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Helper B Cells Promote Cytotoxic T Cell Survival and Proliferation Independently of Antigen Presentation through CD27/CD70 Interactions

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CD8-expressing cytotoxic T cell (CTL) interactions with APCs and helper T cells determine their function and ability to survive. In this study, we describe a novel interaction independent of Ag presentation between activated CTLs and bystander CD19-expressing B lymphocytes. Ag-stimulated CTLs serially engage autologous B lymphocytes through CD27/CD70 contact that promotes their survival and proliferation. Moreover, these interactions induce the release of proinflammatory cytokines that follows two general patterns: 1) an epitope-dependent enhancement of cytokine release, and 2) a previously undiscovered coordinate release of cytokines independent of epitope exposure. The latter includes chemokine targets for activated T cells. As a result, activated T cells are attracted to B cells, which exert a “helper” role in lymphatic organs or in areas of inflammation. This observation provides a mechanistic explanation to previously reported experimental observations suggesting that B cells are required for T cell priming in vivo. The Journal of Immunology, 2008, 180: 1362–1372.

Antigen-specific CD8 T cells mature and expand upon interaction with APCs and CD4 T lymphocytes (1). Previous reports based on in vivo experimental observations pointed to the possible participation of B cells in the context of some virally (2) or parasite-induced T cell responses (3). Moreover, clinical observations suggest that B cells may modulate T cell effector function in the context of allograft rejection (4) and autoimmune disorders such as multiple sclerosis (5). These findings suggest that B cells sustain T cell function in inflamed tissues although, to date, no mechanistic explanation has been provided.

We observed that circulating CTLs induced by melanoma Ag-specific immunization display a quiescent phenotype in vivo that could be reversed in vitro by combined exposure of PBMCs to Ag recall and rIL-2, resulting in their rapid and specific expansion (6). Interestingly, expansion of Ag-specific CD8 T cells in vitro required not only Ag recall and rIL-2, but the whole PBMC population because purified CD8 T cells alone proliferated less efficiently in identical conditions. Additional help by CD4-expressing T cells to CD8 T cell cultures only partially restored the ability of Ag-specific CTLs to proliferate, suggesting that other cells included in the whole PBMC population could play a helper role in CD8 activation/proliferation. This observation revealed the necessity to better understand the kinetics of efficient memory T cell activation.

To further explore the requirements for in vitro activation/expansion of CTLs, we studied the kinetics of proliferation of HLA-A*0201-restricted, Flu M1:58-66-specific CTLs as a well-characterized human memory CD8 T cell model. Using this model, we identified a novel interaction between activated CTLs and CD19-expressing B lymphocytes. Ag-stimulated CTLs serially engage autologous B lymphocytes through CD27/CD70 contact that promote their survival and proliferation. This cross-talk is independent of Ag presentation because it occurs in cultures depleted of the relevant epitope. Moreover, these interactions induce a broad release of proinflammatory cytokines that follows two general patterns: 1) an epitope-dependent enhancement of cytokine release, and 2) a previously undiscovered coordinate release of cytokines independent of epitope exposure. The latter includes chemoattractant chemokines that target specifically activated T cells expressing the chemokine receptors CXCR3 and/or CCR4. As a result, activated T cells are attracted to B cells, which exert a “helper” role significantly enhancing CTL survival and proliferation. This finding may explain previous in vivo observations in mice and humans suggestive of a role for B cells as modulators of T cell function in the context of viral infection, autoimmune pathology, and alloraft rejection.

Materials and Methods

Cell sorting

Magnetic cell sorting of CD8, CD19, and CD4 subpopulations were performed by negative selections (Miltenyi Biotec) on autoMACS separator. Median purity (plus 1st and 3rd quartile) of sorted cells was 84% (77.3–88.8) for CD8 cells, and 93.4% (90.6–96.7) for CD19 cells, respectively. Where indicated, sorting was performed by high speed flow cytometry (FACSVantage SE; BD Biosciences); Median purity of R4 was 97.2% (94–97.4). A logical gate on SSC and live/dead staining with 4′,6-diamidino-2-phenylindole were used to make sure that all sorted cells were indeed live events and not debris or clumped cell artifacts. The sorting was always done in the normal-R mode, which optimizes for cell purity (>98%), as confirmed by re-analysis of the sorted populations. The actual coupling of cells in the sorted Flu “CFSE” population was evidenced by
direct imaging of cells using a fluorescence microscope immediately after sorting, as well as by time-lapse analysis of immune-labeled cells.

In vitro sensitization
PBMCs were obtained by leukapheresis from HLA-A*0201 normal volunteers. HLA-A*0201 expression was documented by sequence-based typing as previously described (7). After overnight panning, cells were pulsed at day 1 with 1 μM peptide (Flu M1:58-66, or EBV BMLF1:280-288 or CMV pp65:495-503; Princeton Biomolecules); 300 IU/ml rIL2 (Chiron) were then added to the culture from day 2, and every 48 h thereafter. Unless differently indicated, cultures were performed for 9 days. For consistency with the expansion method, we performed all in vitro sensitization (IVS) in the upper chambers of a Transwell culture system (cell culture inserts; BD Falcon). We expanded 1.5 × 10^6 or 7 × 10^6 cells in the upper chambers (24- and 6-well size, respectively) and, when indicated, 3 × 10^6 cells in the lower chamber (24-well size). CTL/b cell cocultures were set by adding freshly isolated CD19^+ B cells to T cell cultures 2 days after exposing the T cells to the antigenic peptide to assure its complete degragation by peptidases (8). When specified, cells were stained at day 1 with CFSE (6). Cell expansion of FuI registered by CD8^+ and CD19 cells is expressed as doubling cell number. Cells were counted before and after IVS, by multiplying cell numbers by the percentages of their specific cell fraction calculated on CD8^+ sorted after 9 days of IVS), or to R4 cells added 1:1 with autologous CD19 cells, the ratio was 1:1.

The previously characterized 1520-tumor-infiltrating lymphocyte clone (1520-TIL) (9) was used to assess CTL requirements for B cell contact in the absence of possibly contaminating cells. This clone specifically recognizes the gp100-melanoma differentiation Ag and proliferates in culture in the presence of rIL-2 without the need for Ag-specific stimulation. The TIL was expanded for 4 days alone, or with B cells, in the same culture conditions as fresh CTLs, with the exception of the rIL2 concentration, which was 6000 IU/ml for TILs.

