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Activation induced deaminase-mediated class switch recombination is blocked by anti-IgM signaling in a phosphatidylinositol 3-kinase-dependent fashion

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Abstract

Activation-induced deaminase (AID) is expressed in activated B lymphocytes and initiates somatic hypermutation and class switch recombination. To determine if different stimuli affect the expression and function of AID, we monitored AID activity in murine B cells stimulated *ex vivo* with various ligands. AID was rapidly expressed at both the RNA and protein levels following stimulation with LPS, LPS plus IL-4, and anti-CD40 plus IL-4, but was delayed after stimulation with anti-IgM plus IL-4. By day 4, AID was expressed in all groups; however, cells stimulated with anti-IgM plus IL-4 did not undergo switch recombination. These cells expressed normal levels of $\gamma 1$ germline transcripts, implying that the $\gamma 1$ switch region was accessible. Furthermore, switching was suppressed by the addition of anti-IgM to cells stimulated with LPS plus IL-4 or anti-CD40 plus IL-4, even though AID was expressed. The lack of class switch recombination could be reversed by inhibition of phosphatidylinositol 3-kinase (PI3K). This suggests that activation through the B cell receptor induces PI3K, which interferes with the function of AID.

Keywords

B cells; Antibodies; Activation-induced deaminase; Cell activation

1. Introduction

Immunoglobulin diversity is critical for an effective humoral immune response. The immunoglobulin genes in B lymphocytes are modified by somatic hypermutation, class switch recombination and gene conversion, which are all initiated by the activation-induced deaminase (AID) protein (Arakawa et al., 2002; Harris et al., 2002; Muramatsu et al., 2000; Revy et al., 2000). In biochemical assays, AID has been shown to deaminate cytosine to uracil in single-stranded but not double-stranded DNA (Bransteitter et al., 2003; Chaudhuri et al., 2003; Dickerson et al., 2003; Pham et al., 2003; Ramiro et al., 2003; Sohail et al., 2003). The processing of these uracils can produce both hypermutation in variable and switch regions, and DNA strand breaks which cause class switch recombination and gene conversion. Mutational analyses have shown that the C-terminus of AID is necessary for class switch recombination

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but not for somatic hypermutation and gene conversion (Barreto et al., 2003; Ta et al., 2003). This suggests that different proteins may be recruited by AID to induce alternate outcomes of recombination or mutation.

The regulation of AID is important because it is potentially mutagenic. Over-expression of AID generates mutations that result in tumorigenesis (Okazaki et al., 2003). AID also initiates translocation of IgH to the *c-myc* locus, which is etiologic in Burkitt's lymphoma (Ramiro et al., 2004). Thus, AID needs to be tightly regulated. Although little is known about how AID is targeted to the immunoglobulin locus, there is information on how its activity is controlled at various levels. At the transcriptional level, the AID gene locus contains binding sites for Pax5 (paired box gene 5) and E2A, which regulate B cell development (Dedeoglu et al., 2004; Sayegh et al., 2003; Yadav et al., 2006). Pax5 is expressed in a pattern similar to AID (Gonda et al., 2003) and E2A stimulates hypermutation in the chicken DT40 cell line (Schoetz et al., 2006). Several signaling pathways have been implicated in AID expression, including the JAK (Janus kinase)-STAT (signal transducer and activator of transcription) and NF- κ B pathways (Dedeoglu et al., 2004; Zhou et al., 2003). At the subcellular level, AID contains a nuclear export signal that allows it to exit the nucleus into the cytoplasm. In fact, the majority of AID is found in cytoplasm, and when the export signal sequence is removed, AID accumulates in the nucleus (Brar et al., 2004; Ito et al., 2004; McBride et al., 2004). At the post-translational level, AID is phosphorylated by protein kinase A (Basu et al., 2005; McBride et al., 2006; Pasqualucci et al., 2006), which regulates its activity in B cells. At the protein interaction level, several proteins have been shown to bind to AID. Replication protein A (Chaudhuri et al., 2004) binds to AID and may stabilize the single-strand DNA that is formed during transcription. AID also binds to RNA polymerase II (Nambu et al., 2003), although the functional relevance of this interaction has yet to be determined, and to MDM2 (MacDuff et al., 2006), which has no apparent effect on AID activity.

