Published in final edited form as:

Clin Immunol. 2008 October ; 129(1): 10–18. doi:10.1016/j.clim.2008.06.009.

Concurrent detection of secreted products from human lymphocytes by microengraving: cytokines and antigen-reactive antibodies

Elizabeth M. Bradshaw¹, Sally C. Kent¹, Vinay Tripuraneni², Tihamer Orban³, Hidde L. Ploegh^{4,5}, David A. Hafler^{1,5*}, and J. Christopher Love^{2*}

¹Division of Molecular Immunology, Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115

²Department of Chemical Engineering, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139

³Section of Immunology and Immunogenetics, Joslin Diabetes Center, Harvard Medical School, Boston, MA 02115

⁴Whitehead Institute for Biomedical Research, 9 Cambridge Center, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

⁵The Broad Institute of MIT and Harvard, Seven Cambridge Center, Cambridge, MA 02139

Abstract

Cell surface determinants, cytokines and antibodies secreted by hematopoietic cells are used to classify their lineage and function. Currently available techniques are unable to elucidate multiple secreted proteins while also assigning phenotypic surface-displayed markers to the individual living cells. Here, a soft lithographic method, microengraving, was adapted for the multiplexed interrogation of populations of individual human peripheral blood mononuclear cells for secreted cytokines (IFN- γ and IL-6), antigen-specific antibodies, and lineage-specific surface-expressed markers. Application of the method to a clinical sample from a recent onset Type 1 diabetic subject with a positive titer of anti-insulin antibodies showed that ~0.58% of circulating CD19⁺ B cells secreted proinsulin-reactive antibodies of the IgG isotype and 2–3% of circulating cells secreted IL-6. These data demonstrate the utility of microengraving for interrogating multiple phenotypes of single human cells concurrently and for detecting rare populations of cells by their secreted products.

Keywords

Human; microengraving; Type 1 diabetes; single cell analysis; autoantibodies; cytokines; proinsulin

*Correspondence should be addressed to D.A.H. (dhafler@rics.bwh.harvard.edu) or J.C.L. (clove@mit.edu). David A. Hafler, M.D., Jack, Sadie and David Breakstone Professor of Neurology (Neuroscience), Harvard Medical School, Brigham and Women's Hospital, NRB, Room 641, 77 Avenue Louis Pasteur, Boston, MA, 02115, Phone 617-525-5330, Fax 617-525-5333. J. Christopher Love, Ph.D., Texaco-Mangelsdorf Career Development Professor, Department of Chemical Engineering, Massachusetts Institute of Technology, 77 Massachusetts Ave., Bldg. 66-456, Cambridge, MA 02139, Phone : 617-324-2300, Fax: 617-258-5042.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

INTRODUCTION

Identifying the secreted products of lymphocytes is critical to characterizing immunological responses. For example, quantification of secreted cytokines and antigen-specific immunoglobulins is important for distinguishing immune responses to pathogens, allergens, or self-antigens in autoimmune diseases. Subsets of T cells are often classified into populations (Th1, Th2 or Th17) according to the cytokines they secrete [1;2], and perturbation of the delicate balance between these subtypes can contribute to autoimmune and allergic diseases. Antigen-presenting cells, such as monocytes and dendritic cells, also produce cytokines, which can directly contribute to cellular damage or dictate the differentiation of T cells. Finally, B cells can secrete both cytokines and immunoglobulins (Ig) [3;4]. In human autoimmune diseases, autoreactive immunoglobulins often predict the onset of diseases [5;6;7].

Several techniques exist to study secreted proteins, including enzyme-linked immunosorbent assay (ELISA), enzyme-linked immunosorbent spot (ELISpot), intracellular staining, and cytokine capture. The common immunoassays that directly measure secreted proteins on a solid support (ELISA and ELISpot) fail to correlate the captured analytes with the lineage of the producing cell. ELISA also requires relatively large volumes (50–200 μ L) of supernatant from a collection of cells, instead of individual cells. Intracellular staining, followed by fluorescence-activated cell sorting (FACS), can identify populations of single cells that exhibit both a certain phenotype and intracellular quantities of an analyte, but it requires rendering the cells nonviable after permeabilization. Furthermore, the detection of analytes in intracellular stores and the actual quantities secreted by a cell may not be well correlated under natural stimulation conditions [8]. Chemical derivatization of the surfaces of cells, or complete encapsulation within hydrogels, make it possible to capture analytes close to the surface of the cell secreting them, but these modifications can also damage the cells' viability [8]. It remains a challenge, therefore, to determine both the lineage and functional role (i.e., secreted factors) of a single cell.

