Inducible Tertiary Lymphoid Structures, Autoimmunity, and Exocrine Gland Inflammation in C57BL/6 Mice

Michele Bombardieri, Francesca Barone, Davide Lucchesi, Saba Nayar, Wim B. van den Berg, Gordon Proctor, Christopher D. Buckley and Costantino Pitzalis

J Immunol 2012; 189:3767-3776; Prepublished online 31 August 2012;
doi: 10.4049/jimmunol.1201216
http://www.jimmunol.org/content/189/7/3767

Supplementary Material

http://www.jimmunol.org/content/suppl/2012/09/04/jimmunol.1201216.DC1

References

This article cites 48 articles, 17 of which you can access for free at:
http://www.jimmunol.org/content/189/7/3767.full#ref-list-1

Why The JI? Submit online.

• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Fast Publication! 4 weeks from acceptance to publication

*average

Subscription

Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2012 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Inducible Tertiary Lymphoid Structures, Autoimmunity, and Exocrine Dysfunction in a Novel Model of Salivary Gland Inflammation in C57BL/6 Mice

Michele Bombardieri,*1 Francesca Barone,*7,1 Davide Lucchesi,* Saba Nayar,† Wim B. van den Berg,‡ Gordon Proctor,§ Christopher D. Buckley,† and Costantino Pitzalis*

Salivary glands in patients with Sjögren’s syndrome (SS) develop ectopic lymphoid structures (ELS) characterized by B/T cell compartmentalization, the formation of high endothelial venules, follicular dendritic cell networks, functional B cell activation with expression of activation-induced cytidine deaminase, as well as local differentiation of autoreactive plasma cells. The mechanisms that trigger ELS formation, autoimmunity, and exocrine dysfunction in SS are largely unknown. In this article, we present a novel model of inducible ectopic lymphoid tissue formation, breach of humoral self-tolerance, and salivary hypofunction after delivery of a replication-deficient adenovirus-5 in submandibular glands of C57BL/6 mice through retrograde excretory duct cannulation. In this model, inflammation rapidly and consistently evolves from diffuse infiltration toward the development of SS-like periductal lymphoid aggregates within 2 wk from AdV delivery. These infiltrates progressively acquire ELS features and support functional GL7+ activation-induced cytidine deaminase+ germinal centers. Formation of ELS is preceded by ectopic expression of lymphoid chemokines CXCL13, CCL19, and lymphotoxin-β, and is associated with development of anti-nuclear Abs in up to 75% of mice. Finally, reduction in salivary flow was observed over 3 wk post-AdV infection, consistent with exocrine gland dysfunction as a consequence of the inflammatory response. This novel model has the potential to unravel the cellular and molecular mechanisms that regulate ELS formation and their role in exocrine dysfunction and autoimmunity in SS. The Journal of Immunology, 2012, 189: 3767–3776.

