Published in final edited form as:

Biotechnol Bioeng. 2011 June ; 108(6): 1430–1440. doi:10.1002/bit.23055.

Long-term immunologically competent human peripheral lymphoid tissue cultures in a 3D bioreactor

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Abstract

Peripheral lymphoid organs (PLOs), the primary sites of development of adaptive immune responses, display a complex structural organization reflecting separation of cellular subsets (e.g. T and B lymphocytes) and functional compartments which is critical for immune function. The generation of *in vitro* culture systems capable of recapitulating salient features of PLOs for experimental, biotechnological and clinical applications would be highly desirable, but has been hampered so far by the complexity of these systems. We have previously developed a three-dimensional bioreactor system for long-term, functional culture of human bone marrow cells on macroporous microspheres in a packed-bed bioreactor with frequent medium change. Here we adapt the same system for culture of human primary cells from PLOs (tonsil) in the absence of specific exogenous growth factors or activators. Cells in this system displayed higher viability over several weeks, and maintain population diversity and cell surface markers largely comparable to primary cells. Light microscopy showed cells organizing in large diverse clusters within the scaffold pores and presence of B cell-enriched areas. Strikingly, these cultures generated a significant number of antibody-producing B cells when challenged with a panel of diverse antigens, as expected from a lymphoid tissue. Thus the three-dimensional tonsil bioreactor culture system may serve as a useful model of PLOs by recapitulating their structural organization and function *ex vivo*.

Keywords

bioreactor; lymph node; lymphocytes; tissue engineering; antibodies

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Introduction

Peripheral lymphoid organs, including the spleen, lymph nodes (LNs), Peyer's patches and other types of organ-associated lymphoid tissue are the primary sites for the development of adaptive immune responses in mammals (Goodnow 1997; Junt et al. 2008; Randall et al. 2008). Despite organ-specific differences, their overall anatomical and histological organization share a number of key common features, most prominently the subdivision in specialized areas, including separate regions enriched for B or T lymphocytes harboring a number of distinct zone- and sometimes organ-specific types of stromal and immune accessory cells (such as specialized subsets of macrophages, as well as dendritic, follicular dendritic and stromal cells) (Drayton et al. 2006; Goodnow 1997; Junt et al. 2008). Because of this complexity, secondary lymphoid organ development is a tightly orchestrated process depending on a cascade of cytokines and chemotactic factors (Drayton et al. 2006; Randall et al. 2008).

This complex architecture reflects critical requirements for the development of immune responses, including the regulated access of antigen to immune effectors via professional antigen-presenting cells (APCs), the selective homing and egress of circulating cell populations via specialized blood and lymphatic vessels, and the orchestrated migration of cells within the organs themselves during the development of the immune response (Okada and Cyster 2006; Phan et al. 2009). Underscoring the importance of peripheral lymphoid organs and their architecture in immune function is the frequent association of defects in lymphoid tissue formation and organization with various degrees of immune deficiency of both genetic and infectious origin (e.g. lymph node alterations accompanying the progression of HIV infection) (Fauci et al. 1996; Junt et al. 2008; Junt et al. 2006; Karrer et al. 1997; Kursar et al. 2005; Pantaleo et al. 1993; van Grevenynghe et al. 2008).

Functional studies of peripheral lymphoid tissue organization and function in humans are understandably limited by the lack of suitable experimental systems, and would greatly benefit by the development of artificial culture methods capable of replicating basic features of lymphoid organs. Such system should display several features. First, it should reproducibly maintain diverse populations of human peripheral lymphoid organ cells with viability comparable to the intrinsic life spans of the relevant cell types, in the absence of non-physiological concentrations of exogenous activators and costly cytokines/growth factors. Second, the cultured cells should closely resemble their *in vivo*, fresh counterparts without aberrant and non-physiologic phenotypes or functional properties. Third, the culture system should display architectural features typical of lymphoid organs that are critical for individual applications. Finally, such lymphoid organ cultures should be immunologically competent and replicate key immunological activities of natural lymphoid tissue.

Although conventional flask culture systems, primarily from blood-derived mononuclear cells or bone marrow, have been extensively used to study human lymphocyte development, cell biology and immunology, two-dimensional cultures are intrinsically deficient in reprising the three-dimensional organization of lymphoid tissues (Tan and Watanabe 2010). For these reasons, significant efforts have been spent in recent years to develop suitable 3D culture systems (Hitchcock and Niklason 2008; Tan and Watanabe 2010). Among these advances, a peripheral blood mononuclear cell-based perfusion bioreactor system was developed by Giese and coworkers, in which dendritic cell differentiation/expansion can be detected after an 11-day protocol treatment with GM-CSF, IL4 and TNF α (Giese et al. 2006). The same system was shown most recently to display enhanced cytokine secretion and generation of plasma cell-like components upon administration of cytokines and activators, and/or supplementation with separately generated mature activated dendritic cells (Giese et al. 2010; Hitchcock and Niklason 2008). Similarly, a "peripheral tissue equivalent"

model can be used to generate activated dendritic cells from peripheral blood precursors which can induce antibody responses from autologous B and T cells *in vitro* (Ma et al. 2010; Randolph et al. 1998). Other studies have focused on the engineering of lymphoid tissue for transplantation purposes, including functional artificial murine lymph node organoids generated by the implantation of stromal cell-seeded scaffolds under the kidney capsule of recipient mice (Okamoto et al. 2007; Suematsu and Watanabe 2004), and a transplantable tissue engineered spleen system in rats (Grikscheit et al. 2008). The success of these transplant systems clearly show that functional peripheral lymphoid tissue can be generated using bioengineering strategies and have highlighted requirements for tissue organization and cell repopulation, but their immediate applicability to *in vitro* culture of human functional lymphoid tissue remains undetermined.