Here we demonstrate the adaptation of a soft lithographic method, called microengraving [9], to detect concurrently both cytokines (IFN- γ and IL-6) and antibodies (IgG and IgM) secreted from individual human peripheral blood mononuclear cells (PBMCs). Microengraving uses a planar array of microwells to isolate individual cells, and print protein microarrays where each element corresponds to the captured secretions from a cell isolated in one microwell. We also show how extending the method to include immunofluorescence of the segregated cells makes it possible to determine their lineages *in situ*. The experiments described establish both the utility of the technology for analyzing the same population of primary cells for multiple parameters (cytokines, antibodies, surface markers), and an approach to identify rare cells (~1 in 10,000) of interest—here, proinsulin-reactive CD19+ B cells present in the blood of a Type 1 diabetic patient. Further advancement of this technology should improve the ability to study variations in cellular immune responses for human immunology, especially where samples are of limited size, and perhaps, enable the discovery of new diagnostic indicators for the pathogenesis of autoimmune diseases and others.

MATERIALS AND METHODS

Antibodies

Monoclonal antibodies certified for ELISpot were used to capture IFN- γ and IL-6 (R&D Systems or Peprotech). Polyclonal goat-anti-human antibodies were used to capture IgG and IgM (Zymed). For detection of antibodies produced by B cells from a patient with Type 1 diabetes, recombinant human insulin (Sigma-Aldrich) or recombinant human proinsulin (Eli Lilly, Indianapolis, IN) was used. Differentially-labeled, isotype-specific antibodies were used to identify IgG and IgM (Jackson Immunoresearch). For detection of IL-6 and IFN- γ , affinity-

purified polyclonal antibodies (Peprotech or R&D Systems) were labeled by conjugating the antibodies with NHS-ester activated fluorescent dyes, and purified by spin column (Invitrogen or Pierce). The average degree of labeling with the commercial kits was 3–4 dyes per antibody. Alternatively, for detection of IL-6, biotinylated antibodies (R&D Systems) and fluorescent streptavidin (Alexa 647, Invitrogen) were also used.

Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Venous blood was drawn from a healthy control and an adult recent onset Type 1 diabetic (T1D) subject (Joslin Diabetes Center, Boston, MA) into green-capped, lithium heparin tubes (Kendall). PBMCs were separated using density centrifugation on Ficoll-Paque PLUS (GE Healthcare). PBMCs were frozen at a concentration of 3×10^7 in 10% dimethylsulfoxide (Sigma-Aldrich)/90% fetal calf serum (Atlanta Biologicals). After thawing the PBMCs were washed in phosphate-buffered saline (PBS). All blood samples were drawn with institutional Internal Review Board Approval.

Stimulation of PBMCs

PBMCs were stimulated in HL-1 media, supplemented with 2 mM L-glutamine, 5 mM HEPES, and 100 U/ml penicillin and 100 µg/ml streptomycin, 0.1 mM each non-essential amino acids, 1 mM sodium pyruvate (all from Lonza), and 5% heat-inactivated human male AB serum (Omega Scientific), for 18 hrs with plate-bound anti-CD3 (OKT3, 1 µg/ml) and anti-CD28 (3D10, 1 µg/ml).

Antigen reactivity and cytokine detection experiment

PBMCs, collected 4 months after diagnosis from a recent onset T1D subject with an IAA titer of 1642.0 nU/ml, were thawed, washed in PBS with 1% human serum and stimulated with B cell receptor (BCR) crosslinking using 5 µg/ml AffiniPure F(ab')₂ fragment goat anti-human IgA + IgM + IgG (H+L) (Jackson ImmunoResearch Laboratories) and 1 µg/ml CD40L (R&D Systems) for 3 hours at 37°C. The B cells were stained with CD19-FITC (HIB19), CD138-APC (MI15) (BD BioSciences) and FACS sorted (FACS Aria, BD Biosciences) into tubes. Alternatively, total PBMCs were stained with CD19-PE (HIB19), CD138-FITC (MI15) (BD BioSciences), and stimulated with BCR crosslinking and CD40L for 40 min.

Preparation of microwell arrays

Dense arrays of microwells (0.1–1 nL each) were fabricated by a combination of photolithography and replica molding of monolithic slabs of poly(dimethylsiloxane) (PDMS). The slabs were designed to fit on a 1"×3" glass slide, and were approximately 20×50 mm in size. Each array comprised 40 × 40 blocks of microwells ($50 \times 50 \times 50 \mu\text{m}^3$) set in a 4×13 arrangement with one empty block used to orient the sample (81,400 total wells). The microwell array was treated with an oxygen plasma (PDC-32G, Harrick) for 30 s. Suspensions of activated cells (2×10^5 cells/ml in serum-free media or media supplemented with 10% fetal bovine serum) were deposited on the surface of the PDMS containing the microwells. After the cells settled (5 to 10 min), the excess suspension was aspirated from the surface. Visual inspection of the slabs by microscopy confirmed that the wells contained ~1–2 cells/well with a loading efficiency of 50–70%.