The formation of ectopic lymphoid structures (ELS), defined as aggregates of lymphoid cells forming ectopically in nonlymphoid locations and characterized by B/T cell segregation, differentiation of high endothelial venules (HEV), and development of follicular dendritic cells (FDC) networks supporting a germinal center response, has been observed in chronic inflammatory conditions of both autoimmune and microbial origin (1–3). In Sjögren’s syndrome (SS), a chronic autoimmune disease characterized by circulating high-affinity, class-switched autoantibodies against nuclear Ags and ribonucleoproteins such as Ro/SSA and La/SSB (4), and development of oral and ocular dryness (sicca syndrome) resulting from immune cell infiltration in the exocrine glands (5), salivary ELS develop in 30–40% of patients (6, 7). The development of ELS in SS is thought to be regulated by the ectopic production of lymphoid chemokines CXCL13 and CCL21 (7–9), which physiologically regulate the recirculation and positioning of CXCR5+ and CCR7+ immune cells within secondary lymphoid organs (SLO). Recently, we and others have shown that ELS in SS salivary glands acquire structures typical of germinal centers in SLOs and are capable of supporting the selection and expansion of autoreactive B cell clones as demonstrated by local expression of activation-induced cytidine deaminase (AID) and differentiation of autoreactive plasma cells (10–12). Moreover, prospective data in a large cohort of SS patients provided evidence that the presence of ELS in the salivary glands is an independent predictor of a more aggressive disease phenotype and development of salivary B cell lymphomas (13), suggesting that ELS exert a central pathogenic role in SS and might represent a potential therapeutic target. However, our current understanding of the key cellular and molecular events triggering and regulating the formation of ELS in the salivary glands is inadequate and limited to cross-sectional analysis in SS patients and spontaneous murine models of SS such as NOD mice (14). In addition, it is also unclear why ELS form in some patients but not others and whether these represent discrete disease subsets driven by different triggering agents or different evolutionary stages (15). In this article, we present a new inducible model of salivary denitis developing in the salivary glands of wild-type C57BL/6 animals in response to selective submandibular gland administration of a replication-defective adenovirus 5 (AdV5), which reca-
pilulates several features of SS, such as formation of ELS, ectopic expression of lymphoid chemokines, and functional B cell activation. Importantly, AdV5 infection not only reproduces the phenotypic features of SS, but also functionally leads to the development of humoral autoimmunity to nuclear Ags and decrease in salivary flow. Thus, this novel model offers the unique possibility to dissect the cellular and molecular mechanisms regulating breach of tolerance, autoimmunity, and ELS formation in the salivary glands.

Materials and Methods

Animal collection

All procedures were performed with approval from the local Animal Ethics and Welfare Committee and under a Home Office project license according to Home Office regulations (Local Research Ethics Committee). Young adult female C57BL/6 mice (Harlan Labs, Loughborough, U.K.), aged between 10 and 13 wk at the start of the experiments, were housed under standard conditions.

AdV preparation and intrasalivary gland delivery

Concentrated stocks of E1-E3 replication-deficient human AdV5 encoding for firefly luciferase (LucAdv), generated and characterized as previously described (16) or for bacterial β-galactosidase (LacZAdv, a kind gift of Dr. Gnudi, King’s College London, London, U.K.), were used to produce bulk volume of viral particles via HeK293 cell infection followed by purification using a discontinuous CsCl gradient. AdV were then dialyzed against a solution containing 1 mM MgCl2, 10 mM Tris (pH 7.4), and 150 mM NaCl with 10% glycerol (v/v) overnight on a Side-a-lyzer dialysis cassette (Ambion, Paisley, U.K.) for gene expression profiling. NaCl with 10% glycerol (v/v) overnight on a Side-a-lyzer dialysis cassette (Ambion) for cryosectioning and partly stored in RNA Later (Thatcham, U.K.) for gene expression profiling of ELS-related genes in the salivary glands. Concentrated stocks of E1-E3 replication-deficient human AdV5 encoding for firefly luciferase (LucAdv), generated and characterized as previously described (16) or for bacterial β-galactosidase (LacZAdv, a kind gift of Dr. Gnudi, King’s College London, London, U.K.), were used to produce bulk volume of viral particles via HeK293 cell infection followed by purification using a discontinuous CsCl gradient. AdV were then dialyzed against a solution containing 1 mM MgCl2, 10 mM Tris (pH 7.4), and 150 mM NaCl with 10% glycerol (v/v) overnight on a Side-a-lyzer dialysis cassette (Ambion) for cryosectioning and partly stored in RNA Later (Thatcham, U.K.) for gene expression profiling of ELS-related genes in the salivary glands.