A human *in vitro* artificial lymph node system would have significant biotechnological potential, from the screening of vaccine and immunomodulatory drug candidates to the generation of fully human monoclonal antibodies. Furthermore, long-term culture of lymphoid biopsy samples from individual could be used in the clinic to assist in diagnosis or prognosis, or to predictively evaluate responses to different drug regimens for personalized therapy.

We previously described a three-dimensional bioreactor system employing macroporous microspheres as scaffolds for cell attachment and growth. The 3D culture system is capable of supporting long-term, multilineal *in vitro* hematopoiesis by both human and murine bone marrow samples (Mantalaris et al. 2004; Mantalaris et al. 1998; Panoskaltsis et al. 2005; Sun et al. 2009; Wang et al. 1995). Here we show that this system can be adapted to the culture of human tonsil cells. In contrast to the traditional flask or dish culture, the 3D human tonsil culture system maintains many salient features of lymphoid organs, including immunological competence.

Materials and Methods

Bioreactor design and construction

The bioreactor was fabricated using polycarbonate plates as described previously [Mantalaris, 2004]. Briefly, the bioreactor consists of two chambers, the upper medium chamber and the lower culture chamber. The culture chamber (5 mm *H* × 8 mm *W* × 8 mm *L*) was packed with 0.01 g of the highly porous microcarriers. Cellsnow™-EX, type L (low ion-charged), macroporous cellulose microcarriers (Kirin, Japan; 1–2 mm diameter; 100–200 μm pore size; 95% porosity) were used throughout the experiment. The packed-bed of microcarriers in the culture chamber was overlaid with culture medium. The medium chamber (13 mm *H* × 8 mm *W* × 19 mm *L*) contained 0.6 ml medium. A Teflon™ membrane (50 μm thickness) was fabricated into the bottom of the culture chamber to facilitate gas exchange.

Tissue preparation

Human tonsil tissue samples were discarded pathology material from routine tonsillectomies with general lack of significant pathology. All human subject studies were reviewed and approved by the University of Rochester IRB. First, tonsil tissues were mechanically disrupted and digested with collagenase type IV (2 mg/mL) (Worthington, Lakewood, NJ) and DNase (1 mg/mL) (Sigma, St. Louis MO) for 1 hr at 37°C. Then red blood cells were lysed by incubation in hypotonic lysing solution (0.15M NH₄Cl, 10mM KHCO₃, 0.1mM EDTA, pH7.4) (5 min at room temperature) and live cells were separated from tissue debris by filtration through a cell strainer (BD Falcon). Resulted single cell suspension was spun down and resuspended in OPTI-MEM medium (Gibco Invitrogen) supplemented with 5%

NCTC 109 (Gibco Invitrogen), 7.5% human AB serum (Sigma Aldrich), 50 μ M β -mercaptoethanol, penicillin 100U/ml, streptomycin 100 μ g/ml and amphotericin B 0.5 μ g/ml (“complete OPTI-MEM”). A portion of prepared cells was immediately aliquoted, viably frozen in medium supplemented with 10% DMSO/40% fetal calf serum and used later as a control for flow cytometric analysis.

Culture conditions

To seed bioreactors, tonsil cells (10^7 cells/100 μ L of complete OPTI-MEM) were mixed with porous cellulose microspheres in the culture chamber of the bioreactor, incubated for 2 hr at 37°C, and then 600 μ L of complete OPTI-MEM were added to the medium chamber. Medium in the medium chamber was replaced daily with fresh warm complete OPTI-MEM throughout the entire culture period. Bioreactors were incubated at 37°C in humidified atmosphere with 5% CO₂. At the end of the culture, the medium from the bioreactor was removed and the cells were dislodged from microcarriers by washing the culture chamber with cold 2mM EDTA in PBS (phosphate buffered saline) and harvested. This method, while yielding a less complete harvest than other approaches (e.g. collagenase digestion of microcarriers), minimizes damage to cells and, in pilot experiment comparisons with collagenase digestion, does not introduce any bias in cell population recovery based on flow cytometry profiles.

