Attenuation of HIV-associated human B cell exhaustion by siRNA downregulation of inhibitory receptors

Lela Kardava,[1] Susan Moir,[1] Wei Wang,[1] Jason Ho,[1] Clarisa M. Buckner,[1] Jacqueline G. Posada,[1] Marie A. O'Shea,[1] Gregg Roby,[1] Jenny Chen,[1,2] Hae Won Sohn,[3] Tae-Wook Chun,[1] Susan K. Pierce,[3] and Anthony S. Fauci[1]

[1]Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases (NIAID), NIH, Bethesda, Maryland, USA.
[2]Howard Hughes Medical Institute–NIH Research Scholars Program, Chevy Chase, Maryland, USA.
[3]Laboratory of Immunogenetics, NIAID, NIH, Bethesda, Maryland, USA.

Chronic immune activation in HIV-infected individuals leads to accumulation of exhausted tissue-like memory B cells. Exhausted lymphocytes display increased expression of multiple inhibitory receptors, which may contribute to the inefficiency of HIV-specific antibody responses. Here, we show that downregulation of B cell inhibitory receptors in primary human B cells led to increased tissue-like memory B cell proliferation and responsiveness against HIV. In human B cells, siRNA knockdown of 9 known and putative B cell inhibitory receptors led to enhanced B cell receptor–mediated (BCR-mediated) proliferation of tissue-like memory but not other B cell subpopulations. The strongest effects were observed with the putative inhibitory receptors Fc receptor–like–4 (FCRL4) and sialic acid–binding Ig-like lectin 6 (Siglec-6). Inhibitory receptor downregulation also led to increased levels of HIV-specific antibody-secreting cells and B cell–associated chemokines and cytokines. The absence of known ligands for FCRL4 and Siglec-6 suggests these receptors may regulate BCR signaling through their own constitutive or tonic signaling. Furthermore, the extent of FCLR4 knockdown effects on BCR-mediated proliferation varied depending on the costimulatory ligand, suggesting that inhibitory receptors may engage specific pathways in inhibiting B cell proliferation. These findings on HIV-associated B cell exhaustion define potential targets for reversing the deleterious effect of inhibitory receptors on immune responses against persistent viral infections.

Introduction

Accumulation of a functionally impaired subpopulation of $CD20^{hi}CD27^{-}CD21^{lo}$ tissue-like memory B cells in the peripheral blood of HIV-viremic individuals is a consequence of persistent HIV viremia and is likely induced by chronic immune activation (1, 2). These cells exhibit features of exhaustion, similar to those described in association with persistent viral infections known to induce virus-specific T cell exhaustion (3–6). These features include increased expression of multiple inhibitory receptors, as well as poor proliferative and effector responses to a variety of stimuli.

Inhibitory receptors containing immunoreceptor tyrosine-based inhibitory motifs (ITIMs) are differentially expressed during lymphocyte activation and differentiation. These receptors recognize distinct ligands and trigger the activation of specific intracellular signaling pathways, and thus play an important role in the regulation of immune responses (7–9). Although inhibitory receptors are critical for the normal function of the immune system, their persistent expression can result in decreased cell function, anergy, and exhaustion, as has been observed in persistent viral infections and autoimmune diseases (4–6, 10).

In HIV-viremic individuals, a unique feature of peripheral blood tissue–like memory B cells is the overexpression of the putative inhibitory receptor Fc receptor–like–4 (FCRL4), previously described as the defining phenotype of a distinct subpopulation of tonsillar memory B cells (11). Although its ligands, if any, are currently unknown, functional analyses of the ITIM-containing intracellular domain of FCRL4 indicated that FCRL4 had a profound negative regulatory effect on B cell receptor (BCR) signaling by inhibiting BCR-mediated calcium mobilization, tyrosine phosphorylation of several intracellular proteins, and activation of MAPK Erk and protein kinase B Akt pathways (12). Given its potent immunoregulatory potential, FCRL4 could possibly be a key inhibitory receptor in the B cell dysfunction associated with persistent HIV viremia. However, in addition to FCRL4, tissue-like memory B cells also express at high levels other well-known ITIM-bearing receptors that can serve as negative regulators of BCR-mediated activation. These include FcγRIIB (CD32b), a low-affinity receptor for IgG; CD22 (Siglec-2), a sialic acid–binding Ig-like lectin; CD85j and CD85d, members of the leukocyte Ig-like receptor (LILR) family; and other B cell inhibitory receptors, such as CD72, leukocyte-associated Ig-like receptor–1 (LAIR-1), and variably expressed programmed cell death 1 (PD-1) (2). The increased expression of these multiple inhibitory receptors on tissue-like memory B cells may contribute to their low proliferative capacity and poor effector function and, as such, possibly be involved in the inefficiency of HIV-specific Ab responses in viremic individuals (13). These receptors are thus attractive target candidates for reversing B cell exhaustion.

The purpose of the present study was to investigate the role of inhibitory receptors in HIV-induced B cell exhaustion by downregulating their expression using RNAi technology and evaluating the effect of such downregulation on proliferative and effector

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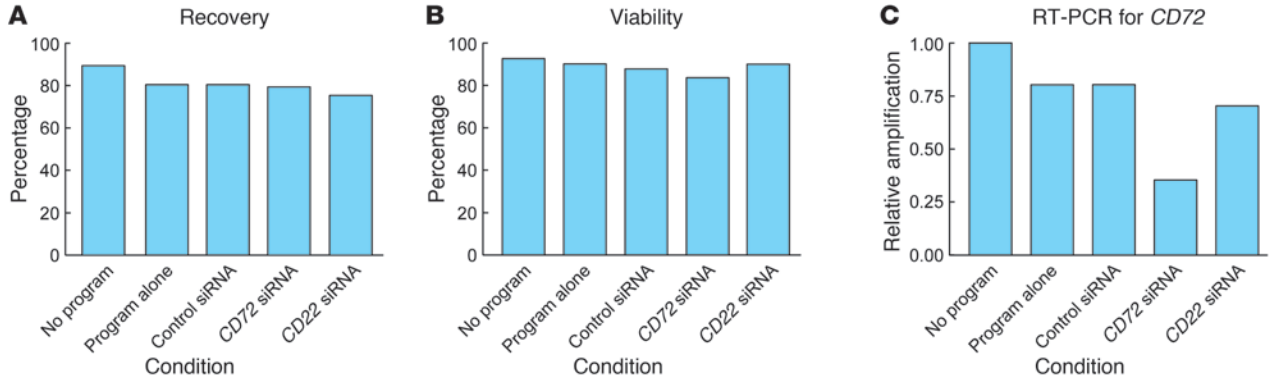

Figure 1

Downregulation of inhibitory receptor CD72 following siRNA transfection into B cells of an HIV-uninfected individual. (**A**) Cell recovery, (**B**) cell viability, and (**C**) relative PCR amplification of *CD72* mRNA at 24 hours after nucleofection into unfractionated B cells. Treatment conditions included cells incubated with nucleofection buffer (no program); cells incubated with nucleofection buffer and submitted to a nucleofection program (program alone); and cells nucleofected with control siRNA or siRNA targeting *CD72* or *CD22*. Percent cell recovery was calculated relative to input number of cells. Levels of *CD72* mRNA are reported relative to amplification in cells subjected to the "no program" condition.

functions. RNAi-mediated sequence-specific post-transcriptional gene silencing triggered by siRNA is a powerful tool for studying the functional attributes of a particular gene and for implementing gene-specific therapeutics (14, 15). Although primary human B cells are largely refractory to most gene transfer techniques, we achieved adequate transfection efficiency and cell viability by nucleofection using a 96-well plate shuttle system, and in the process, we designed a universally applicable strategy involving a nonviral gene delivery method for transfer of siRNA oligonucleotides into primary human B cells. With this approach, we demonstrate that downregulation of several B cell inhibitory receptors in tissue-like memory B cells leads to increased BCR-mediated proliferation and effector function, strongly suggesting a role for multiple inhibitory receptors in B cell exhaustion induced by chronic HIV viremia.

