ELC); CCL19 derives from macrophages and dendritic cells.[28] Both CCL21 and CCL19 effectively regulate T-cell movement *in vitro*.[24]

The organization of B-cell follicles is closely correlated with the action of CXCL13. Mice deficient for the CXCL13 receptor, CXCR5, have profound abnormalities in trafficking of mature B cells to lymphoid follicles and lack splenic follicles, inguinal lymph nodes, and most Peyer's patches.[29] Aberrant expression of CXCL13 in pancreatic islets of transgenic mice leads to the development of lymphocyte clusters with organized T-cell and B-cell zones, but these clusters lack follicular dendritic cells and, thus, do not represent classical GCs.[30] *In vitro*, CXCL13 is a strong B-cell attractant.

An important role in lymphoid organogenesis has been assigned to cytokines of the tumor necrosis factor family. Mice deficient in lymphotoxin (LT)-α or LT-β show profound abnormalities in the organization of lymphoid organs and are impaired in the production of CXCL13, CCL19, and CCL21.[31,32] Tumor necrosis factor-α and its receptor, tumor necrosis factor-R1, are also needed for normal development of lymphoid structures and the ordered expression of chemokines necessary for the recruitment of lymphocytes into the T-cell and B-cell zones.[33,34] It has been suggested that CXCL13 is instrumental in maintaining follicular dendritic cell networks[35] and that CXCL13-attracted B cells that home to the follicles are the source of LT-α1β2, which is critical in the generation and maintenance of established follicles.[36,37]

Ruddle and colleagues[38] were the first to point out the connection between inflammation and the formation of ectopic lymphoid microstructures. They were able to induce aberrant expression of fully organized GCs by introducing an LT-α gene under the control of the rat insulin promoter. Transgenic expression of CXCL13 in a similar system was associated with the formation of compartmentalized lymphoid infiltrates, but there was no establishment of follicular dendritic cell (FDC) networks.[30] A central role of LT has been supported by the observation that LT-α can induce the expression of adhesion molecules and an array of chemokines[39] and that ectopic expression of CXCL13 and CCL21 can be achieved in animals transgenic for LT-α.[40]

Armengol and colleagues[1] support the concept that these murine studies have relevance for lymphoid neogenesis in humans. Both CXCL13 and CCL21 were expressed in thyroid tissue with ectopic GCs. CXCL13 expression has also been described for gastric mucosa-associated lymphoid structures induced by *H. pylori*[41] and for rheumatoid synovitis.[42] In a recent study, we carefully evaluated the contribution of homing chemokines and LT-α and LT-β in rheumatoid arthritis (RA)-associated lymphoid microstructures. The advantage of rheumatoid synovitis lies in the heterogeneity of lymphoid arrangements formed in the inflamed synovial membrane, which allows for a direct comparison of different lymphoid architectures.[43,44] Tissues with GC reactions have 10- to 20-fold higher transcription levels for CXCL13 and CCL21; however, these two chemokines can also be detected in tissues lacking GCs.[42] Other chemokines, including CCL18 and CCL2, are abundant in rheumatoid synovitis with no correlation between their transcription and the resulting lymphoid microstructure. LT-α and LT-β were produced at increased rates in follicle-forming tissue samples but multivariate analysis identified only LT-β as a predictor for GC reactions, suggesting that LT-α1β2 and not LT-α3 predisposes for follicular synovitis. Surprisingly, LT-β and CXCL13 could substitute for each other in promoting ectopic GC reactions, stressing our lack of understanding of how chemokines and cytokines function in concert.

The likelihood that the chain of events culminating in the formation of extranodal GCs can be explained at the level of a single molecule is extremely low. We have barely begun to take a look at the complexity of mediators, cells, signals, and interactions required to orchestrate the events leading to the generation of lymphoid organs. New experimental models will need to be developed to mimic the complexity of cellular interactions within a topographical organization. And, as always, studying human disease can be particularly fruitful in recognizing basic principles of biology and pathophysiology.