Thus, multiple layers of regulation exist to control the activity of AID. To examine which cell stimuli regulate its activity, we studied the expression and function of AID in murine B cells stimulated with various ligands. A delay in expression and lack of class switching was found in cells stimulated through the B cell receptor (BCR) compared to stimulation through Toll-like receptor 4 or CD40. Furthermore, signaling through the BCR blocked class switching in cells stimulated through the Toll-like receptor and CD40. The IgM-mediated inhibition of switching was reversed by inhibition of phosphatidylinositol 3-kinase (PI3K), indicating that PI3K suppresses the function of AID.

2. Materials and methods

2.1. Purification of murine splenic B cells

Single cell suspensions of splenic lymphocytes were prepared from 2- to 4-month-old BALB/c mice. All animal procedures were reviewed and approved by the NIA Animal Care and Use Committee. Cells were suspended to 10^8 cells per 500 μ l, and labeled with magnetic microbeads coupled to anti-CD43 and anti-CD11b antibodies (Miltenyi Biotech, Auburn, CA) to remove T cells and macrophages. Following the manufacturer's directions, the labeled cells were passed over a LS+ column, and flow-through B cells were collected. Purity of the B lymphocytes was >95% as assessed by flow cytometry.

2.2. Activation of B cells

For activation with ligands, B cells were resuspended to 1 to 2×10^6 cells per ml in RPMI supplemented with 10% FCS, 2 mM L-glutamine, 10 mM HEPES, 55 μ M β -mercaptoethanol, 100 U/ml penicillin, and 100 U/ml streptomycin (Invitrogen Gibco, Carlsbad, CA). Cells were placed in a 24-well plate and exposed to the following in various combinations: IL-4 (50 ng/

ml, R&D Systems, Minneapolis, MN), LPS (50 µg/ml, *E. coli* 0111:B4, BD Diagnostics [Difco], Sparks, MD), anti-CD40 (αCD40, 1C10, 10 µg/ml, eBioscience, San Diego, CA), anti-IgM F(ab')₂ (αIgM, 10 µg/ml, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), or LY294002 (1 or 2.5 µM, Sigma, St. Louis, MO).

2.3. Proliferation assays

For proliferation, B cells were plated at 5×10^5 cells per 100 µl well in a 96-well plate, and activated as described above. After 2 days, cells were pulsed for 12-16 hours with [³H] thymidine (1 µCi, specific activity 6.7 Ci/mMole, Perkin Elmer NEN, Waltham, MA), harvested, and ³H incorporation was evaluated by scintillation counting.

2.4. RT-PCR analysis

Complementary DNA was synthesized from 0.5 to 1 µg of total RNA using Superscript III reverse transcriptase (Invitrogen) and an oligo-dT primer in a total volume of 20 µl. Serial dilutions of cDNA were used in a semi-quantitative PCR assay using Pfx Taq Polymerase (Invitrogen). The AID and β-actin primers were: AID(F) 5'-GGAATTCGGATATGGACAGCCTTCTG-3', AID(R) 5'-CGGGATCCCCACGTGCCGGGCACAGTCATAG-3', β-actin(F) 5'-CCTAAGGCCAACCGTGAAAAG-3', and β-actin(R) 5'-TCTTCATGGTGCTAGGAGCCA-3'. Amplification conditions were 95°C for 30 seconds, 55°C for 30 seconds, and 68°C for 1 minute by 35 cycles after denaturation for 2 minutes at 95°C. The PCR products were separated on 1.2% agarose gels, and the images were captured by an Alpha Imager 2200 documentation and analysis system (Alpha Innotech Corporation, San Leandro, CA) or a Typhoon PhosphoImager (Amersham Biosciences, Piscataway, NJ).