Flow cytometry

Surface phenotyping was performed on cell suspensions (for bioreactor cultures, the entire well content was harvested, and cells from 2-3 bioreactors were pooled) by staining with the fluorochrome-conjugated mouse monoclonal antibodies following standard procedure. Briefly, cells were incubated with the antibody mixture containing 5% mouse normal serum (Jackson ImmunoResearch) for 20 min on ice, washed, incubated with LIVE/DEAD Fixable Aqua Dead Cell Stain kit (Molecular Probes) for additional 20 min on ice, washed again and fixed in 1% paraformaldehyde/PBS. In some cases, 7-AAD (BD Biosciences) was used instead LIVE/DEAD Fixable Aqua Dead Cell Stain kit. Except where noted, all antibodies were obtained from BD Biosciences (San Diego, CA). The antibody used for this study included CD3-PerCp-Cy5.5 (cat# 552852), CD4-PE (cat# 347327), CD4-APC-Alexa750 (cat#270049 eBioscience), CD45RA-FITC (cat# 555488), CD8-PE-Cy5 (cat# 555636), CD45RO-APC (cat# 559865), CD3-PE-Cy5.5 (cat# MHCD0318, Caltag), CD16-PerCp-Cy5.5 (cat# 338433), CD19-PerCp-Cy5.5 (cat# 340950), CD19-APC-Cy7 (cat# 557791), CD34-PerCp-Cy5.5 (cat# 347213), CD14-Alexa700 (cat# 557923), CD11c-PE-Cy7 (cat# 301608 Biolegend), CD303-APC (cat# 130-090-905 Miltenyi), CD27-APC (cat# 17-0279 eBioscience), CD38-Pacific Blue (BD custom conjugate), and IgD-FITC (cat# 555778). Samples were run on Becton Dickinson LSR II 11-colors or FACSCalibur flow cytometers and analyzed using FlowJo version 8.8.4 software (Tree Star, Ashland, OR). To ensure consistency of gating profiles longitudinally over several weeks, for each set of experiments the analysis gates were drawn based on the staining pattern of newly thawed frozen tonsil cells from the original sample, stained and run as reference in parallel with the cultured samples.

Histology and immunohistochemistry

Paraffin Embedding and H&E staining—The medium in the bioreactor with unstimulated tonsil cells (day 9 of culture) was replaced with warm (37°C) 2% low melting agarose/PBS. After 30 min of incubation at 37°C, the bioreactor was placed on ice to solidify agarose, and agarose plug was fixed in 3.7% neutral-buffered formaldehyde/PBS at 4 °C over night, dehydrated in graded ethanol solutions, immersed in xylene, and embedded

in paraffin. Paraffin sections (5- μ m thick) were stained with hematoxylin and eosin using standard protocol.

Gelatin Embedding and IHC staining—Gelatin embedding was done according to a protocol previously described by Brown and coworkers (Brown et al. 2005) slightly modified as follows. The medium in the bioreactor with unstimulated tonsil cells (day 9 of culture) was replaced with warm (37°C) 10% gelatin/PBS. After 30 min of incubation at 37°C, the bioreactor was placed on ice to solidify gelatin. Gelatin plug was fixed in 3.7% neutral-buffered formaldehyde/PBS at 4°C overnight, cryopreserved by incubation in 5% sucrose at 4 °C overnight and snap frozen in liquid nitrogen.

For immunohistochemistry (IHC) staining, cryostat sections (10- μ m thick) were mounted on Superfrost Plus glass slides, air dried for 1 hr at room temperature and stained with antibodies against human CD19 and CD3 using LSAB2 kit (cat# K0673, DakoCytomation, Carpinteria, CA) following manufacture's protocol. First, sections were sequentially treated with mouse anti-human CD19 (cat# 555415, BD Pharmingen, San Jose, CA), biotinylated goat anti-mouse antibody, streptavidin-horse radish peroxide (HRP) and DAB substrate-chromogen solution. Then, the same sections were sequentially treated with polyclonal rabbit anti-human CD3 (cat# N1580, DakoCytomation), biotinylated goat anti-rabbit antibody, streptavidin-alkali phosphatase (AP) (cat# D0396, DakoCytomation) and AP substrate (Fuchsin - cat# K0624, DakoCytomation). Stained sections were mounted in Permount permanent mounting medium (cat# SP15-100, Fisher Scientific, Pittsburgh, PA) and visualized using Olympus BX-40 microscope and digital image analysis software Image-Pro Plus, version 4.0 (Media Cybernetics, Silver Spring, MD).

Immunization of bioreactor cultures and detection of antibody-secreting cells

Immunization was carried out by addition of antigen (1 ng/mL) plus LPS from *Salmonella typhimurium* (1 μ g/mL)(cat# L7261, Sigma, St. Louis, MO) to culture medium on week 1, 2 and 3. Each time antigen/LPS were present in culture medium for 2 days and then replaced with medium without stimuli. Antigens used in this study included recombinant protein HIV-1 gp160MN (cat# 2000MN, Protein Sciences, Meriden, CT), recombinant hemagglutinin (HA) from H3N2 and H1N1 influenza viruses (cat# IT-003-0041 Δ TMp and IT-003-SW1 Δ TMp, Immune Technology, New York, NY), keyhole limpet hemocyanin (KLH) (cat#374805, Calbiochem, La Jolla, CA), and tetanus toxin (TT) peptide (cat#8750-1530, AbD Serotec, Oxford, UK).

