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Cytoplasmic FOXO1 identifies a novel disease-activity associated B cell phenotype in SLE

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ABSTRACT

Systemic lupus erythematosus (SLE) is a manifestation of hyperactivated lymphocytes and results, in part, from the loss of normal tolerance checkpoints. FOXO1 is a transcription factor involved at critical early and late B cell development checkpoints; however, its role in regulating peripheral B cell tolerance is not fully understood. We have applied our published approach for using imaging flow cytometry to study native FOXO1 localisation in human lymphocytes to peripheral blood samples from healthy individuals versus patients with SLE. We report, here, on dramatic cytoplasmic localisation of FOXO1 in two peripheral B cell SLE subsets: IgD-CD27+ (class-switched memory) B cells and IgD-CD27- (atypical memory) B cells. The latter, so-called 'Double Negative' (DN) B cells have previously been shown to be increased in SLE and enriched in autoreactive clones. Cytoplasmic-predominant FOXO1 (CytoFOX) B cells are significantly increased in patients with SLE as compared to healthy controls, and the levels of CytoFoOX DN B cells correlate directly with SLE disease activity. The highest abundance of CytoFox DN B cells was observed in African American females with SLE Disease Activity Index (SLEDAI)≥6. The phenotype of CytoFOX DN B cells in SLE includes uniquely low CD20 expression and high granularity/side scatter. As FOXO1 phosphorylation downstream of B cell receptor-dependent signalling is required for nuclear exclusion, CytoFOX B cells likely represent a high state of B cell activation with excess signalling and/or loss of phosphatase activity. We hypothesise that CytoFOX B cells in lupus represent a novel biomarker for the expansion of pathological, autoreactive B cells which may provide new insights into the pathophysiology of SLE.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a manifestation of hyperactivated lymphocytes and results, in part, from the loss of normal tolerance checkpoints.[1–3] FOXO1 is a transcription factor involved at critical early and late B cell development checkpoints; however, its role in regulating peripheral B cell tolerance is not fully understood. We have applied our published approach for using imaging flow cytometry (IFC)[4] to study native FOXO1 localisation in human lymphocytes to peripheral blood samples from healthy individuals versus patients with SLE. We report, here, on dramatic cytoplasmic localisation of FOXO1 in two peripheral B cell SLE subsets: IgD-CD27+ (switched memory) B cells and IgD-CD27- (atypical memory) B cells. Cytoplasmic-predominant FOXO1 (CytoFOX) B cells are significantly increased in patients with SLE as compared with healthy controls, and the levels of CytoFOX double negative (DN) B cells correlate directly with SLE disease activity. CytoFOX B cells likely represent a high state of B cell activation. We hypothesise that CytoFOX B cells in lupus represent a novel biomarker for the expansion of pathological, autoreactive B cells which may provide new insights into the pathophysiology of SLE.

RESULTS

Imaging flow cytometry (IFC) reliably and quantitatively assesses changes in FOXO1 localisation in subpopulations of primary human B cells

At baseline, total B cells and B cell subsets have predominantly nuclear FOXO1 (figure 1A,C and online supplementary figure 1). After BCR stimulation with αIg F(ab')$_2$, FOXO1 moves to the cytoplasm in all B cell subsets (figure 1B), shown by the significant decreases in FOXO1 mean similarity (figure 1C,D and data not shown) at the 30 and 60 min time points. These findings are consistent with studies indicating that cytoplasmic FOXO1 localisation accompanies B cell activation due to PI3K/AKT signalling downstream of the BCR.[5 6] We conclude that IFC is a reliable and reproducible method for detecting dynamic changes in native FOXO1 localisation within user-defined subsets of peripheral human B cells.