For real-time quantification of transcripts, the cDNA was diluted 10-fold in distilled, deionized water, and 5 µl was used as a template in a 20 µl reaction. Primers for β-actin, AID, and γ3 germline transcripts (GLT) were: β-actin(F) 5'-CTAAGGCCAACCGTGAAAAG-3', β-actin(R) 5'-CCATCACAATGCCTGTGGTA-3', AID(F) 5'-AGAAAGTCACGCTGGAGACC-3', AID(R) 5'-CTCCTCTTACCACGTAGCA-3', γ3 GLT(F) 5'-CAGAGAAGAGGTGGCCAGAG-3', and γ3 GLT(R) 5'-GTCACCGAGGATCCAGATGT-3'. The γ1 GLT primers have been previously described (Lumsden et al., 2004). Reactions using iTaq SYBR Green Supermix with ROX were set up according the instructions provided by the manufacturer (Bio-Rad Laboratories, Hercules, CA), and carried out on a 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA). Amplification conditions were as follows: 50°C for 2 minutes; 95°C for 3 minutes; 40 cycles of 95°C for 15 seconds; and 60°C for 45 seconds. Sequence Detection Software version 1.3.1 was used to determine threshold cycle, and C_t values and calculated normalized values as described by Livak and Schmittgen (Livak and Schmittgen, 2001).

2.5. Western blot

Whole cell lysates were prepared from 2×10^7 B cells activated for 4 days with either LPS plus IL-4 or αIgM plus IL-4, using 1% Triton, 20 mM Tris (pH 7.5), 150 mM NaCl supplemented with the complete protease inhibitor mixture (Sigma, St. Louis, MO), 1 µM sodium fluoride, and 1 µM sodium orthovanadate. Protein concentrations were determined with the BCA protein assay (Pierce, Rockford, IL). 30 to 40 µg of sample were loaded into each well, separated by gel electrophoresis, transferred to a polyvinylidene difluoride membrane (Invitrogen), and probed with antibodies. Antibodies used were rabbit anti-AID (a generous gift from Janet Stavnezer, University of Massachusetts (Schrader et al., 2005)), mouse anti-AID (Cell Signaling, Danvers, MA), rabbit anti-AKT (Cell Signaling), rabbit anti-phospho (Ser473, Cell Signaling), and mouse anti-β-actin (Clone AC-74, Sigma). Bound antibodies

were detected by ECL Plus, exposed to film (Amersham Biosciences), and images were acquired on an Alpha Imager 2200 documentation system.

2.6. Class switch flow cytometry

Cells were harvested at varying time points, and labeled using combinations of PE anti-IgG1 (A85-1, BD Pharmingen), PE goat F(ab')₂ anti-mouse IgG1 (Southern Biotech), FITC anti-IgG3 (R40-82, BD Pharmingen), FITC anti-CD45R (B220, eBiosciences), and PE anti-CD45R (B220, eBiosciences). Cells were analyzed on a Becton Dickinson FACScan or FACSCalibur and data analyzed with FlowJo (Treestar, Ashland, OR).

2.7. Digestion circularization-PCR

Digestion circularization (DC)-PCR was performed as described (Chu et al., 1992; Wuerffel et al., 2001). Briefly, DNA was isolated from stimulated cells, digested with EcoRI, and ligated. The ligated DNA was subjected to nested PCR using primers specific for switch (S) μ and S γ 1 regions. As a control, the acetylcholine receptor (AChRe) gene was amplified as described (Chu et al., 1992).

3. Results

3.1. Delay in AID expression following activation through the Ig receptor

Splenic B cells from 2 to 4 month old mice were stimulated alone or in various combinations of the following activators: IL-4, LPS, α IgM, and α CD40. As shown in Fig. 1A, with the exception of IL-4 alone, B cells proliferated in response to all the activators as measured by tritium uptake. Cells activated with LPS with or without IL-4 proliferated similarly, whereas cells stimulated with α IgM and α CD40 proliferated more robustly when IL-4 was added. Next, cells were examined for mRNA expression of AID by RT-PCR on days 2 through 4. The results from multiple experiments show that there is a delay in AID expression in cells activated by α IgM plus IL-4 compared to cells activated with LPS, LPS plus IL-4, or α CD40 plus IL-4 (Fig. 1B). This delay was confirmed by quantitative RT-PCR illustrating the approximately 24 hour delay in AID mRNA expression (Fig. 1C). To confirm the RNA analysis, western blots were used to detect AID protein. By day 4 cells stimulated with α IgM plus IL-4 expressed similar levels of AID protein to those stimulated with LPS plus IL-4 (Fig. 1D).