Immobilization of antigens on glass slides

Capture antibodies or specific antigens were immobilized on glass slides functionalized with epoxide-bearing silanes (SME-2, TeleChem) overnight at 4°C in a humidified chamber (100–200 µg/mL total protein concentration in PBS). The slides were rinsed with PBS and blocked with bovine serum albumin (1% w/v in PBS) for 1 h at room temperature, washed three times with PBS, dipped in water, and spun dry.

Microengraving

An array of microwells loaded with cells was placed face down on a glass slide bearing immobilized anti-IL-6, anti-IFN- γ , anti-IgG/M, insulin or proinsulin; this configuration confined the cells to discrete, closed compartments with volumes of ~0.1 nL each. The device was held together under light compression in a hybridization chamber (Die-Tech DT-1001), and incubated for 1 h at 37 °C. After the incubation, the array of microwells was removed and the glass slide placed into a blocking buffer (0.5% BSA, 0.01% Tween 20 in PBS) for 1 h at 25 °C. The cells in the wells were rinsed gently with fresh serum-free media between prints and reused immediately to generate a second microarray of alternative secreted products.

Staining captured antibodies or cytokines on glass slides

The glass slides bearing the captured antibodies and cytokines were interrogated with appropriate antibodies conjugated to fluorescent dyes (Cy3/Cy5 or Alexa 532/Alexa647) to highlight immunoglobulin, autoantigen-reactive antibodies or specific cytokines. After blocking, the glass slides were washed (3xPBS, dipped in water), and dried by centrifugation. Appropriate detection agents were applied to the slide for 30–60 min at room temperature in the dark (1–5 μ g/mL). After incubation, the slides were washed (3xPBS/0.05% Tween, 3xPBS, dipped in water), and spun dry. Images of the microarrays were collected on laser-based microarray scanners (Genepix 4000B and 4200AL), and analyzed using the accompanying software (Genepix Pro, Molecular Devices).

Post-engraving treatment of the cells

Cells were either stained with antibodies of interest (CD19-FITC/PE (HIB19), CD138-APC/FITC (MI15), CD3-Alexa 647 (UCHT1), and CD14-Alexa 488 (M5E2) (BD BioSciences) and CD20-rhodamine (H299, Beckman Coulter), or fixed with 4% paraformaldehyde and then stained for postponed imaging. Samples not imaged immediately were stored at 4 °C in PBS. After a gentle rinse with PBS, arrays of microwells were covered with a thin layer of PBS and placed face down onto the surface of a clean glass slide. All images were acquired on an inverted epifluorescence microscope (Nikon Eclipse TE2000-E) equipped with an XCite EXFO illumination source, a Hamamatsu Orca AG camera, an automated x–y translation stage (Prior Instruments), and emission/excitation filter wheels (Sutter) with appropriate filters (Chroma). The dichroic was a triple-bandpass (DAPI/FITC/Texas Red) from Chroma. Overlapping images were acquired for the entire array, or subregions, using the Scan Slide module in the MetaMorph software package (v7.1, Molecular Devices).

RESULTS

Detection of cytokines and antibodies by sequential microengraving

Generating multiple microarrays by sequential microengraving that measure multiple secreted factors from one set of cells should allow highly multiplexed profiling of those cells. Microengraving was developed originally to assess the specificity of antibodies secreted from mouse hybridomas [9]. We modified the assay to detect both cytokines and immunoglobulins secreted from PBMCs (Figure 1a). We tested the general approach (described in Figure 1a) with PBMCs stimulated *ex vivo* with anti-CD3/CD28. The cells were loaded into microwells at a density of ~1–2 cells/well on average, and then the array of microwells was used to print on glass slides coated with anti-human cytokine antibodies (IFN- γ and IL-6) (Figure 1b). Based on the fraction of positive elements observed for the microarray (weighted by the loading efficiency of cells in wells, here ~50%), we estimated the frequencies of cells secreting IFN- γ and IL-6 were ~3% and 0.3%.

Examination of the cells by phase-contrast microscopy after a single print showed that the PBMCs retained a bright, refractory appearance similar to that seen upon their initial loading, and suggested that the cells remained viable after the first print. Therefore the same array of cells was rinsed gently with fresh media, and used to print on a second glass slide coated with antibodies specific for human IgM and IgG to detect spontaneous and stimulated antibody secretion (Figure 1c); total Ig (IgG, M, and A) secretion following polyclonal T cell stimulation has previously been described [10]. This observation was confirmed by ELISA for antibody subtypes in supernatants of stimulated cultures (data not shown).