T2 cells were used as HLA-A*0201-expressing cells in functional assays as described later. These cells stem from the fusion of a B cell line with a T cell lymphoma (10) and express several B cell markers including CD19. In other experiments, HLA-A*0201-matched and mismatched heterologous EBV transformed lymphoblastoid cells lines (LCL) were used for CTL stimulation in the presence or absence of Ag. HL-A-A*0201-expressing CD19 expressing autologous B cells, T2 cells, or LCL were pulsed with Flu M1 58–66 peptide, when used as APCs. One ×10^6/ml cells was exposed for 2–3 h to 1 μM peptide at 37°C. Cells were gently shaken every 15′ during peptide exposure, and washed 2 times before being added to effector cells. In every culture where CD8^+ cells were mixed with CD19 cells, the ratio was 1:1.

Secretion of a soluble variety of CD27 (sCD27) by B cells was assessed by ELISA on culture supernatants from PBMCs, B cells, and PBMCs cultured together with autologous B cells in the presence or absence of Flu M1:58–66 peptide plus rIL-2. Concentration of sCD27 was tested in supernatants with the human sCD27 ELISA instant kit (R&D Systems). The specificity of the requirements for CD27 stimulation of T cell proliferation by B cells through CD27 was tested by cross-linking human recombinant CD27 (R&D Systems) 10 ng/ml, anti-human CD70 mAb (Ancell), or anti-CD3/OKT3 mAb (Ortho Biotech) to culture plates. After overnight incubation, CD8 T cells were added in the presence or absence of Flu M1: 58–66 peptide and fold increase of Flu-specific CTLs was calculated as described in the previous section.

Functional assays
Cytotoxicity assays were performed and CFSE-stained (6) T2 cells, pulsed with 1 μM Flu: M1 58-66 peptide to R4 cells (tFlu^+ CD8^+ cells sorted after 9 days of IVS), or to R4 cells added 1:1 with autologous CD19 cells, after 1 day of culture. Four hours, or overnight after T2 addition, cells were counted and analyzed by flow cytometry. Numbers of T2 cells were calculated on CD8^+ , tFlu^+ , CFSE^+ fractions. Cytokine release detection assay was performed on supernatants collected after 36 h of culture, quickly centrifuged, and frozen at −20°C. Analyses were performed by Pierce Biotechnology, with SearchLight multiplex assay method. Caspase generation in target cells exposed to CTLs was measured using the PE-conjugated rabbit anti-caspase-3 mAb (BD Pharmingen) after permeabilization of target cells 3 h following the exposure to the effector cells at 37°C and 5% CO2. Captothecin was added at a final concentration of 20 μM to Jurkat cell suspensions at a density of 1 × 10^6 cells/ml. After 3 h incubation, cells were washed and resuspended in equal volume of Fix & Perm Medium A (Caltag Laboratories) and cell containing solution, and incubated for 15 min at room temperature. The cells were then washed in phosphate-buffered saline containing 5% FCS at 1500 rpm (600 × g) for 5 min. After aspiration of the supernatant, 20 μl of PE-conjugated affinity-purified polyclonal rabbit anti-active caspase-3 Ab were added. After 30 min of incubation at room temperature, the cells were washed and active caspase-3 staining was assessed by FACS analysis; control consisted of Jurkat T cells induced to apoptosis with camptothecin. The cytotoxic activity of Flu-specific CTL was then tested, plating at a 1:1 E:T ratio Flu-CTL in the presence or absence of equal amounts of B cells and T cells loaded with the Flu M1:58–66 peptide, or an irrelevant peptide consisting of the melanoma Ag epitope gp100:209-217. Active caspase-3 was measured as the fold increase in percent of caspase-3 expressing T2 loaded with the relevant Flu peptide, compared with background staining of T2 cells treated with isotype control, or T2 cells pulsed with irrelevant peptide.

Blocking experiments were performed with mAbs obtained from Ancell. Anti-CD70 IgG1 clone BU69 was used against TILs, and anti CD80 IgM clone BB1 was used against B cells. Both Abs and the relative IgG1 and IgM controls were incubated with cells for 45 min at 4°C, at 10 μg/ml (in 200 μl). Cells (2 × 10^6–6/ml) were then cultured for 4 days in the upper chamber of the Transwell system at a 1:1 TIL/B cell ratio.

Abs and fluorescent imaging
Cells were labeled with the following fluorescent mAbs: tFlu (FLU M1 iTag MHC tetramer; Beckman Coulter), CD8, CD4, CD137, CD137-L, CCR4, CXCR3, and CD19 Abs (BD Pharmingen). Indirect Ab staining was performed just for microscope imaging (secondary Abs anti-mouse IgG1, and IgG2a from Invitrogen). Microscope fluorescent images were taken with an Axiovert 200M inverted microscope (Zeiss).

Statistical analyses
Significant p values are shown referring to two-tailed paired Student’s t test.

RNA isolation and amplification and cDNA arrays
Total RNA was isolated with RNeasy minikits (Qiagen) and amplified into anti-sense RNA as previously described (11, 12). First strand cDNA synthesis was accomplished in 1 μl of SUPERaseIn (Ambion) and ThermoScript RT (Invitrogen) in 2 μg of BSA. RNA quality was verified by Agilent Technologies. Anti-sense RNA was labeled with Cy5-dUTP (Amersham Biosciences) and cohybridized with reference pooled normal donor PBMC labeled with Cy3-dUTP to custom made 17k cDNA microarrays. Arrays were scanned on a GenePix 4000 (Molecular Devices) and analyzed using BRB-ArrayTools version 3.3 (Cluster and Tree View software).

The entire microarray dataset will be available upon request at www.ncbi.nlm.nih.gov/geo/.

Results
Expansion of CTLs
We first characterized the kinetics of expansion of HLA-A*0201 expressing PBMC exposed to the HLA-A*0201-associated epitope Flu M1: 58-66. During the 3 wk of expansion, CFSE-labeled CD8 T cells segregated into four regions of the FACS scatter plot (Fig. 1A). A first region (R4) indicating tetrameric (t)Flu^+ (Flu-specific/nonproliferating) cells; R6 and R7 regions indicating respectively tFlu^-CFSE^- (Flu-specific/proliferating) cells; a second one (R5) consisting of tFlu^-CFSE^- (Flu-specific/nonproliferating) cells; R6 and R7 regions indicating respectively tFlu^-CFSE^- (non specifically proliferating) and tFlu^-CFSE^- (nonproliferating) cells. Enumeration of CTLs of the four regions indicated that while the R7 population was not increasing in number during the 3-wk period, the R5 region surprisingly followed the kinetics of expansion typical of proliferating CTLs (proliferation during the first week of culture, steady state during the second week and contraction phase during the third week) (13–15) similarly to the R4 and R6 regions (Fig. 1B). In particular, the highest proportion of CD8-expressing T cells with the R5 phenotype was observed at day 9 paralleling the peak expansion in the R4 and R6 populations.

