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Judith F. Ashouri and Arthur Weiss

Distinguishing true Ag-stimulated lymphocytes from bystanders activated by the inflammatory milieu has been difficult. Nur77 is an immediate early gene whose expression is rapidly upregulated by TCR signaling in murine T cells and human thymocytes. Nur77-GFP transgenes serve as specific TCR and BCR signaling reporters in murine transgenic models. In this study, we demonstrate that endogenous Nur77 protein expression can serve as a reporter of TCR and BCR specific signaling in human PBMCs. Nur77 protein amounts were assessed by immunofluorescence and flow cytometry in T and B cells isolated from human PBMCs obtained from healthy donors that had been stimulated by their respective Ag receptors. We demonstrate that endogenous Nur77 is a more specific reporter of Ag-specific signaling events than the commonly used CD69 activation marker in both human T and B cells. This is reflective of the disparity in signaling pathways that regulate the expression of Nur77 and CD69. Assessing endogenous Nur77 protein expression has great potential to identify Ag-activated lymphocytes in human disease. The Journal of Immunology, 2017, 198: 657–668.
signaling than CD69 in human T and B cells, respectively. This is reflective of differences in the requirements of upstream-specific signaling events between Nur77 and CD69. These results have important implications for identifying Ag-specific T and B cells in a variety of human diseases.

Materials and Methods

Collection and processing of human PBMCs

Whole human blood was collected from healthy volunteers in 60-ml syringes containing heparin as the anticoagulant and gently mixed. Samples were immediately diluted with 15 ml of PBS. Leukocytes were isolated by density gradient centrifugation (400 × g for 30 min) over Histopaque 1077 solution (Sigma-Aldrich). PBMCs harvested from the interface were washed with PBS and resuspended in RPMI 1640 medium with 10% FBS. Cell count and viability were determined using a Vi-CELL cell counter (XR 2.03; Beckman Coulter). Cells were either prepared for stimulation assays or cryopreserved in complete medium with 10% DMSO.

Mice

Thymocytes from C57BL/6 mice were used as a positive control for Western blot analysis. All mice were housed and bred in specific pathogen-free conditions in the Animal Barrier Facility at the University of California San Francisco according to the University Animal Care Committee and National Institutes of Health guidelines.

Lysate preparation and Western blotting

Murine thymocytes and Jurkat cells were stimulated with PMA (20 ng/ml) and ionomycin (1 μM) or C505, an anti-Vβ8 mouse mAb (IgM) that reacts with the Jurkat TCR β-chain (21), for 2 h at 37 °C. Cells were lysed directly in SDS-PAGE sample buffer after stimulation. Proteins were separated on SDS-PAGE gels, transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore) by standard immunoblotting techniques. Primary staining was performed with the following Abs: Nur77 (eBioscience) and GAPDH (Santa Cruz Biotechnology). HRP-conjugated secondary Abs were from SouthernBiotech and visualized with SuperSignal ECL reagent or SuperSignal West Femto maximum sensitivity substrate (Pierce Biotechnology) on a ChemiDoc Image Lab station (Bio-Rad).

PBMC stimulation assays

Fresh or thawed cell suspensions were washed in complete medium, counted, and 0.7–10 × 10^5 cells per well were seeded in round-bottom 96-well culture plates in RPMI 1640 culture medium supplemented with 10% FBS. Cells were stimulated with indicated Abs or immunostimulants. Stimuli included Leu4 (IgG1) mouse mAb directed against human CD3ε purified from ascites, goat anti-human IgM F(ab′)2 (Jackson ImmunoResearch Laboratories), staphylococcal enterotoxin enterotoxin E (SEE; Toxin Technologies), CpG oligodeoxynucleotides (ODNs), ODN 2216 for T cells and CpG ODNs 2006 and 1826 for B cells (InvivoGen), IFN-α (gift from PBL Biotech) was used in all stains.