AdV preparation and intrasalivary gland delivery

Concentrated stocks of E1-E3 replication-deficient human AdV5 encoding for firefly luciferase (LucAdv), generated and characterized as previously described (16) or for bacterial β-galactosidase (LacZAdv, a kind gift of Dr. Gnudi, King’s College London, London, U.K.), were used to produce bulk volume of viral particles via HeK293 cell infection followed by purification using a discontinuous CsCl gradient. AdV were then dialyzed against a solution containing 1 mM MgCl2, 10 mM Tris (pH 7.4), and 150 mM NaCl with 10% glycerol (v/v) overnight on a Side-a-lyzer dialysis cassette (Ambion) for cryosectioning and partly stored in RNA Later (Thatcham, U.K.) for gene expression profiling of ELS-related genes in the salivary glands. Concentrated stocks of E1-E3 replication-deficient human AdV5 encoding for firefly luciferase (LucAdv), generated and characterized as previously described (16) or for bacterial β-galactosidase (LacZAdv, a kind gift of Dr. Gnudi, King’s College London, London, U.K.), were used to produce bulk volume of viral particles via HeK293 cell infection followed by purification using a discontinuous CsCl gradient. AdV were then dialyzed against a solution containing 1 mM MgCl2, 10 mM Tris (pH 7.4), and 150 mM NaCl with 10% glycerol (v/v) overnight on a Side-a-lyzer dialysis cassette (Ambion) for cryosectioning and partly stored in RNA Later (Thatcham, U.K.) for gene expression profiling of ELS-related genes in the salivary glands.

Gene expression profiling of ELS-related genes in the salivary glands

Total RNA was extracted from submandibular glands using the RNeasy Mini kit (Qiagen), with on column DNase I digestion to avoid genomic DNA contamination. RT-PCR ThermoScript System for first-strand cDNA synthesis (Invitrogen Life Technologies) was used to produce cDNA from 1 μg total RNA. Primers and probes for quantitative TaqMan real-time evaluation of CXCL13, CCL21, CXCR5, CCR7, lymphotaxin α1β2 (Ltβ1), LtβR, AID, BAFF, IL-4, and IL-21 were obtained from Applied Biosystems (Table II). Samples were run in triplicate at 10 ng cDNA/well, detected using the ABI PRISM 7900HT instrument, and results analyzed using the ABI PRISM 7900HT sequence detection system version 2.1. Relative quantification was assessed using the comparative threshold cycle method with cDNA from vehicle-cannulated glands used as calibrator.

Assessment of salivary exocrine function

Secretory function was assessed in AdV-treated and contralateral submandibular gland under terminal anesthesia (pentobarbitone 75 mg/kg i.p.). Each submandibular duct was exposed by dissection from the ventral surface through the mylohyoid muscle. Individual submandibular ducts were cut, cannulated, and saliva collected for 10 min after onset of secretion after stimulation with pilocarpine (0.5 mg/kg i.p.). After collection into weighed Eppendorf tubes, the tubes were weighed and the volume of saliva calculated (1 μg = 1 μl saliva). Results were expressed as mg saliv/ 10 min/g body weight.

Anti-nuclear Ab detection

Twelve-spot microscope slides (CA Hendley, Essex, U.K.) were seeded with Hep-2 cells at 5 × 104 cells/well and left in culture overnight. When ready, slides were washed in PBS and fixed in acetone. Animal sera were serially diluted in PBS (1:100, 1:160, 1:320), and 15 ml diluted serum was deposited on each well. Serial dilutions of pooled sera from anti-nuclear Ab-negative (ANA+) and positive (ANA−) control patients were used as positive and negative controls. Sample and control sera were incubated for 1 h at room temperature, washed twice in PBS, and incubated with FITC anti-mouse IgG (Sigma, Poole, U.K.) for 30 min in the dark. Slides were washed twice in PBS, mounted, and observed at fluorescence microscope for ANA staining.

Detection of anti-Adv Abs by Western blot

To assess the production of antiviral Abs in sera of AdV-cannulated mice at different time points, LucAdv- or LacZAdv-infected (or untreated) HEK293 cells were lysed in RIPA buffer, and the protein concentration was determined by BCA assay (Pierce, Cramlington, U.K.). A total of 25 μg/lane of the different lysesates was separated by SDS-PAGE on a 4–12% Novex polyacrylamide gel (Invitrogen, Paisley, U.K.) and transferred on
nitrocellulose membrane using an iBlot device (Invitrogen) following manufacturer’s instructions. Membrane strips were blocked with 5% milk in TBST for 4 h at room temperature and then incubated with individual serum from AdV- or vehicle-treated mice at 1:100 in block buffer overnight at 4°C. A rabbit anti-AdV antiserum (Abcam, Cambridge, U.K.) was used as positive control. Membrane strips were then incubated with HRP-conjugated anti-mouse (Sigma, Poole, U.K.) or anti-rabbit (DAKO, Ely, U.K.) IgG Ab, and peroxidase activity was visualized using ECL reagents (Amersham, Amersham, U.K.) following manufacturer’s instructions.