3.2. No class switch recombination in cells stimulated with α IgM plus IL-4 despite normal levels of γ 1 germline transcripts

Functional class switching by the activated cells was examined by two different methods. First, flow cytometry was used to detect surface expression of different heavy chain isotypes. By days 4 and 5 (Fig. 2A), cells activated with LPS plus IL-4 expressed surface IgG1, and cells stimulated with LPS alone expressed surface IgG3. In contrast, B cells stimulated with α IgM plus IL-4 did not switch to either heavy chain class. This was not due to differences in viability as all cells from all activation conditions displayed similar levels of survival as measured by propidium iodide staining (data not shown). Second, a more sensitive technique, DC-PCR, was used to detect switch recombination between S μ and S γ 1 according to the procedure described by Chu and colleagues (Chu et al., 1992). At day 5 (Fig. 2B), cells stimulated with LPS and LPS plus IL-4 had recombination products between S μ and S γ 1, whereas cells stimulated with α IgM plus IL-4 had no evidence of recombination. S γ 3 recombination products were found only in cells stimulated with LPS (data not shown). Germline transcripts of the S γ 1 and S γ 3 regions were measured to determine the accessibility of the locus for class switch recombination. These transcripts represent the spliced product of transcription from a promoter preceding the S γ 1 or S γ 3 regions through the C γ 1 or C γ 3 gene (Xu and Stavnezer, 1990). Cells stimulated with IL-4 plus α IgM, LPS, or α CD40 had similar levels of γ 1 germline transcripts

(Fig. 2C). The expression of germline transcripts was not seen at the IgG3 locus except in cells activated with LPS only (Fig. 2C). Thus, the lack of switch recombination in cells stimulated by α IgM plus IL-4 is not due to inaccessibility of the switch locus.

3.3. Suppressed class switch recombination in cells stimulated with α IgM plus LPS and IL-4 or α CD40 and IL-4

The finding that stimulation with α IgM does not promote class switching raises the possibility that this signaling pathway might suppress the switch mechanism induced by LPS and α CD40. We therefore stimulated the cells with α IgM plus IL-4 and either LPS or α CD40. As shown in Fig. 3A, the addition of α IgM inhibited the class switch recombination that normally occurs in cells stimulated with LPS plus IL-4 or α CD40 plus IL-4. To determine if AID transcript levels were also delayed, quantitative RT-PCR was performed using mRNA purified from cells activated for either 2 or 4 days. As previously noted, AID expression was induced by day 2 in B cells stimulated with LPS plus IL-4 or α CD40 plus IL-4. Addition of α IgM signaling reduced AID transcript levels in α CD40 plus IL-4 activated cells, but had little effect on AID levels in LPS plus IL-4 stimulated cells on day 2 (Fig. 3B). However, on day 4, α IgM super-induced AID in cells stimulated with LPS plus IL-4, whereas α IgM somewhat reduced AID in cells stimulated with α CD40 plus IL-4. Therefore, absence of switch recombination does not appear to be due to lack of AID expression, since the levels of AID were relatively high in all groups by day 4.

3.4. Restored class switch recombination by blocking PI3K signaling

The suppressive effect of α IgM on class switch recombination that was induced by two different activators, LPS and α CD40, suggested that signaling by the BCR was regulating this event. To determine if BCR signaling was involved, splenic B cells were pretreated with LY294002, an inhibitor of PI3K, followed by activation with LPS plus IL-4, LPS plus α IgM plus IL-4, or α IgM plus IL-4. Following three days in culture, cells were assessed for switching to IgG1. As seen in Fig. 4A, blocking PI3K restored switch recombination in cells activated with LPS plus α IgM. In addition, the restored switch recombination was dose dependent, with switching to IgG1 seen with as little as 1 μ M of inhibitor. Switching was also increased in cells activated with LPS plus IL-4, suggesting there is an inhibitory effect of PI3K signaling in these cells, which has been described previously (Omori et al., 2006). Thus, the inhibition of LPS-induced switching by α IgM signaling is PI3K-dependent. However, the PI3K inhibitor did not restore class switching in cells activated only with α IgM plus IL-4.