Three days after last antigen stimulation cells were harvested as described above and frequency of total and antigen-specific IgM and IgG producing cells was determined by ELISPOT following standard protocol. Briefly, ELISPOT plates (cat#MSIPN4W50, Millipore, Billerica, MA) were coated either with antigen or unlabeled antibodies specific to human IgM or IgG (cat#AHI0601 and AHI0301, Biosource, Camarillo, CA) (3-10 μ g/mL in PBS) overnight at 4°C. Then wells were filled with OPTI-MEM medium supplemented with 7.5% fetal calf serum for 1 hr at 37°C to reduce non-specific binding, and after that harvested cells were added to the wells in OPTI-MEM medium supplemented with NCTC109, 7.5% fetal calf serum, β -mercaptoethanol, and antibiotics. After overnight incubation at 37°C plates were washed with 0.2% Tween 20/PBS, and sequentially treated with AP-conjugated goat anti-human IgM (cat#AHI0605, Biosource, Camarillo, CA) or goat anti-human IgG (cat#A80-104AP, Bethyl Laboratories, Montgomery, TX) for 1 hr at 37°C and Vector Blue AP substrate kit III (cat#SK-5300, Vector Laboratories, Burlingame, CA) following manufacture's protocol. Plates were scanned and analyzed using ImmunoSpot Analyzer plate reader (CTL, Shaker Heights, OH).

Statistical analysis

Statistical comparisons between culture cell subpopulations were conducted by Student's T-test. Analysis of differences in cell viability curves in 3D bioreactor vs. 2D cultures was carried out using the generalized estimation equation (GEE) methods (Liang and Zeger 1986) to study the effect of time (week) and group (2D vs 3D) on the % of live cell of each subset (CD19⁺, CD3⁺, and CD3⁻19⁻). All analyses were implemented in SAS® 9.1 (SAS Institute Inc, Cary, NC). The significance level α was set at 0.05.

Results

3D bioreactor design and culture conditions

The packed-bed, batch-fed 3D bioreactor system consists of a culture chamber topped by a medium chamber as described in the Materials and Methods section. The culture chamber was packed with macroporous microspheres for cells to lodge and form a three-dimensional, tissue-like structure. Initial testing revealed that a seeding density of $5\text{--}20 \times 10^6$ human tonsil cells per bioreactor and the presence of at least 5% human AB serum (7.5% was used in our experiments) in the medium are critical for the consistent performance of the culture.

Extended survival of B lymphocytes in the 3D bioreactor system

We first assessed the ability of 3D bioreactor culture system to support long-term maintenance of B and T lymphocytes compared to the conventional 2D T-flask or dish culture. Figure 1 shows representative examples and summary results of survival of CD19⁺ (B cells) CD3⁺ (T cells), and CD19⁻/CD3⁻ non-B/non-T cells in 2D vs. 3D cultures. Viability was quantified based on dye exclusion using the LIVE/DEAD stain kit as described in Materials and Methods. In conventional 2D T-flask cultures, peripheral T cells maintain reasonably good viability *in vitro*, while resting B cells undergo more extensive cell death within days ($58 \pm 4\%$ CD3⁺ T cells alive at week 2, vs $8 \pm 6\%$ CD19⁺ B cells, Fig. 1b,c). In sharp contrast, in the 3D bioreactor system extended viability was observed for B lymphocytes, with $28 \pm 14\%$ live B cells at week 2 and $22 \pm 9\%$ at week 5 ($49 \pm 9\%$ live at week 2, and $20 \pm 14\%$ live at week 5 for T cells) (Fig. 1b,c). Non-B/non-T cells (CD3⁻/CD19⁻) showed a marked initial decline in viability, after which the fraction of live cells in culture remained relatively constant, with a slightly better viability for cells in 3D cultures vs 2D cultures between weeks 1 to 4 (Fig. 1c). Over the entire course of the experiments, the differences in viability in 3D bioreactors vs 2D cultures were highly significant for B cells ($p < 0.0001$, GEE method), and marginally significant for non-B/non-T cells ($p = 0.04$, GEE method), while the differences for T cells was not significant ($p = 0.21$, GEE method). To quantify cell recoveries, cell yields harvested at week 5 in 2D vs 3D bioreactor cultures were also compared. 3D bioreactors yielded significantly more CD19⁺ B cells than conventional cultures ($6 \pm 3\%$ of initially loaded cells in 3D vs $0.4 \pm 0.2\%$ in 2D, $p < 0.002$), while yields of CD3⁺ T cells ($7 \pm 3\%$ in 3D vs $6 \pm 4\%$ in 2D) and non-B/non-T cells ($4 \pm 2\%$ in 3D vs. $8 \pm 4\%$ in 2D) were not different in the two systems. Note however that because of the bioreactor and scaffold design, and harvesting method (cold PBS/EDTA rinses, to minimize cell manipulation for later procedures), values from the 3D cultures likely represent an underestimate of the total cells in the system.

Phenotypes of 3D culture cells

We further characterized the cells in 3D bioreactor culture based on surface expression of key lymphocyte markers using flow cytometry. T cells of both major subsets, CD4⁺ and CD8⁺ are maintained, although a slight skewing for the former subset is observed (Fig. 2a). T cells of both CD45RA⁺ and CD45RO⁺ phenotypes are observed even late in the cultures

(Fig.2a), suggesting persistence of both naïve and antigen-experienced populations, respectively.