Results

siRNA-mediated downregulation of inhibitory receptors in primary B cells. To investigate the role of inhibitory receptors in B cell exhaustion associated with persistent HIV replication (2), we considered the possibility of measuring functional outcomes after downregulating the expression of various inhibitory receptors in primary human B cells. Attempts to modulate the expression of genes in human B cells by conventional nonviral gene transfer methods have been unsuccessful due to a high rate of cell death and low transfection efficiencies. Recently, it was shown that the death of primary human B cells following transfection by nucleofection correlated with the size and structure of the nucleic acids being transfected (16). The use of siRNA to downregulate gene expression could potentially overcome this limitation given the small size of the RNA molecules involved. In addition to limitations imposed by the size of the nucleic acid molecules, transfection of human B cells by nucleofection is relatively inefficient when compared with transfection of human T cells (up to 40% vs. 70% per Amaxa literature; refs. 17, 18). However, 96-well plate platforms have led to improved efficiency, viability, and versatility when compared with cuvette-based methods (data not shown). Accordingly, we combined siRNA and 96-well plate–based nucleofection technologies in an attempt to downregulate the expression of several known and putative B cell inhibitory receptors that were previously shown to be overexpressed on tissue-like memory B cells of HIV-infected individuals (2). Successful knockdown of target genes was observed 24 hours after transfection as determined by quantitative real-time RT-PCR (Figure 1). As illustrated for *CD72* transcripts in B cells of 1 representative of 6 healthy donors, the nucleofection of siRNA specific for *CD72* led to a median decrease in expression of 62% when compared with control siRNA (Figure 1C; $P = 0.0313$). Similar differences were observed when *CD72* siRNA–treated cells were compared with cells treated with program control or siRNA directed against *CD22*, an unrelated inhibitory receptor (Figure 1C; $P = 0.0313$ for both comparisons). Furthermore, viabilities and recoveries of transfected cells were routinely greater than 80% at 24 hours after treatment (Figure 1, A and B, and Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI45685DS1).

We previously identified and examined in the present study 5 known or putative B cell inhibitory receptors that were either overexpressed or abundantly expressed on tissue-like memory B cells, including CD22, CD72, CD85j, FCRL4, and LAIR-1 (2). In addition, we examined CD32b, a well-characterized B cell inhibitory receptor (19); PD-1, recently shown to be involved in B cell function in a simian model of SIV infection (20); as well as CD85d and Siglec-6. These latter receptors were upregulated on tissue-like memory B cells of HIV-viremic individuals in terms of both transcript (data not shown) and protein expression on the cell surface (Figure 2), when compared with their classical memory and naive counterparts. Increased expression of *SIGLEC6* has also been shown by DNA microarray analyses of similar B cells in rheumatoid arthritis (21). The median decrease in expression of each inhibitory receptor following nucleofection with gene-specific siRNA in unfractionated B cells of healthy donors ranged from 42% to 69% when compared with control siRNA (Supplemental Table 1). Cell viabilities for nucleofected B cells of healthy donors ranged from a median of 80%–90% at 24 hours after transfection. Similar levels of downregulation were achieved in tissue-like memory B cells of HIV-infected individuals (Supplemental Table 1; median ranges from 28% to 66%), although viabilities were generally not as high as those achieved for healthy donors (Supplemental Table 1; median ranges from 64% to 74%). These data show

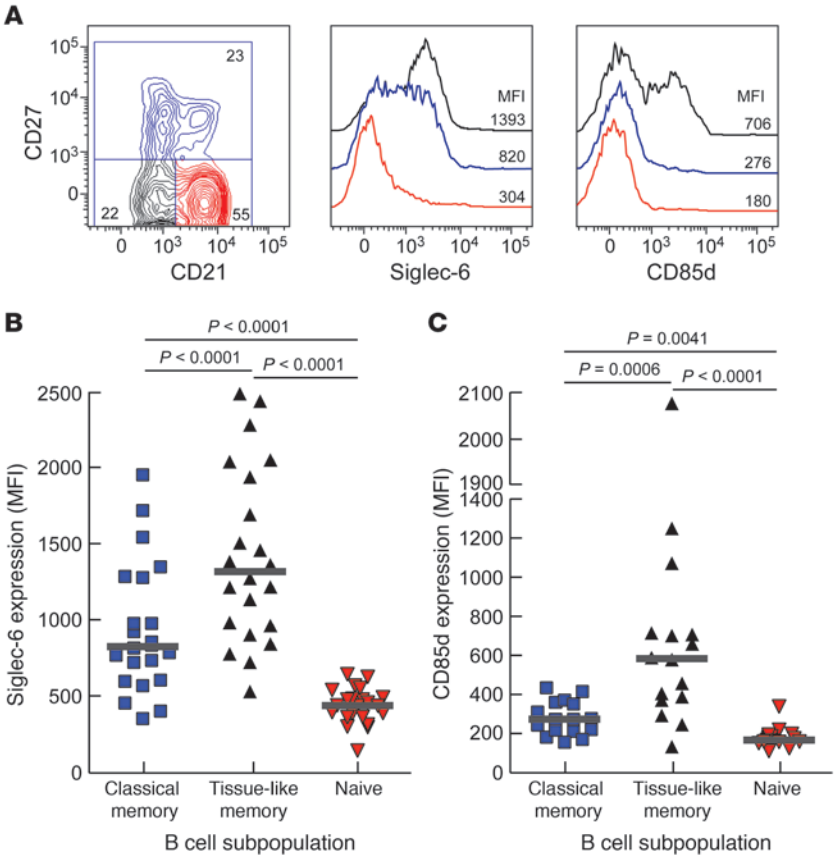

Figure 2
Expression of Siglec-6 and CD85d on tissue-like memory B cells in the peripheral blood of HIV-viremic individuals. (**A**) Representative expression of Siglec-6 and CD85d on B cell subpopulations identified by color coding: $CD27^{-}CD21^{lo}$ tissue-like memory (black), $CD27^{+}$ classical memory (blue), and $CD27^{-}CD21^{hi}$ naive (red) B cells. Expression of (**B**) Siglec-6 and (**C**) CD85d on mature B cell subpopulations of 16–22 HIV-viremic individuals. Horizontal bars indicate medians. Numbers in dot plot in **A** represent percentages. All stains included CD19 to establish the B cell gate and CD10 to exclude immature/transitional B cells.

that a 96-well plate–based nucleofection method can be used for delivering siRNA into primary human B cells with high efficiency and specificity and relatively low toxicity.