To understand the paradoxical observation of CFSE-stained proliferating CTLs, we purified by high-speed cell sorting the four different regions. Whereas sorting of R4, R6, and R7 yielded in all
experiments pure populations, R5 consistently split into 3 different phenotypes representative of the original R4, R5, and R7 groups (Fig. 1C). This was best explained by the presence of tFlu+/CFSE− cells coupled to tFlu−/CFSE+ cells in the parental R5 population that were separated by the shear force of the sorting process. Interestingly, sorting of the R7 population did not yield the same hybrid results. This suggested that coupling of CD8 with CFSE+ cells is mostly pertinent to activated T cells in the first 10 days following Ag exposure. This phenomenon could also be observed in PBMCs stimulated with IL-15 but not IL-7 and using...
other viral epitopes from EBV and cytomegalovirus (data not shown).

**Characterization of CFSE\(^+\) coupled cells**

The nature of CTLs couplets was suggested by transcriptional profiling. CD8\(^+\) T cells sorted from the four regions were processed for messenger RNA amplification and hybridization to CDNA microarrays (11). Several statistical comparisons were performed to identify gene signatures specific for each region, which yielded comparable results. Surprisingly, among the immune-related signatures, Ig transcripts, and other private B cell signatures such as CD19, CD20, and CD79, were most consistently represented in region 5 (Fig. 1D). Phenotyping of the R5 subregions (r4, r5, and r7, mimicking respectively R4, R5, and R7) corroborated the transcriptional data demonstrating that while the r4 population consisted purely of CD8\(^+\)/CD19\(^-\) cells, the r5 population included double positive CD8\(^+\)/CD19\(^+\) cells that had remained coupled after sorting (Fig. 1E). Interestingly, the r7 subfraction of R5 included a small proportion of CD8\(^-\)/CD19\(^+\) cells. Subsequent analyses demonstrated that these were CD4\(^+\), CD56\(^+\) but not CD14\(^+\) cells, participating to a minor degree to couplet formation. The participation of CD4\(^+\) T cells was secondary to the CTL/B cell coupling and occurred at a later stage (Fig. 1F), around the 8th day of culture, while CD19\(^+\) cells were observable in the R5 region from the 6th day on. At day 12, cells staining for all three markers (CD4, CD8, and CD19) were observed suggesting that all three populations participated at that point in the interaction.

Timed fluorescence microscopy demonstrated a progressive association between CD19\(^+\) cells and CD8\(^+\)/tFlu\(^+\) cells that resulted in tight cellular interactions in which CD19 capped close to the T cell membrane (Fig. 1, G to K). Slow motion movies taken at intervals of 5 min per frame demonstrated that coupling was T cell-dependent as B cells predominantly remained motionless being progressively "visited" by wandering CTLs (Video 1). These interactions were selective as CTLs discriminated among B cells (additional clips available upon request). Moreover, CTLs controlled the kinetics of this interaction, as a 10-fold depletion of CD19\(^+\) cells did not significantly decrease the proportion of CTL/B cell couplets (data not shown). These observations suggest that a specific proportion of proliferating CTLs possess the ability to interact with subsets of B cells.

**CTL/B coupling is independent of Ag presentation by B cells**

To dissect the dynamics of CTL/B cell coupling, we mixed R4 CTL sorted on the 9th day of culture without further re-exposure to Ag with freshly isolated autologous CD19\(^+\) cells. This sorting procedure resulted in high degree of purity of flu specific CTLs (\(\sim 99\%\)). Overnight culture in IL-2 consistently reproduced the coupling between B cells and R4-derived CTLs (Fig. 2A). When fresh autologous B cells exogenously loaded with Flu peptide were mixed with R4-derived tFlu\(^+\) CTLs, complete killing of all B cells occurred and rare couplets were observed. Thus, the coupling between CTLs and B cells was a bystander phenomenon independent of Ag presentation by B cells that, in fact, were totally eliminated when loaded with Flu peptide. Moreover, the CTLs cultures were likely depleted of cognate epitope during the 9 days in culture, since it has been shown that the half life of synthetic nonamer peptides in vitro falls within a range of seconds due to peptidase degradation (8). Furthermore, minuscule amounts of remaining peptide should have been washed off during the steps associated with the sorting procedure, and no additional epitope administration was necessary to reproduce the coupling between previously Ag-exposed CTLs and fresh B cells. The coupling represented a general characteristic of Ag-activated CTLs, as it could be equally observed whether CTLs were induced in vitro with HLA-A*0201 associated epitopes other than Flu, such as EBV BMLF-1:280–288, or CMV pp65:495–503 (Fig. 2B).

**CTL/B coupling does not enhance CTL cytotoxic activity, but stimulates cytokine release by B cells**

RNA profiling demonstrated that genes associated with CTL effector function were expressed in common between R4 and R5, and were not significantly affected by B cell interactions (Fig. 2C). Concordantly, cytotoxic activity of R4 cells sorted at day 9 was potent and not affected by the addition of CD19 cells (Fig. 2D). Thus, no obvious differences were noted in the ability of CTLs to lyse their targets. However, subtle differences in potency could not be directly addressed with this assay, and therefore, we applied a caspase-3 generation assay to identify finer differences in the potency of CTLs that had been cultured in the presence or absence of CD19-expressing B cells (Fig. 2E). The results corroborated the conclusion drawn by the cytotoxicity assay that T cells/B cell interactions did not affect the cytotoxic function of Flu-specific CTL. In fact, in three consecutive experiments no difference was noted in caspase-3 activation in Flu peptide bearing T2 cells exposed to Flu-specific CTL in the absence or presence of B cells.

