Purinergic Signalling (2007) 3:59–69
DOI 10.1007/s11302-006-9037-8

REVIEW

Activation of transcription factors by extracellular nucleotides in immune and related cell types

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Received: 22 February 2006 / Accepted: 13 March 2006 / Published online: 3 January 2007

Abstract Extracellular nucleotides, acting through P2 receptors, can regulate gene expression via intracellular signaling pathways that control the activity of transcription factors. Relatively little is known about the activation of transcription factors by nucleotides in immune cells. The NF-κB family of transcription factors is critical for many immune and inflammatory responses. Nucleotides released from damaged or stressed cells can act alone through certain P2 receptors to alter NF-κB activity or they can enhance responses induced by pathogen-associated molecules such as LPS. Nucleotides have also been shown to regulate the activity of other transcription factors (AP-1, NFAT, CREB and STAT) in immune and related cell types. Here, we provide an overview of transcription factors shown to be activated by nucleotides in immune cells, and describe what is known about their mechanisms of activation and potential functions. Furthermore, we propose areas for future work in this new and expanding field.

Key words AP-1 · CREB · mast cell · microglia · NFAT · NF-κB · osteoclast · P2 receptor · STAT · T-cell

Abbreviations

AP-1	activator protein 1
ATF	activating transcription factor
ATPγS	adenosine-5′-*O*-(3-thiotriphosphate)
BzATP	2′3′-*O*-(4-benzoylbenzoyl)-ATP
CaM kinase	Ca^{2+}/calmodulin-dependent protein kinase
CBP	CREB-binding protein
ChIP	chromatin immunoprecipitation
COX-2	cyclooxygenase-2
CRE	cyclic AMP-response element
CREB	CRE binding protein
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal-regulated kinase
IκB	inhibitor of NF-κB
IL	interleukin
iNOS	inducible nitric oxide synthase
JAK	Janus kinase
LPS	lipopolysaccharide
MAP kinase	mitogen-activated protein kinase
MEK	MAP kinase/ERK kinase
NFAT	nuclear factor of activated T-cells
NF-κB	nuclear factor κB
RSK2	ribosomal S6 kinase 2
STAT	signal transducer and activator of transcription
TNF-α	tumor necrosis factor-α

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Introduction

Extracellular stimuli regulate gene expression via intracellular signaling cascades that control the activity of transcription factors. Nucleotides such as ATP are now considered essential autocrine/paracrine mediators that influence cell behavior through activation of P2 receptors. Release of nucleotides from injured or stressed cells may serve as ‘danger signals’ to cells of the immune system [1]. In addition, nucleotides acting through P2 receptors often potentiate responses to other mediators, such as neurotransmitters, growth factors or cytokines [2, 3]. P2 receptors are expressed in many cell types, including cells of the

immune system. There are two distinct subclasses of P2 receptors: P2X are ligand-gated ion channels permeable to Na^+, K^+ and in many cases Ca^{2+}; whereas P2Y are G protein-coupled receptors [4]. Multiple P2X and P2Y receptors are often expressed on the same cell type and couple to diverse signaling pathways.

Gene expression is regulated principally at the level of initiation of transcription. Mammalian genes consist of a protein coding sequence, a proximal upstream promoter (sequence to which basal transcription factors bind) and distant enhancer and/or silencer elements (sequences to which inducible transcription factors bind). Basal transcription factors are proteins that assemble with RNA polymerase to form an initiation complex. Binding of inducible transcription factors to their response elements enhances or sometimes represses formation of this initiation complex. In addition, gene expression can be regulated indirectly via interactions among transcription factors. Some transcription factors recruit chromatin remodeling and modification proteins such as histone acetylases. Acetylation of histones weakens the interaction between the nucleosome and DNA permitting assembly of the initiation complex. Alternately, recruitment of histone deacetylases leads to transcriptional repression [5]. The activity of inducible transcription factors can be regulated by several mechanisms, such as phosphorylation or dephosphorylation, binding of activating or inhibitory factors, or *de novo* synthesis.

Transcription factors play critical roles in the development and function of the immune system [reviewed in 6–9]. Although the regulation of transcription factors by nucleotides has been studied in detail in several neuronal and muscle cell types, relatively little is known about the responses of immune cells.[1] Here, we review evidence for the regulation of transcription factors by nucleotides in immune and related cell types, as summarized in Table 1. We begin with a discussion of the role of P2 receptors in the regulation of NF-κB in macrophages and macrophage-like cell types—perhaps the best understood system. This is followed by brief descriptions of the regulation of AP-1, NFAT, ATF/CREB and STATs.

Methods for assessing activation of inducible transcription factors

Several approaches can be used to detect the activation of transcription factors. DNA binding can be directly monitored using the electrophoretic mobility shift assay (EMSA), also known as a gel shift assay. The basis of EMSA is that protein-DNA complexes migrate more slowly than free DNA when subjected to nondenaturing gel electrophoresis. To assess transcription factor activity, nuclear or whole-cell extracts are combined with labeled double-stranded DNA sequences containing one or more specific response elements. Retardation of DNA mobility indicates binding of protein. Specificity can be confirmed by including an antibody specific for that transcription factor, which will either block DNA binding or 'super-shift' the complex. Recently, protein-DNA array technology has been developed that permits profiling of the DNA binding activities of large numbers of transcription factors in one assay [21].

The chromatin immunoprecipitation (ChIP) technique allows analysis of protein-DNA interactions in living cells. The technique involves *in vivo* cross-linking of chromatin-associated proteins to DNA, its fragmentation, immunoprecipitation with specific antibodies and analysis of the DNA sequences obtained. Thus, ChIP permits the characterization of protein interactions with chromatin in its native conformation and reveals the effects of protein-protein interactions that may alter DNA binding [22]. In contrast, EMSA assesses the binding of proteins to short fragments of DNA *in vitro*. Reporter gene assays are also used to assess transcription factor activity in living cells. Vectors are constructed encoding the appropriate response elements upstream of a gene for an indicator protein, such as β-galactosidase or luciferase. Constructs are then expressed in the cell system of interest. Activation of the transcription factor results in quantifiable changes in the expression of the indicator protein.