Determining the lineages of cells in microwells by immunofluorescence

Because the cells are retained in the microwells after printing, we developed a protocol to stain the cells in situ for certain surface-expressed markers, and then imaged them by immunofluorescence. Following the generation of a microarray of captured IgG and IgM, we labeled a set of cells with antibodies specific for CD3, CD14, and CD20. Visual correlation of the data for a subset of the captured antibodies and labeled cells showed that elements detected on the microarrays that were positive for either IgG or IgM mapped to CD20⁺ B cells in the microwells (Figure 1d). For a region of the microwells analyzed in this experiment (1066 wells), there were 72 CD20⁺ cells detected. In this same region, there were 3 IgG⁺ and 16 IgM⁺ spots on the microarray. All but one of the IgG⁺ spots correlated to a well containing at least one CD20⁺ cell. (For that one IgG⁺ spot, there was a cell present in the corresponding microwell, but it did not stain for CD20.) These data suggest ~25% of the CD20⁺ B cells were producing antibodies in sufficient quantity for detection (~3% IgG⁺ and 22% IgM⁺); these values are similar to the expected frequencies of secreting B cells previously determined [10]. Inspection of the images gathered by immunofluorescence also revealed that a large percentage of the wells with cells contained CD3⁺ T cells (62%) and a small percentage contained CD14⁺ monocytes (14%). These cells did not correlate to IgG/M⁺ elements in the microarrays as expected.

Detection of antigen-specific immunoglobulin and cytokine secretion from PBMCs from a recent onset T1D subject

Autoantigen-reactive antibodies are used clinically as predictive indicators of Type 1 diabetes (T1D) [5;6;7;11]. The diagnosis of diabetes is determined by elevated levels of glucose in the blood during periods of both fasting and nonfasting, as well as low or undetectable levels of C-peptide; the disease is also associated with titers of autoantibodies against insulin, GAD65 or IA-2 as measured by solution-phase radioimmunoassays [11;12;13;14]. Both traditional solid-phase assays and spotted microarrays of autoantigens have been employed successfully to detect autoreactive antibodies in the sera of patients with (or at risk for) autoimmune disease, though the results from these assays have less specificity and are not generally acceptable for clinical use [11;15;16;17;18;19;20].

We hypothesized that microengraving could identify autoreactive, insulin-specific B cells in the circulation of individuals with a positive titer of insulin autoantibodies (IAA) in their serum. We screened PBMCs by microengraving from a patient with adult recent-onset T1D within 4 months of diagnosis who had not received insulin injections. Cells from the patient were stimulated for 3 h using BCR cross-linking and CD40L. We expected the number of insulin-reactive B cells to be small so CD19⁺ and CD19⁺/CD138⁺ PBMCs were isolated first by FACS. The sorted cells were deposited into microwells, and used for microengraving on a glass slide coated with recombinant human proinsulin. We reasoned that proinsulin, which is larger than insulin, might retain more conformational epitopes on the solid support. The resulting microarray was labeled for captured IgG and IgM, and the cells were imaged for CD19 and CD138 (Figures 2a and 2b).

The number of proinsulin-reactive IgG spots observed on the microarray was 39 out of 13,500 elements analyzed. Based on the percentage of B cells present in the microwells (93% of total cells loaded) and the percentage of wells filled with cells in this experiment (~53%), we estimate the frequency of circulating CD19⁺ B cells secreting anti-proinsulin IgG antibody was 0.58% for the patient. Similar measurements by flow cytometry or antigen-specific ELISpot suggest frequencies of autoreactive B cells range from 0.03–0.55% for human autoimmune diseases [21;22;23]. Interestingly, there were no anti-proinsulin antibodies of the IgM isotype detected, and we did not detect any B cells that secreted an antibody (IgG or IgM) reactive with recombinant insulin in this assay (tested by generating a second print from the same cells, data not shown). We are interested in retrieving the B cells that secrete antigen-reactive antibodies from wells by micromanipulation, and we are presently pursuing the generation of monoclonal antibodies to validate their antigen reactivity [24;25]. Together, these observations suggest that the proinsulin-reactive B cells circulating in this individual resulted from the autoimmune response and are isotype-switched B cells.

Total PBMCs from the same individual were also tested for secretion of IL-6. The cells were stimulated with a BCR crosslinking antibody and CD40L for 40 min, dispersed into microwells, and then applied sequentially to glass slides coated with either proinsulin or anti-IL-6. There were 300 IL-6⁺ elements detected out of 22,000 spots analyzed on the microarray. With ~50% of the microwells filled with 1.4 cells per well, we estimate the percentage of PBMCs secreting IL-6 was approximately 2–3%. None of the analyzed wells that correlated to IL-6⁺ spots contained single cells that were CD19⁺ (Figure 2c). There were also proinsulin-reactive B cells present (data not shown), which were not the same cells as those secreting IL-6.

DISCUSSION

Studies of human immunology have been hampered by an inability to enumerate accurately the cells participating in an immune response in a high-throughput manner that assigns both multiple cell surface markers and multiple secreted products to the same cell. The experiments described here show how the combination of microengraving and immunofluorescence can identify specific types of cells by their surface markers and both their secreted antibodies (total and antigen-specific) and cytokines (IFN- γ or IL-6).