Strategy for evaluating the effect of downregulating inhibitory receptors on function of tissue-like memory B cells. We previously demonstrated that tissue-like memory B cells of HIV-infected individuals manifested a reduced proliferative capacity when compared with their naive and classical ($CD27^{+}$) memory B cell counterparts (2). In that previous study, we evaluated proliferation by thymidine incorporation rather than by dilution of the cell division tracking dye CFSE, in part because the former method required relatively few fractionated cells. In addition, the CFSE method was not amenable to the analysis of a slowly proliferating subpopulation, such as tissue-like memory B cells, within an unfractionated preparation of B cells. Furthermore, low expression of CD21, a defining feature of tissue-like memory and other similar abnormal B cells (21), is also observed in vitro when B cells begin to die (22). Nonetheless, when combined with an approach that isolates the subpopulation of interest, CFSE remains a superior method for measuring proliferative capacities and distinguishing live from dead cells (23). Based on these considerations, we devised a method that incorporated purification of B cells by negative selection, enrichment of $CD27^{-}CD21^{lo}$ tissue-like memory B cells by magnetic bead–based fractionation, CFSE staining, nucleofection, and culturing with B cell stimuli (Figure 3A). We used the combination of $F(ab')_2$ pan-BCR polyclonal Abs and type B synthetic CpG oligodeoxynucleotides (CpG-B) as stimuli based on the following criteria: (a) the reduced proliferative response of tissue-like memory B cells to this combination of B cell stimuli (2); (b) the fact that CpG is an excellent survival factor for B cells that could help minimize the stress of nucleofection (24–26); and (c) the fact that many inhibitory receptors have an impact on BCR-driven stimulation (8). In preliminary attempts, we used a previously described double-fractionation scheme to isolate 3 subpopulations of B cells: $CD27^{+}$ classical memory, $CD27^{-}CD21^{lo}$ tissue-like memory, and $CD27^{-}CD21^{hi}$ naive B cells (2). However, this approach overly reduced the yield of viable cells and prompted the single fractionation method shown in Figure 3A to isolate tissue-like memory B cells. We also attempted to sample cultures after 24 hours to establish the percent downregulation for each targeted inhibitory receptor; however, this step induced a high percentage of cell death and was abandoned. Finally, given that setting up duplicate cultures to measure nucleofection efficiency in parallel with assessment of cellular proliferation required a prohibitively large number of cells, we monitored inter-assay nucleofection efficiencies by nucleofecting cells from the "control" $CD27^{+}CD21^{hi}$ fraction (Figure 3A) with *CD72* siRNA and control siRNA and measuring *CD72* transcripts at 24 hours after treatment. In addition, when sufficient numbers of cells in the $CD27^{-}CD21^{lo}$ (tissue-like memory) B cell fraction were available, we evaluated gene-specific siRNA downregulation at the protein level. As illustrated in Figure 3B for one representative downregulation of *SIGLEC6* from a total of 7 assays performed, the median decrease in MFI of receptor expression compared with control siRNA was 44% ($P = 0.0156$), similar to levels found at the transcript level (Supplemental Table 1).

Increased proliferation of tissue-like memory B cells following downregulation of B cell inhibitory receptors. Initial assays to evaluate a gain of function in tissue-like memory B cells following transcrip-

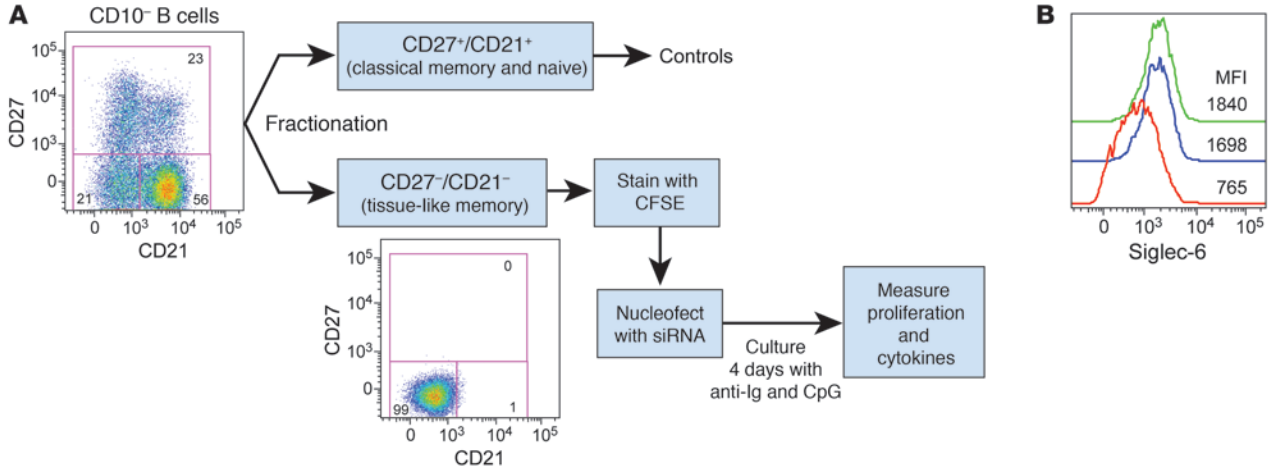

Figure 3
Strategy for evaluating functional responses of tissue-like memory B cells following treatment with siRNA. (**A**) Scheme represents a one-step fractionation to isolate CD27⁻CD21^{lo} tissue-like memory B cells that were then stained with CFSE, nucleofected with gene-specific or control siRNA, and cultured for 4 days. Proliferation was assessed by flow cytometry, and cytokine/chemokine secretion was measured by a multiplex CBA assay. Non-tissue-like memory B cells were used as controls for gene knockdown efficiencies. (**B**) Representative expression of Siglec-6 on tissue like memory B cells following treatment with control siRNA (blue line) or *SIGLEC6*-specific siRNA (red line) or no treatment (green line). Numbers in dot plots in **A** represent percentages.

tional downregulation with siRNA were performed with pools of inhibitory receptor–specific siRNAs. The goal was to diminish the expression of receptors involved in the inhibition of proliferation and/or identify inhibitory receptors that had synergistic inhibitory effects on proliferation. However, this approach led to excessive cell death when optimal levels for each siRNA in a pool were used, and it was difficult to apply equivalent levels of diversified control siRNAs (data not shown). Thus, an approach that targeted multiple individual genes was used, requiring the use as study subjects of HIV-infected individuals from whom we could obtain tissue-like memory B cells in sufficient quantities to test all 9 inhibitory receptors in parallel. These were all individuals who were chronically HIV infected and either viremic or recently viremic at the time of study. Within this group, the geometric mean viral load was 2,096 copies of HIV RNA per milliliter plasma, and the median CD4⁺ T cell count was 427 cells/μl. Individuals with low CD4⁺ T cell counts were generally excluded because they manifest an overabundance of immature/transitional B cells, especially of the most immature type in the CD21^{lo} B cell compartment (27), that could also potentially contaminate the fractionated tissue-like memory B cells, as we have previously reported (2). As shown in Figure 4A for tissue-like memory B cells of a representative HIV-infected individual, downregulation of *FCRL4* by *FCRL4*-specific siRNA led to a 1.8-fold increase in proliferation when compared with treatment with control siRNA. A similar increase in proliferation was observed when a total of 15 individuals were analyzed: the median increase in proliferation after downregulation of *FCRL4* was a stimulation index (SI) of 1.88 when compared with treatment with control siRNA (Supplemental Table 2). In contrast, there was almost no effect of downregulating *FCRL4* in other B cell fractions,