There are also several indirect approaches for monitoring the activation of inducible transcription factors. These include: (a) monitoring phosphorylation of transcription factors by immunoblotting using phospho-specific antibodies; (b) assessment of translocation of factors from the cytosol to the nucleus using various techniques, such as immunoblots of cytosolic and nuclear fractions, or *in situ* immunolabeling; or (c) quantification of levels of transcription factors (for those regulated by *de novo* synthesis) or regulatory molecules.

Nuclear factor κB (NF-κB) family of transcription factors

The transcription factor nuclear factor κB (NF-κB) plays an important role in many types of immune cells [reviewed in 23, 24]. NF-κB was first identified as a protein that binds to a specific DNA sequence (κB element) within the enhancer region of the immunoglobulin κ light chain gene in mature B cells [25]. NF-κB regulates the expression of a wide variety of genes that are involved in the regulation of

[1] Since preparation of this manuscript, an excellent review has been published on the regulation of MAP kinases and transcription factors by P2 receptors in microglial cells [53].

Table 1 Regulation of transcription factor activity in immune and related cell types by extracellular nucleotides

Transcription factor	Change[a]	Cell type	Agonists	Receptor	References
NF-κB	↑	Murine microglial cell lines N9 & N13	ATP, ATPγS, BzATP	P2X7	[10]
	↑	Murine macrophage cell line RAW 264.7	BzATP	P2X7	[11]
	↑	Rabbit and murine osteoclasts	BzATP	P2X7	[12]
	↑	Rabbit and rat osteoclasts	UDP, INS48823	P2Y6	[13]
	↑	J774 murine macrophage cell line	UTP+LPS	P2Y6	[14–16]
	↓	Jurkat human T lymphoblastoid cell line	ATP	P2X7	[17]
AP-1	↓	Murine microglial cell lines N9 & N13	ATP	P2X7	[10]
	↑	Jurkat human T lymphoblastoid cell line	ATP	P2X7	[17]
	↑	J774 murine macrophage cell line	UTP	P2Y6	[14]
NFAT	↑	Murine microglial cell line N9	ATP, BzATP	P2X7	[18]
ATF/CREB	↑	Murine microglial cell line BV-2	ATP, 2-chloro-ATP	P2Y1	[19]
STATs	↑	Murine mast cells	ATP	P2X7	[20]

[a] Change refers to whether transcription factor is activated (↑) or suppressed (↓)

immune and inflammatory responses, proliferation, tumor growth and cell survival. This family of transcription factors consists of five members in mammals: p65 (RelA), c-Rel, RelB, NF-κB (p50/p105) and NF-κB2 (p52/p100) [23]. They all contain an N-terminal DNA binding region known as the Rel homology domain and bind as homo- or heterodimers to κB elements. The Rel homology domain is 300 amino acids in length and, in addition to DNA binding, is responsible for dimerization and interaction with inhibitory IκB proteins. Transcriptional activation domains are found in the C-terminal region of p65, c-Rel, and RelB, and are important for inducing target gene expression. Due to lack of these domains, homodimers of p50 and p52 have no intrinsic ability to drive transcription. Both p50 and p52 are synthesized as longer precursors—p105 and p100, respectively—which are cleaved by the proteasome [26]. The other three members of the NF-κB family are synthesized as mature proteins.

In the classical activation pathway, NF-κB is maintained as an inactive complex in the cytoplasm when bound to inhibitory IκB proteins (which include IκBα, IκBβ, IκBε and IκBγ). Activation of NF-κB involves phosphorylation of IκB by an IκB kinase in response to a variety of stimuli, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) or lipopolysaccharide (LPS) [23]. Phosphorylation of IκB is followed by its ubiquitination and degradation, unmasking a nuclear import signal on NF-κB and hence permitting its translocation to the nucleus, where it regulates expression of target genes. In the alternative pathway for NF-κB activation, an IκB kinase phosphorylates inactive p100, triggering its processing and generating active p52/RelB heterodimers.

Ferrari and coworkers showed that extracellular ATP led to the activation of NF-κB in the N9 and N13 murine microglial cell lines [10]. Activation induced by ATP or LPS (used as a positive control) was monitored by EMSA to detect binding activity of total cell extracts to κB-specific oligonucleotide probes (Fig. 1a). Interestingly, relatively high concentrations of ATP were required to activate NF-κB, with maximal effects observed at 3 mM (Fig. 1b), consistent with involvement of the P2X7 receptor, which has a relatively low affinity for ATP. In contrast to its effects on NF-κB, ATP suppressed DNA binding activity of the transcription factor AP-1 (Fig. 1c), indicating that ATP has specific effects on different transcription factors.

Involvement of the P2X7 receptor in mediating ATP-induced activation of NF-κB was supported by the agonist specificity of the response. Responses were elicited by ATP, ATPγS or BzATP (a relatively potent P2X7 agonist), but not by the P2Y receptor agonists ADP or UTP. Moreover, activation of NF-κB was inhibited by the P2X7 receptor antagonist, oxidized ATP [10]. However, it is now known that oxidized ATP is not specific for the P2X7 receptor and can suppress NF-κB activation in cells lacking the P2X7 receptor [27, 28]. The mechanism through which ATP causes activation of NF-κB is not clear. However, Ferrari and coworkers did show that activation was sensitive to inhibitors of proteasomal degradation and caspase activity. Furthermore, scavengers of reactive oxygen species were found to inhibit ATP-induced activation of NF-κB [10].