Dendritic cells were phenotyped based on their lack of expression of the lineage markers CD3, CD16, CD19 and CD34, and further gated from CD14⁺ monocytes into the two subsets CD14⁻CD4⁺CD11c⁻CD303⁺ (plasmacytoid DCs) and CD14⁻CD4⁺CD11c⁺CD303⁻ (myeloid DCs). While dendritic cells were maintained overall, preferential survival of myeloid dendritic cells was observed, while plasmacytoid dendritic cells rapidly disappeared from culture (Fig.2b). Whether this is related to intrinsic life span properties of different resident dendritic cell populations or to the culture conditions remains to be determined.

CD19⁺ B cell subsets were evaluated based on differential expression of the CD38, IgD and CD27 markers, which have been shown to differentiate at least 5 major B cell populations in the human tonsil (Bohnhorst et al. 2001; Sanz et al. 2008): naïve B cells (IgD⁺CD38⁻CD27⁻), pre-germinal center (GC) B cells (IgD⁺CD38^{low}), GC B cells (IgD⁻CD27⁺CD38⁺), plasmablasts/plasma cells (PB/PC) (IgD⁻CD38^{high}CD27^{high}) and memory B cells (mainly IgD⁻CD27⁺CD38⁻) (Fig.3). Our data show that GC B cells are relatively short lived in 3D culture, decreasing from 20±7% at day 0 to 3±1% at week 2 (p=0.01, 2-tailed pairwise T-test). This is expected because these cells have short life spans *in vivo* and are critically dependent on continuing interactions within the GC structure that are largely disrupted during tissue preparation for seeding. CD27⁺ IgD⁻ memory cells are well maintained within the cultures, and in fact tend to accumulate over time, from 7±3 % at day 0 to 19±9% at week 2 (p=0.03, 2-tailed pairwise T-test) and 27±21% at weeks 5/6 (p=0.04, 2-tailed pairwise T-test). Whether this is due to preferred survival or differentiation of cells into memory from other compartments remains to be established. Most importantly, however, IgD⁺ naïve B cells are preserved in high numbers through the culture, from 47±7% at week 0 to 43±18% at weeks 5/6.

Altogether, these data show that 3D bioreactor cultures allow survival of diverse populations of B and T lymphocytes including, critically, naïve resting subsets, over long periods of time, and that these cells maintain surface phenotypes comparable to those of their *in vivo* counterparts.

Self-organization of tonsil cells within 3D bioreactors

We next investigated how cells seeded onto 3D bioreactors distribute themselves over the scaffold. Hematoxylin-eosin stain showed that the cells in 3D cultures are not randomly distributed, but mostly form isolated, recognizable highly packed clusters of live cells, numbering from few hundreds to likely several thousands (Fig.4a-c). These clusters harbor heterogeneous populations, including cells with lymphocyte or myeloid morphology, cells with fine dendritic structure as well as large cells with stroma-like morphology (Fig.4c).

Immunohistochemistry with antibodies to CD19 and CD3 revealed that B cells are predominantly found in small to medium size clusters, often found at the periphery of the larger cell aggregates, while T cells are dispersed, and found mainly within the main body of the aggregates (Fig.4d-f).

Remarkably, this non-random distribution is reminiscent, although in much simplified form, of the general organization of most peripheral lymphoid organs, in which B cells and T cells are segregated and B cell follicles usually appear in a cortical/exterior position compared to the T cell-rich zones, as well as of the more rudimentary tertiary lymphoid structures which can form within inflamed tissues. Thus, cells in 3D cultures can to a significant extent self-organize within the bioreactor scaffold along the lines of their *in vivo* counterparts.

In vitro immune responses to antigens in the 3D bioreactor system

As the bioreactor cultures display several of the features of lymphoid tissues, such as the presence of diverse populations, including naïve and antigen-experienced B and T cells, with well-preserved phenotypes and a degree of histological order, we tested the possibility that the cultures may indeed be immunologically functional. To this end, we supplemented the cultures with antigens and LPS as an immune adjuvant. The Elispot assay was used to measure immune responsiveness. Alternative administration patterns, combinations and concentrations of antigens and adjuvants were tested empirically to determine optimal conditions. A range of antigens were chosen, including likely recall antigens such as tetanus toxoid C fragment and H3N2 influenza virus hemagglutinin, and presumptive neo-antigens, such as keyhole limpet hemocyanin, H1N1 influenza virus hemagglutinin and gp160 of HIV. Although it was not known whether the patients had been previously exposed to any of the antigens tested, this panel of antigens should provide an assessment of the range of immune responses inducible in the 3D bioreactor system, and their response patterns.

Figure 5 shows a set of typical Elispot results obtained with different antigens. As internal negative controls for non-specific activation and background, cells from cultures immunized with a different antigen from the one used for plate coating were used. Thus, higher numbers of IgM or IgG antibody-secreting cells in the specific antigen wells should reflect actual immunization-dependent enrichment for and differentiation of antibody-secreting cells. Cells were pooled from 3 immunized bioreactor wells before counting and plating in each test well in duplicate. Strikingly, a significant number of cultures displayed detectable, and in many case vigorous antigen-specific immune responses after immunization. While in most cases both IgG- and IgM-secreting cells were detected (e.g. Fig.5b), in others primarily IgM or IgG secretion was observed (Fig.5a,c).

