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Involvement of calmodulin and calmodulin kinase II in tumor necrosis factor alpha-induced survival of bone marrow derived macrophages

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ABSTRACT

We previously showed that survival signaling in TNFα-treated, human THP1-derived macrophages (TDMs) has an obligatory requirement for constitutive Ca^{2+} influx through a mechanism involving calmodulin/calmodulin kinase II (CAM/CAMKII). We also demonstrated that such requirement also applies to the protective actions of TNFα in murine bone marrow-derived macrophages (BMDMs) and that TRPC3 channels mediate constitutive Ca^{2+} influx. Using a pharmacological approach we here examined if in BMDMs, similarly to TDMs, TNFα-induced survival signaling also involves CAM/CAMKII. In BMDMs, TNFα induced rapid activation of the survival pathways NFκB, AKT and p38MAPK. All these routes were activated in a PI3K-dependent fashion. Activation of AKT and NFκB, but not that of p38MAPK, was abrogated by the CAM inhibitor W7, while KN-62, a CAMKII inhibitor, prevented activation of AKT and p38MAPK but not that of NFκB. Inhibition of CAM or CAMKII completely prevented the protective actions of TNFα. Our observations indicate that in BMDMs CAM and CAMKII have differential contributions to the components of TNFα-dependent survival signaling and underscore a complex interplay among canonical survival routes. These findings set a signaling framework to understand how constitutive Ca^{2+} influx couples to macrophage survival in BMDMs.

1. Introduction

Macrophages are critical players during inflammation resolution and as such, they are determinant to the progression of inflammatory vascular disease such as atherosclerosis [1]. In fact, within the context of the cellular and molecular events that underlies atherosclerotic lesion formation and progression, the balance between survival and apoptosis of lesional macrophages and their timely clearance from the lesion site by resident phagocytes – efferocytosis – shape the lesion cellularity and thus its progression and fate. A rapidly growing area of research in the field is focused on characterizing mechanisms that regulate macrophage survival, apoptosis and efferocytosis during atherogenesis with the hope of identifying novel targets to develop alternative strategies to manage the disease [1,2]. In cells other than macrophages some of the typical cell survival pathways – i.e., the phosphatidylinositol-3-kinase (PI3K)/AKT axis and nuclear factor kappa B (NFκB) – are modulated, directly or indirectly, by Ca^{2+} influx [3–5]. While studying the actions of the atherorelevant cytokine tumor necrosis factor alpha (TNFα) on macrophage survival and apoptosis, we recently showed for the first time that in human, THP-1-derived macrophages (TDMs), as in other cell types, Ca^{2+} influx is also critical to support survival signaling [6]. In that instance we found that constitutive, non-regulated Ca^{2+} influx couples to cell survival through a calmodulin/calmodulin-dependent kinase II (CAM/CAMKII) axis [6]. In TDMs however, TNFα-induced survival signaling is the result of a compensatory response of the macrophages against the pro-apoptotic actions of the cytokine. In more recent work using murine bone marrow-derived macrophages (BMDMs), in which TNFα exerts an unambiguous pro-survival effect [7,8], we found, once again, that a Ca^{2+} influx dependent mechanism exists and that TRPC3, a member of the TRPC family of Ca^{2+}-permeable cation channels [9,10], is the channel responsible for mediating constitutive Ca^{2+} influx in these cells [11]. However, if a CAM/CAMKII-dependent mechanism also operates in BMDMs remains to be determined. To examine this, in the present work we explored TNFα-dependent activation of survival pathways in BMDMs and the impact of selective inhibitors of CAM and CAMKII on cell survival and apoptosis.

Abbreviations: CAM, calmodulin; CAMKII, calmodulin dependent kinase II; GSK3β, glycogen synthase kinase 3 beta; PI3K, phosphatidylinositol-3-kinase; NFκB, nuclear factor kappa B; M-CSF, macrophage-colony stimulating factor; TNFα, tumor necrosis factor alpha; TRPC, transient receptor potential canonical.

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2. Materials and methods

2.1. Preparation of bone marrow-derived macrophages

In our recent studies on the role of TRPC3 channels in macrophage constitutive Ca^{2+} influx we used BMDMs derived from 129SvTrpc3$^{-/-}$ mice, while BMDMs from 129SvTrpc3$^{lox/lox}$ mice were used as control cells. Although in pilot studies we did not find any significant differences in the phenotype of macrophages from 129SvTrpc3$^{lox/lox}$ mice compared to those from wild-type animals, for consistency, in the present work all BMDMs used for experiments were obtained from 129SvTrpc3$^{lox/lox}$ (provided by Dr. Lutz Birnbaumer, NIEHS, NC). Generation and full characterization of these mice have been previously described [12]. All animal procedures were approved by University of Toledo IACUC. Bone marrow-derived macrophages were obtained essentially as described by us in [11]. Briefly, femurs and tibias were flushed with sterile RPMI (containing 2% FBS + 5 U/ml heparin + 1% penicillin/streptomycin) and cells were plated with L929-conditioned medium for 7 days (37 °C, 5% CO_2 atmosphere); after that, cells were recovered with ice-cold phosphate-buffered saline solution (PBS) and replated in 6- (immunoblots) or 96-well (TUNEL) plates for experiments. Macrophage phenotype was confirmed as we described in [6] (not shown). Immunoblotting conditions were as we described in [6,11]. Briefly, following cell lysis, solubilized proteins were separated in 10% acrylamide gels, electrotransferred to PVDF membranes and immunoblotted with the indicated primary antibody. After incubation with appropriate HRP-conjugated secondary antibodies, immunoreactive bands were visualized by ECL (Amersham, PA). Phosphorylation of IκBα is immediately followed by its degradation; therefore, immunodetection of phospho-IκBα was normalized against GAPDH (see also [6]). Primary antibodies used were: cleaved PARP (Asp214, clone 7C9), phospho-IκBα (Ser32/36, clone 5A5), phospho-AKT (Ser473, clone 587F11), total AKT, phospho-GSK3β (Ser9, clone D85E12), total GSK3β (clone 27C10), phospho-p38 MAPK (Thr180/Tyr182, clone D3F9), total p38MAPK, all from Cell Signaling (MA); anti-GAPDH (clone 0411) was from Santa Cruz (CA).