DN and switched memory B cells from patients with SLE have significantly more cytoplasmic FOXO1 compared to healthy donors

We compared four subsets of peripheral B cells in patients with SLE and healthy donors: naïve (IgD +CD27-), DN atypical-memory (IgD-CD27-), switched-memory (IgD-CD27+) and unswitched-memory (IgD+CD27+) (online

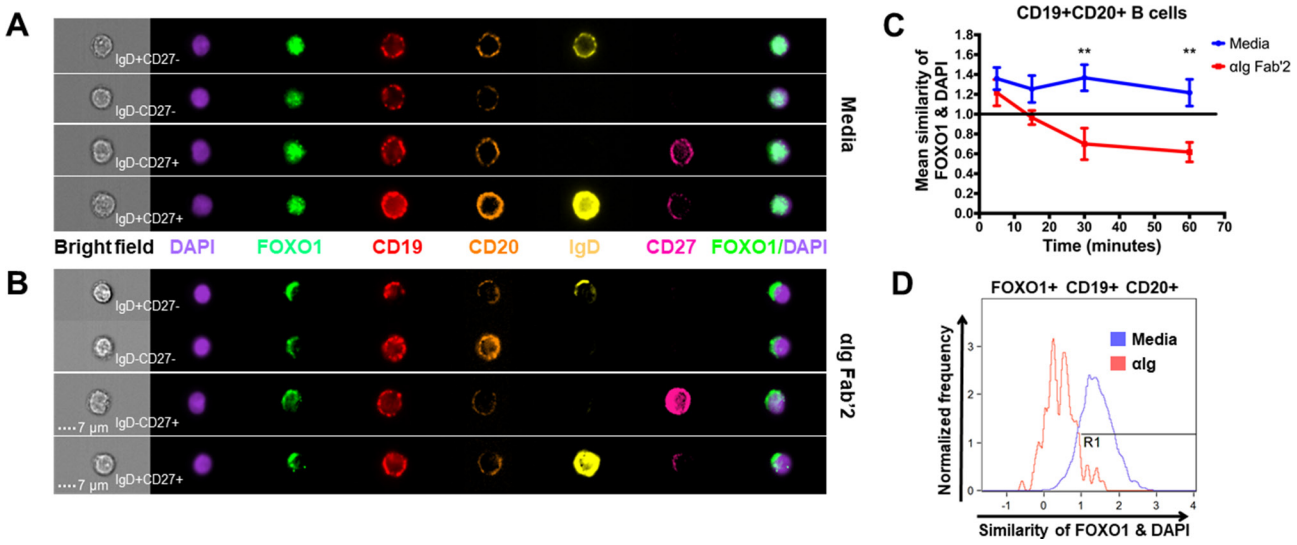

Figure 1 IFC can reliably detect dynamic changes in native FOXO1 localisation in primary human B cell subsets. PBMCs from healthy donors were exposed to either media (A) or a BCR crosslinker (αIg Fab'2) (B) for 5, 15, 30 and 60 min, stained for CD19, CD20, IgD and CD27 (surface) and intracellularly for FOXO1 and the nucleus (DAPI) and then analysed via IFC. Overlay images show that at baseline all B cell subsets have nuclear FOXO1 (A). However, with αIg Fab'2, FOXO1 mean similarity decreases, that is, FOXO1 localises to the cytoplasm (B,D). This effect is kinetic: FOXO1 mean similarity decreases over time with the BCR activation in total B cells at both 30 and 60 min ($p<0.01$) (C). Average of three separate experiments. Mean similarity ≥1 (black line or R1 gate) indicates nuclear FOXO1. Representative images (60×) and histograms from 30 min. Error bars depict SE of the mean; Student's t-test with posthoc Holm Sidak multiple comparisons analysis. IFC, imaging flow cytometry; PBMC, Peripheral Blood Mononuclear Cells.

supplementary table 1). In healthy donors, all peripheral B cells had nuclear FOXO1 (figure 1A). In patients with SLE, however, DN B cells had a significantly lower mean similarity of FOXO1 and DAPI compared with healthy volunteers ($p<0.0001$) indicating that FOXO1 is predominantly cytoplasmic in DN B cells of many patients with SLE (CytoFOX) (figure 2A,E). Patients with SLE also have significantly more CytoFOX DN B cells than healthy controls (~80% vs ~25%) ($p<0.0001$) (figure 2F). Switched-memory and to a lesser extent naïve B cells, but not unswitched-memory cells also displayed a statistically significant increase in cytoplasmic FOXO1 (figure 2B,C,D,F).

