Adenosine triphosphate acts as a paracrine signaling molecule to reduce the motility of T cells

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Abstract

Organization of immune responses requires exchange of information between cells. This is achieved through either direct cell–cell contacts and establishment of temporary synapses or the release of soluble factors, such as cytokines and chemokines. Here we show a novel form of cell-to-cell communication based on adenosine triphosphate (ATP). ATP released by stimulated T cells induces P2X4/P2X7-mediated calcium waves in the neighboring lymphocytes. Our data obtained in lymph node slices suggest that, during T-cell priming, ATP acts as a paracrine messenger to reduce the motility of lymphocytes and that this may be relevant to allow optimal tissue scanning by T cells.

Keywords adenosine triphosphate; calcium wave; cell migration; imaging; T cells

Subject Categories Immunology; Signal Transduction

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Introduction

Extracellular adenosine-5′-triphosphate (ATP) is an important secondary messenger with roles in many cellular processes including cell proliferation, apoptosis and differentiation (Popper & Batra, 1993; Vandewalle et al., 1994; Atarashi et al., 2008). ATP mediates its signaling activities through activation of the P2 purinergic family of receptors (Burnstock, 2006). Based on pharmacological and functional data, protein topology and gating properties, P2 purinergic receptors are divided into P2X and P2Y subfamilies. Stimulation of both of these receptors leads to an increase in intracellular calcium although via two distinct mechanisms. Ionotropic P2X receptors (P2X1—7) are ligand-gated cation channels that, upon ATP binding, form non-selective pores allowing ion influx from the extracellular space (Burnstock, 2006). In contrast, metabotropic P2Y receptors (P2Y1, 2, 4, 6, 11–14) are coupled to G-proteins and ATP binding to these receptors results in phospholipase C–β (PLCβ) activation, IP3 production and the consequent release of intracellular calcium from ER stores (Burnstock, 2006).

In T cells, ATP is released through gap junction pannexin-1 pores during lymphocyte activation (Schenk et al., 2008; Woehrle et al., 2010) and it enhances interleukin-2 (IL-2) production by binding to purinergic receptors P2X1, P2X4 and P2X7 in an autocrine manner (Yip et al., 2009; Woehrle et al., 2010). The binding of ATP results in calcium entry, which subsequently activates the nuclear factor of activated T cells (NFAT), the mitogen-activated protein kinase (MAPK) signaling and IL-2 expression, providing feedback regulation to T-cell activation (Schenk et al., 2008; Yip et al., 2009). Extracellular ATP was also shown to have synergistic effect on the proliferation of peripheral blood lymphocytes stimulated with phytohemagglutinin (Baricordi et al., 1996). On the other hand, antagonists to purinergic receptors or ATP degradation were shown to inhibit T-cell capacitative calcium entry and favor T-cell anergy (Schenk et al., 2008; Yip et al., 2009). More recently, ATP was shown to inhibit immunosuppressive functions of regulatory T cell and to promote their conversion to T helper 17 cells in a murine model of inflammatory bowel disease (Schenk et al., 2011).

Adenosine triphosphate released by stimulated cells may also act as a paracrine messenger involved in intracellular calcium mobilization activated by purinergic receptors (Ospichuk & Cahalan, 1992; Zsembry et al., 2003; Yegutkin, 2008). Thus, ATP-induced intercellular spreading of calcium signals—the so called calcium waves—has been observed in many cell types such as mast cells, epithelial cells, astrocytes and hepatocytes (Cotrina et al., 1998; Guthrie et al., 1999). Calcium waves represent a dynamic intercellular signaling mechanism that allows spatiotemporal information to be rapidly propagated in tissues (Arcuino et al., 2002; Weissman et al., 2004; Dupont et al., 2007). For example, the propagation of signals between brain cells depends on the formation of calcium waves (Nedergaard, 1994), whereas normal hearing function depends on calcium waves in the cochlear of the ear (Zhang et al., 2005b; Anselmi et al., 2008).