The primary advantage of microengraving over other techniques for quantifying the frequencies of antigen-reactive cells (ELISpot, FACS) is that it allows multiplexed detection of analytes from the same population of cells, with minimal stimulation and no expansion (Table 1). This feature makes the technique well-suited for studying human immunology because rare cells in clinical samples of limited size could be extensively characterized with this method. For example, analysis of total PBMCs for secretion of IL-6 using ELISpot requires ~250,000 cells/well and one or two replicates to validate the number of apparent spots. In contrast, microengraving only requires 50,000–100,000 cells for analysis. Although the analysis of the datasets is limited presently by visual inspection and manual correlation, we expect that the realization of automated image analysis will enable the construction of profiles that correlate all individual cells to their secreted products for a set of experiments. Furthermore, it should be possible to retrieve identified cells of interest by micromanipulation to assess genetic variants within rare populations [9].

The applications for the method presented here can be extended to analyze populations of cells derived from other tissues, and the analyses refined by first presorting classes of cells by flow cytometry according to known markers of interest for maturation, activation, adhesion, or homing. We believe further developments should make it possible to monitor the immune responses of patients involved in clinical trials for new therapies. In addition, examination of at-risk, prediabetic subjects—first degree relatives to a subject with Type 1 diabetes, with

serum autoantibody(s) but without diagnosis of clinical disease—for frequency and phenotype of B cells secreting specific autoantibodies could yield important information regarding the role of these cells in the development of the disease.

Abbreviations

PBMCs, peripheral blood mononuclear cells; T1D, Type 1 diabetes; PDMS, poly (dimethylsiloxane); FACS, Fluorescence-activated cell sorting; BCR, B cell receptor; IAA, Insulin autoantibodies; ELISA, Enzyme-linked immunosorbent assay; ELISpot, Enzyme-linked immunosorbent spot.

ACKNOWLEDGMENTS

Support for these studies was provided by grants from the NIH Autoimmunity Prevention Center and the Broad Institute of MIT and Harvard (H.L.P. and J.C.L.), S.C.K. (Juvenile Diabetes Research Foundation International), E.M.B. (NIH AI651003), National Institutes of Health Grants R01 AI39229, R01 AI44447, and P01 AI39671 (to D.A.H.); and a Jacob Javits Merit Award (NS2427 to D.A.H.) from the National Institute of Neurological Disorders and Stroke and a grant from the National Multiple Sclerosis Society. The authors thank Khadir Raddassi for helpful discussions.

REFERENCES

1. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 1986;136:2348–2357. [PubMed: 2419430]
2. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, Weaver CT. Interleukin 17-producing CD4⁺ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nature Immunology* 2005;6:1123–1132. [PubMed: 16200070]
3. Duddy ME, Alter A, Bar-Or A. Distinct profiles of human B cell effector cytokines: a role in immune regulation? *Journal of Immunology* 2004;172:3422–3427.
4. Lund FE, Garvy BA, Randall TD, Harris DP. Regulatory roles for cytokine-producing B cells in infection and autoimmune disease. *Curr Dir Autoimmun* 2005;8:25–54. [PubMed: 15564716]
5. Eisenbarth GS. Update in type 1 diabetes. *J Clin Endocrinol Metab* 2007;92:2403–2407. [PubMed: 17616634]
6. Verge CF, Gianani R, Kawasaki E, L Y, Pietropaolo M, Jackson RA, Chase HP, Eisenbarth GS. Prediction of type I diabetes in first-degree relatives using a combination of insulin, GAD, and ICA512bdc/IA-2 autoantibodies. *Diabetes* 1996;45:926–933. [PubMed: 8666144]
7. Verge CF, Gianani R, Kawasaki E, Yu L, Pietropaolo M, Chase HP, Eisenbarth GS. Number of autoantibodies (against insulin, GAD or ICA512/IA2) rather than particular autoantibody specificities determines risk of type I diabetes. *J Autoimmun* 1996;9:379–383. [PubMed: 8816974]
8. Hernandez-Fuentes MP, Warrens AN, Lechler RI. Immunologic monitoring. *Immunol Rev* 2003;196:247–264. [PubMed: 14617209]
9. Love JC, Ronan JL, Grotenbreg GM, van der Veen AG, Ploegh HL. A microengraving method for rapid selection of single cells producing antigen-specific antibodies. *Nature Biotechnology* 2006;24:703–707.
10. Amoroso K, Lipsky PE. Frequency of human B cells that differentiate in response to anti-CD3-activated T cells. *J Immunol* 1990;145:3155–3161. [PubMed: 2146316]
11. Liu E, Eisenbarth GS. Accepting clocks that tell time poorly: fluid-phase versus standard ELISA autoantibody assays. *Clin Immunol* 2007;125:120–126. [PubMed: 17904423]
12. Wilkin T, Palmer J, Bonifacio E, Diaz JL, Kruse V. First international workshop on the standardization of insulin autoantibodies. *Diabetologia* 1987;30:676–677.
13. Wilkin T, Palmer J, Kurtz A, Bonifacio E, Diaz JL. The second International Workshop on the Standardisation of Insulin Autoantibody (IAA) measurement. Held in New York, 27–30 October 1987. *Diabetologia* 1988;31:449–450.
14. Williams AJ, Bingley PJ, Bonifacio E, Palmer JP, Gale EA. A novel micro-assay for insulin autoantibodies. *J Autoimmun* 1997;10:473–478. [PubMed: 9376075]