CTL/B cell coupling primarily affected cytokine release. Transcriptional analysis clearly identified three cytokine signatures (Fig. 3A): a CTL-specific expression pattern that was retained by couplets (IFN-\(\gamma\), IL-4, IL-15, CXCL12/SDF-1, CCL1/7-309, CCL2/MCP-1, CCL3/MIP1-\(\alpha\), CCL4/MIP1-\(\beta\), CCL5/RANTES, CCL19/MIP3\(\beta\), CCL20/MIP3\(\alpha\)), a second signature specific for B cells and retained by couplets (IL-6, IL-16, IL-24, ANG-2, CXCL1/GRO-\(\alpha\), CXCL3/GRO-\(\gamma\), CXCL8/IL-8, CXCL9/Mig, CXCL11/TAC, CCL13/MCP-4), and a third one unique for the couplets (IL-7, CXCL1/Lymphotactin, CCL22/MDC). Interestingly, the expression of individual cytokine transcripts by one population was in several cases associated with the complementary expression of the corresponding receptors by the opposite population (IFN-\(\gamma\)/IFN-\(\gamma\R; MIP-3\(\alpha\)/CCR6; MIP-3\(\beta\)/CCR7; fractalkine/CX3CR1 for signature-1 cytokines, and CXCL9, CXCL10, and CXCL11/CXCR3 and MCP-4/CXCR2 for signature-2 cytokines). This observation suggested an active cross-talk between T and B cells.

Save for the third cytokine expression pattern, it did not appear that CTL/B cell coupling dramatically altered cytokine transcription; rather, respective expression levels were maintained. However, coupling strikingly affected the secretion of several of these cytokines. R4 CTLs were sorted at day 9 and exposed to freshly isolated autologous CD19\(^+\) cells. After 36 h of coculture, supernatants were tested on a protein array platform. Two patterns of cytokine secretion were observed: 1) an epitope-dependent cytokine release that was enhanced by the presence of autologous CD19\(^+\) cells (type A; IFN-\(\gamma\), GM-CSF, TNF-\(\alpha\), IL-4, IL-7, CXCL8/IL-8, CCL1/7-309, IL-10, CCL3/MIP1-\(\alpha\), CCL4/MIP1-\(\beta\), and 2) an in promptu previously undiscovered coordinate release of cytokines (type B; IL-6, CXCL9/Mig, CXCL10/IP-10, CXCL11/TAC, ANG2, CCL17/TARC, CCL22/MDC), occurring independently of epitope exposure, that was further enhanced by the presence of Ag (Fig. 3B). Interestingly, the majority of cytokines whose release was dependent upon epitope stimulation (type A cytokines) belonged to the first transcriptional pattern, with the exception of IL-8, whose mRNA levels were higher in B cells. Cytokines following the epitope-independent pattern of secretion (type B) belonged exclusively to the second and third transcriptional patterns.

The type-A pattern of cytokine release could be best characterized by exposing R4 CTLs to HLA-A*0201-expressing T2 cells.
T2 cells stem from the fusion of a B cell line with a T cell lymphoma. They express several B cells markers, including CD19 and selectively express HLA-A*0201 HLA class I allele. In this model, a consistent release of type-A cytokines by CTLs occurred only when T2 cells were loaded with Flu, but not when R4 CTLs were exposed to T2 cells alone, with or without autologous CD19⁺ cells confirming that the protein release was tightly dependent upon Ag exposure. Similarly, coupling of R4 CTLs with autologous B cells alone did not induce cytokine release significantly above background levels.

The type-B pattern of cytokine release was directly induced by R4 CTL/B cell interactions without need for Ag exposure. R4 CTLs cocultured with autologous CD19⁺ cells consistently induced secretion of cytokines ≥3 times over background independently of exogenous loading with Flu. Since T2 cells represent an hybrid cell population of T cell/B cell derivation, they could have
FIGURE 3. Cytokine mRNA expression and protein release by CTL/B cell couplets. Supervised clustering of cytokine and respective receptors (color coded) after 9 days of IVS. A, Signature-1 RNA includes cytokine transcripts up-regulated by R4 CTLs and corresponding receptors; signature-2 and -3 RNA include cytokine transcripts and receptors up-regulated by B cells, and CTL/B cell couplets respectively. B, Cytokine release by R4 CTL and autologous B cell couplets in the absence (black box) or presence (blue box) of Ag stimulation. Cytokine names are color coded (as in A). In red and bold are values ≥3-fold the sum of spontaneous release by individual cell populations relevant to each combination. For instance, the background release for the R4/B combination was calculated by adding spontaneous release values of R4 CTLs and B cells cultured alone. Ag presentation was provided by HLA-A*0201-expressing T2 cells exogenously exposed to Flu peptide (T2F). Type A proteins include cytokines significantly released by R4 CTL/B cell couplets only in the presence of CTL epitope stimulation; type B proteins include cytokines significantly released by R4 CTL/B cell couplets without need for epitope stimulation. Averages ± SEM of four independent experiments are represented. Graphs display representative experiments for two type 1 and type 2 cytokines. Values indicate picograms per milliliter. C, Expression of CCR4 (receptor for CCL17/TARC and CCL22/MDC) CXCR3 (receptor for CXCL9/Mig, CXCL10/IP-10, and CXCL11/I-TAC) was analyzed in tFlu CD8 cells, tFlu CD8*CD19* couplets, and CD19* cells after 9 days of IVS (n = 5).
participated in the secretion of the type B cytokines (Fig. 3B); indeed, the release of type B cytokines was minimally enhanced when CTL/B cell couplets were cocultured with T2 cells alone, and it was further enhanced when cocultured with Flu-loaded T2 cells. Thus, T2 cells may display some of the characteristics of B cells and may produce type B chemokines when exposed to CTLs alone. As discussed later, B cells can induce CTL proliferation through CD27 (expressed by B cells)/CD70 (expressed by T cells) interactions. We therefore tested the expression of CD27 and CD70 by T2 cells to determine whether the ability to secrete type B cytokines could be mediated through the same interactions. T2 cells uniformly expressed high levels of CD70 but did not express any detectable amounts of CD27, suggesting that chemokine secretion by B cells exposed to CTL follows a different pathway than the one inducing CTL proliferation. It thus appears that B cells constitutively express at the transcriptional level several cytokines that are released only upon interaction with activated CTLs. Importantly, CXCL9/Mig, CXCL10/IP-10, and CXCL11/I-TAC, whose release was strongly enhanced by CTL/B cell coupling, interact with the same receptor CXCR3. CXCR3 is a pivotal modulator of central memory T cell differentiation and was typically expressed by R4 CTLs (16) (97.2 vs. 6.5% in CD19, Fig. 3C). Consistently, CXCR3, but not CCR4 (receptor for CCL17/TARC and CCL22/MDC), surface expression was highly expressed by R4 CD8 cells and was expressed by almost 100% CD8 cells coupled with B cells in the R5 region. Moreover, CD8 T cells belonging to the R7 region that were not reacting to Ag exposure expressed significantly lower levels of CXCR3 (data not shown). This data suggests that CXCR3 and its ligands might play a predominant role in specifically attracting activated CTLs to B cells. Chemotactrant cytokines released by CTL/B couplets represent a strong proinflammatory signal capable of amplifying immune responses by attracting T lymphocytes (15, 17, 18). To our knowledge, however, none of these chemokines had known effects on T cell proliferation and/or survival, with the exception of IL-7, whose release was not significantly affected by coupling. In addition, both transcriptional analysis and SearchLight Multiplex Assay for cytokine detection identified secretion of other putative cytokines associate with TH1 or TH2 polarization. Thus, in these in vitro conditions, B cells do not appear to modulate T cell activation and/or expansion through known classical polarization pathways.