Lymphoid Neogenesis—Apples and Oranges

Armengol and colleagues[1] have carefully analyzed the cellular components of the intrathyroidal follicles to con-

firm their resemblance to tonsillar or nodal follicles. When studying inflammatory infiltrates, it can be very difficult to distinguish between different forms of lymphoid microstructures and to identify the physiological analog in secondary lymphoid structures. This is exemplified in rheumatoid synovitis. In the majority of patients with RA, T cells infiltrate the synovial membrane in a seemingly random manner;[14,44] B cells and plasma cells may be present. A strict correlation between the superficial synovial layer or capillary networks and the T-cell infiltrates suggest that the process of tissue infiltration is far from being nonorganized. More intriguing are aggregates composed of a fixed ratio of B and T lymphocytes.[14,44] Such aggregates contain interdigitating dendritic cells, lack FDCs, and have only few proliferating B cells. These aggregates are not GCs but can easily be mistaken for follicles. The analog of synovial T cell-B cell aggregates in lymphoid organs has not yet been determined. Finally, a subset of patients with RA forms classical ectopic GCs. Patterns of lymphoid microstructures generated by patients with RA are stable within a given patient, are consistent in distinct joints, and are maintained throughout several years (C. M. Weyand and J. J. Goronzy, unpublished observation). When analyzing the role of cytokines and chemokines in forming sophisticated microarrangements at extranodal sites, the intrinsic heterogeneity of the disease process must be kept in mind.

A particularly interesting form of ectopic lymphoid microstructures is extranodal marginal zone B cell lymphoma of the mucosa-associated lymphoid tissue (MALT) type. MALT lymphomas are neoplasms of postfollicular memory B cells that arise in ectopic lymphoid tissue that is recruited to anatomical sites containing immune reactions to specific pathogens [*H. pylori* in the stomach[45] or *B. burgdorferi* in the skin[46]] or that represent targets of autoimmune disorders [Hashimoto thyroiditis[47] or myoepithelial sialadenitis of Sjogren syndrome[48]]. Even though the ectopic lymphoid tissue in which MALT lymphomas arise frequently contains GCs, the cells of MALT lymphomas are different from GCs. The cells of MALT lymphomas do not spontaneously form GCs composed of neoplastic cells, but can colonize pre-existing follicles.[49] They are negative for CD10[50] and bcl-6.[51] They contain immunoglobulin gene-point mutations in a pattern indicating a postfollicular memory B cell.[52] In gastric MALT lymphomas, the specificity of the immunoglobulin produced by the neoplastic cells is not directed toward *H. pylori*, but rather the cells seem to represent an outgrowth of autoreactive B cells.[53] Finally, the microarchitecture of GCs is not necessary to maintain the growth of MALT lymphomas. Rather, in those from the stomach, T cells that recognize specific *H. pylori* strains are necessary to maintain *in vitro* growth of the neoplastic B cells.[54] Therefore, development of MALT lymphoma seems to result when clonal autoreactive B cells in ectopic sites escape the GC or other control mechanisms, are stimulated to proliferate by antigen-specific T cells, and acquire stable transforming genetic abnormalities. The mere placement of GCs in nonlymphoid tissue sites seems to be insufficient to give rise to B-cell malignancies. In support of this concept, tertiary lymphoid structures in rheumatoid joints have not been associated with MALT lymphomas. The lack of neoplastic transformation in GCs associated with RA argues that antigen-driven T cell-B cell interaction in nonlymphoid tissue sites is not necessarily permissive for the escape of B lymphocytes from normal growth regulation. The synovial membrane may possess regulatory mechanisms that prevent B-cell neoplasia, or RA-associated GCs may be fundamentally different from MALT.