15. Brooking H, Ananieva-Jordanova R, Arnold C, Amoroso M, Powell M, Betterle C, Zanchetta R, Furmaniak J, Smith BR. A sensitive non-isotopic assay for GAD65 autoantibodies. *Clin Chim Acta* 2003;331:55–59. [PubMed: 12691864]
16. Merbl Y, Zucker-Toledano M, Quintana FJ, Cohen IR. Newborn humans manifest autoantibodies to defined self molecules detected by antigen microarray informatics. *J Clin Invest* 2007;117:712–718. [PubMed: 17332892]
17. Drouvalakis KA, Bangsaruntip S, Hueber W, Kozar LG, Utz PJ, Dai H. Peptide-coated nanotube-based biosensor for the detection of disease-specific autoantibodies in human serum. *Biosens Bioelectron.* 2007
18. Graham KL, Vaysberg M, Kuo A, Utz PJ. Autoantigen arrays for multiplex analysis of antibody isotypes. *Proteomics* 2006;6:5720–5724. [PubMed: 17068762]
19. Robinson WH, DiGennaro C, Hueber W, Haab BB, Kamachi M, Dean EJ, Fournel S, Fong D, Genovese MC, de Vegvar HE, Skriner K, Hirschberg DL, Morris RI, Muller S, Pruijn GJ, van Venrooij WJ, Smolen JS, Brown PO, Steinman L, Utz PJ. Autoantigen microarrays for multiplex characterization of autoantibody responses. *Nat Med* 2002;8:295–301. [PubMed: 11875502]
20. Li QZ, Zhou J, Wandstrat AE, Carr-Johnson F, Branch V, Karp DR, Mohan C, Wakeland EK, Olsen NJ. Protein array autoantibody profiles for insights into systemic lupus erythematosus and incomplete lupus syndromes. *Clin Exp Immunol* 2007;147:60–70. [PubMed: 17177964]
21. Leyendeckers H, Voth E, Schicha H, Hunzelmann N, Banga P, Schmitz J. Frequent detection of thyroid peroxidase-specific IgG+ memory B cells in blood of patients with autoimmune thyroid disease. *Eur J Immunol* 2002;32:3126–3132. [PubMed: 12385033]
22. Leyendeckers H, Tasanen K, Bruckner-Tuderman L, Zillikens D, Sitaru C, Schmitz J, Hunzelmann N. Memory B cells specific for the NC16A domain of the 180 kDa bullous pemphigoid autoantigen can be detected in peripheral blood of bullous pemphigoid patients and induced in vitro to synthesize autoantibodies. *J Invest Dermatol* 2003;120:372–378. [PubMed: 12603849]
23. van Helden PM, Kaijen PH, Fijnvandraat K, van den Berg HM, Voorberg J. Factor VIII-specific memory B cells in patients with hemophilia A. *J Thromb Haemost* 2007;5:2306–2308. [PubMed: 17958750]
24. Burgoon MP, Keays KM, Owens GP, Ritchie AM, Rai PR, Cool CD, Gilden DH. Laser-capture microdissection of plasma cells from subacute sclerosing panencephalitis brain reveals intrathecal disease-relevant antibodies. *Proc Natl Acad Sci U S A* 2005;102:7245–7250. [PubMed: 15883366]
25. Tiller T, Meffre E, Yurasov S, Tsuiji M, Nussenzweig MC, Wardemann H. Efficient generation of monoclonal antibodies from single human B cells by single cell RT-PCR and expression vector cloning. *J Immunol Methods* 2008;329:112–124. [PubMed: 17996249]
26. Arif S, Tree TI, Astill TP, Tremble JM, Bishop AJ, Dayan CM, Roep BO, Peakman M. Autoreactive T cell responses show proinflammatory polarization in diabetes but a regulatory phenotype in health. *J Clin Invest* 2004;113:451–463. [PubMed: 14755342]
27. Mallone R, Kochik SA, Reijonen H, Carson B, Ziegler SF, Kwok WW, Nepom GT. Functional avidity directs T-cell fate in autoreactive CD4+ T cells. *Blood* 2005;106:2798–2805. [PubMed: 16030184]
28. Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F. Interleukins 1 β and 6 but not transforming growth factor- β are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* 2007;8:942–949. [PubMed: 17676045]
29. Reijonen H, Mallone R, Heninger AK, Laughlin EM, Kochik SA, Falk BA, Kwok WW, Greenbaum C, Nepom GT. GAD65-specific CD4+ T-cells with high antigen avidity are prevalent in peripheral blood of patients with type 1 diabetes. *Diabetes* 2004;53:1987–1994. [PubMed: 15277377]
30. Chen ZJ, Shimizu F, Wheeler J, Notkins AL. Polyreactive antigen-binding B cells in the peripheral circulation are IgD+ and B7. *Eur J Immunol* 1996;26:2916–2923. [PubMed: 8977286]
31. Townsend SE, Goodnow CC, Cornall RJ. Single epitope multiple staining to detect ultralow frequency B cells. *J Immunol Methods* 2001;249:137–146. [PubMed: 11226471]
32. Amanna IJ, Slifka MK. Quantitation of rare memory B cell populations by two independent and complementary approaches. *J Immunol Methods* 2006;317:175–185. [PubMed: 17055526]