It is well established that many inflammatory cells, including microglia respond to LPS through activation of NF-κB. LPS, which was used by Ferrari and coworkers as a positive control, induced NF-κB activation within 15 min. In contrast, activation of NF-κB was not observed until 3 h following stimulation with ATP [10]. This delay suggested an indirect effect of ATP, perhaps mediated via release of IL-1β or other cytokines. However, the possible role of soluble factors such as IL-1β was excluded using a series of approaches including media transfer experiments. Activation of NF-κB by LPS and ATP also differed in that LPS led to formation of the classical p65/p50 heterodimers and p50 homodimers, whereas ATP induced formation of p65 homodimers. Different NF-κB dimers have distinct actions

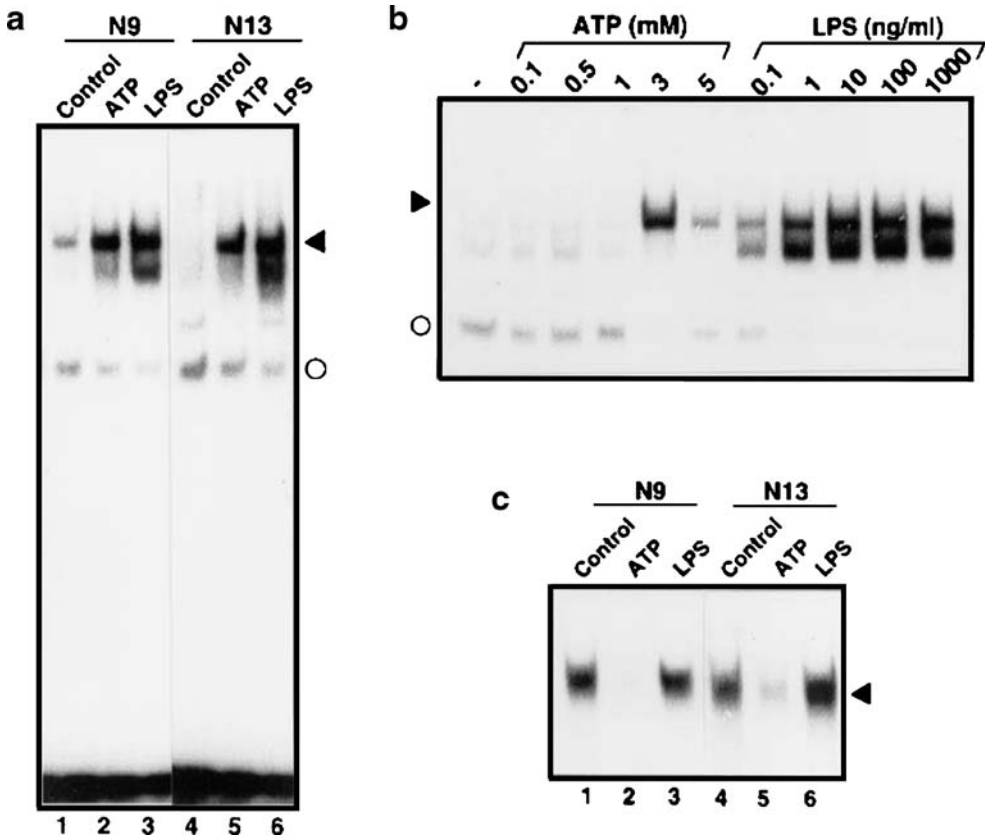

Fig. 1a–c ATP induces NF-κB activation in microglial cell lines. **a** The microglial N9 cell line (*lanes 1–3*) or N13 cell line (*lanes 4–6*) were either left untreated (*Control*) or treated with ATP (3 mM) or LPS (100 ng/ml) as a positive control. After 3 h, total cell extracts were prepared and analyzed with an NF-κB-specific oligonucleotide by EMSA. The NF-κB-DNA complex is indicated by *filled arrowheads* in **a** and **b**. A faster migrating nonspecific complex is marked by *circles*. **b** Dependence of NF-κB activation on the concentration of ATP or LPS. N9 cells were treated for 3 h with the indicated concentrations of ATP or LPS and analyzed by EMSA. **c** The effect of ATP and LPS on the DNA-binding activity of AP-1. The same cellular extracts as in **a** were analyzed with an AP-1-specific oligonucleotide by EMSA. The AP-1-DNA complex is indicated by the *filled arrowhead*. In contrast to its effects on NF-κB, ATP suppressed the DNA binding activity of AP-1. Reproduced from [10], with copyright permission of The Rockefeller University Press

on gene expression. Thus, activation of NF-κB by ATP in microglial cells likely has different transcriptional effects than NF-κB activation by LPS or other established proinflammatory mediators.

BzATP has also been shown to regulate NF-κB signaling in the murine macrophage cell line RAW 264.7 [11]. Individually, LPS and BzATP were found to increase NF-κB DNA binding activity of nuclear extracts (measured using EMSA), while treatment with both agonists resulted in cooperative activation of NF-κB. To examine the mechanism, Aga and coworkers monitored levels of the NF-κB inhibitory protein IκBα by immunoblot (Fig. 2). Within 15 min, LPS alone rapidly induced degradation of IκBα, levels of which returned to normal within 30–45 min. Concomitant stimulation with BzATP delayed the reappearance of IκBα, consistent with more sustained NF-κB activity. The mechanism underlying these effects of nucleotides was thought to involve cross-talk with the Ras/MEK/ERK pathway. Although responses were attributed to P2X7 activation, it is possible that other P2 receptors were involved, since BzATP can activate a number of P2 receptor subtypes in addition to P2X7 [29]. In bacterial infections where both LPS and nucleotides are present, cooperative activation of NF-κB in macrophages may enhance inflammatory responses.

Recent work has shown direct involvement of the P2X7 receptor in mediating ATP-induced activation of NF-κB in osteoclasts. Osteoclasts, the large multinucleated cells responsible for bone resorption, form by the fusion of precursors of the monocyte-macrophage lineage [30]. The essential role of NF-κB in osteoclast development was discovered in genetically modified mice lacking both the p50 and p52 subunits of NF-κB, in which osteoclasts failed to develop, resulting in severe osteopetrosis [31]. Osteoclasts are terminally differentiated cells that cannot be isolated in sufficient numbers or purity to permit assess-