**CTL/B coupling promotes survival and proliferation of CTLs through CD27/CD70 interactions**

We observed that sorted R4 CTL exposed to fresh autologous CD19 cells survived better in culture (Fig. 4A), suggesting that B cells could participate in the modulation of CTL survival. To evaluate the weight of this phenomenon and test whether PBMC depleted of B cells could support CTL proliferation, we attempted to deplete PBMC populations of B cells. This was done using either bead-based selection of CD19-expressing B cells, or by providing Rituximab to the PBMC cultures containing fresh plasma that, although aimed at the destruction of CD20 expressing B cells, could have decreased the number of CD19 expressing-B cells. In either case, however, we failed to completely remove CD19-expressing B cells from the PBMC cultures, and comparable numbers of CTL/B cell couplets could still be seen in the in vitro conditions tested even after several days. Thus, the B cell depletion
experiments provided inconclusive results (data not shown). Therefore, to further evaluate the role of B cells in CTL proliferation/survival, we stimulated with Flu peptide PBMC or freshly isolated CD8\(^+\) cells in the presence or absence of autologous CD19\(^+\) cells (Fig. 4B). The number of Flu-specific CTLs was significantly increased after 9 days when CD19\(^+\) cells were added to CD8 cells, demonstrating that CTL/B cell interactions contribute to CTL proliferation. Because B cells were added two days after in vitro stimulation of the CD8 cells with the Flu-M1:58-66 peptide, and after careful washing of supernatant, the resulting proliferation of CTLs in the presence of bystander B cells could not be due to a costimulatory effect during Ag presentation.

To test whether the increased proliferation of Flu-specific CTL in the presence of B cells resulted from paracrine cytokine release or direct cell-to-cell contact, we exposed CD8\(^+\) cells to IVS adopting a trans-well system. Proliferation of Flu\(^+\) CD8\(^+\) cells increased significantly when they were cocultured with fresh autologous B cells in the same chamber (dark-gray bar, Fig. 4C). However, increased proliferation was observed also when CD8\(^+\) T cells alone placed in the upper chamber were exposed to soluble factors passing through the membrane from the lower chamber where B cells were in direct contact with CD8 cells (light-gray bar). Interestingly, the proliferation of the upper chamber CTLs mediated by soluble factors coming from the lower chamber required CD8\(^+\) T cell/B cell contact in the lower chamber, as it was not observed with B cells alone in the lower chamber (black bar). Thus, cell-to-cell interactions and soluble factors may co-operate in inducing CTL survival/proliferation. Because significant differences were noted between the two conditions (direct contact of CTL/B cell in the upper chamber compared with indirect effects due to soluble factors trespassing the membrane from the lower chamber containing CTL and B cells to stimulate the upper chamber containing only CD8 cells), it is likely that direct contact significantly contributes to the proliferative effects.

**FIGURE 5.** Function of TILs/B cell couplets. A, Representative expression of CD27, CD28, CD70, and CD80 in TILs, and in fresh CD19\(^+\) cells. B, F.I. of TILs after a 4-day expansion alone or in the presence of fresh heterologous B cells. TILs, or CD19 cells, were blocked at the beginning of the culture with anti-CD70 (dashed bar), and anti-CD80 (diamond bar) mAbs, respectively, or with the proper Ig control (light-gray bar is the control for CD70 block, dark-gray bar for CD80 block) (n = 4). C, F.I. of CTL after 9 days culture alone or after cross-linking of human recombinant CD27, anti-CD70 mAb, or a combination of the two. The anti-CD19 mAb used as an isotype matched negative control, whereas the pan T cell stimulator OKT3 mAb was used as positive control. PBMC were expanded in presence of rIL-2 with (CD8\(^+\) Flu, black bars) or without (CD8 alone, white bars) Ag-specific stimulation with the Flu peptide. Student's t test p values refer to significant differences between OKT-3 stimulated cultures and CD8 alone cultures (* < 0.05, ** < 0.01); all other conditions did not yield significant differences compared with CD8 alone cultures.
To further understand the mechanism responsible for CTL proliferation in this context, we chose an experimental model, where we could study the function of an established CTL clone that did not require Ag stimulation for expansion. We selected 1520-TIL that we have previously characterized as a monoclonal CD8\(^+\) T cell recognizing the GP100:209-217 peptide sequence of the melanoma-associated Ag GP100/PMel17 (9). This TIL can display a prolonged expansion in vitro in the presence of rIL-2 requiring only occasionally Ag re-stimulation. As we observed with fresh CD8 cells, 1520-TIL proliferated better when exposed to fresh CD19\(^+\) B cells (Fig. 4D). Interestingly, proliferation was enhanced only when 1520-TIL were in direct contact with B cells in the trans-well system. This suggests that a mixed CD8 T cell population may behave differently from this clonal model by producing soluble factors when admixed to B cells that may contribute to increased survival. This model was, therefore, deemed ideal for the characterization of the cell-to-cell interaction between CD8 T and B cells that leads to increased survival/proliferation. Because of their independence from Ag exposure, experiments with 1520-TIL supported the helper role of B cells in promoting CTL proliferation as a mechanism separate from classical costimulation during Ag presentation. This model also excluded artefactual effects on CTL proliferation due to a minimal number of contaminant cells that might have been present in the experiments with sorted CD8 T cells (less than <1% in the fresh CD8 T cell preparations).

