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Continual signaling is responsible for constitutive ERK phosphorylation in B-1a cells

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ABSTRACT

B-1a cells constitutively express phosphorylated, activated ERK, but the origin of pERK in B-1 cells has not been determined. To address this issue, we examined specific mediators of intracellular signaling in unmanipulated B-1a cells. We found that constitutive pERK was rapidly lost from B-1a cells following addition of metabolic inhibitors that block src kinase, Syk, PI-3K, and PLC function. We examined Syk and PLC in more detail and found rapid accumulation of phosphorylated forms of these molecules in B-1a cells, but not B-2 cells, when phosphatase activity was inhibited, and this change occurred in the majority of B-1a cells. Further, we showed that inhibition of src kinase activity eliminated "downstream" pSyk and pPLC accumulation in phosphatase-inhibited B-1a cells, indicating a pathway connection. CD86 expression is greater on B-1 than B-2 cells and plays a role in antigen presentation by B-1 cells to T cells. We found that when Syk or PI-3K was inhibited, CD86 expression was diminished in a reversible fashion. All together, these results indicate that continual activation of intracellular signaling leads to constitutive activation of ERK in B-1 cells, with attendant consequences for co-stimulatory molecule expression.

1. Introduction

B-1a cells constitute a unique set of B lymphocytes, initially distinguished from conventional splenic B (B-2) cells by expression of the pan-T cell surface glycoprotein, CD5. Additional well-recognized identifying phenotypic characteristics include IgMhi, IgDlo, B220lo, Mac1+, CD23−, and CD43+ (reviewed in Hardy and Hayakawa, 2001; Kantor and Herzenberg, 1993; Rothstein, 2002). B-1b cells represent a companion B-1 cell subset that is phenotypically similar to B-1a cells but lacks CD5 expression. B-1a cells appear first in ontogeny, after which B-1a cells decline in relative number over time as B-2 cell production proceeds (Hayakawa et al., 1983; Lalor et al., 1989). Although B-1a cells are numerically much less abundant than B-2 cells in adult animals (both in mouse and human), B-1a cells maintain themselves as a distinct population through self-renewal, in contrast to B-2 cells that are replenished from early progenitor cells (Hayakawa et al., 1986; Kantor et al., 1992; Lalor et al., 1989). The distinctions between B-1a and B-2 cells extend beyond phenotype to include structural differences in terms of differential expression of various transcription factors, transcripts and proteins, as well as metabolic and functional differences such as variations in mitogenic responsiveness, antigen presentation and skewing of T cell differentiation (Frances et al., 2006; Morris and Rothstein, 1993, 1994; Rothstein and Kolber, 1988b, Fischer, 2001; Zhong, 2007).

B-1a cells provide a unique element in the foundation of immune defense. B-1a cells spontaneously secrete immunoglobulin (Ig) and are responsible for the majority of non-immune serum IgM and substantial amounts of "resting" IgA (Forster and Rajewsky, 1987; Kroese et al., 1993; Sidman et al., 1986; Tumang et al., 2005). The inherent and constitutive secretion of Ig by B-1a cells, in the absence of direct stimulation, distinguishes B-1a cells from B-2 cells, and separates the native functional activities of these two B cell populations. B-1a cell-derived Ig generally adheres more closely to the germline state than B-2 cell Ig, as a result of diminished somatic mutation and reduced length of non-templated N-insertions, and is thus repertoire restricted (Forster et al., 1988; Gu et al., 1990). B-1a cell Ig is often found to recognize microbial cell wall determinants, such as phosphorylcholine derived from *S. pneumoniae*, against which it is protective (Boes et al., 1998; Haas et al., 2005; Klinman and Holmes, 1990). This has led to the accepted notion that B-1a cells produce "natural" antibody, representing a set of broadly reactive specificities encoded in the germline and evolutionarily retained that provides (at low affinity) serological protection against a range of microorganisms prior to the immunization that accompanies microbial pathogenesis. Evidence that natural Ig plays a key role in limiting microbial and viral dissemination and insuring the survival of infected animals has produced a new appreciation

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of the importance of B-1a cells to the overall scheme of immunity and a renewed emphasis on understanding the nature of B-1a cells (Baumgarth et al., 2000; Boes et al., 1998; Briles et al., 1982; Forster and Rajewsky, 1987; Haas et al., 2005).