Lymphoid Neogenesis—The Host's Decision

Given the stability and diversity of inflammation-associated lymphoid neogenesis, the question arises as to which factors ultimately control the process. A critical element is antigen, but equally important seem to be host response patterns with a likely contribution of genetic risk factors. Almost certainly, multiple contributing factors are necessary to transform an inflammatory infiltrate into an organized lymphoid structure that has the capability to acquire novel functions and direct the course of autoimmune responses (Figure 1). Multivariate logistic regression analysis examining the power of different parameters in predicting the formation of GCs in rheumatoid synovitis identified three independent determinants, CXCL13, LT-β, and CCL21.[42] Other cytokines and chemokines produced in the lesion did not appear to be primary determinants in the process of lymphoid organogenesis.

Antigen

The work of Armengol and colleagues[1] strongly suggests that the antigens to which the intrathyroidal GCs are committed are components of the thyroid gland. There is also evidence that B cells contributing to gastric mucosa-associated lymphoid structures associated with *H. pylori* infection are specific for bacterial antigens.[55] The nature of the antigen in RA remains unresolved; however, primary follicles are not present in synovial lesions, and all follicles with FDC networks have ongoing GC reactions, which lends strong support to the concept that antigen recognition precedes (and drives) the generation of ectopic lymphoid tissue. One of the most interesting questions is whether lymphoid neogenesis can only be induced by a restricted panel of antigens. Miklos and colleagues[56] have reported a strong bias in the repertoire of VH genes used by MALT lymphomas in myoepithelial sialadentis and a nonrandomness in the formation of the immunoglobulin CDR3 regions. These data suggest that shared antigens are driving the process of lymphoid neogenesis in Sjögren's syndrome, including the neoplastic transformation of the tissue-invading B cells.

CXCL13-Producing Nonlymphoid Cells

Inflammation-associated lymphoid neogenesis is a nonrandom process preferentially occurring at selected tissue sites, such as thyroid, thymus, salivary glands,

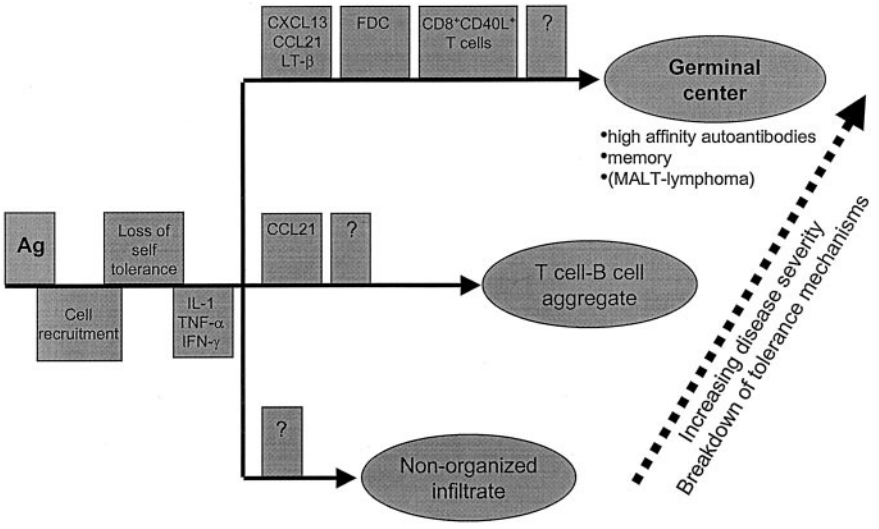

Figure 1. Emergent pathways of lymphoid organization in autoimmune disease. We propose that distinct pathways of lymphoid neogenesis can be set in place by an antigen-driven immune response occurring in extranodal tissue. Selection of the pathway depends on modulating host factors, including the aberrant expression of cytokines/chemokines and the recruitment of cellular components necessary for certain types of lymphoid microstructures. The organization of the lymphoid infiltrate may confer capabilities that fundamentally affect the quality and quantity of the pathological immune response and thus have direct implications in accelerating the breakdown in self-tolerance.

gastric mucosa, and synovial membrane. It is conceivable that the tissue site reacts to inflammatory injury with a unique response program that determines organ specificity for downstream events. It will be interesting to discover which cells in the thyroid provide CXCL13 and CCL21. In rheumatoid synovitis, antibodies to CXCL13 identified synoviocytes and endothelial cells in addition to FDCs in established follicles. This raises the interesting possibility that blood vessels and mesenchymal cells of the synovial membrane contribute to the decision process and, in part, determine the nature of the evolving lymphoid microstructures as lymphocytes invade this tissue site.