To address which molecular mechanism may be involved in promoting CTL proliferation, we screened several molecules known to be associated with T cell activation and the corresponded ligand (4-1BB/4-1BBL, CD27/CD70, and CD28/CD80) for their expression on the surface of 1520-TIL and fresh B lymphocytes. After a first screening, we excluded 4-1BB and its ligand because they were barely expressed. Conversely, CD27, CD70, and CD80 were expressed by either 1520-TIL or B cells; in particular, CD70 was expressed universally by 1520-TIL while CD27 was expressed by approximately half of the CD19-expressing B cells (Fig. 5A); this pattern of expression was representative of the expression of the same surface molecules on Flu-specific activated CTL (data not shown). It has been observed that CD27 is required for generation and long term maintenance of T cell immunity (19), and collectively with CD28 contributes to primary and memory CD8 T cell responses in experimental animal models (20). By blocking CD27/CD70 interaction, with a blocking Ab directed to CD70 expressed by 1520-TIL, we observed an almost complete abrogation of the survival advantage provided by B cell contact. Interfering with CD28/CD80 interactions instead did not affect the proliferation of 1520-TIL (Fig. 5B). Thus, CD27/CD70 interactions were necessary to the stimulation of CTL proliferation by B cells; however, cross-linking experiments suggested that such interactions alone are not sufficient. Cross-linking of human recombinant CD27, anti-human CD70 mAb, or a combination of the two to a solid surface could not induce a significant increase in Flu-specific CTL proliferation compared with CTL proliferation in their absence. A significant increase was noted, however, when the pan T cell stimulator OKT3 mAb was used as a control (Fig. 5C). It is therefore possible that a soluble factor may serve as a co-stimulator during this interaction, as suggested by the trans-well experiments. In an attempt to identify it, we first tested B cell supernatants in different culture conditions, including in the presence or absence of CD8 T cells, Flu-peptide, and rIL-2 in different combinations for the secretion of sCD27. In no circumstance could we identify evidence of secretion of sCD27, suggesting that factors other than this molecule may be responsible for the costimulation (data not shown). We then tested the same supernatants with the SearchLight Multiplex Assay, which failed to identify any cytokine specifically produced by B cells, save for those chemokines described in Fig. 3B which have no proliferative effects. We are presently attempting with discovery-driven approaches to identify which factor may be responsible for this costimulation.

Because 1520-TIL expressed high levels of CXCR3, we also tested whether its ligands CXCL9/Mig, CXCL10/IP-10, and CXCL11/I-TAC could affect its proliferation. As expected, exogenous exposure of 1520-TIL to chemokine concentrations similar to those detected in supernatants of CTL/B cell cultures (Fig. 3B) did not affect CTL survival. Survival was also not affected by exogenous administration at 1 or 2 logs higher concentrations, or when the three chemokines were collectively provided (data not shown). Similar results were obtained providing the same chemokines to sorted Flu\(^+\) CTLs (data not shown). Thus, the release of Type-B cytokines by CTL/B cell coupling represents a relevant phenomenon that follows the coupling, and may serve to amplify the coupling process by attracting de novo activated CTLs that may be subsequently activated by direct cell-to-cell contact.

**Discussion**

Homeostatic cytokines determine survival of central memory T cells (T\(_{CM}\)) by inducing survival of selected cells from a pool of expanded T cells, among which effector memory T cells (T\(_{EM}\)) prevail (21). Accordingly, lymphocyte development is accompanied by programmed changes in cytokine receptor expression (22). Moreover, chemokine receptor expression characterizes functional phenotypes of differentiated T cells. For instance, the expression of CXCR3 (receptor for CXCL9/Mig, CXCL10/IP-10, and CXCL11/I-TAC) or CCR4 (receptor for CCL17/TARC and CCL22/MDC) determines CD4 T cells polarization respectively toward a Th1 or Th2 phenotype (16). Similarly, CCR4 expressed by recently activated T cells promotes migration toward CCL22/MDC-producing dendritic cells. CXCR3 also plays a major role in modulating activated CD8 T cell physiology, particularly memory CD8 T cells at an intermediate level of differentiation (23). It has been suggested that CD8 T cell infiltration to inflamed areas such as the cerebrospinal fluid in multiple sclerosis (24), atherosclerotic plaques (18), the lung of HIV patients with T cell alveolitis (17), or allografts (25, 26) is predominantly mediated by chemoattractant cytokines of the CXCL9/10/11 family attracting CXCR3 expressing T cells. Although it is generally believed that CXCR3 ligand chemokines are produced by activated dendritic cells, it is possible that the same mechanisms may be used by B cells through CXCR3 and/or CCR4 signaling (27) to attract and retain activated T cells (28) in secondary lymph nodes, or during formation of tertiary lymphoid organs (29). Generally, these interactions are considered from a B cell-centric point of view with helper CD4 T cells migrating to germinal centers to promote B cell maturation (22). This study indicates that the interaction between activated CTL and B cells leads to the release of chemoattractant cytokines, which in turn amplify the inflammatory signal by attracting additional immune cells. As T cells enter into contact with B cells, they are stimulated to proliferate. This information suggests a novel dimension to T/B cell interactions in which B cells play a helper role to support CD8 (and possibly CD4) T cells survival and expansion. Moreover, this observation corroborates others’ findings that B cells may function as central immune modulators through a reciprocal regulation of polarized cytokine production through T cell interactions (30). These interactions predominantly affect CTL while B cells play a helper role. In contrast to CD4 cells, only CXCR3 was expressed by CTLs paralleling the Th1 polarization of CXCR3\(^{-}\) expressing CD4\(^{+}\) cells (16). Thus, it is possible that
CTL/B cell coupling may attract activated CTL in areas of inflammation through the coordinate release of CXCR3 ligands. The release of CCR4 ligands may secondarily attract Th2 type regulatory CD4 cells at a later stage (31) (hypothesis not tested by this study). The concomitant enhancement of CTL survival and proliferation through CD27/CD70 contact suggests that B cells can further regulate CTL responses by increasing CTL number during the expansion phase and survival during the contraction phase (32).