One of the more curious distinguishing features of naïve B-1a cells lies in constitutive expression of phosphorylated and activated ERK (Wong et al., 2002). B-1a cell expression of pERK distinguishes B-1a cells from naïve B-2 cells, which do not normally express pERK, whereas ERK phosphorylation is induced in B-2 cells following B cell receptor engagement. It has been suggested that B-1a cells show signs of previous activation, which might provide some explanation for constitutive pERK. Although some findings are consistent with this idea (e.g., elevated CD44 expression), many other markers of lymphocyte activation (e.g., elevated CD69 expression) are lacking. Thus, B-1a cells cannot be categorized as an activated form of B-2 cells, and this has been confirmed by the recent identification of a distinct B-1 cell progenitor establishing that B-1a cells constitute a distinct B cell lineage (Montecino-Rodriguez et al., 2006), as was proposed years ago (Herzenberg, 2000). Further, the transcriptional "signature" of "resting" B-1a cells is not the same as that of anti-Ig-stimulated B-2 cells, further confirming that B-1a cells are not similar to activated B-2 cells (unpublished observations). Finally, constitutive B-1a cell expression of pERK is not accompanied by constitutive expression of activated forms of signaling mediators that would be expected if pERK were produced by B-1a cell "activation". For these reasons, the presence of pERK in B-1a cells has been considered to reflect isolated ERK activation, possibly as a result of aberrant MAPKK activity, or as a reflection of previous activation events that have long since run their course and are no longer present.

We have now evaluated the origin of B-1a cell pERK. In B-2 cells, the pathway leading to BCR-triggered ERK phosphorylation begins with src kinase activation and propagates via Syk kinase and a collection of intermediaries termed the signalosome that includes phosphoinositide-3-kinase (PI-3K), and phospholipase Cgamma2 (PLCγ2) (Fruman et al., 2000). Inhibition of these mediators blocks BCR-induced ERK phosphorylation in B-2 cells (Jacob et al., 2002). We considered the possibility that dynamic operation of this pathway might, in fact, be responsible for the presence of phosphorylated ERK in "resting", unstimulated B-1a cells, despite the fact that B-1a cells fail to express many criteria of "activation". To address this issue, we examined the template of BCR-triggered intracellular signaling to query the basis for constitutive pERK in B-1a cells (Morris and Rothstein,

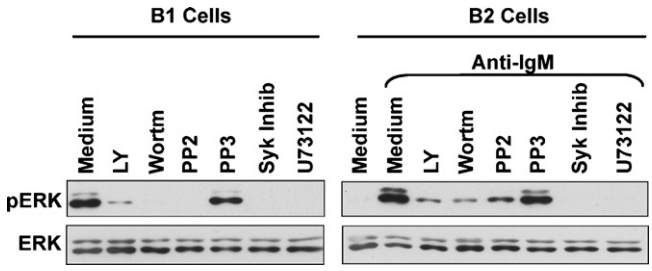

Fig. 1. Constitutive pERK is rapidly lost in B-1 cells exposed to inhibitors of key signaling elements. (Left-hand panel) B-1 cells were cultured in medium alone (medium); were treated with PP2 (20 μM), PP3 (20 μM), Syk inhibitor (Syk inhib, 10 μM), LY294002 (LY, 10 μM), wortmannin (wortm, 25 nM), or U73122 (0.6125 μM), for 1 h. Whole cell extracts were prepared and Western blotted with anti-pERK antibody. Blots were stripped and reprobed with anti-ERK antibody to verify equal loading. (Right-hand panel) B-2 cells were cultured in medium alone (medium); were stimulated by anti-IgM (aIgM, 15 μg/ml) for 10 min; or were treated inhibitors for 1 h as described above followed by anti-IgM for 10 min. Whole cell extracts were prepared and Western blotted as above. One of three comparable experiments is shown.