CD45RO (UCHL1) (BioLegend), and IgM Fab fragment (Jackson Immunotechnologies), CD4 (RPA-T4), CD45RA (HI100), CD45RO (UCHL1), and CD8 (I293) (BioLegend), and PE-Cy7, Pacific Blue, allophycocyanin or Alexa Fluor (ImmunoResearch Laboratories) were conjugated to FITC or Alexa Fluor (Invitrogen). Primary Abs and other reagents

Lysis, permeabilization, and analyses were performed using FlowJo (Tree Star). One-way ANOVA with the Bonferroni post-test was performed and graphs were generated using GraphPad Prism version 5.0f for Mac (GraphPad Software, La Jolla, CA). Differences were considered significant at p < 0.05.

In vitro lymphocyte stimulation (with and without inhibitor)

Single-cell suspensions of lymphocytes were plated at a concentration of 1 × 10^6 cells/ml in complete RPMI 1640 and were incubated in the presence of stimuli as indicated. For inhibitor experiments, cells were preincubated with various inhibitors or vehicle control (DMSO) for 30 min at the doses indicated above followed by stimulation with Leu4, anti-IgM (ab′)2, cytokine (IFN-α or IL-4), or TLR ligand (CpG or LPS) at 37 °C.

Intracellular Nur77 and pSTAT staining

Live cells were washed and fixed for 10 min with 2% (v/v) fresh paraformaldehyde at room temperature. Cells were then stained with cell surface markers as indicated. Cells were fixed again with 2% paraformaldehyde followed by permeabilization with 0.1% Triton X-100 (Calbiochem) for 10 min at room temperature. After permeabilization, cells were stained with anti-Nur77 Ab. Cells were washed twice between each step. For pSTAT staining, live cells were fixed, stained for surface markers, and fixed again as per above. They were then permeabilized with 90% ice-cold methanol, vortexed, and maintained on ice for 30 min. After permeabilization, cells were stained with the appropriate pSTAT Ab.

Results

Endogenous Nur77 protein expression reflects the strength of TCR signaling in human peripheral T cells

Three independent fluorescent Nur77 reporter lines have been generated in mice and show that Nur77 is a robust marker of specific Ag receptor signaling (1, 19). We wanted to determine whether this held true for endogenous Nur77 induction in human peripheral lymphocytes. Substantial homology exists between the mouse and human NRR41 genes. Given the 91% shared amino acid identity between the two (30), as well as limited available anti-human Nur77 reagents for flow cytometry, we determined whether anti-mouse Nur77 Ab could bind human Nur77 protein. To address this, Western blotting was performed using lyses from unstimulated Jurkat cells, a human leukemic T cell line, or cells stimulated with the Jurkat-specific TCR Vβ8 Ab C505 (21). Nur77 induction in murine thymocytes in response to PMA and ionomycin was used as a positive blotting control. In Western blots, a band with appropriate mobility for endogenous Nur77 could be detected in stimulated but not unstimulated Jurkat cells using the anti-mouse Nur77 Ab in response to TCR stimulation (Fig. 1A). We confirmed these results in flow cytometry studies in primary human T cells after we adapted the use of this Ab for immunofluorescence and flow cytometry. Polyclonal TCR stimulation of primary human T cells with anti-CD3ε Ab induced Nur77 expression detectable by intracellular staining in a dose-dependent manner (Fig. 1B). In vitro TCR stimulation induced endogenous Nur77 in both CD4 and CD8 primary human T cells, and the percentages of responding cells correlated with the concentration of stimulating anti-CD3ε mAb (Fig. 1B). Furthermore, this correlated well with CD69 induction.