To determine the α IgM-specific effect on class switch recombination, we examined the phosphorylation status of AKT, since AKT has been shown to negatively regulate class switch recombination (Omori et al., 2006). As shown in Fig. 4B, LPS plus IL-4 treatment induced AKT phosphorylation, and this inducible phosphorylation was increased by the addition of α IgM (compare lanes 4 vs 2). The increased phosphorylation was not due to an increase in total AKT levels, since they were comparable in these two groups. As expected, both inducible phosphorylations were sensitive to LY204002 pretreatment (lanes 3 and 5). The higher degree of phosphorylation by BCR signaling could explain the fact that BCR-induced inhibition of class switching still existed at lower doses of LY294002, while at higher doses, the inhibitory effect of BCR signaling was completely blocked (Fig. 4A). Collectively, these data suggest that the inhibition of class switch recombination by α IgM is dependent on PI3K.

4. Discussion

Multiple factors influence the induction of AID expression and subsequent class switch recombination, including the cytokines and ligands used to activate the B cell as well as the signaling pathways used by these external stimuli. In this report, we observed that cells

stimulated with α IgM plus IL-4 had delayed AID expression and lacked class switch recombination, as compared to cells stimulated with LPS or α CD40. Splenic murine B cells were stimulated ex vivo with a variety of canonical ligands known to induce AID (Muramatsu et al., 1999) and cause heavy chain class switching (Snapper et al., 1995; Snapper and Paul, 1987), such as LPS and α CD40 in the presence or absence of IL-4. As previously reported, AID transcripts were evident by day 2 after stimulation (Fig. 1), which is when DNA breaks can be detected in the switch regions (Rush et al., 2004; Schrader et al., 2005). On the other hand, there is no information on AID activity in B cells stimulated through the BCR, even though this receptor binds antigen in vivo and initiates the cascade of AID-induced antibody diversity. Previous studies showed that cells stimulated with α IgM proliferate, but do not switch to other isotypes (Parker et al., 1980; Rush et al., 2004; Snapper et al., 1995), which could be interpreted as lack of expression of AID. However, our results demonstrate the presence of both AID mRNA transcripts and protein, although with delayed kinetics compared to cells stimulated with LPS and α CD40.

Although AID transcripts were eventually expressed in all groups, cells stimulated with α IgM plus IL-4 did not undergo class switching. As assessed by two techniques, flow cytometry of surface IgG protein and DC-PCR, α IgM activated cells had no evidence of switching to IgG1 or IgG3, whereas LPS and LPS plus IL-4 activated cells readily switched to these isotypes. Transcription of the switch regions precedes recombination, so it is possible that the $S\gamma$ regions were not transcribed in cells activated through the BCR. However, $\gamma 1$ germline transcripts were detected in these cells, which indicated that the $S\gamma 1$ region was accessible. Further, $\gamma 3$ germline transcripts were not found in cells activated with α IgM plus IL-4, suggesting proper targeting of factors required for germline transcription. Therefore, the AID protein that is expressed following α IgM plus IL-4 is not sufficient for class switch recombination.

It is possible that class switch recombination following cellular activation by α IgM plus IL-4 requires additional factors not induced by this activation. To address this idea, α IgM was added to both the LPS plus IL-4 and α CD40 plus IL-4 activations. Surprisingly, the addition of α IgM suppressed the class switch recombination observed in both activations, which has also been observed by others in cells stimulated through the CD40 receptor (Rush et al., 2002; Thomas et al., 2007). We show here that this was not due to lack of AID, since AID was expressed in all activations. These results suggest that signaling through the BCR plays a regulatory role in class switch recombination.

Suppression of class switch recombination following stimulation through the BCR may be a result of multiple different pathways. Recently, Omori and colleagues defined a role for PI3K in class switch recombination in cells activated with LPS plus IL-4 or α CD40 plus IL-4 (Omori et al., 2006). To determine if PI3K was involved in BCR-induced suppression of class switch recombination, cells were treated with the PI3K inhibitor LY294002. Inhibiting PI3K activity restored class switch recombination in cells stimulated with LPS plus IL-4 in the presence of α IgM. Inhibition of PI3K also increased class switching in cells stimulated with LPS plus IL-4. To determine the contribution from BCR signaling, we investigated the status of phospho AKT. Addition of α IgM increased AKT phosphorylation in cell stimulated with LPS plus IL-4, which could explain the fact that restoration of class switch recombination by LY294002 was dose dependent. Interestingly, inhibiting PI3K did not restore class switch recombination in cells activated only by α IgM plus IL-4. This suggests the following: (1) activation through the BCR induces a regulatory signal through PI3K capable of downregulating class switch recombination, and (2) α IgM plus IL-4 stimulation requires additional factors to initiate class switch recombination.