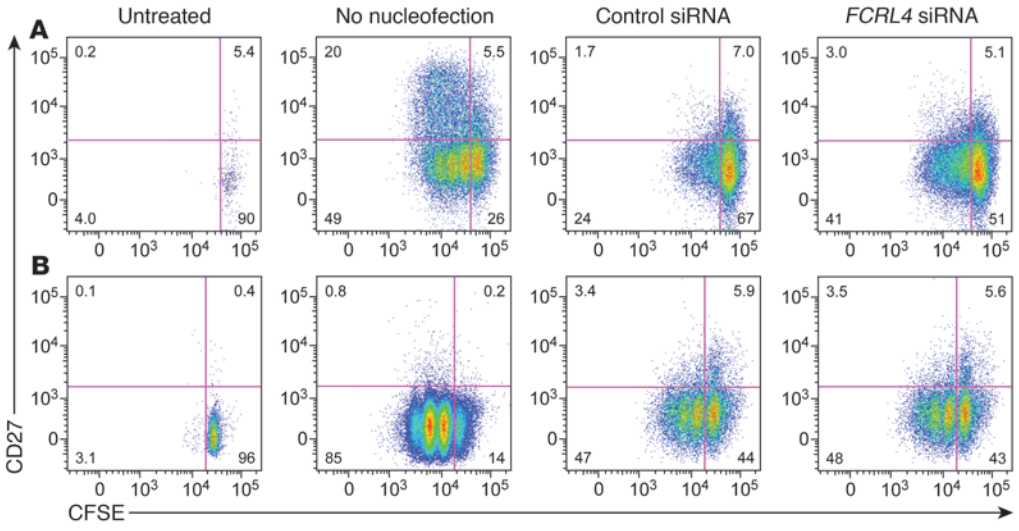

Figure 4
Proliferation of B cells following downregulation of expression of *FCRL4* with siRNA. (**A**) CD27⁻ CD21^{lo} tissue-like memory and (**B**) CD27⁻ naive B cells of 2 different HIV-infected individuals were cultured without any treatment; without nucleofection but with B cell stimuli ($F[ab']_2$ anti-IgG/A/M and CpG); with control siRNA and B cell stimuli; and with *FCRL4*-specific siRNA and B cell stimuli. Numbers in each quadrant represent percentages.

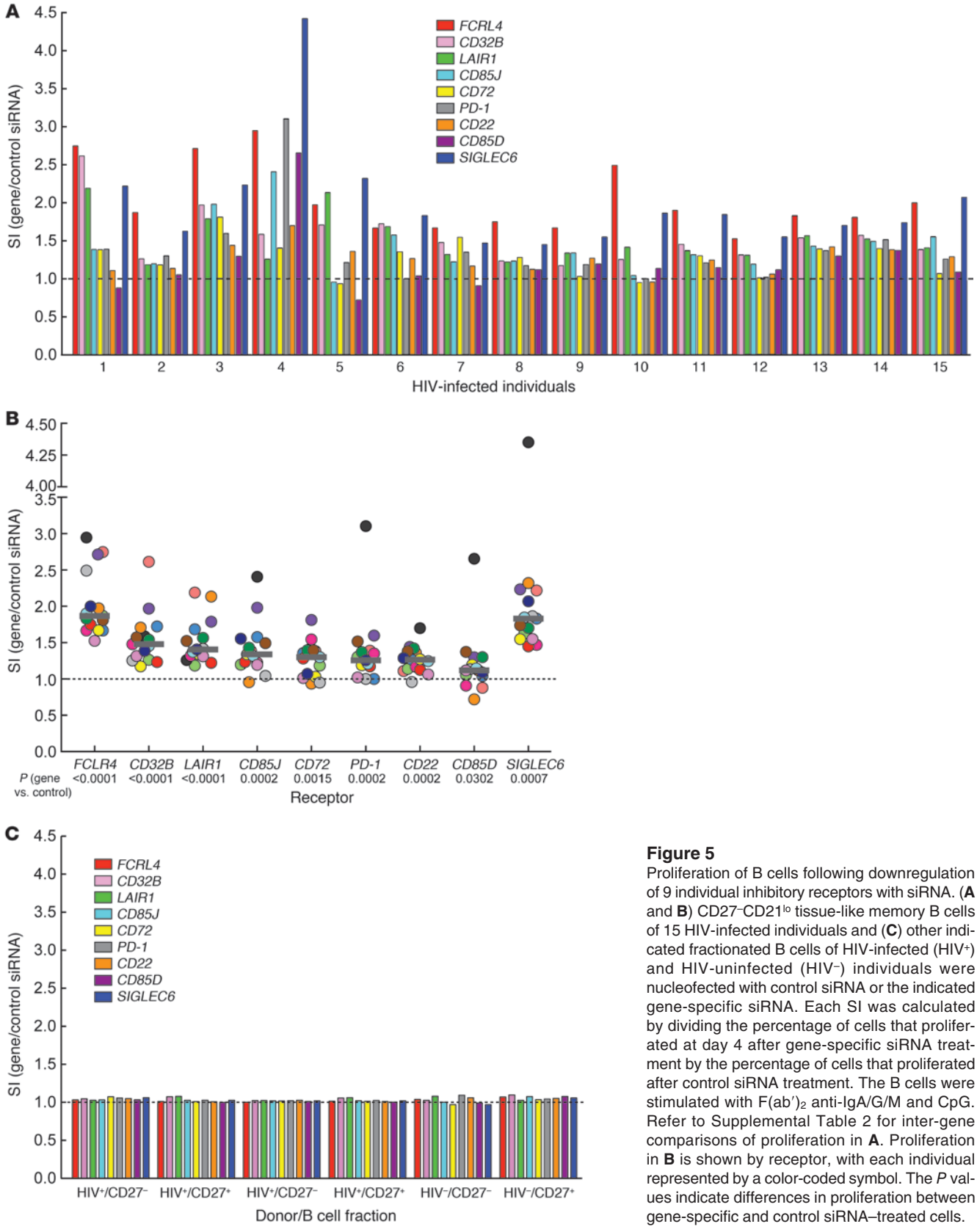

Figure 5
Proliferation of B cells following downregulation of 9 individual inhibitory receptors with siRNA. (**A** and **B**) CD27^{-}CD21^{lo} tissue-like memory B cells of 15 HIV-infected individuals and (**C**) other indicated fractionated B cells of HIV-infected (HIV^{+}) and HIV-uninfected (HIV^{-}) individuals were nucleofected with control siRNA or the indicated gene-specific siRNA. Each SI was calculated by dividing the percentage of cells that proliferated at day 4 after gene-specific siRNA treatment by the percentage of cells that proliferated after control siRNA treatment. The B cells were stimulated with F(ab′)$_2$ anti-IgA/G/M and CpG. Refer to Supplemental Table 2 for inter-gene comparisons of proliferation in **A**. Proliferation in **B** is shown by receptor, with each individual represented by a color-coded symbol. The *P* values indicate differences in proliferation between gene-specific and control siRNA–treated cells.