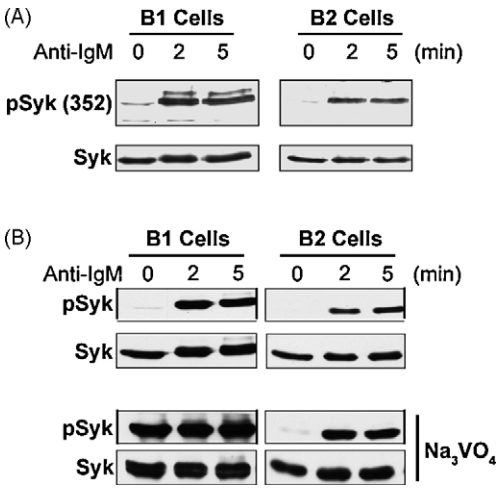

Fig. 2. B-1 cells accumulate pSyk in the presence of phosphatase inhibition. (A) B-1 and B-2 cells were unstimulated (0) or were stimulated with anti-IgM (15 μg/ml) for 2 or 5 min. Whole cell lysates were then prepared and Western blotted sequentially with antibodies specific for tyrosine phosphorylated Syk (Tyr352) and for unphosphorylated Syk. One of five comparable experiments is shown. (B) B-1 and B-2 cells were unstimulated (0) or were stimulated with anti-IgM as described above (top panels); and, B-1 and B-2 cells were treated with Na_3VO_4 (2 mM) for 30 min in the absence or presence of anti-IgM for 2 and 5 min. Whole cell lysates were then prepared and Western blotted with anti-pSyk352 antibody. Blots were stripped and reprobed with anti-Syk antibody to verify equal loading. One of five comparable experiments is shown.

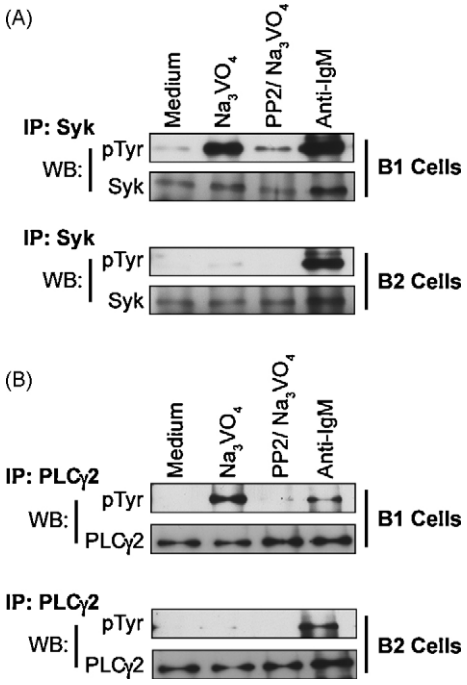

Fig. 3. Inhibition of src kinase activity blocks orthovanadate-enhanced accumulation of pSyk and pPLC. B-1 and B-2 cells were either untreated (medium) or were treated with Na_3VO_4 (2 mM, 15 min); PP2 (20 μM, 1 h) followed by Na_3VO_4 (2 mM, 15 min); or anti-IgM (65 μg/ml, 2 min). Whole cell lysates were prepared, pre-cleared with protein G, and then immunoprecipitated with anti-Syk (A) or anti-PLCγ2 (B) antibody and protein G agarose. Immunoprecipitates were Western blotted with anti-phosphotyrosine antibody 4G10. Blots were stripped and reprobed with anti-Syk (A) or anti-PLCγ2 (B) to verify equal loading. One of three independent experiments is shown.