Patients with SLE have higher percent DN B cells compared to healthy donors and CytoFOX DN B cells correlate with disease activity

When comparing the percentages of each B cell subset as part of a total typical human B cell compartment, we found a number of variations in the B cell subsets in patients with SLE. As in previous studies,[7 8] we found that patients with SLE have a higher percentage of the DN B cell population compared with healthy donors ($p=0.0006$) (figure 3A), and a significantly decreased percentage of unswitched-memory B cells ($p=0.001$) (online supplementary figure 2) and switched-memory B cells ($p=0.03$) (online supplementary figure 2).[7 8] Total and naïve B cell percentages did not significantly differ between patients with SLE and healthy donors (online supplementary figure 2). The per cent DN B cells in patients with SLE did not correlate with SLEDAI (online supplementary figure 3), also consistent with previous work.[7] However, we did find a strong positive correlation between per cent CytoFOX DN B cells and disease activity in patients with SLE (figure 3B).

CytoFOX DN B cells from patients with SLE have lower CD20 and increased granularity

We examined a number of phenotypic markers to further characterise the CytoFOX population of DN B cells in patients with SLE and healthy donors. CD20 is expressed on all mature B cells and pre-B cells, but is downregulated in plasma cells.[9] Mean intensity of CD20 expression was determined in IDEAS (V.6.2) for the B cells in each of the four subsets analysed above. IgD-CD27- DN B cells had a significantly lower CD20 intensity in patients with SLE compared with healthy controls, in particular CytoFOX DN B cells ($p=0.0191$) (figure 3C). CD20 intensity did not significantly differ between patients with SLE and healthy donors in naïve, switched-memory or unswitched-memory B cells, independent of FOXO1 localisation (online supplementary figure 4). Within patients with SLE, CytoFOX DN B cells had significantly decreased CD20 expression when compared with nuclFOX DN B cells ($p=0.0353$) (online supplementary figure 4); however, they do not stain positive for plasma cell marker CD38 (data not shown).

In flow cytometric analysis, activated B cells and plasmablasts have higher Side Scatter (SSC), indicating increased granularity.[10] Thus, we assessed SSC in the DN B cells of the patients with SLE and healthy donors. Cells were gated based on higher or lower SSC in the total CD19+ CD20+ population (intensity and visual