Statistical analysis
Differences in quantitative variables were analyzed by the Mann–Whitney U test when comparing two groups and by the Kruskal–Wallis with Dunn’s posttest when comparing multiple groups. χ2 test with Yates’s correction when required or Fisher’s exact test when appropriate was used to evaluate associations of qualitative variables in the different groups. All the statistical analyses were performed using GraphPad Prism version 5.01 for Windows (GraphPad, San Diego, CA). A p value <0.05 was considered statistically significant.

Results
Dose–response time-course analysis of AdV gene transfer
Delivery of replication-deficient AdV5 in murine submandibular salivary glands via retrocannulation of the excretory duct has previously been described as a suitable and efficient tool for transient local gene transfer (20). We adapted a retrograde cannulation technique that we previously developed in rats (18) using customized glass cannulae, and it proved to be a reliable method for specific delivery of small volumes (up to 50 μl) in the submandibular glands (Fig. 1A, 1B). As shown in Fig. 1C, this technique allows precise delivery and full permeation of the cannulated submandibular gland of C57Bl/6 mice, but not the adjacent sublingual gland or the contralateral gland. Using this approach, we next performed a time-course and dose–response analysis of gene transfer using a replication-defective human AdV5, encoding for the firefly luciferase gene (LucAdV). As shown in Fig. 1D, increasing luciferase activity was observed using increasing doses of LucAdV within the first week pc, which then decreased over the next 2 wk.

Formation of SS-like aggregates in submandibular glands is dependent on AdV dose and lack of viral clearance in ductal epithelial cells
Histological analysis of the cannulated glands demonstrated that AdV doses of 105 PFU/gland, but not vehicle control (or doses <107 PFU, data not shown), induced from week 2 pc the formation of lymphomonocytic infiltrates organized as periductal aggregates, which by week 3 pc strongly resembled lymphocytic foci found in SS patients (Fig. 2A–F).

The formation of lymphoid aggregates was invariably preceded by a rapidly induced diffuse inflammatory infiltrate, which resolved within the first week pc (Fig. 2D, 2G) to be replaced by typical periductal focal infiltration by week 2 pc. Importantly, this phenomenon was independent from the reporter gene used, because it was also triggered by same amounts of LacZAdV (Fig. 2G–I) with no significant difference in terms of prevalence and size of the periductal foci comparing the two viruses. The mean ± SEM of the number of periductal foci developing in the salivary glands after delivery of either LucAdV or LacZAdV at the different time points is reported in Fig. 2J.

The use of the LacZAdV allowed us to investigate the relationship between viral localization and immune cell infiltration at the different time points analyzed. As shown in Fig. 2G–I, staining for bacterial β-galactosidase demonstrated early widespread distribution of the virus within the gland (Fig. 2G). By week 2 pc, the LacZAdV was almost exclusively localized within ductal epithelial cells surrounded by initial inflammatory aggregates (Fig. 2H). Confirmation of selective colocalization of AdV particles within ductal epithelial cells was demonstrated by double immunostaining with an anti-cytokeratin Ab (Supplemental Fig. 1). Although we could not detect residual β-galactosidase staining in ducts surrounded by large aggregates at week 3 pc (Fig. 2I), luciferase activity was still detectable in all infiltrated glands at this time point (Fig. 2K). Overall, in the presence of residual luciferase activity, from week 2 pc onward, 100% of cannulated glands displayed focal periductal aggregates, demonstrating high reproducibility and disease penetrance of our model.