A “B cell-helper” hypothesis also finds support in in vivo observations. More than a decade ago, Schultz et al. (2) observed that B cells play a significant role in modulating in vivo T cell responses to Friend virus-induced leukemia. Mice studies suggest that B cells play an important role in the initiation of T cell responses to *Chlamydia trachomatis* (mouse pneumonitis) lung infection (3). Moreover, the presence of B cell signatures in human renal allografts tightly correlates with acute rejection (a predominately T cell mediated phenomenon) (4). Finally, depletion of B cells in the cerebrospinal fluid of multiple sclerosis patients by the anti-CD20 Ab Rituximab results in secondary reduction of cerebrospinal fluid T cells (5). Taken together these observations suggest that B cells may play a role in attracting, sustaining, and regulating T cell responses in inflamed tissues in experimental animal models and in human pathology. At present, however, it remains unknown whether, and in what circumstances, CTLs would encounter in vivo and be attracted to non Ag-bearing B cells. As a consequence, we cannot speculate whether this potential helper B cell effect is more likely to represent another facet of a disturbed immune reactivity (such as in autoimmunity or during acute transplant rejection), or represent a physiologic mechanism for the maintenance and modulation of the homeostatic milieu in response to immune stimulation.

The mechanisms determining memory T cell differentiation into TCM vs TEM remain poorly understood in humans (16, 32–34). CD8 memory cells bearing the same TCR-β clonotype share features specific for TCM or TEM cells (35), suggesting that the two phenotypes can be displayed by the same T cell clone, depending upon unequal stimulation of sister cells (36) or progressive differentiation (1, 14) during the contraction phase of the immune response. In accordance, mouse studies suggest that a fraction of CD8 T TEM convert to TCM yet, the mechanisms responsible for this conversion or “reversion” (37) are not clearly understood. By increasing survival of activated CTLs, B cells may participate in the process that promotes such conversion. In particular, it has been observed that CD27 is required for generation and long term maintenance of T cell immunity (19), and promotes survival of activated CD8 T cells. Collectively with CD28, it contributes to the primary and memory CD8 T cell responses to influenza virus in a mouse model (20). In addition, CD27 and its ligand CD70 have been described as markers and mediators of B cell responses in the germinal center, suggesting a role for B cell expansion (38).

However, no report has to our knowledge described a CD27/CD70-mediated cross talk between activated CD8 T cells and B cells that results in enhanced survival and proliferation of the T cells.

As CD48 cells program CD8 memory cells through cell-to-cell interactions (39), it is possible that B cells may participate in these interactions providing additional chemotraction and survival signals for CXCR3-expressing T cells. In fact, irradiated autologous or heterologous EBV-transformed lymphoblastoid B cell lines have long been used as “feeder cells” for the clonal expansion of CTLs (40). Their beneficial effect on CTL proliferation has generally been attributed to costimulatory property of lymphoblastoid B cell line in the context of Ag presentation in autologous settings, or through nonself interactions in heterologous settings. In this study, we clearly show that CTL/B cell interactions occur independently of Ag presentation and in autologous settings strongly supporting a helper role of bystander B cells.

In summary, we discovered that B cells play a helper role upon coupling with activated CTLs; production of chemoaatractive cytokines is enhanced promoting a positive feed back to retain and/or attract CTLs in areas of inflammation. Although CTL/B cell coupling is independent of Ag presentation by B cells, it appears to favor Ag-activated CTLs. Moreover, the secretion of chemokines is associated with increased proliferation and survival of these CTLs that is at least in part mediated through CD27/CD70 contact. The possible role of a soluble cofactor is presently under investigation.

**Online supplemental material**

Video1.mov shows contacts between tetramer Flu+CD8+ cells, and autologous CD19+ cells during a 2.5-h period of culture. CD8 cells move through the field, and engage interactions with rather static B cells.

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**Disclosures**

The authors have no financial conflict of interest.

**References**


B CELLS HELP CYTOTOXIC CD8 T CELLS


ONLINE SUPPLEMENTAL MATERIAL

**Video 1.** Movie of FLU+ CD8+ cells interactions with B cells. Tetramer Flu+/CD8+ cells after 9 days of IVS, and fresh autologous CD19+ cells were labeled with tFlu, CD8 and CD19 antibodies. Cells were sorted by high-speed flow cytometry, mixed at 1:1 ratio in a poly-D-lysine/fibronectin coated gridded culture dish (MatTek Corporation), and cultured o.n. in 10% human serum Iscove’s medium, supplemented with 300 IU/ml rIL2, and DAPI.

The cells were incubated at 37°C in 5%CO2 using an automatically controlled environmental chamber (Incubator XL) mounted on an Axiovert 200M inverted microscope (Zeiss) and video-recorded during o.n. culture. The movie clip shows a 2.5-hour sequence of snapshots captured at 5-minute intervals using a 25X Plan-Neofluar (Phase-2, multi-immersion) objective under phase-contrast illumination of the Axiovert 200M inverted microscope (Zeiss). The original images were automatically acquired at 1,344 x 1,024 pixel resolution and 12-bit grayscale depth using an ORCA-ER CCD camera (Hamamatsu) controlled by a customized OpenLab time-lapse imaging automation (Improvision). The final images depicting the field of interest over the indicated time frame were cropped at 596 x 716 pixel resolution and the movie clip was exported using a Sorenson video compression method into a QuickTime format. The full 15-hour video over the entire imaged field at the original pixel resolution is available upon request.

**Keys:**

Fluorescent labels: green (AlexaFluor 488) = CD19; blue (AlexaFluor 647) = CD8; red (PE) = FLU M1 iTAg MHC Tetramer; yellow = DAPI.
MIAME array description

Microarrays Experiment Design

- **Keys:**
  tFLU= tetrameric HLA-FluM1:58-66 complex-(tFlu)-staining cells
  IVS= In *vitro* stimulation of peripheral blood mononuclear cells, with 1 μM FluM1:58-66 peptide, and 300 IU/ml rIL2 for 9 days.