We then examined the temporal characteristics of endogenous Nur77 protein expression in anti-CD3ε–stimulated human CD4

Flow cytometry and data analysis

Cells were stained with Abs of the indicated specificities and analyzed on a BD LSRFortessa flow cytometer (BD Biosciences). Flow cytometry plots and analyses were performed using FlowJo (Tree Star). One-way ANOVA followed by a Dunnett multiple comparison test or a paired two-tailed Student’s t test was performed and graphs were generated using GraphPad Prism version 5.0f for Mac (GraphPad Software, La Jolla, CA). Differences were considered significant at p < 0.05.
and CD8 peripheral blood T cells. Nur77 protein levels peaked between 2 and 4 h after stimulation with anti-CD3ε in both CD4 (Fig. 2A) and CD8 (Fig. 2B) T cells, faster than the peak response of CD69 upregulation. Endogenous Nur77 expression declined after 4 h, somewhat faster than the decline of CD69, in the presence of a robust TCR stimulus in vitro, yet expression continued to remain above basal levels at 40 h (Fig. 2). Nur77 induction after TCR stimulation was further characterized in both the naive (CD45RA⁺RO⁻) and memory (CD45RA⁻RO⁺) T cell populations. Nur77 production is rapid and robust in both CD4 naïve and memory T cell subsets in response to TCR ligation. Its induction in the memory subset was followed by a more rapid decline after 4 h of in vitro stimulation (Fig. 2A). In contrast, the naïve T cells exhibited an initial decline in Nur77 at ∼6 h but had more persistently elevated levels at late time points. These results were mirrored in the CD8 memory and naive subsets, although the CD8 memory subset exhibited a more rapid decline in intracellular Nur77 (Fig. 2B). Because the CD8⁺CD45RA⁺ T cell gate will also capture terminally differentiated CD8 effector memory T cells, CD8 T cells were further distinguished based on CCR7 gating to more accurately resolve the naïve and memory subsets (31–33). There did not appear to be a large difference between the CD8 naive (RA⁺CCR7⁺), central memory (RA⁻CCR7⁺), and effector memory (RA⁻CCR7⁻) subsets in the dose response and temporal induction of Nur77 in response to TCR stimulation (Supplemental Fig. 1). However, the induction of Nur77 in terminally differentiated (RA⁺CCR7⁻) CD8 T cells in response to TCR stimulus appeared to be dampened (Supplemental Fig. 1). Overall, Nur77 was induced rapidly after TCR stimulation, consistent with its known regulation as an immediate early gene. However, its amounts declined more rapidly than CD69, a commonly used marker of early T cell activation, implying that either its transcript is turned off or its protein half-life is shorter. Both have been reported in other systems (34, 35). We conclude that elevated intracellular Nur77 protein expression is a sensitive marker for recent Ag encounter in human T cells, as previously demonstrated in murine reporter mice (20).

**Nur77 is a marker of Ag-specific signaling in human T cells**

To further validate these results using a more physiologic approach, we stimulated primary human PBMCs with SEE, which drives polyclonal activation of Vβ5.1 and Vβ8-expressing T cells through interactions with the TCR in an APC-MHC-dependent manner (36–38). After overnight SEE stimulation, we observed induction of endogenous Nur77 in a small percentage of the unfractionated CD4 T cell population (Fig. 3A). However, when we gated on Vβ5.1 and Vβ8, we observed strong induction of Nur77 and CD69 in a dose-dependent manner (Fig. 3B, Supplemental Fig. 2A). Importantly, these responses were specific for the responding Vβ5.1 and Vβ8 populations, as they were not observed in the vast majority of the CD4⁺Vβ5.1⁻ or CD4⁺Vβ8⁻ populations (Fig. 3C, Supplemental Fig. 2B). In the superantigen system, we observed a robust and sustained induction of Nur77 at 16 h in contrast to the rapid decline of Nur77 when using TCR ligation with anti-CD3ε mAb. These results might reflect the retention of superantigen on the APC/TCR complex, thereby providing continuous TCR signaling.

**Nur77 is not induced by inflammatory stimuli in human peripheral T cells**

Murine studies previously revealed that Nur77-GFP was not sensitive to mitogenic stimulation, unlike CD69 (1). To address whether Nur77 is a more specific marker of Ag-receptor signaling than CD69 in human T cells, mixed human PBMC populations were stimulated in vitro with various inflammatory stimuli, including CpG ODN, IFN-α, LPS, and zymosan (a fungal cell wall component). Each of these stimuli induced CD69 but not Nur77 expression in human CD4 and CD8 T cells (Fig. 4A, 4B and data not shown). We also did not observe Nur77 induction in human T cells following treatment with high doses of IL-6 or IL-2 (data not shown), with the latter being consistent with published mouse studies (1, 20). These results confirm the specificity of Nur77 as a marker of Ag receptor signaling in T cells and suggest that Nur77 expression may serve as a useful tool to identify Ag-activated T cells in clinical disease settings.