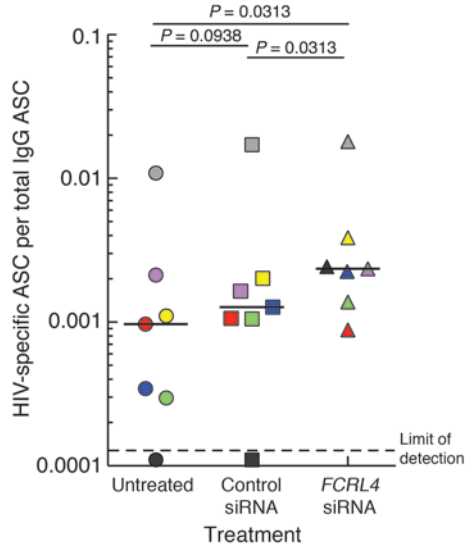

Figure 6
HIV-specific responses by tissue-like memory B cells following siRNA-mediated downregulation of the inhibitory receptor *FCRL4*. Tissue-like memory B cells of 7 HIV-infected individuals were either untreated or treated with control or *FCRL4*-specific siRNA and stimulated in the presence of CD19-depleted autologous PBMCs with $F(ab')_2$ anti-IgA/G/M, CpG, and IL-21 for 4 days prior to measurement of B cell response by ELISPOT assay. Each individual is represented by a color-coded symbol. Horizontal bars indicate medians.

as shown for $CD27^-$ B cells (94% naive and 6% tissue-like memory B cells) of 1 representative of 6 HIV-infected individuals (Figure 4B; median SI of 0.993 and $P = 1.0$) and CD21- or CD27-fractionated B cells of a group of either HIV-uninfected or HIV-infected individuals with few tissue-like memory B cells (Figure 5C). Of note, the effect of siRNA nucleofection was more deleterious to tissue-like memory compared with naive B cells, as evidenced by the differences in percentage of cells that did not proliferate in the presence versus absence of nucleofection (compare Figure 4, A and B). Finally, comparison of the level of proliferation between tissue-like memory B cells and naive B cells confirmed previous observations that tissue-like memory B cells underwent fewer cell divisions in response to the same B cell stimuli, consistent with their low proliferative capacity (Figure 4 and ref. 2).

As shown in Figure 5, downregulation of the 9 inhibitory receptors led to consistent increases in proliferation for tissue-like memory (Figure 5A) but not other B cell fractions (Figure 5C). These differences can also be appreciated from the CFSE profiles shown in Supplemental Figure 1 for two of the individuals from Figure 5, clearly demonstrating increases in proliferation of tissue-like but not naive B cells following downregulation of the 9 inhibitory receptors. When each receptor was analyzed individually for the 15 individuals, significant differences between genes were found (Friedman test: $P < 0.0001$ for the entire set of genes and individual comparisons shown in Supplemental Table 2). As shown by gene in Figure 5B, the highest SI was observed for *FCRL4* (1.88), followed by *SIGLEC6* (1.84) and *CD32B* (1.46). For the rest of the inhibitory receptors tested, the SI values ranged from 1.12 to 1.36 (Figure 5B). As shown by the inter-gene comparisons in Figure 5A, downregulation of *FCRL4* and *SIGLEC6* resulted in levels of proliferation that were significantly higher when compared with all other genes studied, although there was no difference between these two genes. In contrast, while downregulation of other genes, including *CD32B*, *LAIR1*, *CD85J*, and *PD-1*, showed significant increases in proliferation relative to some of the other genes (Supplemental Table 2), the effects were more limited when compared with those observed for *FCRL4* and *SIGLEC6*. When the effect of downregulating each receptor was compared individually against control siRNA, proliferation of tissue-like memory B cells was significantly increased for all inhibitory receptors investigated (*P* values shown in Figure 5B). We also evaluated the effect of downregulating *FCRL4* and *SIGLEC6* together but found no evidence of an additive effect on proliferation (data not shown). Finally, reproducibility of the approach was evaluated by retesting the tissue-like memory B cells of the same HIV-infected individual at a second time point and for whom there was no change in disease status. The patterns shown in Figure 5A were very similar for all 3 individuals who were retested, in that the relative differences between the receptors were maintained, although small differences in SI were observed that reflected differences in efficiency of nucleofection between the duplicate assays (data not shown). Taken together, these data indicate that downregulation of several inhibitor receptors in tissue-like memory but not other B cell subpopulations reproducibly leads to enhanced proliferation in response to BCR stimulation, with the most potent effects observed after downregulation of *FCRL4* and *SIGLEC6*.

Increased effector function of tissue-like memory B cells following downregulation of B cell inhibitory receptors. Next, we considered whether increased proliferation following downregulation of B cell inhibitory receptors was accompanied by an increase in effector function. While Ig secretion is the effector function of choice for B cells, such an evaluation was not feasible given that the anti-BCR reagents used to stimulate B cells interfere with assays used to detect Ig secretion, including most multiplex and ELISA-based assays. However, in separate assays with conditions aimed at maximizing B cell terminal differentiation while maintaining adequate viability (28, 29), we evaluated the effect of downregulating B cell inhibitory receptors on Ab secretion measured by ELISPOT. As shown in Figure 6, following downregulation of *FCRL4* in tissue-like memory B cells, frequencies of HIV-specific Ab-secreting cells (ASCs) per total Ig ASC levels were significantly increased when compared with either untreated or control siRNA–treated counterparts. Absolute values for both total and HIV-specific ASC frequencies are shown in Supplemental Figure 2; there was no significant difference between treatments. Of note, and in contrast to the strong deleterious effect of siRNA treatment on B cell proliferation (see Figure 4 and Supplemental Figure 1), terminal differentiation was not negatively impacted by siRNA treatment, and in some individuals ASC frequencies were increased by the siRNA treatments (Supplemental Figure 2).

In addition, we measured the secretion of various cytokines that are typically produced by B cells (refer to Methods for the entire list of cytokines measured). It should be noted that the cytokine data were more variable compared with the proliferation data; this was not surprising given that cytokine secretion, but not proliferation, is strictly dependent on the concentration of cells in each well along with the viability of the cells. Nonetheless, as shown in Figure 7 for CCL-3 and IL-6, downregulation of *FCRL4* and *SIGLEC6* led to significantly higher levels of these two cytokines by the tissue-like memory B cells when compared with all other inhibitory