- **Goals and experimental design:**
  1. *Characterization of CFSE+ coupled cells.*
     To understand the paradoxical observation of CFSE-stained proliferating CTLs we performed 6 independent IVS experiments and we purified by high-speed cell sorting the following FACS scatter plot regions: R4, R5, R6, R7. Regions respectively indicate tFlu+/CFSE- (Flu-specific/proliferating), tFlu+/CFSE+ (Flu-specific/"non-proliferating") tFlu-/CFSE- (non specifically proliferating) and tFlu-/CFSE+ (non-proliferating) CD8-expressing T cells (see manuscript fig.1 A-C).
  2. *Further characterization of CTL/B couplets.*
     When transcriptional profile of R5 revealed that CTLs were coupling with B cells, we repeated other 6 independent IVS experiments to compare the transcriptional profile of R4 and R5 with the one of high-speed sorted CD19+ expressing B cells (B) uncoupled at the end of the stimulation (see Fig. 2C, 3A, and 4C).

- **Quality control steps:**
  We tested routinely 5 slides per printing set for quality assurance. The slides were hybridized using identical samples with dye swap. Genes with dye inconsistencies were excluded from further analysis (see Jin Pet al. Selection and validation of endogenous reference genes using a high throughput approach. *BMC Genomics*, 5 (1): 55, 2004).

- **Links to the publication:** the entire microarray dataset will be available upon request at [http://www.ncbi.nlm.nih.gov/geo/](http://www.ncbi.nlm.nih.gov/geo/)
Samples and labeling

- **Origin:**
  Samples consisted of peripheral blood mononuclear cells obtained by leukapheresis from HLA-A*0201 normal volunteers (age 31-61, mixed gender).

- **Cell preparation and labelling:**
  Cells were stimulated in vitro with 1 μM FluM1:58-66 peptide, and 300 IU/ml rIL2 for 9 days, and sorted by high-speed flow cytometry.
  After sorting, total RNA was isolated with RNeasy minikits (Qiagen, Germantown, MD) and amplified into anti-sense RNA as previously described (Wang, E., Miller, L., Ohnmacht, G.A., Liu, E. & Marincola, F.M. High fidelity mRNA amplification for gene profiling using cDNA microarrays. *Nature Biotech* 17, 457-459; 2000). First strand cDNA synthesis was accomplished in 1μl SUPERase•In (Ambion) and ThermoScript RT (Gibco BRL, Gaithersburg, MD) in 2 μg bovine serum albumin. RNA quality was verified by Agilent technologies (Palo Alto, CA). Anti-sense RNA was labeled with Cy5-dUTP (Amersham, Piscataway, NJ) and co-hybridized with reference pooled normal donor peripheral blood mononuclear cells (PBMC) labeled with Cy3-dUTP to custom made 17K-cDNA microarrays.

Hybridization procedures and parameters

Samples were treated according to the following protocol:

**Target labeling by reverse transcription**

4μl First strand buffer
1μl dN6 primer (8μg/μl; Boehringer Mannheim Cat# 1034731 re-suspended in 250ul DEPC H2O)
2μl 10X low T - dNTP (5mM A, C and GTP, 2mM dTTP)
2μl Cy-dUTP (1mM Cy3 or Cy5)
2μl 0.1 M DTT
1μl RNasin 1μl RNAsin (Promega Cat# N2111)
3μg amplified aRNA in 8μl DEPC H2O

Mix well and heat to 65°C for 5min then cool down to 42°C.
Add 1μl SSII (Gibco BRL Cat# 18064-071). Incubate for 30 min at 42°C and add another 1μl SSII for 40 min at 42°C. Add 2.5μl 500mM EDTA and heat to 65°C for 1min. Add 5μl 1M NaOH and incubate at 65°C for 15 min to hydrolyze RNA. Add 12.5μl 1M Tris immediately to neutralize the pH. Bring volume to 70μl by adding 35μl of 1xTE.

**Target clean up**

Prepare Micro Bio-spin 6 Columns (BioRad Cat#732-6221) and run target solution through it. Collect flow through and add 200μl 1 x TE to it. Concentrate target to ~20μl using Microcon YM-30 column (Millipore Cat# 42410).

**Hybridization**

Combine Cy3 and Cy5 labeled target and concentrate to 16μl by speed vacuum. Add 1μl 50x Denhardt’s blocking solution (Sigma Cat# 2532), 1μl poly dA (8μg/μl Pharmacia Cat# 27-7988-01), 1μl yeast tRNA (4mg/ml Sigma Cat# R8759), 1μl Human Cot I DNA (10mg/ml Gibco BRL Cat# 15279-011) and 2.6μl 20X SSC. Heat for 2 min at 99°C and add 0.6μl 10% SDS. Cool to RT. Apply target mixture to array slide, add coverslip, place in humidified hybridization chamber, and hybridize at 65°C over night.

**Washing**

1. Wash with 2x SSC + 0.1% SDS to get rid of the cover slide.
2. Wash with 1x SSC for 1 min.
3. Wash with 0.2 x SSC for 1 min.
4. Wash with 0.05x SSC for 10 second
5. Centrifuge slide at 80-100 g for 3 min. (Slide can be put in slide rack on microplate carriers or in 50ml conical tube and centrifuged in swinging-bucket rotor.)
Measurement data and specifications

- **Data**
  Arrays were scanned on a GenePix 4000 (Axon Instruments, Union City, CA). Data were uploaded to National Institute of Health, NCI/CCR μArray database ([http://nciarray.nci.nih.gov](http://nciarray.nci.nih.gov)). Raw data were retrieved in BRB-ArrayTools format for analysis and normalization. Following filter criteria was applied to the data set:
  1. spots were excluded if intensity < 100, and spot size less than 25μm
  2. genes were filtered out if >50% of experiment with missing data values.
  3. log2 transformed data were normalized using lowess smoother, and intensity ratios were truncated if greater than 64.

  Class comparison statistical analyses were performed with F-test, or T-test (with random variance model) with the following statistical parameters:
  - Nominal significance level of each univariate test: 0.001
  - Confidence level of false discovery rate assessment: 95 %
  - Maximum allowed number of false-positive genes: 10
  - Maximum allowed proportion of false-positive genes: 0.1

  Average correction was applied to data before Tree View visualization.
  Please find enclosed as Excel files the raw data, and the average corrected data for each figure.

**Array Design:**

The array consists of 16,914 cDNA clone-fragments amplified by PCR from Research Genetics clone sets and custom printed on glass slides in 32x24x23 configuration, using OmniGrid printer. The slides were poly-lysine coated in house and used after at least two weeks of post-coating. PCR products were purified using Centri-Sep 96 Multi-well Filter Plates (Princeton Separations), and resuspended in 3xSSC at about 0.5ug/ul. The array feature annotation table is attached as Excel file (Table 1).

Principal array organism: Homo sapiens