Overall, anywhere between 20-50% of tested individual donors displayed detectable responses by Elispot (defined as >2-fold enrichment of antigen-secreting cells over irrelevant antigen after background subtraction) in their tonsil cultures. Figure 6 shows a summary of all the immunization experiments conducted. Note that in most cases, antigens can elicit both IgM and IgG responses, the only significant deviation being observed for the H1N1-HA “swine flu” antigen, which seems to induce IgM responses more often than IgG.

Thus, the 3D bioreactor system appears to be remarkably capable of supporting the differentiation of detectable numbers of antibody-secreting cells in an antigen-specific manner, indicating that the cells in the system are immunologically competent.

Discussion

The generation of an in vitro system capable of replicating key properties of human peripheral lymphoid organ function would represent a significant advance in tissue engineering aimed at providing a unique tool for experimental immunology, as well as biotechnological and clinical applications. Here we show that a 3D bioreactor system with a relatively simple design can support the maintenance of functional primary human tonsil cell populations. Specifically, we have shown that 3D bioreactor tonsil cultures are able to: a) maintain diverse populations of viable cells for several weeks without exogenous activators or specific growth factors (other than human serum); b) harbor cell populations with phenotypes largely comparable to their primary ex vivo counterparts, including naïve and memory B and T lymphocytes; c) support cellular organization in large, diverse cell aggregates with identifiable separation of B-rich vs T-rich areas; and finally d) can generate antigen-specific antibody-secreting cell populations upon stimulation with a number of antigens tested and addition of adjuvant.

Several key aspects of using human tonsil cells for constructing ex vivo lymphoid tissue models should be considered. First, tonsils from routine tonsillectomies represent the most easily accessible source of human peripheral lymphoid tissue. There is little regulatory concern for using these surgically removed samples that are otherwise discarded. However, some caveats should be considered to interpret results from tonsil cultures. For instance, young children under the age of five, which may have immature immune systems, are overrepresented among tonsillectomy patients (>70% in our samples). Another concern is that tonsillectomy samples may include chronically stimulated, inflamed tonsils not fully representative of normal lymphoid organs. However, this concern is lessened because comparison between tonsillectomy samples and tonsil biopsy material from healthy subjects has not revealed major differences in cell subsets and phenotypes (J. Anolik, personal communication). Finally, their anatomical location and the post-surgical handling of the specimens render these samples susceptible to harboring microorganisms which may lead to culture contamination. Possible alternatives to tonsils are lymphoid tissues from splenectomies and surgical lymph node removal procedures, or from organ donors. These samples are obviously not as easily available and hence have not been tested in our system.

The second important set of considerations relates to the culture conditions. In our empirical testing, we found that cultures do not survive in the absence of human serum, despite the addition of serum-free media such as NCTC109. Thus, human AB serum was added as a critical medium additive. For both standardization purposes and for a number of applications (e.g. ELISA detection of antibodies which may have detectable pre-existing titers in human serum), it will be valuable in the future to identify the components in human serum that are key to bioreactor culture maintenance in order to develop serum-free media. Differential physico-chemical conditions provided by the 3D bioreactor culture system over the conventional 2D culture may also account for better cell viability in the 3D culture (Fig. 1). The 3D culture system is subject to diffusion limitation, leading to the formation of chemical gradients, such as those of oxygen and CO₂, as well as other nutrients and metabolic wastes. Diffusion limitation in the 3D culture system thus creates heterogeneous microenvironments or “niches” for various cell populations normally found in the tonsil. In addition, the 3D scaffold prevents cell flattening at the culture surface and is conducive to cell-cell interactions in multiple dimensions. Thus the tissue formation in the 3D bioreactor culture system is a fairly complex process as the chemical gradient can lead to the formation of heterogeneous cellular niches and the formation of a particular cell aggregate can alter the chemical gradient. The diffusion barrier and three-dimensional cell-cell and cell-matrix interactions may more closely mimic those encountered by the cells *in vivo* and explain why lymphocytes in the 3D culture are better maintained. Specifying these interplaying factors in the 3D culture system is inevitably complicated as it is *in vivo*. However, important insights may be gained by studying the dependence of cell viability and function on oxygen concentration. This can be done by varying the oxygen concentration in the headspace to create an oxygen gradient through the depth of the 3D packed-bed bioreactor and determine if particular cell niches (e.g. those of B-cells and T-cells shown in Figure 4, or stromal cells), through serial thin-sectioning, are dependent on the oxygen profile measured by an oxygen microprobe. It would also be interesting to determine if the ability of the 3D tonsil culture to mount an immune response is oxygen dependent. Additional insights on the critical role of cell-cell interactions can be obtained if well-characterized tonsil stromal cell lines become available as discussed below.