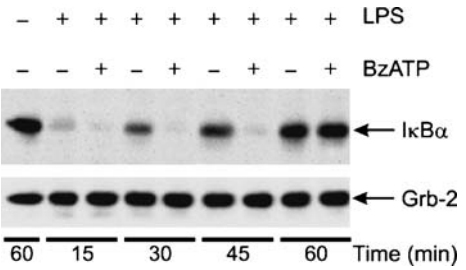

Fig. 2 Effects of the P2X7 agonist BzATP and LPS on levels of IκBα in a murine macrophage cell line. RAW 264.7 cells were treated with LPS (100 ng/ml) or BzATP (250 μM) alone or in combination for the specified times. IκBα levels were assessed by immunoblot analysis of whole-cell lysates. Within 15 min, LPS alone rapidly induced degradation of IκBα, levels of which returned to normal within 30–45 min. Concomitant stimulation with BzATP delayed the reappearance of the inhibitory protein IκBα, consistent with enhancement of NF-κB activity. Equivalent protein loading was verified by reprobing membranes with anti-Grb-2 antibodies. Modified from [11], with permission of the American Physiological Society

ment of transcription factor activation using conventional assays such as EMSA. To overcome this limitation, Korcok and coworkers used immunofluorescence to monitor localization of the p65 subunit of NF-κB, which upon activation translocates from the cytoplasm to the nucleus [12]. BzATP was applied to osteoclasts isolated from wild-type and P2X7 receptor knockout mice. In wild-type osteoclasts, BzATP increased the proportion of cells with nuclear localization of NF-κB within 30 min (Fig. 3a,d); whereas, BzATP had no effect on osteoclasts lacking the P2X7 receptor (Fig. 3c,e). This finding confirmed the involvement of P2X7 receptors in mediating the actions of ATP on NF-κB. Interestingly, in contrast to mouse osteoclasts, which showed maximal effects 30 min following exposure to BzATP, rabbit osteoclasts showed maximal nuclear localization of NF-κB 3 h after exposure to nucleotide [12], similar to the delayed response observed by Ferrari and coworkers in microglial cells [10]. The reason for these differences in activation kinetics among cell types and species of origin is not clear. Ferrari and coworkers found that ATP induced formation of p65 homodimers in microglial cells [10]; whether p65 translocated as a homodimer or as heterodimer in osteoclasts was not investigated [12].

In addition to P2X7, P2Y6 receptors have been implicated in the activation of NF-κB in osteoclasts and macrophages. To examine P2Y receptor-mediated activation of NF-κB in osteoclasts, immunofluorescence was again used by Korcok and coworkers [13]. Rabbit osteoclasts were exposed to various P2Y receptor agonists. UDP, an agonist at P2Y6 receptors, increased the proportion of osteoclasts displaying nuclear localization of NF-κB with maximal effects at 3 h (Fig. 4a,d). Control osteoclasts showed low levels of nuclear NF-κB for up to 4 h (Fig. 4b). The extent of NF-κB translocation was dependent on UDP concentration, with maximal effect observed at 10–100 μM UDP (Fig. 4e). Lactacystin, a proteasome inhibitor, abolished the UDP-induced activation of NF-κB. In contrast to its action on osteoclasts, UDP did not induce NF-κB translocation in bone marrow stromal cells (Fig. 4c), indicating specificity for cell type.

In addition to UDP, significant translocation of NF-κB was induced by INS48823 [13], a selective agonist at the P2Y6 receptor [32]. In contrast, no response was elicited by 2-methylthio-ADP (P2Y1 receptor agonist), UTP (P2Y2 receptor agonist) or low concentrations of ATP sufficient to activate multiple P2 receptors including P2Y2 and P2X4, but not P2X7. Thus, the agonist specificity for NF-κB activation indicates the involvement of P2Y6, but not other P2Y receptor subtypes known to be present on osteoclasts.

UDP and INS48823 enhanced survival of osteoclasts *in vitro* [13], presumably by inhibiting apoptosis—the predominant form of cell death for osteoclasts [33]. NF-κB has been implicated in controlling the survival of a number of cell types [34], including osteoclasts [35]. In this regard, a cell-permeable peptide inhibitor of NF-κB was found to block the effect of P2Y6 activation on osteoclast survival [13]. Enhancement of osteoclast survival through the P2Y6 receptor is consistent with data from 1321N1 human astrocytes, in which activation of P2Y6 receptors prevented TNF-α-induced apoptosis [36].

The predominant P2Y receptor expressed by the J774 murine macrophage cell line has been shown to be P2Y6 [15]. Exposure of these cells to UTP alone, which presumably leads to activation of P2Y6 receptors, does not activate NF-κB; however, UTP does potentiate LPS-induced activation of NF-κB as well as expression of inducible nitric oxide synthase (iNOS) [14]. In a series of studies, Chen and coworkers found that UTP increased LPS-induced phosphorylation and degradation of IκBα, by enhancing activation of IκB kinase [16]. Enhancement of IκB kinase activity appeared to involve several signaling pathways, including P2Y6-mediated elevation of cytosolic free Ca^{2+} and subsequent activation of Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase) [14, 16].

In summary, NF-κB is critical for immune and inflammatory responses by regulating the expression of key cytokines, growth factors and effector enzymes. The NF-κB pathway is highly conserved among species [37] and plays crucial roles in the development, activity and survival of osteoclasts [31, 35, 38]. Nucleotides released from damaged or stressed cells can alter gene expression on their own or regulate responses induced by LPS, thus playing potentially important roles in innate immunity. It will be of interest to examine whether nucleotide signaling through P2 receptors modulates NF-κB activation induced by other inflammatory mediators in addition to LPS.