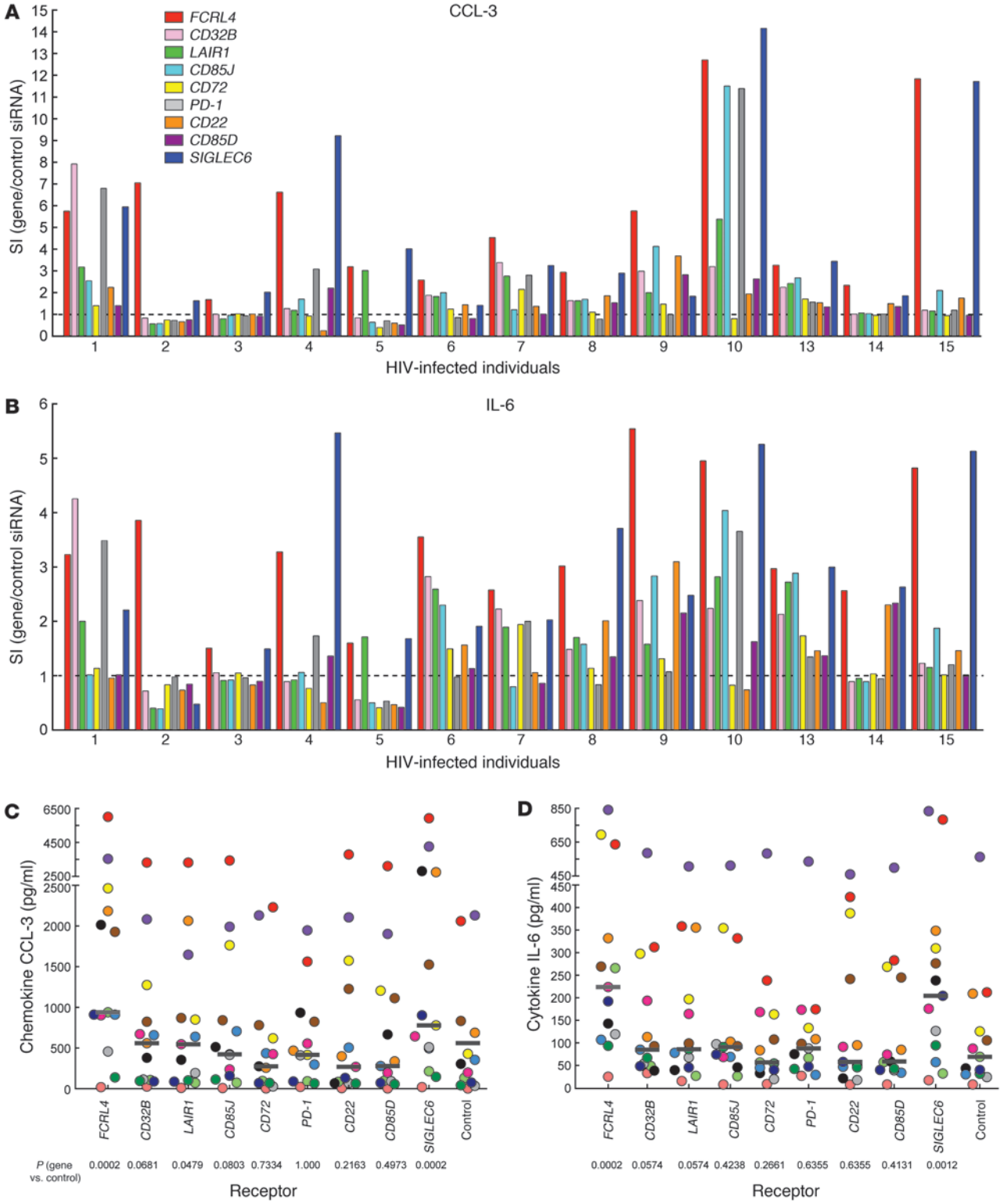

Figure 7

Cytokine and chemokine secretion by tissue-like memory B cells following siRNA-mediated downregulation of inhibitory receptors. (**A** and **C**) CCL-3 and (**B** and **D**) IL-6 secretion from day-4 culture supernatant of tissue-like memory B cells of 13 HIV-infected individuals. Stimulation indices in **A** and **B** were calculated by dividing the levels of cytokines secreted from cells treated with gene-specific siRNA by the levels of cytokines secreted from cells treated with control siRNA. The B cells were stimulated with $F(ab')_2$ anti-IgA/G/M and CpG. Refer to Supplemental Tables 3 and 4 for inter-gene comparisons of CCL-3 and IL-6 secretion, respectively. Absolute values of secretion of CCL-3 (**C**) and IL-6 (**D**) are shown by receptor, with each individual represented by a color-coded symbol. The *P* values indicate differences in cytokine or chemokine secretion between gene-specific and control siRNA-treated cells.

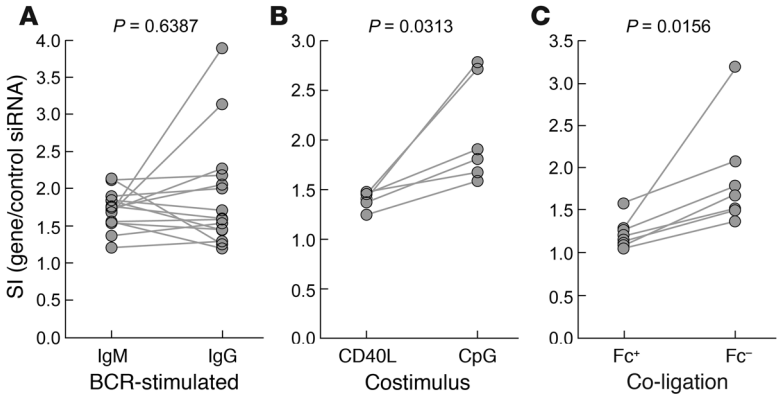

Figure 8
Factors affecting proliferation of tissue-like memory B cells following siRNA-mediated downregulation of *FCRL4*. siRNA-treated tissue-like memory B cells of (**A**) 15 HIV-infected individuals stimulated for 4 days with $F(ab')_2$ anti-IgM or anti-IgG and CpG; (**B**) 6 HIV-infected individuals stimulated with $F(ab')_2$ anti-IgA/G/M and either CD40L or CpG; and (**C**) 7 HIV-infected individuals stimulated with anti-IgM containing an Fc portion (Fc^+) or not (Fc^-) and CpG. The stimulation indices were calculated as described in Figure 5.

receptors studied, although there was no difference between these two genes (*P* values shown in Supplemental Tables 3 and 4). In addition, downregulation of *CD32B*, *LAIR1*, and *CD85J* led to significantly greater secretion of CCL-3 when compared with *CD72*, whereas the difference was restricted to *CD32B* for IL-6 (Supplemental Tables 3 and 4). To be consistent with the proliferation data in Figure 5, we have reported the data in Figure 7, A and B, as the SI of cytokine levels secreted by cells treated with receptor-specific siRNA over levels from cells treated with control siRNA. However, absolute values are shown in Figure 7, C and D, and include *P* values showing that levels were significantly higher for *FCRL4* and *SIGLEC6* when compared with control siRNA. Similar profiles were obtained for cytokines IL-10, TNF-α, and lymphotoxin-α, although cytokines levels were generally lower than those measured for IL-6 and CCL-3 (data not shown). Collectively, these data demonstrate that FCRL4 and Siglec-6 have a dominant effect on both the proliferation and effector functions of tissue-like memory B cells.

Response to different B cell stimuli following downregulation of FCRL4. Next, we considered the effect of different B cell stimuli on the proliferative response of tissue-like memory B cells following downregulation of *FCRL4*. We chose to focus on FCRL4 given its dominant effect on both proliferation and cytokine secretion. First, we attempted to evaluate non-BCR-based stimuli in order to determine whether enhanced proliferation following downregulation of inhibitory receptors required signaling through the BCR. Memory ($CD27^+$) B cells have been shown to proliferate robustly in response to CD40 ligand (CD40L) and cytokines IL-2/-10, although relatively little proliferation was induced in the case of tissue-like memory B cells (2). When tissue-like memory B cells were treated with *FCRL4*-specific or control siRNA, proliferation in response to CD40L and cytokines was too weak to measure (data not shown). Furthermore, attempts to perform these assays with a very potent stimulus such as SAC were also unsuccessful because of a very high rate of cell death.