To define the characteristics of the divergent pathways contributing to induction of CD69 via non–TCR-mediated pathways, we treated CpG, IFN-α, and LPS-stimulated human PBMCs with small molecule inhibitors. Because type I IFNs and cytokines activate JAK signaling pathways in murine and human T cells (39, 40), cells were preincubated with JAK or Src family kinase (SFK) inhibitors tofacitinib and PP2, respectively. Type I IFN induction of CD69 on CD4 and CD8 T cells proved to be dependent on the JAK signaling pathway, with complete inhibition in the presence of tofacitinib, but not PP2 (Fig. 4C, 4D, Supplemental Fig. 3A). We also found that both the SFKs and JAKs were important in mediating LPS and CpG stimulation of CD69.
induction of CD69 on T cells (Fig. 4C, 4D). The JAK inhibitor sensitivity suggests that the effects of the TLR ligands were acting indirectly, perhaps through monocyte-derived cytokine release in the mixed PBMC population (41), because TLRs have not been shown to signal through JAKs, consistent with published mouse data (2). The SFK inhibitor sensitivity coincided with published mouse and human data demonstrating TLR-MyD88–independent LPS and CpG SFK signaling in macrophages resulting in inflammatory cytokine production (42–44).

Interestingly, we observed a late, but not early, effect of JAK inhibitor on TCR-induced Nur77 induction (Fig. 4C, 4D, Supplemental Fig. 3B). This demonstrates that direct TCR-mediated Nur77 induction after 4 h of stimulation is SFK- but not JAK-dependent as expected. It also suggests that the effects of tofacitinib on the anti-CD3ε induction of Nur77 and CD69 at later time points (16 versus 4 h) (A and B). Data are representative of at least three independent experiments.

Nur77 is a specific reporter of BCR signaling strength in human B cells

The Nur77-GFP transgene has been used as a reporter of murine B cell signaling events, revealing developmental checkpoints, stages of maturation, and in vivo Ag receptor signaling in germinal center B cells (19, 45). In humans most newly generated B cells and a significant fraction of mature B cells display some features of autoreactivity (46). Furthermore, this fraction is markedly expanded in patients with autoimmune disease (46–50). Therefore, detection of autoreactive human B cells could be diagnostically important in patients. We asked whether endogenous Nur77 could be stained on a single cell basis in B cells and whether its induction reflected BCR stimulation and signaling strength. We found that Nur77 was strongly induced in human B cells stimulated with anti-IgM in a dose-dependent manner (Fig. 5A). As in T cells, it was robustly induced at early time points, peaking between 2 and 4 h (Fig. 5B). The induction of Nur77 was similar between naive (CD27−) and memory (CD27+) IgM+ B cell populations, although the memory population appeared to be more sensitive to lower doses of Ag receptor signaling (Fig. 5A, 5B), consistent with reports describing comparable early biochemical events despite robust differences in downstream events (51). Additionally, basal Nur77 levels correlated with surface IgM expression in human B cells, which likely reflects differences in tonic B cell signaling (Fig. 5C), consistent with published functional studies in human B cells showing that anergic autoreactive B cells express lower surface IgM (52).