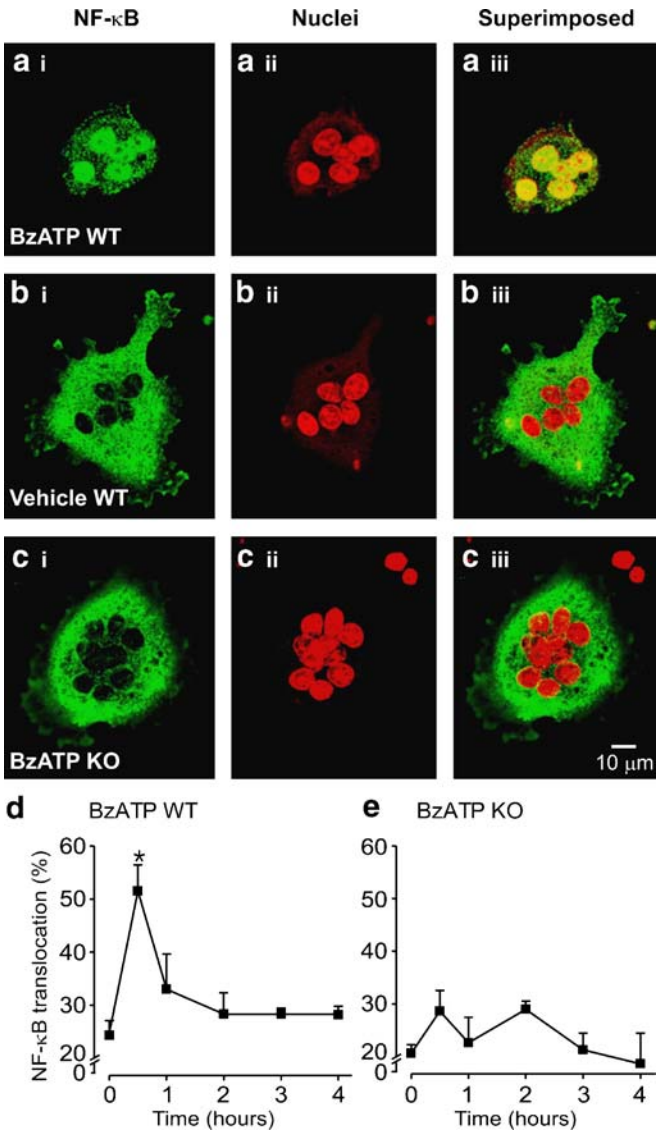

Fig. 3a–e BzATP acts through P2X7 receptors to induce nuclear translocation of NF-κB in murine osteoclasts. Osteoclasts isolated from wild-type (*WT*) and P2X7 receptor knockout (*KO*) mice were treated with BzATP (300 μM) or vehicle for 0–4 h. **a**–**c** The p65 subunit of NF-κB was visualized by immunofluorescence (*green, left*). All nuclei were stained with TOTO-3 (*red, middle*), with superimposed images of NF-κB and TOTO-3-stained nuclei at right. **a** BzATP-treated WT osteoclasts showed nuclear localization of NF-κB at 30 min (evident as *yellow staining* in **a***iii*). **b**, **c** In contrast, vehicle-treated WT and BzATP-treated KO osteoclasts showed cytoplasmic localization of NF-κB. **d** WT osteoclasts treated with BzATP exhibited a significant increase in nuclear translocation of NF-κB at 30 min compared with time 0 (**P*<0.05). **e** In contrast, KO osteoclasts did not show a significant change in NF-κB translocation at any time point after BzATP treatment. Data are the percentage of osteoclasts with nuclear localization of NF-κB. Reproduced from [12], with permission of the American Society for Bone and Mineral Research

Activator protein 1 (AP-1) family of transcription factors

AP-1 is composed of structurally and functionally related members of the Jun and Fos protein families. Fos proteins (Fos, FosB, Fra-1 and Fra-2) heterodimerize with members of the Jun family, whereas Jun proteins (Jun, JunB, JunD) can form homodimers as well as heterodimers with Fos proteins. Some members of the ATF/CREB family of transcription factors can also participate in AP-1 complexes. With many possible combinations of heterodimers and homodimers comprising the AP-1 complex, a broad diversity in gene regulation can be achieved [39, 40].

AP-1 translates extracellular signals in immune and related cell types into changes in the expression of specific target genes, which in turn control proliferation, differentiation and apoptosis [39]. AP-1 activity can be regulated by *de novo* synthesis of subunits, dimer composition, post-translational modifications and interactions with other proteins. Two of the components of AP-1 (Jun and Fos) were first identified as viral oncoproteins, so their role in tumorigenesis is also well established.

Budagian and coworkers examined the effects of ATP on AP-1 activity in the Jurkat human T-lymphoblastoid cell line [17]. Using immunoblots, they found that expression of Jun and Fos increased 30–60 min following exposure to ATP. Effects were observed only at ATP concentrations of 1 mM or higher, consistent with involvement of the P2X7 receptor. In keeping with increased expression of Jun and Fos, EMSA of nuclear extracts indicated that AP-1 DNA binding activity increased within 60 min following exposure to ATP and continued to intensify for at least 3 h. Supershift analysis confirmed that the AP-1 complex contained Jun and Fos. Surprisingly, ATP transiently suppressed NF-κB DNA

binding activity within 1 h of exposure, with recovery by 3 h. Thus, ATP has opposite effects in Jurkat cells compared with its effects in microglial cells, in which ATP activates NF-κB and suppresses AP-1 [10]. Nevertheless, the effects of ATP were presumably mediated by P2X7 receptors in both cell types [10, 17].

In Jurkat cells, the mechanism mediating the effect of ATP on AP-1 was shown to involve the tyrosine phosphorylation and activation of $p56^{lck}$ [17] (a tyrosine kinase essential for signal transduction through the T-cell receptor [41]). In Jurkat cells, but not JCaM1 cells (a mutant Jurkat cell line that lacks $p56^{lck}$), ATP activated ERK and Jun N-terminal kinase and enhanced AP-1 activity, indicating an essential role for $p56^{lck}$ in regulating AP-1. It is known that AP-1 stimulates expression of IL-2 in T lymphocytes [42, 43]. In this regard, Budagian and coworkers found that ATP stimulated IL-2 expression in Jurkat cells but not in JCaM1 cells, consistent with a role for AP-1 in mediating this response to ATP.

In addition to examining the effects of UTP on NF-κB signaling, Chen and coworkers assessed the effects of P2Y6 receptor activation on AP-1 activity in the J774 macrophage cell line [14]. Using EMSA, they found that UTP and LPS each induced AP-1 activation and that their effects were additive.