Although the autocrine signaling of ATP has been extensively investigated in lymphocytes, it is not known whether ATP is used by T cells to communicate with neighboring lymphocytes in lymphoid tissues. Thus, the aim of our study was to investigate ATP paracrine signaling in T lymphocytes and to determine its impact on T-cell physiology.
Results

Identification of an ATP-induced paracrine calcium signaling among lymphocytes

In T cells, the interaction of the T-cell receptor (TCR) with cognate antigen results in the activation of phospholipase C–γ, which generates inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG) from phosphatidylinositol 4,5 bisphosphate (PIP2) in the plasma membrane (Lewis, 2001). IP3 induces Ca2+ release from the endoplasmic reticulum (ER), which, in turn, stimulates activation of the Ca2+-permeable calcium release activated channels (CRAC) Orai1/CRAM1 in the plasma membrane, resulting in a sustained influx of Ca2+ from the extracellular space into the cytosol, termed capacitative Ca2+ entry (CCE) (Putney & Bird, 1993; Zhang et al., 2006; Feske et al., 2005a; Prakriya et al., 2006; Vig et al., 2006; Yeromin et al., 2006).

Using a cell membrane permeable caged-IP3 (Li et al., 1998), we triggered single-cell Ca2+ influx in human peripheral blood CD4+ T lymphocytes using a short UV laser pulse (Fig. 1). Interestingly, we clearly observed calcium signal propagation to bystander T cells that were not in contact with the triggered one (Fig 1 A and Supplementary Video S1), suggesting that calcium waves were propagated through a soluble mediator (Suadicani et al., 2006). In T cells, Ca2+ influx induces ATP synthesis and release (Schenk et al., 2008), and, on the other hand, extracellular ATP is known to induce Ca2+ influx in several cell types (Osipchuk & Cahalan, 1992; Schlosser et al., 1996; Guthrie et al., 1999; Gomes et al., 2006). Thus, we speculated that the calcium waves observed in T cells might be caused by paracrine ATP signaling. To verify the involvement of extracellular ATP in this process, we repeated the experiment in the presence of the ATPase enzyme apyrase (Fig 1 B and Supplementary Video S2), and we found that in the absence of extracellular ATP, the percentage of calcium-influxing T cells was significantly reduced (Fig 1C). The analysis of the kinetic of the calcium waves indicated that it was a function of the distance from the triggered cell, suggesting that it depended on ATP diffusion (Fig 1D). Limited calcium waves were obtained using the Jurkat T-cell line, too (not shown).

The effects of apyrase on changes in single-cell intracellular calcium concentration [Ca2+]i were also analyzed (Fig. 1E and F). Apyrase reduced calcium influx in the cell stimulated by UV exposure (Fig 1E), indicating that extracellular ATP sustains calcium signaling in an autocrine manner (Yegutkin et al., 2006; Yip et al., 2009). Notably, in agreement with data shown in Fig 1B and C, the

Figure 1. Identification of adenosine triphosphate (ATP) paracrine signaling.

A An intercellular Ca2+ wave initiated by flash photolysis of caged-IP3 of ROI in one human CD4+ T cell (arrowhead) propagates to non-adhering bystander CD4+ T cells. Increase in cytosolic calcium was indicated by the increase in green fluorescence intensity (Fluo-4). The time-lapse video is illustrated in Supplementary Video S1.
B Calcium waves were inhibited by the ATPase apyrase (5 U/ml). Arrowhead marks the uncaged cell. Scale bar 10 μm, time is indicated in seconds. See also Supplementary Video S2.
C The number of bystander cells showing calcium influx after the uncaging of one central cell was quantified from each imaging frame. In the control condition, approximately 65% of the bystander cells would respond to extracellular ATP released within the 3-min time-lapse videos, and apyrase significantly reduced this percentage (n = 6, ***P < 0.001).
D The relationship between the distance of bystander cells from the uncaged one versus the time of calcium level increase.
E Graph showing ratiometric measurements of cytosolic calcium levels in the uncaged cells in control and in the presence of 5 U/ml active or heat-inactivated (HI) apyrase. The control cells responded to UV uncaging with increased cytosolic calcium. In the presence of apyrase, the increase was markedly reduced (n = 11, Wilcoxon signed rank test P < 0.001). Heat-inactivated apyrase did not significantly alter the cytosolic calcium increase after uncaging (n = 4, P > 0.05).
F The ratiometric measurements of cytosolic calcium levels showed that in the bystander cells, the intracellular calcium oscillated significantly and increased with time in the control condition (n = 48 cells), whereas in the presence of apyrase, the intracellular calcium level remained flat in the bystander cells (n = 20, Wilcoxon signed rank test P < 0.001). Heat-inactivated apyrase did not prevent the calcium responses in the bystander cells (n = 14).
cells nearby the uncaged one no longer responded respond in the presence of apyrase (Fig 1F). Individual traces of calcium influx in response to ATP for each cell are shown in Supplementary Fig S1.