Interestingly, we find that endogenous Nur77 is a much more specific reporter of Ag receptor signaling in human B cells than CD69. CD69, but not Nur77, could be efficiently induced by IFN-α, TLR-4 (LPS), and TLR-9 (CpG) signaling in a mixed PBMC population (Fig. 5D). One possible explanation for the observed differential induction of Nur77 and CD69 in B cells is that TLR stimulation of mixed PBMCs may trigger cytokine-dependent CD69 induction whereas Nur77 induction appears to be independent of JAK/Stat pathways (Supplemental Fig. 3C and data not shown) (1). This is in contrast to what had previously been reported in Nur77-GFP reporter mice (19) and LPS-induced *Nr4a1* gene expression in murine B cells (53). The delay in CD69 induction by CpG and LPS suggests a delayed or indirect effect on primary human B cells in our assay. The effects of LPS are most likely mediated through TLR4 receptors on monocytes in our cultures because TLR4 receptors are not substantially expressed on human B cells from healthy individuals (54–56).
contrast, the early CD69 induction by IFN-α argues for a direct B cell effect, signaling through the JAK/Stat pathway directly (Supplemental Fig. 3C and data not shown).

Biochemical pathways contributing to Nur77 induction reflect T and B cell differences in Ag receptor signaling

To determine which Ag receptor–induced signaling pathways contribute to Nur77 induction in human T cell subsets and B cells, we treated PBMCs stimulated with anti-CD3ε or anti-IgM with a range of small molecule inhibitors in vitro. These experiments demonstrated a nearly complete dependence on SFKs in T cells and Syk in B cells, as expected and previously shown (Figs. 6, 7) (19). Multiple biochemical pathways are important for Nur77 expression in CD4 T cells. Significant levels of dependency were observed for pathways downstream of PI3K and MEK as probed with small molecule inhibitors GDC-0941 and PD0325901, respectively. We also observed significant sensitivity to MK-2206 and INK128 in memory CD4 T cells, inhibitors of Akt and mTOR complex (mTORC) 1 and 2, respectively, and less sensitivity to rapamycin, inhibitor of mTORC1 alone. Moreover, both memory and naive T cell induction of Nur77 after anti-CD3ε stimulation exhibited similar dependency on the TCR signaling pathways probed (Fig. 6, Supplemental Fig. 4).

Interestingly, CsA, which inhibits calcineurin, a key regulator of the NFAT pathway, did not play a significant or substantial role in Nur77 protein expression after TCR signaling, but it did in the induction of CD69 in CD4 (Fig. 6C–F) and CD8 (Supplemental Fig. 4) T cells. However, Nur77 protein levels in memory CD4 and CD8 T cells may have been more sensitive to CsA than naive CD4 T cells, which can be better appreciated in histograms (Fig. 6A, 6B, Supplemental Fig. 4A, 4B), consistent with a published report (57), although this did not reach statistical significance. Interestingly, JAK signaling contributed substantially to CD69 protein levels after TCR stimulation in CD4 naive and memory T cells (Fig. 6D, 6F), whereas JAK signaling does not have a significant effect on Nur77 induction in response to 4 h of TCR stimulation. These results most likely reflect early cytokine release in these mixed PBMC assays contributing to induction of CD69 and reflecting the relative insensitivity of Nur77 to JAK/Stat signaling (Figs. 4C, 4D, 5D, Supplemental Fig. 3A, 3C, and data not shown) (1).

We did not observe a strong dependence of Nur77 induction on protein kinase C (PKC) signaling using PKC inhibitor Go-6983 (general PKC inhibitor, targeting multiple PKC isoforms) or Go-6976 (classical PKC inhibitor targeting calcium-sensitive isoforms, i.e., PKCα and PKCβ), coinciding with a published report of calcium-dependent, but PKC-independent, induction of Nur77 (58). This contrasts with what was previously described in murine reporter mice (19). The control of Nur77 transcription can vary depending on cell type (59). Our findings do not rule out a role for