Focal lymphocytic infiltration after AdV delivery displays progressive features of ELS: B/T cell segregation and development of FDC networks
We next evaluated the progressive acquisition of SLO features by the inflammatory foci, such as T/B cell segregation and differentiation of FDC networks. Double-immunofluorescence sequential section analysis of submandibular gland infiltrates was performed using CD3/B220 and B220/FDC-M1 to assess the presence of T/B cell compartmentalization and FDC network formation (19). A list of the Abs used for immunofluorescence is presented in Table I. Initial infiltrates (week 1) were mostly diffuse and characterized by a predominance of T cell infiltration followed by a progressive influx of B cells and the development of highly organized lymphoid structures with T and B lymphocytes localizing in discrete areas (weeks 2–3; Fig. 3A–C). Thus, on AdV delivery, T and B cells serially enter the glands first with a non-segregated pattern and then gradually develop an organized segregated disposition in up to 70% of the aggregates (Fig. 3G). Furthermore, we observed differentiation of FDC networks within
The B cell-rich areas in the context of segregated infiltrates in >60% of the mice at week 3 pc (Fig. 3D–F, 3H).

Ectopic expression of lymphoid chemokines

Production of lymphoid chemokines CXCL13, CCL21, and CCL19 is of pivotal importance in the development of ELS in chronic autoimmune diseases, including SS (2, 15). Induction and secretion of lymphoid chemokines has been shown to rely on the expression of the heterotrimetric Ltβ (21–23). To verify the involvement of these regulators of ELS in our model, we assessed the ectopic expression of CXCL13 and CCL19 and their cognate receptors CXCR5 and CCR7, as well as the Ltβ/LtβR axis in the cannulated salivary glands. A list of the TaqMan probes used for expression analysis is presented in Table II.

As shown in Fig. 4A–F, CXCL13/CXCR5 and CCL19/CCR7 mRNA transcripts were abundantly upregulated in AdV-treated mice, as compared with vehicle-treated mice, and their ectopic expression was consistent with the progressive detection of ELS in the salivary glands, reaching their peak of expression between week 2 and 3 pc. Of relevance, Ltβ, CXCL13/CXCR5, and CCL19/CCR7 mRNA displayed significant upregulation within the first week pc, suggesting very early involvement of these factors in the formation of ELS in this inducible model of lymphoid neogenesis.

To confirm the presence and expression pattern of lymphoid chemokines at a protein level, we used multicolor confocal microscopy to detect CXCL13 or CCL21 together with B220/CD3. As shown in Fig. 4G to 4N, in the context of segregated aggregates, CXCL13 and CCL21 retained their expression pattern within the B and T cell-rich areas, respectively, suggesting a prominent role in directing T/B cell segregation.

Inducible ectopic lymphoid aggregates progressively acquire characteristics of functional germinal centers

We next assessed the presence of features typically associated with germinal center function such as differentiation of germinal center B cells and expression of AID. As shown in Fig. 5, sequential section analysis (Fig. 5A–D) demonstrated that T/B-segregated aggregates characterized by the differentiation of HEV and T cells) and FDCs within the B cell follicle support the activation of numerous GL7+ germinal center B cells.

The appearance of GL7+ germinal centers was strongly associated with functional B cell activation as demonstrated by the detection of high levels of miRNA transcripts for AID (Fig. 5E), the enzyme required for Ig somatic hypermutation (SHM) and class-switch

| Table I. List of primary and secondary Abs used for immunofluorescence |
|-----------------------------|------------------|-------------------|-----------|
| Clone/Catalog Number | Specificity | Host | Source |
| 500A2 | Biotin anti-mouse CD3ε | Hamster | BD Pharmingen |
| RA3-6B2 | PE anti-mouse B220 | Rat | BD Pharmingen |
| MECA-79 | Biotin anti-mouse PNA | Rat | BioLegend |
| AF2125 | Anti-mouse Lyve-1 | Goat | R&D Systems |
| FDC-M1 | Anti-mouse FDC | Goat | BD Pharmingen |
| AF470 | Anti-mouse CXCL13 | Goat | R&D Systems |
| AF457 | Anti-mouse CCL21 | Goat | R&D Systems |
| S-32354 | Streptavidin Alexa 488 | Rabbit | Invitrogen |
| S-32355 | Streptavidin Alexa 555 | Goat | Invitrogen |
| A-11078 | Anti-goat Alexa 488 | Rabbit | Invitrogen |
| A-21434 | Anti-rat Alexa 555 | Goat | Invitrogen |
| A-11034 | Anti-rabbit 488 | Goat | Invitrogen |
| A-11090 | Anti-FITC | Rabbit | Invitrogen |
| A-11055 | Anti-goat Alexa 488 | Donkey | Invitrogen |
recombination (CSR). Of relevance, ectopic induction of AID was also associated with the expression of several cytokines that are known to cooperate in directly promoting AID expression in B cells, such as IL-4, BAFF, and IL-21 (Fig. 5F–H). Overall, these data demonstrate that post-AdV infection, ELS support the development of functional niches of B cells that acquire the machinery to undergo SHM and CSR at these ectopic sites.