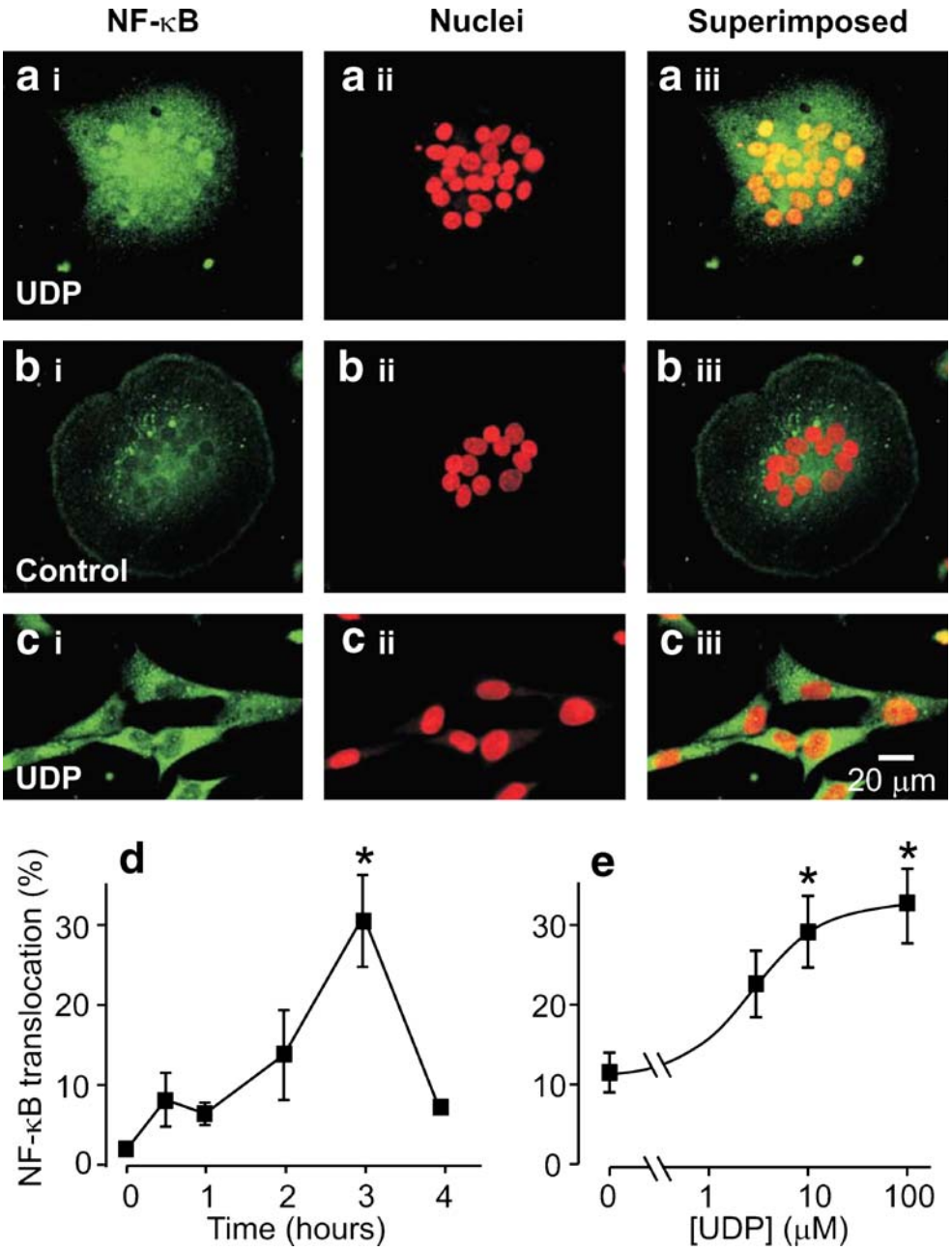

Fig. 4a–e The P2Y6 receptor agonist UDP induces nuclear translocation of NF-κB in rabbit osteoclasts. Osteoclasts were treated with UDP or vehicle. **a–c** The p65 subunit of NF-κB was visualized by immunofluorescence (*green, left panels*), and the nuclei were stained with TOTO-3 (*red, middle panels*). Superimposed images of NF-κB and nuclei are shown at right. **a** Osteoclast treated with UDP (10 μM) showed nuclear localization of NF-κB at 3 h (evident as *yellow staining* in **a***iii*). **b, c** Osteoclast treated with vehicle (*Control*) and stromal cells treated with UDP (10 μM) showed cytoplasmic localization of NF-κB at 3 h. **d** Osteoclasts were treated with UDP (10 μM) for 0–4 h. The data are the percentages of osteoclasts with nuclear localization of NF-κB. Significant nuclear translocation of NF-κB was observed at 3 h compared with time 0 (*P<0.05). **e** Rabbit osteoclasts were treated for 3 h with the indicated concentrations of UDP. Significant nuclear translocation of NF-κB was observed at 10–100 μM UDP (*P<0.05). Reproduced from [13], with permission of the American Society for Biochemistry and Molecular Biology

In summary, the ability of nucleotides to activate AP-1 likely depends upon the subtypes of P2 receptors involved as well as cell type. Furthermore, cross-talk with other signaling pathways and transcription factors likely influences the ultimate effects of AP-1 activation on gene expression.

Nuclear factor of activated T-cells (NFAT) family of transcription factors

NFAT is a family of transcription factors that regulate the differentiation and activation of a number of immune and related cell types, including T-cells and osteoclasts [7, 8]. This family of proteins consists of five members (NFAT1-5), of which four are regulated by calcium signaling [7]. All NFAT proteins contain a highly conserved Rel-homology region that confers common DNA-binding specificity. Inactive NFAT is maintained in the cytoplasm in a hyperphosphorylated state. Elevation of cytosolic free Ca^{2+} stimulates the phosphatase calcineurin, which in turn dephosphorylates multiple serine residues, exposing a nuclear localization signal that permits translocation of NFAT to the nucleus.