It seems highly likely that the survival of cells in our culture system depends on cell-cell interactions and paracrine factor release by stromal cells, and that cells quickly incorporated into the newly formed 3D cell aggregates might have survival advantage over isolated cells. Thus, while the limitations of mass transfer affect the maximum number of living cells within each cluster and globally in the culture, overall survival represents the balance

between the diffusion-limited chemical environment and active cellular adaptation and interactions. This could explain the contrast between the healthy cells in the clusters and the viability in the total harvested cells, which suggests that most dead cells may actually have failed to participate in proper cellular superstructures. This is an important consideration with regard to potential bioreactor modifications to enhance chemical diffusion (e.g. using perfusion systems), in that these changes may in fact hinder intrinsic soluble factor gradients and disrupt supercellular structures. More interestingly in our opinion, the possibility should be considered that facilitation of cell clustering - using more sophisticated scaffold materials incorporating specialized extracellular matrix components, as well as growth and/or chemotactic factors (reviewed in (Place et al. 2009; Tan and Watanabe 2010)), or engineered stromal components - may in fact represent the key goal of future modifications.

Related to the same issues, another set of critical parameters that will require evaluation are the constraints related to the differential maintenance and function of individual cell types in the culture system, and in particular the extent to which differential sensitivity to culture factors and conditions, the presence of clearance mechanisms, and the possibility of spontaneous proliferation of specific cell types (e.g. stromal cells) in culture may affect overall culture viability and immunological competence. For instance, it is possible that the rapid loss of plasmacytoid dendritic cells may negatively affect antigen responses, and specific culture adaptations may be able to overcome this deficiency.

The detection of antigen-specific antibody responses in 3D bioreactors unequivocally demonstrates their immunological competence. The observation of active responses to a diverse array of both recall and neo-antigens in our system is extremely encouraging, since *in vitro* antigen-specific immune responses from primary human cells are extremely hard to generate, and usually rely on presence of primed precursors or other enriched starting populations (Akeson et al. 2000; Akeson et al. 2002; Chin et al. 2007; Chin et al. 1994; Garraud et al. 1997; Ma et al. 2010; Zafiropoulos et al. 1997). The frequency of individuals displaying *in vitro* immune response ranges from 20 to 50%, depending on the antigen, suggesting that additional factors should be considered to further improve the immunization methods. The observed efficiency may be a consequence of a sub-optimal system or immunization protocols, such as requirements for stronger adjuvants, or different antigen administration methods. Notably, other *in vitro* lymphoid culture models rely on independently differentiated and antigen-loaded dendritic cells for immunization (Giese et al. 2010; Hitchcock and Niklason 2008; Ma et al. 2010), as opposed to intrinsic components as in our system. The abundance of autologous dendritic cells obtainable from tonsil explants make it feasible to directly purify these cells for use in analogous approaches using our bioreactor system, and experiments to test the effects of modification on immunization efficiency are underway. Another possibility is that the fraction of antigen-specific precursors, which can be as low as 0.01% even for expanded memory populations (Crotty et al. 2004; Tajiri et al. 2007), could be limiting within each culture. This would particularly be a problem if cell trafficking between and within the cell clusters is limited, thereby reducing the chances of encounter, cognate interaction and antigen presentation between antigen-specific B and T cell precursors. A systematic effort to study these factors may provide solutions to improve immunization efficiency in the bioreactor system. For instance, pre-selection and enrichment of antigen-specific B and T cell precursors may bypass intrinsic stochastic limitations and prove useful in both basic immunology and biotechnological applications (e.g. vaccine antigen design and human monoclonal antibody production).

The tonsil bioreactor system provides a useful model for characterizing immune responses. One interesting question concerns whether germinal center-like structures are generated during the immune response in the bioreactor and the cell types involved. Studies using labeled antigens, HLA tetramer systems (Kwok et al. 2002) for T cells and/or anti-idiotypic

reagents would likely illuminate these issues, while also addressing the question of precursor frequency. Similarly, establishing the occurrence and extent of affinity maturation during the generation of antigen-specific clones will be of interest, especially for certain applications such as the generation of human monoclonal antibodies. While the temporal window available (2-3 weeks from immunization to harvest) is likely limiting for the accumulation of substantial numbers of mutations and extensive clonal selection, we note that every tonsil sample yields cell numbers vastly in excess of those required for primary cultures ($>10^9$ cells/tonsil). These excess cells can be frozen and maintain good viability and phenotypes upon thawing, at least for the established subsets we have analyzed. Therefore, one can easily envision harvesting immunized cells from one bioreactor and seeding them on newly established bioreactor cultures from frozen samples from the same patient, allowing a further cycle of immunization, over several cycles that should in principle result in progressive expansion of clones with increased affinity.

Thus, the tonsil bioreactor system described here both represents, in its current version, a breakthrough in the engineering of artificial human peripheral lymphoid organs, and displays sufficient potential for improvement, flexibility and expandability to suggest potential practical applicability in a number of fields in the near future.

Acknowledgments

We are grateful to James Kobie and Inaki Sanz for useful discussion and for providing antigens for immunization. We also thank Martin Zand and Tina Pellegrin for protocols and help with Elispot assays, and Benjamin Wu for advice on bioreactor preparation for histology. This work was supported by NIH grant N01-AI-50029. JHDW acknowledges support from NIH grant U19 AI-067733-01 and BARDA.