Humoral immune response against AdV infection favors breach of tolerance against nuclear self-Ags

In patients with SS, the formation of immune cells infiltrates in the salivary glands is associated with the presence of circulating autoantibodies against nuclear Ags and ribonucleoproteins (4, 24, 25). Thus, we next investigated whether this model of AdV-induced sialoadenitis was associated not only with the formation of anti-AdV Abs but also with breach of self-immunological tolerance and development of ANAs. As expected, sera from AdV-cannulated mice, but not control C57BL/6, progressively displayed a strongly antiviral activity appearing as early as day 5, with humoral responses prevalently directed against proteins of the viral core and the viral capsid (Fig. 6A). The presence and titer of ANAs was determined using Hep2 cells as substrate. We chose an initial serum dilution of 1:80 because this is considered significant in clinical tests for autoimmunity in patients. As shown in Fig. 6B–E, no sera from AdV-cannulated mice within week 1 pc displayed ANA positivity. Conversely, ANA reactivity was detected in 30.7% of mice by week 2 pc, and this percentage increased to 75% in animals culled at week 3 pc. Interestingly, at week 3 pc, the prevalence of ANA was higher in FDC+ versus FDC− mice (87.5 versus 25%). Finally, age/sex-matched vehicle control C57BL/6 mice did not show any reactivity. Taken together, these observations demonstrate that AdV-induced sialoadenitis in nonautoimmune prone mice is associated with the induction of humoral autoimmunity toward nuclear Ags.

AdV infection induces sustained reduction in salivary flow in the affected but not contralateral gland

SS patients are characterized by an excretory dysfunction of the salivary (and lacrimal) glands that leads to mouth (and eye) dryness, also denoted as sicca syndrome. To assess whether AdV delivery induced exocrine dysfunction in the affected gland, we cannulated each mouse with 10^8 PFU AdV in one the submandibular glands and with vehicle control in the contralateral gland. At appropriate time points after cannulation, pilocarpine-stimulated

Table II. List of primers and probes for TaqMan real-time PCR

<table>
<thead>
<tr>
<th>Gene Product</th>
<th>mRNA Accession Number</th>
<th>Assay ID</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse AID</td>
<td>NM_009645</td>
<td>Mm00507774_m1</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Mouse CXCL13</td>
<td>NM_018866</td>
<td>Mm00444533_m1</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Mouse CXCR5</td>
<td>NM_007551</td>
<td>Mm00432086_m1</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Mouse CCL19</td>
<td>NM_011888</td>
<td>Mm00839967_g1</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Mouse CCR7</td>
<td>NM_007719</td>
<td>Mm00432608_m1</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Mouse BAFF</td>
<td>NM_033622</td>
<td>Mm00446347_m1</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Mouse IL-4</td>
<td>NM_021283</td>
<td>Mm00445259_m1</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Mouse IL-21</td>
<td>NM_021782</td>
<td>Mm00517640_g1</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Mouse Ltβ</td>
<td>NM_008518</td>
<td>Mm00434774_g1</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Mouse LtβR</td>
<td>NM_010736</td>
<td>Mm00440235_m1</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Eukaryotic 18S</td>
<td>Cat N 4319413E</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*aAccession numbers are from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).*
salivary flow was measured from each individual gland separately as described in Materials and Methods.