Ferrari and coworkers examined the effect of ATP on NFAT activation in the N9 murine microglial cell line [18]. A high concentration of ATP (3 mM) activated NFAT, as determined by assaying nuclear extracts using EMSA (Fig. 5). In contrast to the delayed effect of ATP on activation of NF-κB in these cells, NFAT activity was evident within 1 min, reached a maximum after 15 min and diminished in the following 60 min. As shown previously for the effects of ATP on NF-κB [10], activation of NFAT was mediated by the P2X7 receptor [18]. This conclusion was based on agonist specificity, with responses induced by high concentrations of ATP or BzATP, but not by other nucleotides. In addition, ATP did not activate NFAT in a P2X7 receptor-deficient cell clone N9R17, although NFAT could still be activated in these cells by Ca^{2+} ionophore. In this regard, ATP-induced NFAT activation in N9 cells was dependent on the presence of extracellular Ca^{2+} and was prevented by the calcineurin inhibitors cyclosporin A and FK506, consistent with NFAT activation through the canonical signaling pathway.

Using competition and supershift assays in N9 cells, Ferrari and coworkers demonstrated that ATP induced activation of NFAT-1 and NFAT-2, but not NFAT-3 or NFAT-4 [18]. These findings were confirmed by immunoblot analyses of nuclear extracts from cells stimulated with ATP. The functional role of ATP-induced NFAT activation in microglial cells is poorly understood; however, it was proposed that microglial NFAT may play a role in inducible expression of cytokines and, thus, mediate proinflammatory responses to ATP in the central nervous system [18].

Most isoforms of NFAT are activated by Ca^{2+} elevation, and signaling through many P2X and P2Y receptors involves Ca^{2+} increase (by influx or release from stores, respectively). Thus, future studies may reveal NFAT to be downstream of many P2 receptors. The versatility of NFAT as a regulator of gene expression is thought to be due to different binding partners within the nucleus. Thus, the effects of extracellular nucleotides on the activation of NFAT and its various binding partners will be an important area for future research.

Activating transcription factor (ATF)/cyclic AMP response element binding protein (CREB) family of transcription factors

The ATF/CREB family plays important roles in the regulation of a number of cell functions, including proliferation and apoptosis. Members of the ATF/CREB family dimerize with themselves or other family members and bind to the cyclic AMP response element (CRE) on target genes. Activation of CREB involves phosphorylation of a single serine residue (Ser^{133}), which in turn promotes association of CREB with the coactivator CREB-binding protein (CBP), resulting in transcriptional activation [44]. CREB can be activated by multiple kinases, including: protein kinase A, regulated by cyclic AMP; CaM kinase, regulated by cytosolic Ca^{2+}; and ribosomal S6 kinase 2 (RSK2), regulated by MAP kinase pathways [45, 46]. CREB controls the expression of a number of genes, including members of the AP-1 family of transcription factors.

Brautigam and coworkers have examined signaling pathways activated by extracellular nucleotides in the BV-2 murine microglial cell line [19]. LPS induces expression of iNOS and COX-2 in these cells, a process that was inhibited by ATP and 2-chloro-ATP (P2Y1 agonist). ATP also suppressed LPS-induced NO production in this microglial cell line, an effect that was reversed by SB 203580, an inhibitor of p38 MAP kinase. CREB activation was assessed indirectly by monitoring Ser^{133}CREB phosphorylation by immunoblot of whole-cell lysates (Fig. 6). Both ATP (500 μM) and 2-chloro-ATP (10 μM) rapidly induced phosphorylation of CREB and ATF-1 (phosphorylated ATF-1 was also recognized by the antibody used to detect phosphorylated CREB). SB 203580 inhibited ATP-induced phosphorylation of CREB and ATF-1, implicating p38 MAP kinase in their activation. The potential role of CREB in the suppression of iNOS and COX-2 expression is not immediately obvious [47]; however, one possibility considered by Brautigam and coworkers is that activation of CREB sequesters the transcriptional coactivator CBP, preventing its interaction with p65 and thereby inhibiting the expression NF-κB-dependent genes such as iNOS [19].

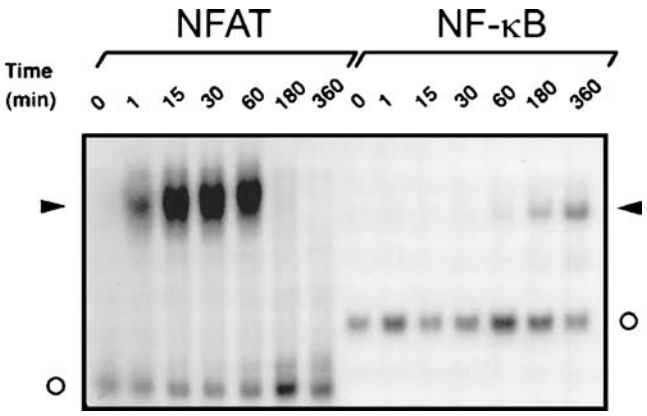

Fig. 5 Kinetics of ATP-induced NFAT and NF-κB activation in a microglial cell line. N9 cells were stimulated for the indicated times with ATP (3 mM) and nuclear extracts were analyzed by EMSA. *Filled arrowheads* indicate positions of the NFAT and NF-κB DNA complexes. Faster-migrating nonspecific complexes are indicated by *circles*. NFAT activity was evident within 1 min following exposure to ATP, reached a maximum after 15 min and diminished following 60 min. In contrast, NF-κB activation was relatively delayed and sustained. Reproduced from [18], with permission of the American Society for Biochemistry and Molecular Biology

Thus, Brautigam and coworkers found that activation of P2Y1 and possibly other P2 receptors suppresses LPS-induced expression of iNOS and COX-2 in a microglial cell line [19]. This is in contrast to the findings of Chen and coworkers that P2Y6 receptors potentiate LPS-induced expression of these inflammatory genes in murine J774 macrophages [14, 15]. Further studies of the effects of nucleotides on CREB activation may be informative. In T lymphocytes, engagement of the T-cell receptor leads to phosphorylation of CREB on Ser^{133} by a pathway that involves activation of $p56^{lck}$, protein kinase C, Ras, Raf-1, MEK and RSK2 [45]. Since ATP has been shown to activate $p56^{lck}$ in Jurkat cells [17], future studies should examine the possibility that ATP also induces activation of CREB in these cells.