**FIGURE 3.** Stimulation with SEE validates Nur77 as a marker of Ag-specific signaling in human T cells. Mixed human PBMCs were stimulated with soluble SEE for 16 h and analyzed by FACS. (A) Overlaid histograms are gated on total CD3+CD4+ T cells and represent endogenous Nur77 expression in unstimulated (light gray–filled histogram), SEE (dark gray dotted line), or 2 h with PMA and ionomycin (dark gray–filled histogram) stimulation. (B) In the upper left panel, the plot demonstrates gating strategy used to identify CD4+Vβ5.1+ subset. In the upper right panel, histograms representative of intracellular Nur77 and surface CD69 levels in response to 3–100 μg/ml SEE as indicated in the CD4+Vβ5.1+ subset. In the bottom panel, plots of Nur77 and CD69 dose response of CD4+Vβ5.1+ subset to SEE at indicated doses are shown. (C) The top plot represents CD3+ T cells stained for CD4 and Vβ5.1 expression to identify double-positive and CD4+Vβ5.1+ subsets. The bottom plots represent Nur77 and CD69 levels in CD4+Vβ5.1- (left) or CD4+Vβ5.1+ (right) subsets stimulated with 3 ng/ml SEE overnight. Data are representative of at least two to three independent experiments.

The Journal of Immunology 661

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PKC signaling in the activation of Nur77 via phosphorylation and translocation, as has been previously described in thymocytes (60). In B cells, Nur77 expression was partially dependent on calcineurin, PKC (Go-6983), MEK, Akt, and mTORC1/2 pathways, and it showed a clear dependence on Syk and PI3K signaling. The biochemical pathways contributing to the induction of Nur77 and CD69 in B cells after BCR crosslinking appear to be similar, although CD69 protein levels are more sensitive to NFAT and MEK inhibition and are less sensitive to mTORC1/2 inhibition (Fig. 7).

**Discussion**

Distinguishing true Ag-stimulated lymphocytes in humans from bystanders activated by the inflammatory milieu has been difficult. Lymphocytes enriched at sites of inflammation become activated...
FIGURE 5. Endogenous Nur77 expression reflects strength of BCR signaling in human B cells. (A and B) Histograms represent intracellular Nur77 and surface CD69 expression in total human CD20+ B cells, naive (CD20+IgM+CD27−) B cells, and memory (CD20+IgM+CD27+) B cells from mixed PBMC populations (A) treated with varying doses of soluble anti-IgM for 2 h (1.25–20 μg/ml in a 5-fold dilution series) or (B) treated with 20 μg/ml soluble anti-IgM from 1 to 16 h as indicated. Data are representative of at least three independent experiments and five biologically different donors. (C) Histograms represent basal Nur77 levels in unstimulated primary human CD20+ B cells (left panel) binned on surface IgM expression levels: highest 15% (red), medium high (blue histogram), medium (green histogram), lowest 15% (gray) (middle panel). Right panel bar graph represents mean Nur77 mean fluorescence intensity (MFI) of B cells based on surface IgM levels and normalized to Nur77 MFI from cells in lowest surface IgM bin ± SEM. Data are accumulative from two independent experiments and three to five biologically different donors. Results are expressed as mean ± SEM. *p < 0.05, **p < 0.01. (D) Mixed human PBMCs were stimulated with various immunostimulants: 12 μg/ml CpG, 20 kU/ml IFN-α, 10 μg/ml (Figure legend continues)
not only through direct Ag stimulation, but also indirectly by other inflammatory mediators. The identification of Ag-specific T and B cell clones has important implications in understanding early events in human diseases. This study describes a novel means of identifying Ag-reactive T and B cells through the detection of endogenous Nur77 protein levels, which serves as a specific reporter of Ag receptor signaling in both primary human T and B cells.

In CD4 and CD8 T cells, endogenous Nur77 expression directly correlates with the strength of TCR signaling (Fig. 1), consistent with previous reports from Nur77-GFP transgenic mice (1, 19). However, the kinetics of endogenous Nur77 could not be accurately defined in these reporter mice due to the extended in vivo half-life of GFP (61, 62). Nur77 is an immediate early gene and indeed is quickly induced upon Ag receptor signaling and depends on new protein synthesis in both T and B cells. Our characterization of Nur77 expression in both memory and naive T cells demonstrates a rapid induction in both populations with an initial bimodal response in both subsets. However, after 4–6 h, the expression levels of Nur77 diverge. Nur77 levels rapidly decline in memory T cells and become unimodal, albeit with persistent lower level expression at late time points. In naive T cells, we observed persistently elevated Nur77 expression.