A significant and acute reduction in salivary flow (Fig. 7) was observed within week 1 pc, in line with the histological evidence of diffuse epithelial cell apoptosis up to day 3 pc (Supplemental Fig. 2). Surprisingly, albeit less drastic, at weeks 2 and 3 pc, a significant reduction in saliva production was still evident in AdV-treated mice characterized by the presence of lymphocytic aggregates in salivary glands with an otherwise reconstituted acinar and ductal architecture. This suggests that within the observed period, immune cell infiltration induces a functional impairment that does not allow the full recovery of the excretory function of the glands.

**Discussion**

The formation of ELS in the target organ of several chronic inflammatory conditions, including SS, has long been recognized (1–3, 7, 26–28). ELS formation is highly relevant to SS because germinal center-like structures in the labial salivary glands are independent predictors of increased disease severity and evolution toward B cell lymphomas (6, 13).

The lack of reliable inducible animal models of ectopic lymphoid neogenesis has limited our understanding of the mechanisms leading to the formation of ELS in the salivary glands. Murine models of inducible sialoadenitis with some features of SS in response to systemic or local viral infection have been reported. However, to induce sialoadenitis, previous work relied either on...
the use of fully infectious and replicating sialotropic viruses in autoimmune prone mice such as lpr/lpr mice (29, 30), or replication-deficient AdV vectors harboring genes encoding for proinflammatory mediators such as IL-17 (31). In addition, no comprehensive demonstration that viral-induced sialoadenitis can progress toward the formation of functional ELS and resemble the main physiopathological characteristic of SS (i.e., the formation of autoantibodies) has, to our knowledge, been as yet provided.

In this article, we present a novel model of inducible lymphoid neogenesis that rapidly develops in the submandibular glands of wild-type C57BL/6 mice in response to local delivery of replication-deficient adenoviral vectors. In this model of sialoadenitis, immune cell infiltration rapidly progresses from a diffuse inflammatory process toward the formation of periductal lymphocytic aggregates that further evolve into highly organized ELS within 3 wk from viral infection. This process is reproducible and synchronous with lymphocytic foci developing in 100% of the mice by week 2 pc and evolving by week 3 pc into fully formed ELS characterized in ~70% of the mice by T/B cell segregation and formation of FDC networks with germinal center-like structures with high similarity to the human counterpart (7).

The formation of ELS has been suggested to be the result of a tightly regulated series of immunological and molecular processes with recapitulation of the main pathways involved in lymphoid organogenesis in prenatal life (32). Embryonic lymphoid organ development is critically dependent on lymphoid chemokines CXCL13, CCL19, and CCL21, which are produced by VCAM-1^ICAM-1^LTβR^ mesenchymal “organizer” cells in response to close interaction with CD3^CD4^CD45^IL-7Ra^RANK^ lymphoid “inducer” cells expressing the membrane-bound heterotrimeric (α1β2) member of TNF family, LTβ (32). The establishment of a positive feedback loop between lymphoid chemokines and LTβ is critical in promoting the development of the stromal and vascular architecture of SLOs, including the differentiation of HEVs (21).
However, evidence from transgenic models of ELS has questioned the relevance of lymphoid inducer cells in ectopic lymphoid neogenesis in adult life (33). Similarly, lymphoid chemokine expression in patients with ELS developing during chronic inflammatory diseases, including SS (7–9, 11, 34), suggests that the lymphoid chemokines/Ltβ pathway in inducible ELS formation is regulated by cellular and molecular mechanisms that are different from the conventional interactions observed during embryonic development.

In this study, we showed that post-AdV delivery, early upregulation of lymphoid chemokines CXCL13, CCL19, and CCL21 mRNA precedes the development of ELS and peaks in concomitance with fully functional ELS. Of interest, lymphoid chemokines retained their discrete expression pattern observed in SLOs, with CXCL13 confined to the B cell-rich areas of the periductal aggregates and CCL21 in the surrounding T cell area. This suggests that the progressive recruitment and positioning of T and B cells subsets post-AdV infection in the salivary glands are not random events but follow tightly regulated chemoattractive gradients and establish this inducible model of ELS as a suitable platform to dissect the dynamic expression, source of production, and hierarchical importance of the critical factors regulating ectopic lymphoid neogenesis.