Figure 1. Microengraving with human PBMCs for concurrent detection of antibodies and cytokines
(a) Schematic illustration of the microengraving method for detection of multiple secreted products from human PBMCs. PBMCs, suspended in media, are deposited onto a large array ($\sim 20 \times 50 \text{ mm}^2$) of microwells ($\sim 0.1 \text{ nL}$ each) molded on a poly(dimethylsiloxane) slab, and allowed to settle from suspension in the microwells at a density of ~ 1 cell/well. The cells adhere loosely to the bottoms of each well. Excess cells are rinsed off the surface of the array, and the microwells are then inverted onto a glass slide coated with a specific capture reagent (e.g., anti-cytokine). After an incubation period ($\sim 1 \text{ h}$), the microwells are removed and applied to a second glass slide coated with a different capture reagent (e.g., anti-IgG and anti-IgM). The resulting microarrays are interrogated with fluorescently labeled reagents for detection and laser-based fluorescence scanners. After printing, the cells in the wells can be stained *in situ* for subsequent imaging by immunofluorescence. **(b)** A fluorescent micrograph of a region of a microarray showing cytokines (IFN- γ blue and IL-6, yellow) captured from PBMCs stimulated with anti-

CD3 and anti-CD28. The arrowheads indicate one example of each captured cytokine. **(c)** A fluorescent micrograph of a region of the microarray of captured antibodies (IgG, red and IgM, green) produced from the same array of PBMCs used in (b). **(d)** Corresponding micrographs showing a region of a microarray of captured antibodies (anti-IgM, anti-IgG) and the phase/fluorescence image of the matching region (white box) of microwells with cells (anti-CD3, red, anti-CD20 (blue) and anti-CD14, green). The arrowheads indicate wells with CD20⁺ B cells (blue, 1d, right) that secreted IgM (green, 1d, left) upon polyclonal stimulation of PBMC. Some of the wells contain multiple cells.

Figure 2. Detection of proinsulin-specific secreted immunoglobulin from single CD19⁺ B cells and cytokine secretion from PBMCs from an adult recent onset T1D subject with a positive IAA titer PBMCs collected from a T1D subject (IAA titer of 1642.0 nU/ml) were stimulated with BCR crosslinking and CD40L for 3 h at 37 °C. The cells were stained with CD19-FITC and CD138-APC, and then sorted by FACS (CD19⁺/CD138⁻ and CD19⁺/CD138⁺ B cells). The collected cells were diluted into the microwells at ~1–3 cells/well (visual inspection) in serum-free media. The microwells were placed in contact with a glass slide coated with proinsulin. After 1 h, the glass slide was removed and incubated with fluorochrome-tagged secondary reagents. **(a)** Fluorescent micrograph of an area of the resulting microarray showing three proinsulin-reactive IgG⁺ spots (denoted with arrowheads). The group of wells highlighted with a white

square is magnified in (b). **(b)** Fluorescent micrograph of a magnified region of the microarray showing proinsulin-reactive antibodies (2b, left) and the matched set of microwells (2b, right). The cells are labeled for CD19 (green) and CD138 (red). An arrowhead indicates the well containing the CD19⁺ B cells corresponding to the anti-proinsulin IgG detected on the left. **(c)** Fluorescent micrograph of a region of a microarray with captured IL-6 and the set of microwells matching to a small region (white box). The total PBMCs are labeled for CD19 (green) and CD138 (red). The cells were stimulated with BCR crosslinking and CD40L for 40 min, diluted into microwells at 1–3 cells/well in serum-free media, and then screened for IL-6 production. After printing for 1 hour, the glass slide was blocked with BSA, and then incubated with biotinylated anti-IL-6, followed by fluorescent streptavidin. Five wells with positive IL-6 secretion are shown (red, 2c, left). On the microwells, the white arrowheads denote three wells with IL-6 secretion (2c, right). Two of the wells contain single cells (CD19⁻/CD138⁻; negative) and the third well contains three cells, one of which is CD19⁺ (green).