2.2. TUNEL assay

Apoptosis was assayed by using the in situ cell death detection kit, TMR red (Roche, IN) as described in [13]. Macrophages were grown on 96-well plates under the culture conditions described above; visualization and analysis was performed by fluorescence microscopy. Hoechst co-staining was used to count total cells. TMR-positive cells in five fields were counted and expressed as % of total cells.

2.3. Statistical analysis

Comparison of mean values was by using a two-tailed *t* test for two means, using Graph Pad InStat version 3.00 for Windows 95 (Graph Pad Software, San Diego CA, www.graphpad.com). All biochemical experiments were repeated at least three times. $P < 0.05$ was considered significant.

3. Results

We recently showed that in THP-1-derived macrophages (TDMs) CAM and CAMKII are critical players within the compensatory survival signaling that takes place in response to the pro-apoptotic actions of TNFα [6]. Contrarily to the actions of this cytokine in TDMs, in bone marrow-derived macrophages (BMDMs) TNFα exerts an unambiguous pro-survival effect [7,8] (see also Section 4). Yet, similar to TDMs, survival signaling in BMDMs also exhibits an obligatory requirement for constitutive Ca^{2+} influx [11] (and see Supplementary Fig. I). To determine if in BMDMs the survival mechanism underlying the protective actions of TNFα is similar to that mediating compensatory survival in TDMs, we first examined the contribution of different survival pathways to the protective actions of TNFα against apoptosis induced by macrophage-colony stimulating factor (M-CSF) withdrawal – i.e., serum free RPMI. BMDMs were incubated for 24 h in complete growth medium, serum free medium (RPMI) or RPMI containing TNFα (10 ng/ml) in the presence or absence of inihibitors that selectively target PI3K/AKT, NFκB or p38MAPK – typical macrophage survival molecules – or in the presence or absence of selective inhibitors of CAM and CAMKII. Following treatments apoptosis was examined by terminal deoxynucleotidyl-transferase-dUTP-nick end labeling (TUNEL) assay, as we described in [6]. As previously shown by others [7,8], TNFα treatment exerted a clear protective action against M-CSF withdrawal-induced apoptosis, manifested by a significant reduction in the number of TUNEL-positive cells when compared to the control (Fig. 1A). Notably, when macrophages were pre-treated with selective inhibitors for PI3-kinase (LY294002, 10 μM), calmodulin (W7, 10 μM), CAMKII (KN62, 25 μM), p38MAPK (SB203580, 10 μM) or the IκBα kinase IKK (hypoestoxide, 50 μM), the protective effect of TNFα was completely abrogated and the number of apoptotic cells increased by 2–4-fold, clearly indicating the involvement of these pathways in both basal and cytokine-dependent survival of the macrophages. Unlike to what we observed upon inhibition of PI3K, CAM, CAMKII or IKK, inhibition of p38MAPK did not affect apoptosis induced by M-CSF withdrawal. All these observations were positively correlated with increased levels of cleaved poly (ADP-ribose) polymerase (PARP; Fig. 1B).

We next examined if treatment of BMDMs with TNFα promoted activation of survival signaling pathways. Canonical survival mechanisms in macrophages, as in other cell types, involve rapid, sometimes transient activation but with a long lasting impact in anti-apoptotic mechanisms [6,11]. BMDMs were exposed to TNFα (10 ng/ml) for 5, 10, 15, 30 or 60 min; following treatments, cells were lysed and cellular proteins processed for immunoblotting to examine the phosphorylation status of typical survival molecules. NFκB is a key regulator of survival gene expression in macrophages [14], and we have previously shown that the phosphorylation status of IκBα, rather than its degradation, is a more reliable indicator of rapid activation of the NFκB route [6,11]. Treatment of BMDMs with TNFα caused a rapid (within 5 min) and transient phosphorylation of IκBα (Fig. 2A). Activation of AKT, another critical regulator of macrophage survival [13,15] occurs in a strictly PI3K-dependent manner and is evidenced by phosphorylation of AKT on Ser473 [15]. Fig. 2B shows that TNFα treatment resulted in a time-dependent activation of AKT (10–15 min) which declined to pre-stimulation levels after 60 min of treatment. No significant changes were observed in the amount of total AKT. The PI3K inhibitor LY294002 (10 μM) completely abrogated TNFα-dependent phosphorylation of AKT (Fig. 2C) confirming activation downstream of PI3K. As we recently described in TDMs [6], pre-treatment of BMDMs with LY294002 abrogated TNFα-dependent IκBα phosphorylation (Fig. 2D) supporting the notion that AKT and NFκB can crosstalk to promote macrophage survival [3]. The mitogen-activated protein kinase p38MAPK also represents an important player in macrophage survival [16]. As shown in Fig. 2E, TNFα treatment promoted a robust and transient (5–15 min) increase in phosphorylation of p38MAPK (Thr180/Tyr182). Total levels of p38MAPK remained unchanged. Similarly to what we observed for AKT and IκBα, inhibition of PI3K with LY294002 resulted in marked reduction of TNFα-dependent phosphorylation of p38MAPK (Fig. 2F).