Temporal differences in Nur77 expression after initial peak induction with anti-CD3ε stimulation in naive and memory T cell subsets may point toward differences in NR4A1 transcription, protein stability, TCR recycling, cell-intrinsic differences in chromatin remodeling (63–65), differences in negative feedback pathways in these cell types, and/or TCR signaling pathways. Disparities in memory and naive T cell–proximal TCR signaling events and effector responses to Ags have been described in both CD4 and CD8 T cells (1, 57, 66–70). Several groups have previously observed greater TCR signaling and calcium mobilization in naive than in memory T cells from humans and mice (68, 70–73), consistent with our Nur77 results. The distinct Nur77 expression patterns in naive and memory T cells are reminiscent of calcium flux differences observed in naive and memory T cells, namely persistent calcium increases in naive T cells over time versus a rapid decline in memory T cells (72).

Despite these differences in Nur77 kinetics, no large disparities were noted in the requirements of the Ag receptor–induced biochemical pathways between naive and memory T cells with TCR stimulation at 4 h, although memory T cells appeared to be more sensitive to Akt and mTORC1/2 inhibitors (Fig. 6, Supplementary Fig. 4). This suggests that Nur77 expression is induced by similar TCR signaling events. Downstream TCR signaling events, such as MAPK signaling, have been shown to be similar in naive and memory CD4 T cells (70). How, then, are the differences in Nur77 expression levels at later time points explained? There are several contributing possibilities. Negative feedback mechanisms may be induced more robustly in memory cell responses to limit immunopathology. The CD45RO isoform expressed in memory T cells is more likely to dimerize and result in dampened TCR signaling compared with the CD45RA isoform expressed in naive T cells (71). Additionally, the strength of early phosphorylation events are reduced in memory compared with naive CD4 T cells (70) and may affect later signaling events and gene transcription. Nur77 transcription and protein levels are sensitive to calcium signaling (74, 75), and prolonged calcium increases in naive T cells may contribute to additional Nur77 transcription. Alternatively, Nur77 may be degraded more rapidly in memory T cells. These mechanisms are not mutually exclusive and can be explored in future studies.

Additionally, we demonstrate that Nur77 levels reflect the strength of BCR signaling in human B cells. The kinetics of Ag receptor–induced Nur77 expression in B cells mirrors that of memory T cells, with a rapid decline in protein levels after 4 h of BCR stimulation with anti-IgM. Much as in T cells, multiple Ag-induced BCR signaling pathways contribute to optimal Nur77 protein expression in B cells (Fig. 7). Interestingly, in addition to Syk signaling, Nur77 induction is completely dependent on the PI3K signaling pathways, a central part of the BCR-triggered signalosome (76, 77). CsA appears to interfere more with Nur77 protein expression in response to BCR signaling in B cells, in contrast to what was observed in T cells (Fig. 6, Supplemental Fig. 4). CsA is an immunosuppressive drug widely used in transplant medicine that inhibits calcineurin activity and thereby abrogates NFAT signaling, although there is increasing evidence that CsA has other cellular targets in addition to NFAT (66). CsA has been shown to interfere with TCR-mediated signaling in T cell hybridomas (78–80) by disrupting Nur77 activation without affecting its transcript or protein levels (80). This is consistent with our findings, namely minimal to no effect of CsA on Ag receptor–induced Nur77 protein levels in T cells (Fig. 6, Supplemental Fig. 4).