Table 1
Comparison of classes of assays used to detect secreted products from lymphocytes.

Assay	Description	Analyte	Number of traits detected ^a	Traits correlated per cell	Measurement	Sensitivity	Advantages	Disadvantages	Example [Reference]
Micro-engraving	Soft lithographic method for printing protein microarrays from ~10 ⁵ individual cells with option of retrieval.	Single cells (10 ⁴ –10 ⁵) and supernatants from single cells (100 pL per test)	Surface markers (3) and cytokines (2) and antibodies (2).	Yes	• Frequency of reactive cells. • Relative amounts of proteins from single cells	1 in 80,000	• Low numbers of cells needed. • Correlation of surface markers with multiple secreted products. • High detection sensitivity. • Cells retained and remain viable after cytokine detection.	• Specialized equipment for imaging.	See results section.
ELISpot	Pooled cells are plated on coated nitrocellulose and interrogated for secreted products.	Supernatants from single cells (10 ³ –10 ⁵).	Cytokines or antibodies (2/sample)	Yes	• Frequency of reactive cells. • Relative amounts of secreted proteins from single cells	1 in 60,000	• High detection sensitivity.	• Semi-quantitative • Quality varies • Cannot recover cells of interest.	PBMCs were stimulated with islet antigen peptides followed by detection of IFN- γ , IL-10 and IL-4 secretion by ELISpot [26].
ELISA	Analytes from supernatants from pooled cells (50–200 μ L per test).	Supernatants from pooled cells (50–200 μ L per test).	Cytokines or antibodies (6–8/sample)	No	• Integrated measure of secreted products.	N/A	• Low cost for degree of sensitivity to a secreted product • Process can be fully automated	• Secreted products cannot be correlated to single cells.	B cell antigen-reactivity was determined using limiting dilution ELISA [27].
Flow cytometry/ FACS with intracellular staining	Cells with fluorescently labeled surface markers or intracellular proteins analyzed by fluorescence while flowing through a capillary.	Populations of single cells (10 ⁴ –10 ⁶).	Surface marker, or cytokine, or antibody (up to 18)	Yes	• Frequency of reactive cells.	1 in 5,000	• Multiplexed detection of surface markers and cytokines from single cells.	• Detection of cytokine renders cells unviable. • Intracellular staining exhibits poor sensitivity for detecting rare cells. • Specialized equipment for analysis.	Induced Th-17 cells are stained with IL-17 and IFN- γ [28].
FACS with cytometric bead array/Luminex	Coded plastic beads supporting cytokine-specific	Supernatants from pooled cells (25–50 μ L per test).	Cytokines (CBA-27; Luminex ~100)	No	• Measure of the amounts of individual secreted products	N/A	• Highly multiplexed detection of secreted cytokines	• Secreted products cannot be correlated to single cells.	Tetramer isolated GAD-specific T cell clones were analyzed for IFN- γ , IL-10, IL-5,

Assay	Description	Analyte	Number of traits detected ^a	Traits correlated per cell	Measurement	Sensitivity	Advantages	Disadvantages	Example [Reference]
FACS with cytokine capture	antibodies mixed with supernatant and analyzed by flow cytometry				present in supernatants.				
	Cells are chemically modified to capture cytokines on their surface and analyzed by flow cytometry	Populations of single cells (10^4 – 10^6).	Surface marker, or Cytokines (up to 18)	Yes	• Frequency of reactive cells. • Relative amounts of secreted proteins from single cells	1 in 5,000	from a population of cells.	• Specialized equipment for analysis.	and IL-4 using CBA [29].
FACS with B cell antigen reactivity									
	Cells fluorescently labeled with surface markers and antigens analyzed by FACS.	Populations of single cells (10^4 – 10^6).	Surface marker, or antigen (up to 18)	Yes	• Frequency of reactive cells.	1 in 10^6	• Simultaneous detection of phenotype and cytokine secretion. • Selection of viable antigen reactive B cells. • B cells do not have to be actively secreting Ab.	• Lacks sensitivity to detect rare cells. • Can damage cell viability. • Specialized equipment for analysis. • Specialized equipment for analysis. • Signal-to-noise can be low depending on the antigen	GAD-specific expansion, tetramer sorting, and stimulation followed by IFN- γ capture assay [27]. Reactive B cells were identified based on their interaction with fluorescently labeled antigen. [21;30;31;32]

^aNumbers indicate independent parameters scored with current embodiments of the assays.