Signal transducer and activator of transcription (STAT) family

The Janus kinase (JAK)/STAT pathway transduces signals from cytokines, growth factors or cellular stress to regulate a number of processes including proliferation, differentiation, cell migration and apoptosis [48]. Binding of a cytokine or growth factor induces dimerization of its receptor. JAK tyrosine kinases are then recruited to these dimerized receptor complexes. JAKs are activated by transphosphorylation and subsequently phosphorylate and activate STATs (latent transcription factors that reside in the cytoplasm until activated). There are seven mammalian STATs that, upon phosphorylation, dimerize and enter the nucleus, where they bind specific regulatory sequences to activate or repress the transcription of target genes [49].

Bulanova and coworkers have recently reported the responses to extracellular ATP of murine bone marrow-derived mast cells and two mast cell lines, MC/9 and P815 [20]. These cells express multiple subtypes of P2X and P2Y receptors. Apoptosis of these cells was induced by ATP (1–3 mM) or BzATP (100 μM), consistent with involvement of the P2X7 receptor. In addition, immunoblotting of whole-cell lysates with phospho-specific antibodies established that ATP (3 mM) induced phosphorylation of STAT6 and weak phosphorylation of JAK2. These effects were rapid and transient with maximal phosphorylation evident 15 min following exposure to ATP. In contrast, there was no apparent change in the other members of the JAK family (JAK1, JAK3, Tyk2) or in STAT1, STAT3 or STAT5, indicating specificity of activation. ATP-induced phosphorylation of JAK2 and STAT6 was abolished by the P2X7 antagonists KN-62 or oxidized ATP. However, due to lack of specificity of these antagonists, it is difficult to interpret these findings. First, although KN-62 is a potent antagonist of the human and mouse P2X7 receptor [50], it is also an inhibitor of CaM kinase II [51]. Second, as mentioned above, oxidized ATP is not a specific P2X7 antagonist [27, 28].

Additional findings of Bulanova and coworkers show that high concentrations of ATP or BzATP enhance the production of several cytokines in mast cells including IL-6 and IL-13, which are known to signal through the JAK/STAT pathway. Therefore, ATP may stimulate the rapid activation and release of cytokines or other signaling molecules that bind to receptors on mast cells in an autocrine fashion to activate JAK/STAT signaling.

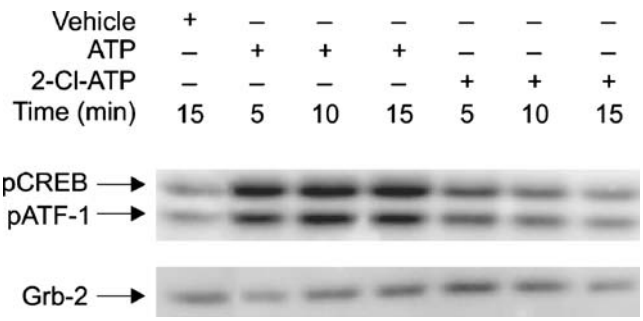

Fig. 6 ATP induces phosphorylation of ATF-1 and CREB in a microglial cell line. BV-2 cells were treated with ATP (500 μM) or the P2Y1 agonist 2-chloro-ATP (2-Cl-ATP, 10 μM) for 5–15 min. Immunoblot analysis of whole-cell lysates revealed levels of Ser^{133}CREB phosphorylation (pCREB), an indicator of CREB activation. The antiSer^{133}CREB antibody cross-reacted with phosphorylated ATF-1 (pATF-1). Both ATP and 2-chloro-ATP rapidly induced phosphorylation of CREB and ATF-1. Grb-2 levels are shown in the *lower panel* as loading controls. Modified from [19], with permission of Elsevier

Concluding remarks

Evidence is accumulating that specific P2 receptors are involved in the regulation of proliferation, differentiation, activity and survival of immune and related cell types through the activation of distinct transcription factors. The lack of selective agonists and antagonists for many subtypes of P2 receptors has made it difficult to assess their specific roles. Development of genetically modified mouse models (in which receptor subtypes are over-expressed or deleted) will provide critical information on the transcriptional effects of specific subtypes of P2 receptors. Such information will be instrumental in determining the roles of these receptors in inflammation and immunity, as well as related processes such as osteoclastic bone resorption [52]. This knowledge may lead to the identification of P2 receptors or components of their downstream signaling pathways as targets for therapeutic intervention in inflammatory and immune disorders.

Leukocytes express multiple subtypes of P2X and P2Y receptors that play diverse roles in regulating the development of the immune system and in modulating inflammatory and immune responses. Although great progress has been made in sorting out many of the initial signaling events triggered by activation of P2 receptors, relatively little is understood about their role in transcriptional regulation. Given the availability of high throughput transcription factor arrays, ChIP and reporter assay screens, rapid progress in this field is expected. An important question to be answered is whether the effects of nucleotides on transcription factor activation are direct—mediated through P2 receptors—or indirect—mediated by autocrine/paracrine factors whose release is stimulated by P2 receptor activation.

Further studies are clearly needed to elucidate the effects of nucleotides on the transcriptional machinery of various immune cell types, and interactions with pathways activated by cytokines, growth factors, extracellular matrix and other stimuli. The transcriptional effects of nucleotides will undoubtedly depend on the nature of the stimulus, P2 receptor expression, cell type and cellular environment. Therefore, the actions of nucleotides on processes such as proliferation and differentiation must eventually be considered within the context of a complex dynamic network of signaling pathways that are activated in spatially and temporally distinct patterns.

Acknowledgements We thank Frank Beier, Suzanne M. Bernier, Andrew Leask and Nattapon Panupinthu (University of Western Ontario) for helpful comments and suggestions. We are also grateful to investigators in this rapidly advancing field for permission to reproduce illustrative material from their previously published work. We apologize to those scientists whose findings were omitted due to space constraints or to oversight. Studies from the authors' laboratories described in this paper were supported by the Canadian Institutes of Health Research (CIHR), the CIHR Institute of Musculoskeletal Health and Arthritis, The Arthritis Society, and the Canadian Arthritis Network (CAN). Souzan Armstrong is supported by Postdoctoral Fellowship from CAN and Jasminka Korcok was supported by Doctoral Research Award from CIHR.

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