We then considered whether BCR-mediated proliferation following downregulation of *FCRL4* was influenced by the Ig class within the BCR being triggered or by the route of costimulation. Tissue-like memory B cells of HIV-infected individuals predominantly secrete IgM and IgG, with a very minor component being IgA (2). A survey of 26 HIV-viremic individuals revealed a mean surface distribution on tissue-like memory B cells of 67% IgM/D, 27% IgG, and 6% IgA, compared with 49% IgM/D, 36% IgG, and 15% IgA for classical memory B cells. These Ig class differences between tissue-like and classical memory B cells were significant (IgM/D and IgA: $P < 0.0001$; IgG: $P = 0.0002$). Given the low expression of IgA on tissue-like memory B cells and recent evidence that signaling through IgG versus IgM BCRs is both qualitatively and quantitatively different (30–32), we concentrated on the effect of FCRL4 expression on IgG or IgM BCR-triggered signaling. When tissue-like memory B cells of a group of 15 HIV-infected individuals were stimulated with an $F(ab')_2$ IgM–specific polyclonal Ab, the median proliferation SI following nucleofection with *FCRL4*-specific and control siRNA was 1.7 (Figure 8A). The SI was 1.6 when the stimulus was an $F(ab')_2$ IgG–specific polyclonal Ab, and there was no significant difference between the two BCR stimuli (Figure 8A). Furthermore, as shown in Figure 8B for 6 individuals tested, triggering TLR9 with CpG had a stronger effect on the enhancement of BCR-mediated proliferation following downregulation of *FCRL4* than did triggering CD40 with CD40L. Finally, we considered the effect of co-ligating a known potent inhibitory receptor, CD32b, on BCR-mediated proliferation following downregulation of *FCRL4*. As shown in Figure 8C, proliferation SIs were significantly diminished when the anti-BCR contained an Fc portion that co-ligated CD32b. Interestingly, CD32b's inhibition of proliferation was not complete (median SI of 1.2 for Fc^+ condition; $P = 0.0156$), suggesting that FCRL4 and CD32b may be signaling through similar pathways. Taken together, our findings show that downregulation of inhibitory receptors leads to enhanced BCR-mediated proliferation and that the costimulatory and coinhibitory pathways modulate this enhancement.

Discussion

In the present study, we demonstrate that siRNA can effectively downregulate the expression of genes in primary human B cells using nucleofection technology. In addition, we demonstrate that the downregulation of several known and putative B cell inhibitory receptors in dysfunctional tissue-like memory B cells, but not other mature B cell subpopulations, led to increased proliferation and effector function. Given the previously described association between increased expression of multiple inhibitory receptors and B and $CD8^+$ T cell exhaustion (2, 4), these data suggest that inhibitory receptors may play an important role in the exhaustion and dysfunction of tissue-like memory B cells in chronically HIV-viremic individuals. The downregulation of putative inhibitory receptors FCRL4 and Siglec-6 was found to have the most potent effects on BCR-mediated proliferation and cytokine secretion, despite similar or even lesser degrees of siRNA-mediated downregula-

tion of expression compared with the other 7 inhibitory receptors investigated. These data suggest that FCRL4 and Siglec-6 have the most potent inhibitory effects on BCR-induced proliferation and effector function of tissue-like memory B cells.

The enhanced functional properties of tissue-like memory B cells following the downmodulation of B cell inhibitory receptors was observed without the addition of exogenous ligands. This raises the question of how these inhibitory receptors act to dampen BCR-mediated proliferation and effector function. For certain inhibitory receptors, such as CD22, PD-1, and members of the CD85 family (8), it is possible that their respective ligands are also expressed on B cells, and thus ligation and signal transduction could occur in *cis* or *trans*. In the case of putative inhibitory receptors FCRL4 and Siglec-6, such considerations can only be speculative, given that their respective ligands, if they exist, have yet to be identified. In addition, it is difficult to reconcile the effects observed with CD32b; its downregulation led to an increase in BCR-mediated proliferation of tissue-like memory B cells without any known ligand being present in the cell cultures. These observations raise the possibility that these inhibitory receptors may dampen BCR-mediated proliferation and effector function in exhausted B cells through ligand-independent or tonic signals, as has been suggested for the BCR in various models of B cell survival (33, 34). One such model suggests that inhibitory receptor–mediated negative signaling counteracts the BCR-mediated positive signaling that would otherwise lead to uncontrolled cellular activation (34). It is tempting to speculate that in the context of chronic systemic immune activation, such as during persistent viral replication in untreated HIV-infected individuals, B cells counteract the deleterious effects of excessive activation by upregulating multiple inhibitory receptors that then dampen the ability of the BCR to transduce activating signals. Such a mechanism would be consistent with observations that tissue-like memory B cells and other exhausted and unresponsive lymphocytes express increased levels of markers associated with migration to sites of inflammation, namely CXCR3, CCR6, and CD11c, as well as decreased levels of markers associated with migration to lymph nodes, namely CCR7, CD62L, and CXCR4 (2, 4, 21). In addition, tissue-like memory B cells have a reduced replicative history compared with their classical memory CD27-expressing counterparts, as measured by levels of κ-deleting recombination excision circles (KRECs) (2), further suggesting that the course of their differentiation has been halted.

The increase in proliferation and effector function following the downregulation of inhibitory receptors was unique to tissue-like memory B cells and not observed to any significant extent in either naive or classical memory ($CD27^+$) B cells, despite the fact that these latter subpopulations of B cells express several of the inhibitory receptors tested, including CD22, LAIR-1, CD72, and CD32b (2). Given the strong inhibitory effects seen with FCRL4 and Siglec-6, neither of which is expressed on naive and resting memory ($CD27^+$) B cells found in the peripheral blood (Figure 2 and ref. 2), it is tempting to suggest that the expression of these two receptors play a major role in the unresponsiveness of tissue-like memory B cells. However, given that downmodulation of inhibitory receptors that are expressed on all B cells, including CD22 and CD32b, only enhanced the proliferation of tissue-like memory B cells, there is more to consider than simply an enhanced expression of unique inhibitory receptors (FCRL4 and Siglec-6). It is thus more likely that the stunted proliferative capacity of tissue-like memory B cells is driven by a cumulative effect of increased expression of multiple inhibitory receptors, with some having a stronger impact than others. It is also possible that there are other intracellular features of tissue-like memory B cells that predispose them to tonic inhibitory effects on BCR-mediated signaling. The observation that downmodulating FCRL4 had a greater effect on CpG- compared with CD40L-mediated costimulation of BCR-induced proliferation suggests that distinct signaling pathways may be targeted by the inhibitory receptors, resulting in the inhibition of proliferation of tissue-like memory B cells. In support of this notion, a recent study demonstrated the importance of the PI3K signaling pathway in tonic BCR-mediated survival of mature B cells and, in contrast, a minimal role for the NF-κB pathway (35). In this regard, BCR-mediated induction of mature B cells involving TLR triggering may also depend on the PI3K pathway (35), whereas CD40 signaling is known to depend on the NF-κB pathway (36). Our data showing a stronger effect of TLR9 ligand CpG than CD40L on the enhanced proliferative effect of downregulating the expression of FCRL4 is consistent with a possible role for the PI3K pathway in tonic BCR-mediated survival of mature B cells as well as of other B cell subsets such as tissue-like memory B cells that arise as a result of excessive immune activation. We also observed that co-ligation of BCR and CD32b, a potent inhibitory receptor that blocks BCR signaling, reversed the enhanced BCR-mediated proliferation associated with FCRL4 downregulation. CD32b functions to inhibit BCR signaling via 5′-inositol phosphatase–mediated (SHIP-1–mediated) hydrolysis of PI3K products (37), although human CD32b may function through the tyrosine phosphatases SHP-1 and SHP-2 (38), which have been implicated in FCRL4 signaling (12). Collectively, these observations support the notion that FCRL4 and other inhibitory receptors may modulate tonic BCR signaling in certain B cell subsets.