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Figure 1. B and T lymphocytes viability in conventional 2D vs. 3D bioreactor human tonsil cultures

Cells were harvested from 2D dish cultures and 3D bioreactors at the time points indicated, stained with fluorochrome-conjugated antibodies and Live/dead stain, and analyzed by flow cytometry. Panel **a** shows distribution of live (Live/dead⁻ gated) T cells (CD3⁺, CD19⁻), B cells (CD19⁺, CD3⁻) and non-T/non-B cells (CD19⁻, CD3⁻) in 2D cultures at week 2, and 3D cultures at weeks 2 and 5. **b**. Quantification of survival of B and T cells by live/dead staining at week 2 and week 5. Cells were gated based on CD3 (left panels) and CD19 (right panels) expression. The numbers indicate percentages of Live/dead⁻ cells in the indicated regions. **c**. Longitudinal comparison of B and T cell viability in 2D (open bars) vs 3D (filled bars) cultures; striped bars represent day 0 freshly isolated cells used for culture seeding. Means and standard deviations of the percentage of live cells of each type (top, CD19⁺ B cells; middle; CD3⁺ T cells, bottom; CD3⁻CD19⁻ non-B non-T cells). Numbers on top of graph bars in the top panel represent number of replicate data points, which is the same for all panels. ND = not done.

Figure 2. T cell and dendritic cell phenotypes in 3D cultures

Cells were harvested from 3D bioreactors at the times indicated, stained for T and dendritic cell markers and analyzed by flow cytometry. **a.** Top row, percentages of CD4 and CD8 T cells in culture. Center and bottom rows, cells were gated for CD4 and CD8 expression, and percentages of CD45RO and CD45RA cells were plotted. **b.** To analyze for DC subsets, cells were first gated based on expression of lineage markers (see text), and Lin⁻ cells were plotted for CD14 and CD4 expression (left plots). Monocyte/macrophage cells are contained in the CD14⁺ cells gate, while CD4⁺, CD14⁻ cells were further typed into CD303⁺ plasmacytoid DCs and CD303⁻ myeloid DCs (right side panels). Note the loss of plasmacytoid DCs in 3D cultures. Plots representative of 3 experiments (T cell typing) or 2 experiments (DC typing).

Figure 3. B cell subset phenotypes in 3D bioreactor cultures

Panels show B cells either uncultured or harvested from 3D bioreactor cultures, gated on CD19⁺ cells and plotted based on CD38 vs IgD and CD27 vs IgD expression (top and bottom panels, respectively). Note the persistence of naive B cells (IgD⁺, CD38⁻ CD27⁻), the loss of activated GC B cells (IgD⁻, CD38⁺) and the relative increase in memory B cells (IgD⁻, CD27⁺). Results representative of >10 experiments.

Figure 4. Three-dimensional organization of cells within bioreactor cultures

Panel **a** shows a 10x-magnified micrograph of a representative H&E-stained section from a 3D bioreactor at week 2 of culture. Note how cells are primarily organized in large, isolated aggregates. Panel **b** shows a 40x magnification of a portion of the field in the previous panel. In a digitally expanded detail from this field, shown in panel **c**, it is possible to identify cells with diverse, characteristic types of morphology, including lymphoid-like (examples marked with white arrows), myeloid-like (yellow), dendritic-like (blue) and stromal-like (red). The lower set of panels (**d-f**) show representative micrographs of 3D bioreactor sections labeled with anti-CD19 (purplish-brown) and anti-CD3 (pink) antibodies. Note how B cells tend to cluster in small areas at the periphery of the larger aggregates.

Figure 5. Generation of antibody-secreting cells in immunized 3D bioreactor cultures

Results are shown from three independent experiments in which bioreactor cultures were immunized with the following antigens: tetanus toxoid (TT) and keyhole limpet hemocyanin (KLH) (experiments a and b), HIV gp160 (experiments b and c) and influenza H3N2 and H1N1 hemagglutinins (experiment c). Duplicate wells loaded with 2×10^4 or 7.5×10^4 total live cells (as indicated) from 3 pooled cultures were analyzed for each experiment. For each set analyzed, the antigen coating the well is indicated on the left of the panels, and the immunizing antigen used for each set is indicated on the right. After overnight incubation on the antigen-coated plates, the cells were washed off and the presence of antigen-specific antibodies bound to the plates was determined using either anti-IgM or anti-IgG labeled antibodies. For every set analyzed, antibody-secreting cells detected with a matched immunization/coating antigen represent specific responders, while antibody-secreting cells detected in mismatched pairs are by definition non-specific (e.g. activated bystanders or assay background). Note that only one irrelevant antigen response was measured as control in each experiment, which usually included multiple parallel antigen immunizations, and therefore the full reciprocal immunization/detection sets (e.g. experiment a) are not always available.

Figure 6. Summary of immunization results

Results from all the bioreactor immunization experiments are shown. Bars represent the fraction of responding individuals (N, number of individuals tested for each antigen, is shown on top of each graph bar). A positive response was arbitrarily defined as one yielding a number of antigen-specific antibody-secreting cells at least 2-fold higher than non-antigen-specific antibody-secreting cells, after background subtraction. All antigens tested were capable of inducing antibody-secreting cell formation in a fraction of the tested individuals, although the rate of response and the IgM (striped bars) vs IgG (white bars) ratios varied by antigen.