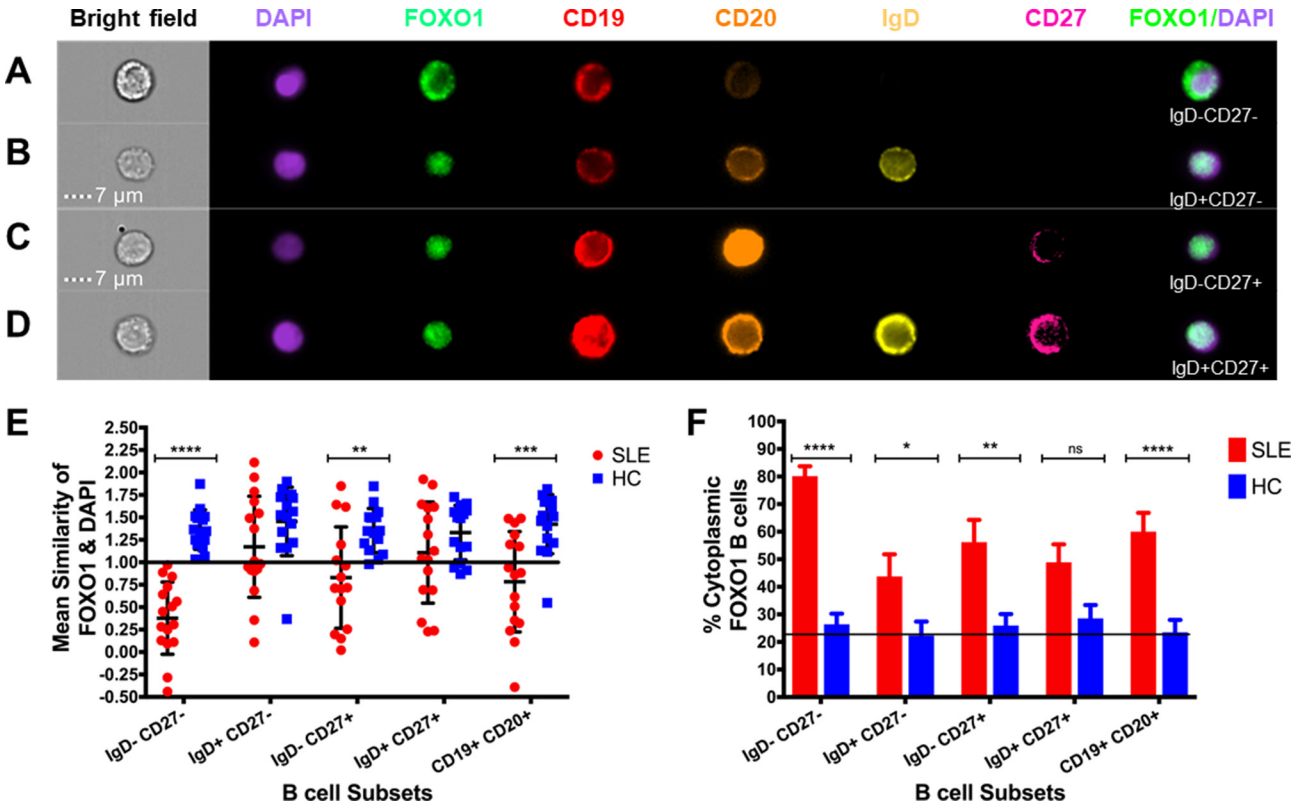

Figure 2 DN B cells from patients with SLE have significantly increased cytoplasmic FOXO1 compared with healthy donors. PBMCS isolated from patients with SLE (n=15) and HC (n=15) were stained with CD19, CD20, CD27 and IgD (surface) and intracellularly for FOXO1 and the nucleus (DAPI) and then analysed via IFC. Cells were gated on focused, single, CD19+ DAPI+ cells and then the CD19+ CD20+ B cells were gated based on IgD and CD27 expression. At least 10 000 single, CD19+ cells were acquired on the Amnis Imagestream Mark IITM and subjected to further analysis in IDEAS. The four subsets of B cells were assessed for FOXO1 localisation using the nuclear translocation wizard in IDEAS (V.6.2). Representative overlay images (60×) from a patient with SLE show that while naïve (B), switched memory (C) and unswitched memory B cells (D) have nuclear FOXO1, DN B cells have cytoplasmic FOXO1 (A). Patients with SLE have significantly lower FOXO1 and DAPI mean similarity in their DN ($p<0.0001$) and switched memory B cells ($p<0.01$) compared with healthy donors (E), indicating more cytoplasmic FOXO1. Additionally, patients with SLE have significantly higher percentages of cytoplasmic FOXO1 (CytoFOX) DN ($p<0.001$), naïve ($p<0.05$) and switched memory B cells ($p<0.01$) compared with healthy donors and well above the average level of CytoFOX in total B cells (black line) (F). Mean similarity ≥1 (black line in E) indicates nuclear FOXO1. Error bars depict SE of the mean; Student's t-test with posthoc Holm Sidak multiple comparisons analysis. DN, double negative; SLE, systemic lupus erythematosus.

inspection confirmed, data not shown). These gates were applied to DN B cells (online supplementary figure 4). Based on these findings, we designed an IFC gating strategy based on CD20 and SSC intensity that allowed us to confidently gate on a population of cells with ~80%–90% CytoFOX (figure 3D). Additionally, the percentage of $SSC^{hi}/CD20^{lo}$ within the DN B cell compartment positively correlated with disease activity in patients with active SLE (SLEDAI ≥4) ($r=0.6245$) ($p=0.0151$) (figure 3E). By gating on $SSC^{hi}/CD20^{lo}$ B cells that are also IgD-/CD27-, we are able to reliably identify the CytoFOX DN B cells, which will allow us to live-sort these cells for future studies.

The CytoFOX DN B cells in SLE had increased granularity, which is sometimes used as a measure of activation.[10] However, in order to more definitely assess the activation state of these CytoFOX DN B cells, SLE (n=6) and HC (n=10) samples were also surface stained with CD95, a classic activation maker, also known as Fas. CD95 is upregulated on active cells and has been shown to be increased on B cells in SLE, including DN B cells.[11] Additionally, the percentage of CD95+ DN B cells correlates with disease activity in patients with SLE.[12] We confirmed that both SLE and healthy donors (online supplementary figure 5) have CD95+ DN B cells. CD95+ DN B cells have more cytoplasmic FOXO1 than CD95- DN B cells in patients with SLE while both CD95+ and CD95- DN B cells from healthy donors have predominantly nuclear FOXO1 (online supplementary figure 5D,E).