The role of the BCR, while central to the analyses described in the current study, remains a source of several unanswered questions. Naive B cells require at least two if not three signals to proliferate, including one that triggers the BCR; in contrast, memory B cells require fewer signals to proliferate and can efficiently be induced to proliferate independently of the BCR (2, 11). In a previous study, we had shown that tissue-like memory B cells proliferated very weakly in response to two signals, similar to naive B cells. We have not been able to increase BCR-independent proliferation following the downregulation of inhibitory receptors. Whether this is due to a dependence of the inhibitory effects on the BCR signal or simply that we are operating below the level of detection remains unclear. In addition, we found no evidence of differences in the effects of downregulating inhibitory receptors on proliferation following the triggering of IgG versus IgM. This observation may suggest that differences in the BCR complex signalosome of altered cells such as tissue-like memory B cells is not influenced by the Ig isotype of the BCR. Alternatively, the early signaling events that have been shown to be Ig isotype specific (30) may not translate into differences in proliferation, or the differences may not be detectable in our system. Nonetheless, our findings clearly demonstrate that tissue-like memory B cells, which arise as a result of HIV-induced persistent immune activation, express multiple inhibitory receptors that dampen BCR-mediated proliferation and effector function. Given that inhibitory receptor–associated unresponsiveness of B cells and T cells may contribute to the persistence of HIV and SIV (3, 20, 39), our findings also suggest that the development of strategies aimed at reversing the deleterious effect of these inhibitory receptors may improve immune responses against such persisting viruses.

Methods

Study subjects. Study subjects were over the age of 18 and recruited both locally and nationwide, although all procedures were performed at the NIH. Leukapheresis products were obtained from HIV-infected and HIV-uninfected individuals. HIV plasma viremia was measured by branched DNA assay (Bayer Diagnostics), with a lower limit of detection of 50 copies per milliliter. The study was approved by the Institutional Review Board of the NIAID, NIH, and all study subjects provided written informed consent.

Phenotypic analysis. PBMCs were obtained by density-gradient centrifugation. Mature ($CD10^-$) B cells were isolated from PBMCs by negative magnetic bead-based selection using a B cell enrichment cocktail that was supplemented with tetrameric anti-CD10 mAb (StemCell Technologies). Phenotypic analyses were performed using mAbs obtained from BD, with the following exceptions: FITC-conjugated anti-human CD21 (Beckman Coulter); PerCP-Cy5-5–conjugated anti-human CD19 and PE-Cy7–conjugated anti–human CD27 (eBioscience); and anti–human Siglec-6 was obtained from A. Varki (UCSD, San Diego, California, USA), and its secondary Ab was PE-conjugated goat anti-mouse IgG (CALTAG Laboratories, Invitrogen). FACS analyses were performed on a FACSCANTO II flow cytometer (BD) using FlowJo software (Tree Star).

B cell fractionation. The enrichment for $CD27^-CD21^{lo}$ cells from mature B cells was performed as previously described (2), or by a one-step magnetic bead–based fractionation with biotinylated anti-CD27 (eBioscience) and anti-CD21 (Ancell) together, followed by anti-biotin microbeads (Miltenyi), according to the manufacturer's specifications. The purity of the $CD27^-CD21^{lo}$ fraction was typically greater than 95%.

siRNA transfection. Transient transfection of B cells was performed using the Lonza nucleofection 96-well plate system according to the manufacturer's specifications. Briefly, 1×10^6 cells were resuspended in 20 μl of nucleofector solution, mixed with 500 nM of control nontargeting or gene-specific ON-TARGETplus SMARTpool siRNAs from Dharmacon, with the exception of *CD22* siRNA, which was obtained from Ambion (Supplemental Table 1). Nucleofection was performed using program EO-117. Cells were rapidly transferred to preheated complete medium (RPMI 1640/10% FBS) and incubated for 24–96 hours at 37°C. Viability of cells was evaluated by vital dye exclusion (Guava Technologies).

Proliferation assay. Cells resuspended in PBS were labeled for 8 minutes at room temperature with 0.5 μM CFSE (Molecular Probes, Invitrogen), followed by addition of RPMI 1640/10% FBS and extensive washing. The cells were then nucleofected and cultured for 4 days at 1×10^6 cells per well of a 48-well flat-bottom plate with different combinations of the following reagents: 10 μg/ml goat $F(ab')_2$ anti-human IgG/A/M, or IgG or IgM (Jackson ImmunoResearch Laboratories); 10 μg/ml rabbit $F(ab')_2$ or full Ab anti-human IgM (Jackson ImmunoResearch Laboratories); 2.5 μg/ml CpG oligodeoxynucleotide type B (CpG-B; Operon); and 500 ng/ml CD40 ligand (40). The effect of stimulation on nucleofected samples was evaluated by FACS analysis on a FACSCANTO II flow cytometer (BD) using FlowJo software (Tree Star).

ELISPOT assay. The assay was preformed as previously described (2), with the following culture modifications. The B cells were cultured with CD19-depleted (anti-CD19 beads; Invitrogen) PBMCs at a ratio of 1:10. The stimuli included 10 μg/ml anti-human IgG/A/M, 2.5 μg/ml CpG, and 50 ng/ml IL-21 (Peprotech).

Multiplexed cytokine assay. Cell culture supernatants were collected from transfected samples after 96 hours and stored at –80°C until assayed. Cytokine and chemokine levels were measured using a multiplex bead array system (Cytometric Bead Array [CBA] Flex Sets) and FACSArray instrumentation and FCAP Array software (BD), according to the manufacturer's specifications. The cytokines and chemokines assayed were IL-6, IL-8, IL-10, lymphotoxin-α, TNF-α, VEGF, and CCL-3.

Quantitative PCR. Total RNA was isolated from 0.5×10^6 to 1×10^6 cells using the RNeasy Micro Kit with on-column DNA digestion (QIAGEN), according to the manufacturer's specifications. Total RNA was reverse transcribed and analyzed using TaqMan probe/primer mix (Applied Biosystems), and assays were performed in triplicate by one-step quantitative PCR (Applied Biosystems 7500 system). Data were normalized to the housekeeping gene *POLR2A* by the comparative ΔΔCt method.

Statistics. Phenotypes of B cell subpopulations (Figure 2) and effects of modulating the expression of the 9 inhibitory receptor genes (Supplemental Tables 2–4) or effects of 3 conditions (Figure 6) were compared simultaneously by the Friedman test, which, if differences were significant, prompted pairwise comparisons by the Wilcoxon signed-rank test. Comparisons between control siRNA and gene-specific siRNA (Figure 5B and Figure 7, C and D) or between treatments in Figure 8 were performed with the Wilcoxon signed-rank test. A P value less than 0.05 was considered significant.

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Address correspondence to: Susan Moir, NIH, 9000 Rockville Pike, Building 10, Room 6A02, Bethesda, Maryland 20892-1576, USA. Phone: 301.402.4559; Fax: 301.402.5920; E-mail: smoir@niaid.nih.gov.

Marie A. O'Shea's present address is: Center for Drug Evaluation and Research, Food and Drug Administration, Silver Spring, Maryland, USA.

Jenny Chen's present address is: Indiana University School of Medicine, Indianapolis, Indiana, USA.

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