LT-$\alpha 1\beta 2^+$ B Cells

The concentration of LT-β sequences in synovial tissue was the strongest predictor for GC reactions.[42] LT-β protein was detected on a small subset of B cells, some in the mantle zone and some in the follicular centers. T cells could also supply LT-β. The critical role of this molecule in the process of ectopic GC reactions immediately raises the question as to which B cells have the potential to express this mediator. Surprisingly, almost all B cells in the peripheral blood express cell surface LT-β (J. J. Goronzy and C. M. Weyand, unpublished data). Therefore, the ability of such B cells to enter the tissue and to continue to produce LT-β becomes a limiting factor. We propose that host differences exist in the expression of LT-β on B cells and the recruitment of such cells to inflammatory sites.

Recruitment of FDCs

Although host heterogeneity for CXCL13 production and homing of LT-β-producing B cells may critically shape the organization of tissue-invading inflammatory cells, additional factors are almost certainly involved. In the absence of CXCL13, CCL21, and LT-β, GCs are not formed; however, the presence of these three mediators is not sufficient to guarantee the successful creation of ectopic GCs.[42] In some instances, high levels of either of the critical chemokines/cytokines are available, yet the infiltrates fail to organize into secondary follicles. Considering the absolute necessity for FDCs to create a functional GC, we propose that host variability exists in recruiting these cells to nonlymphoid tissue sites. Their cellular origin has remained an enigma. The total lack of primary follicles gives evidence that follicular dendritic cells are normally not represented at extranodal sites. Thus, FDCs or their precursors would either need to be recruited or, very unlikely, tissue-residing cells would have to undergo differentiation into FDCs. The hypothesis that the ultimate determinant in allowing for extranodal lymphoid neogenesis is the ability of a host to mobilize FDCs or their precursors and to seed them into nonlymphoid organs is appealing but awaits experimental confirmation.

T Cells

GC reactions that result in the selection of hypermutant immunoglobulins, which give rise to high-affinity IgG antibodies, are absolutely dependent on T-cell help. Antigen-specific T cells encounter antigen on interdigitating dendritic cells in T-cell zones, search for their B-cell partner, and provide helper signals required for B-cell proliferation and differentiation. T cell-B cell communication is facilitated by the CD40-CD40 ligand (CD40L) pathway. Mutations in CD40L have been identified as the underlying defect in patients with hyperIgM syndrome. Such patients cannot generate high-affinity IgG responses and typically lack GC reactions.[57] Observations in mice rendered defective for the major T-cell co-stimulatory molecule, CD28, have also confirmed that secondary follicles can only be generated with intact T-cell help.[58] T-cell help seems even to be required for neoplastic B cells from MALT lymphomas. T-cell co-stimulatory molecules are expressed in low-grade MALT lymphomas *in vivo*, and MALT lymphoma B cells associated with *H. pylori* infection have been reported to require autologous T-cell help.[54]