We also categorised patients based on two of the SLE symptoms associated with poorer outcomes and more active, inflammatory disease–nephritis and presence of double stranded DNA autoantibodies (dsDNA autoAb). Only patients with greater than 58% CytoFOX DN B cells had nephritis (n=8), positive dsDNA autoAbs (n=5) or both (n=4) as compared with those with <58% CytoFOX DN B cells (figure 3F). χ^2 analysis (data

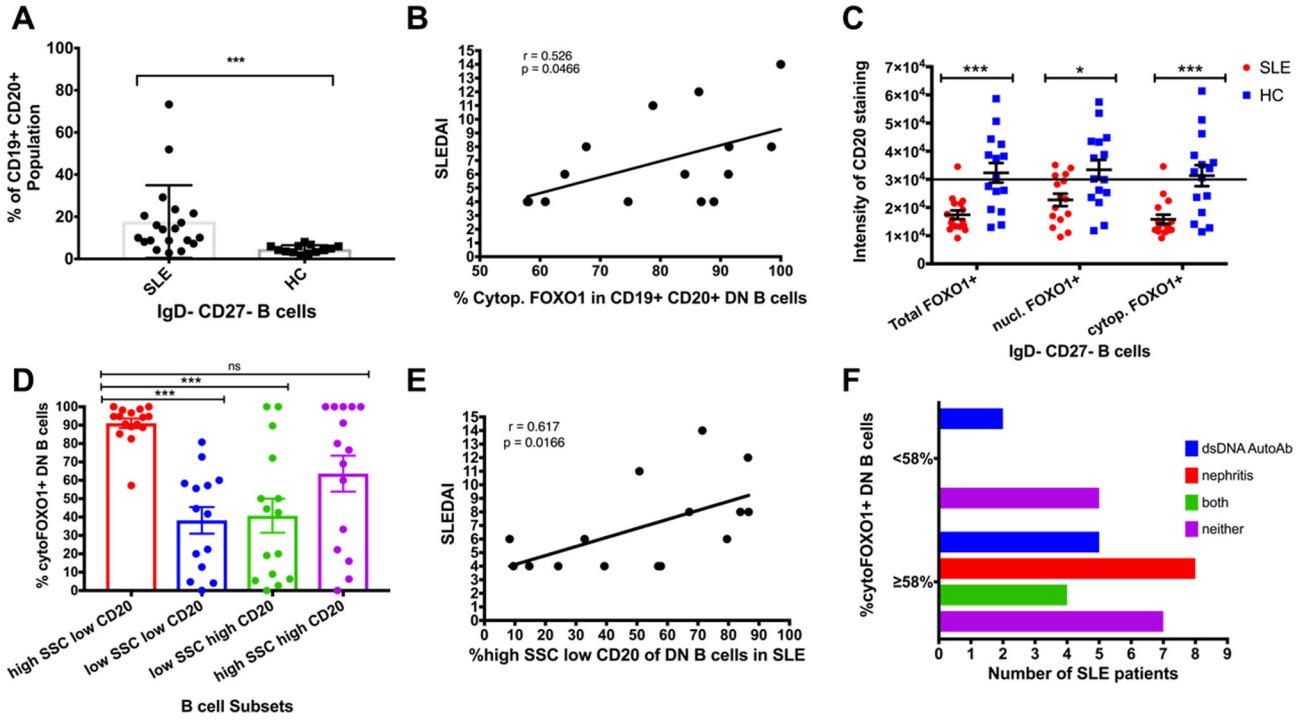

Figure 3 CytoFOX DN B cells correlate with SLEDAI and have lower CD20 and increased granularity**.** PBMCs from patients with SLE (n=20) and healthy donors (n=15) were stained with CD19, CD20, IgD and CD27 and analysed via flow cytometry. Patients with SLE had significantly higher percentages of DN B cells (~20%) compared with healthy volunteers (~4%–5%) (p<0.001) (A). Per cent cytoplasmic FOXO1 DN B cells positively correlates with disease activity (r=0.526) in patients with active SLE (SLEDAI ≥4) (p<0.05) (B). Additionally, SLE FOXO1 +DN B cells have significantly decreased CD20 intensity (p<0.001), especially in the CytoFOX DN subset (p<0.001) compared with healthy donors (C). When the SLE DN B cells are also gated based on granularity (SSC), and CD20 intensity, the $SSC^{hi}CD20^{lo}$ fraction was significantly enriched in CytoFOX cells compared with the other subsets (p<0.001, p<0.001, p=0.058) (D). Additionally, the percentage of $SSC^{hi}CD20^{lo}$ DN B cells positively correlated with SLEDAI in patients with active disease (SLEDAI ≥4) (r=0.617) (p<0.05), (E). Using a cut-off of 58% CytoFOX DN B cells for χ^2 analysis, only patients above that cut-off had more severe disease manifestations such as positive dsDNA antibody, nephritis or both (F). The black line on the CD20 intensity graphs represents the mean intensity of CD20 in total CD19+ CD20+ B cells in healthy donors. Error bars depict SE of the mean. Correlation was assessed via Spearman non-parametric correlation test. The data in (F) were analysed via χ^2 analysis. DN, double negative; SLE, systemic lupus erythematosus; SSC, Side Scatter.

not shown) showed a significant association between frequency of CytoFOX DN B cells and severe SLE disease manifestations.

DISCUSSION

We found that the percentage of CytoFOX DN B cells was positively associated with disease activity in patients with SLE. Furthermore, patients above a certain level of CytoFOX DN B cells were much more likely to have manifestations of more severe disease, including positive anti-dsDNA autoAbs and nephritis.[13 14] Our discovery of increased CytoFOX in DN B cells provides a new biomarker for increased risk of active SLE disease.