Together these data imply that other factors in addition to AID are critical for class switch recombination, and that activation through the BCR plays a role in regulating recombination.

Furthermore, the activity level of PI3K may control the amount of switching. For example, cells stimulated with LPS or α CD40 in the presence of IL-4 do switch to IgG1, whereas the addition of α IgM ablates switching. Inhibiting PI3K with LY294002 can reverse this ablation in a dose-dependent manner. Induction of PI3K activity by α IgM suppresses class switch recombination, possibly by suppressing AID function (Omori et al., 2006). Increased PI3K may affect AID activity by controlling the expression, protein modifications, nuclear localization, or recruitment of co-factors that target AID to the switch region. PI3K activity has also been shown to influence FOXO proteins through AKT (Omori et al., 2006). It will be interesting to determine whether the higher degree of AKT phosphorylation by BCR signaling correlates with its activity. Therefore, PI3K activity influences AID as well as other factors involved in class switch recombination. The difference between cellular activation with LPS plus IL-4, α CD40 plus IL-4, and α IgM plus IL-4 provides an intriguing model to identify the signaling pathways and regulatory factors involved in recombination of immunoglobulin constant genes.

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Fig. 1.

Kinetic differences in AID expression following B cell activation. (A) Cells were activated with the indicated stimulants and assayed for proliferation after 2 days using a standard [³H] TdR assay. Error bars represent the standard deviation of triplicate samples from two independent experiments. (B) RT-PCR of transcripts. Total RNA was isolated from the cells after 2-4 days of culture and subjected to semi-quantitative RT-PCR. Data are representative of three independent experiments. (C) AID expression was quantified using quantitative, realtime PCR, and results were normalized to β-actin expression levels. Error bars represent the standard deviation of triplicate samples from three independent experiments. (D) Western blot for protein expression. Total B cell extracts were prepared from cells stimulated with LPS plus IL-4 or αIgM plus IL-4 after 4 days. Resting cells were from day 0. The blot was stained first with anti-AID, followed by anti-β-actin as a loading control. Daudi whole cell extracts were used as a control for AID protein expression. Data are representative of three independent experiments.

Fig. 2.

Heavy chain class switching to IgG1 or IgG3. (A) Surface expression of IgG. Cells were stained with B220 and either anti-IgG1 or anti-IgG3. The percentage of cells that switched is indicated in the upper right hand quadrant. Data are representative of >5 independent experiments. (B) Switch junctions. DNA was analyzed by DC-PCR with primers specific for S μ and S γ 1. Primers for the AChRe gene were used as a control. Data are representative of three independent experiments. (C) Germline transcripts of S γ 1 and S γ 3. RNA was purified from day 3 activated cells and analyzed by quantitative RT-PCR. Results were normalized to β -actin expression. Error bars represent the standard deviation of triplicate samples from three independent experiments.

Fig. 3. Suppression of class switching by α IgM added to cells stimulated with LPS or α CD40. (A) Surface expression of IgG1. Cells were stained with B220 and anti-IgG1. The percentage of cells that switched is plotted. Data are representative of three independent experiments. (B) AID expression was quantified using quantitative, realtime PCR, and results were normalized to β -actin expression levels. Error bars represent the standard deviation of triplicate samples from three independent experiments.

Fig. 4. Inhibition of PI3K restores class switching in activated B cells. Cells were activated with LPS plus IL-4, LPS plus α IgM plus IL-4, or α IgM plus IL-4, in the presence or absence of the PI3K inhibitor LY294002. (A) Surface expression of IgG1. After three days, cells were stained with B220 and anti-IgG1. The percentage of cells that switched is indicated. Data are representative of three independent experiments. (B) Western blot for AKT protein expression. Total B cell extracts were prepared from cells stimulated for 2 hours with LPS plus IL-4, with or without LY294002 (2.5 μ M), and from cells stimulated with LPS plus IL-4 plus α IgM, with or without LY294002 (2.5 μ M). Control cells were from time 0. The blot was stained first with anti-phospho AKT (Ser473), followed by anti-AKT. Data are representative of three independent experiments.