Evidence has been provided that T-cell help supporting extranodal GCs derives from specialized T-cell subsets. Whereas CD4 T cells regulate proliferation and differentiation of B cells in lymph nodes, GC formation in rheumatoid synovitis has been associated with CD8 T cells. CD8 T cells expressing CD40L have been localized to the perifollicular zone of synovial GCs.[14] Functional studies have established that these $CD8^+CD40L^+$ T cells produce interferon (IFN)-γ and lack the expression of the pore-forming enzyme perforin. Depletion of $CD8^+$ T cells in rheumatoid synovitis, accomplished by treating human synovium-SCID mouse chimeras with T-cell-directed antibodies, abrogates tissue IFN-γ and tumor necrosis factor-α production and also inhibits B-cell activity.[59] Overall, these data suggest a unique role of CD8 T cells in follicular synovitis. Involvement of CD8 T cells instead of CD4 T cells in rheumatoid synovitis would predict that the relevant antigen is not of exogenous origin but derives from an endogenous pool of antigens. Exogenous antigens are internalized by phagocytosis, targeted to lysosomes, digested into oligopeptides, and transported to the cell membrane by HLA class II molecules. CD4 T cells recognize peptide-HLA class II complexes. In contrast, endogenous antigens are degraded in the cytoplasm by the proteasome complex and transported to the rough endoplasmic reticulum where they associate with HLA class I molecules. Peptide-HLA class I complexes stimulate the antigen receptor of $CD8^+$ T cells. The data of Armengol and colleagues[1] would suggest that endogenous antigen, such as thyroglobulin and thyroidal peroxidase, are particularly powerful in driving the formation of GCs. T cells specific for autoantigens may thus be the critical factor in determining whether the host is at risk to develop GCs in nonlymphoid sites.

Ectopic GCs—Taking Autoimmunity into the Fast Line

The work by Armengol and colleagues[1] confirms and extends the critical role of autoantigens in the disease process of autoimmune thyroiditis. They found that most of the intrathyroidal follicles bind thyroglobulin and thyroidal peroxidase. This provides solid support for the horror autotoxicus model, in which one expects pathology whenever forbidden clones escape from surveillance mechanisms and generate immunity against self-antigens. For decades, this model has been in competition with the hypothesis that autoimmunity is not directed against self-components but is ultimately induced by a yet unidentified infectious agent. The commitment of intrathyroidal GCs to thyroglobulin and thyroidal peroxidase would make a microbial antigen an unlikely driver of the pathological immune response unless this response were initiated by an exogenous antigen but targeted to endogenous antigen by a molecular mimicry mechanism.

Considering the sophistication and the functional competence of GCs, it is tempting to speculate that the establishment of these microstructures at ectopic sites, where large amounts of autoantigen are available, is a critical checkpoint in the maintenance and loss of self-tolerance. Zinkernagel and colleagues[60] have forwarded the concept that the initiation of an immune response requires the three-dimensional structures of lymphoid organs, depends on the antigen storage capacity of lymphoid organs, and can only be successfully launched with a critical density of immunocompetent cells.[61] Induction of autoimmunity in animal models by simply providing high numbers of dendritic cells pulsed with antigen support this notion. Establishing lymphoid architecture in the close vicinity of autoantigen production sites would certainly create a new balance of immune responsiveness and tolerance. The intrathyroidal follicles in ATD and the synovial follicles in RA no longer depend on complex transport systems ferrying autoantigens to secondary lymphoid organs; they gain independence from several restrictions and *per se* provide optimal conditions to break self-tolerance.

Not only will ectopic lymphoid microstructures allow for the handling and recognition of autoantigens, they will also optimize the immune response. Through this mechanism, they pose an enormous threat to the host. No longer does the autoimmune response need to work with low-affinity IgM antibodies. Affinity maturation in GCs will guarantee steady improvement in the antibodies and will secure memory for these immune responses. One could not think of a more efficient way to optimize the immune reaction, yet the outcome is harmful and not protective for the host. Outposts of lymphoid organs directly at the site of inflammation may, therefore, not only be a fundamental process in the loss of self-tolerance, but also add a new dimension to the disease process. Considering that the requirements for such a complex process are under genetic control, it is likely that genetic polymorphisms of molecules relevant in lymphoid neogenesis will eventually be identified as shared disease-risk factors in autoimmune syndromes. At a minimum, the process of ectopic GC formation will change the profile of autoantibodies produced and, thus, the phenotype of disease. Understanding the determinants that control the decision to place lymphoid microstructures in nonlymphoid tissue has multiple potentials. Suppressing the process could possibly allow for the treatment of the most severe types of autoimmune syndromes. Determining the genetic polymorphisms and antigenic features involved in the process could lead to the identification of major acceleration factors in autoimmunity.

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