By gating on high $SSC^{hi}/CD20^{lo}$ cells, we were able to identify a population of DN B cells that were 80%–90% CytoFOX. This gating strategy will allow us to live-sort these cells for further ex vivo experiments, including B cell effector function, stimulation of antibody secretion combined with EliSpot to determine antigen specificity, and BCR sequencing to determine potential clonal relationships of the DN B cells subsets to other B cells subsets.

Since CytoFOX DN B cells have lower CD20, higher SSC and higher CD95 expression, this suggests that they are more activated than other B cell subsets. Both BCR and BAFFR signalling have been linked, via PI3K/AKT activation, to phosphorylation and nuclear exclusion of FOXO1.[15] Taken together, our findings suggest that the cytoplasmic localisation of FOXO1 in SLE DN B cells is linked to B cell hyperactivation.

In summary, we have identified a subset of IgD-CD27- (DN) atypical memory B cells with CytoFOX, higher SSC and lower CD20 that are significantly increased in patients with SLE with higher disease activity. These cells bear the hallmarks of excessive activation and it is likely that the cytoplasmic FOXO1 reflects this increased activation. Additionally, CytoFOX DN B cells potentially represent a pool of memory cells that can respond to intracellular autoantigens like RNA and DNA and potentiate the smouldering immune activity that characterises SLE. Future experiments will focus on further characterising the origin and antigen-specificity of these CytoFOX DN B cells and the mechanisms that maintain these unusual cells in the periphery in the setting of SLE inflammation.

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Contributors MKHA designed and performed the experiments, analysed the data, prepared the figures and helped to edit the manuscript. AG designed the experiments, analysed the data, prepared the figures and wrote the manuscript. AG designed the experiments, analysed the data, prepared the figures and wrote the manuscript.

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Competing interests None declared.

Patient consent Not required.

Ethics approval This study involved human subjects. Approval for this study was obtained from the University of Maryland School of Medicine IRB as well as the Baltimore VA Research Office of Human Research Protection. There is no identifiable medical information in this manuscript. All patient identities have been removed. Per our IRB-approved protocol, all participants signed informed consent. All identifiable information has been removed from the reported data.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement There are no unpublished data from the study as we have included all the data either in the main document or in the supplemental information. Also, our previously published manuscript in Journal of Immunological Methods (Hritzo *et al*, 2018) provides very detailed information on how to use the AMNIS ImageStream to visualise FOXO1 in human lymphocytes.

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