Interestingly, we also found that in this model, formation of ELS was invariably associated with the persistence of the transgene.
product within the salivary glands 3 wk after AdV delivery, despite viral inability to replicate. Strikingly, prolonged viral and transgene protein expression was selectively observed within ductal epithelial cells, an observation in keeping with the inability of this cell type to clear viral pathogens as described both in rodent and human salivary glands (35, 36). This is relevant for SS because several viruses have been implicated in SS pathogenesis, and a type-I IFN signature is typical of the disease (37, 38). Thus, in this model, induction of ELS likely derives from lack of viral clearance resulting in prolonged antigenic exposure, which is a potent trigger for ELS formation, as previously suggested in other models of ectopic lymphoid neogenesis such as chronic graft rejection (39) and autoimmune insulitis in NOD mice (19). Thus, we believe that this model has the potential to unravel the intimate physiopathological viral–host interactions that lead to the formation of ELS in SS.

Importantly, in this work, we have also demonstrated that AdV-induced ELS not only recapitulate the cellular and molecular organization of SLOs but also support functional B cell activation with expression of AID, the enzyme that initiates SHM and CSR of the Ig genes (40), leading to affinity maturation and differentiation of memory B and plasma cells (41, 42). Expression of AID in the salivary glands required the formation of ELS acquiring markers of germinal centers such as FDC networks and GL7, similarly to SS patients (10). AID expression and subsequent CSR in naïve B cells can be directly induced by a milieu of cytokines, among which BAFF, IL-4, IL-10, and IL-21, even in the absence of B cell receptor cross-linking (43–47). Accordingly, in this model, AID transcript levels were strictly associated with the expression of BAFF, IL-4, and IL-21 mRNA, suggesting a functional role for these factors in downstream B cell activation.

Because the architectural, cellular, and molecular features of ELS in this model were highly reminiscent of those observed in SS, we next investigated whether typical features of the human disease were also present in this model. Two hallmarks of SS are the breach of self-immunological tolerance toward nuclear Ags with circulating autoantibodies and a progressive exocrine dys- function that results in the classical signs and symptoms of mouth/eye dryness (sicca syndrome). At week 3 pc, 75% of the AdV-cannulated mice developed positivity for IgG ANA; in comparison, none of the vehicle controls displayed evidence of autoimmunity. Interestingly, onset of ANA was preceded by the rapid and abundant induction of epithelial cell apoptosis post-AdV infection, as demonstrated by the presence of pyknotic nuclei and positive TUNEL staining (Supplemental Fig. 2). It is likely that exposure of nuclear material in the context of the ectopic formation of B cell follicles in the salivary glands would be sufficient to trigger autoimmunity. In this regard, it will be interesting to dissect whether ELS are directly involved in the generation of autoantibodies over and above draining lymph nodes, as previously demonstrated for ELS in patients with SS (11, 12).

Finally, we showed that the development of sialoadenitis and ELS is also accompanied by exocrine dysfunction. Although we did not elucidate the pathophysiology of hyposecretion in this model, which might be caused by viral and/or immune-mediated mechanisms, our observations would be in keeping with indications in patients with SS that exocrinopathy in the context of focal sialoadenitis is primarily due to a functional rather than anatomical impairment (48).

In conclusion, we present a novel model of AdV-induced lymphoid neogenesis that has the potential to unravel the critical interactions between viral infection, formation of ELS, breach of immunological tolerance, and exocrine dysfunction in the salivary glands, providing a suitable platform to investigate the mechanisms regulating ELS formation and their role in the development of features typical of SS.

Acknowledgments

We thank Dr. Luigi Gnudi (Professor of Diabetes and Metabolic Medicine, Unit for Metabolic Medicine, Cardiovascular Division, King’s College London) for proving the LacZAdV.

Disclosures

The authors have no financial conflicts of interest.

References