A critically important feature of Nur77 expression in primary human T cells is its specificity for Ag receptor signaling, in contrast to the commonly used lymphocyte activation marker CD69. CD69 surface expression is induced not only after TCR or BCR signaling, but also via type 1 IFNs and other immunostimulators (2, 81–83). This appears to be in large part due to JAK-dependent signaling, as the pan-JAK inhibitor, tofacitinib, completely abrogates type 1 IFN–dependent pSTAT1 and CD69 induction in CD4 and CD8 T cells (Fig. 4C, 4D, Supplemental Fig. 3A). Nur77 also proves to be a more specific marker of BCR receptor signaling in B cells than CD69, as it is also not induced upon cytokine or TLR stimulation of mixed PBMCs (Fig. 5, Supplemental Fig. 3C). This differs from what had previously been published in the Nur77-GFP reporter mice (19). We postulate that several possibilities may explain this, including that species differences, tissue-specific differences, or prolonged GFP half-life may allow for accumulation of small amounts of Nur77 induction in the setting of immunostimulators that are below the threshold of detection when staining for endogenous Nur77 in human PBMCs.

One limitation for the use of Nur77 in some human studies is the need for detection by intracellular staining, making it difficult to isolate live cells for functional and some forms of genomic analyses. For functional analyses, it would be preferable to identify surrogate surface markers that identify cells enriched in Nur77. However, this has proven to be difficult, as many activation markers we have studied can be influenced by inflammatory cytokines. Additionally, surrogate cell surface markers may vary between different diseases. With genomic analyses this may be addressed indirectly by matching NR4A1/Nur77 transcript levels with other response genes of interest. Importantly, recent advancements in genomic methodologies now make it possible to perform gene expression analyses on fixed and permeabilized human cells (84).

In summary, Nur77 can be used as a reporter of Ag-specific signaling in primary human T and B cells. Nur77 is an immediate early gene that is rapidly induced after Ag receptor signaling in both human B and T cells. Elevated levels of Nur77 are likely to represent recent Ag signaling

LPS, or with 20 μg/ml anti-IgM for 4 h (green histograms) or 16 h (blue histograms) or with PMA and ionomycin for 4 h. Filled gray histograms represent unstimulated samples treated with media alone. Data are representative of at least three independent experiments.
FIGURE 6. Nur77 levels reflect the integration of multiple TCR signaling pathways in human PBMCs. Histograms represent Nur77 and CD69 induction in CD4+CD8$^-$ naive (A) and memory T cells (B) treated with anti-CD3ε in the presence or absence of specific inhibitors for 4 h. Data in (A) and (B) are representative of at least five biologically different donors. (C–F) Bar graphs represent Nur77 or CD69 percentage positive cells in CD4+CD8$^-$ naive (CD45RA$^+$RO$^-$) (C and D) and memory (CD45RA$^-$ RO$^+$) T cells (E and F) treated with 1.0 μg/ml anti-CD3ε in the presence or absence of specific inhibitors for 4 h. Horizontal dashed line in (C–F) marks Nur77 and CD69 percentage positive of unstimulated cells. Positive gate was set at highest 5% of unstimulated cells. Values in (C–F) are the mean of five to six biologically different donors ± SEM. One-way ANOVA was used to compare unstimulated samples (treated with DMSO vehicle control) and inhibitor treatment groups to Leu4 plus inhibitor vehicle control (DMSO). *p < 0.05, **p < 0.01, ***p < 0.001. ns, not significant (p > 0.05).
FIGURE 7. Nur77 levels reflect the integration of multiple BCR signaling pathways in human PBMCs. Histograms represent Nur77 and CD69 induction in CD20^{+} IgM^{+} B cells (A) treated with anti-IgM in the presence or absence of specific inhibitors for 4 h. Histograms are representative of at least five biologically different donors. (B and C) Bar graphs represent Nur77 or CD69 percentage positive cells in CD20^{+} IgM^{+} B cells treated with anti-IgM in the presence or absence of specific inhibitors for 4 h. Horizontal dashed lines mark Nur77 or CD69 percentage positive of unstimulated cells. Positive gate was set at the highest 5% of unstimulated cells. Values in Fig. 6B and 6C are the mean of five to six biologically different donors. \( * \) used to compare unstimulated samples (treated with DMSO vehicle control) and inhibitor treatment groups to anti-IgM plus inhibitor vehicle control (DMSO). \( * p < 0.05, ** p < 0.01, *** p < 0.001, \text{not significant (} p > 0.05). \)

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