To estimate the level of ATP released by T cells in our experimental conditions, we stimulated T cells with anti-CD3 plus anti-CD28 antibodies, a condition known to induce ATP release (Schenk et al., 2008; Yip et al., 2009), or UV photolysis of caged-IP3 loaded cells and quantified the ATP released by the cells. The results indicate that the level of ATP released in response to the two stimuli is not significantly different (Supplementary Fig S2A). The level of intracellular calcium increase was also similar when the T cells were stimulated either by anti-CD3 and anti-CD28 antibodies, or extracellular ATP (100 μM, Supplementary Fig S2B).

To verify the existence of T-cell calcium waves in a more physiological environment, we performed experiments similar to those described above using lymph node (LN) slices that had been previously loaded with the calcium indicator Fluo-4 and caged-IP3 (Fig 2, Supplementary Videos S3, S4 and S5). Calcium waves were clearly detected in the LN microenvironment (Fig 2A–B and Supplementary Video S3) and required the presence of extracellular ATP (Fig 2C and Supplementary Video S4).

Altogether, these data demonstrate the existence of an ATP-induced, Ca2+-mediated paracrine signaling among lymphocytes.

Identification of the receptors involved in lymphocyte paracrine ATP signaling

Adenosine triphosphate mediates its signaling activities through activation of the P2X and P2Y purinergic receptors (Burnstock, 2006). On the basis of the distinctive properties of the P2X and P2Y receptors, we tried to identify the subfamily of receptors responsible for paracrine ATP binding by repeating the in vitro IP3-uncaging experiment in the presence (Fig 3A) or in the absence (Fig 3B) of extracellular Ca2+. Indeed, in the absence of extracellular Ca2+, P2X receptors would be unable to signal, whereas P2Y-mediated [Ca2+]i would still be detected. To further confirm the involvement of P2X receptors, T cells were pre-incubated with the P2X antagonist suramin before UV uncaging. Suramin inhibited the bystander cell calcium increase, similarly to apyrase (Fig 3E). Our experiments demonstrated that extracellular Ca2+ is required for ATP-mediated T-cell paracrine signaling (Fig 3), suggesting the involvement of the purinergic receptors belonging to the P2X subfamily.

The P2X family comprises seven receptor subunits (P2X1—7). We analyzed the expression of the seven subunits in Jurkat and human peripheral blood CD4+ T cells by reverse transcriptase PCR (Fig 4A). P2X2 and P2X3 mRNAs were not detectable in both CD4+ and Jurkat T cells (not shown). The only P2X receptors expressed in both cell types were P2X1, P2X4 and P2X7, although they were expressed at very low levels in Jurkat cells as compared to the human peripheral CD4+ T cells (Fig 4A). To further characterize the contribution of these three receptors to ATP paracrine signaling in human T cells, we separated peripheral blood CD4+ T cells in three distinct populations—T naïve, central memory and effector memory (Sallusto et al., 2004)—and quantified the expression of each receptor subunit (Fig 4C), as well as the calcium response induced by paracrine ATP in the different cell populations (Fig 4B). For this, we performed experiments using extracellular ATP to stimulate T-cell calcium influx, detected by flow cytometry. The analysis demonstrated that P2X1 is not expressed in memory T cells, that are, however, characterized by a strong ATP-induced calcium signaling, suggesting that P2X1 is not involved in the bystander activation of T cells. This conclusion was supported by experiments in which pharmacological blockers for P2X1, P2X4 or P2X7 were used to interfere with ATP signaling and that demonstrated that blockage of P2X4 and P2X7, but not of P2X1, inhibited ATP-induced calcium influx in T cells (Fig 4D and E). To provide further support in favor of a role for P2X4 and P2X7, we knocked down in T cells the expression of the two receptors using siRNA technology (Fig 4F) and analyzed the calcium response induced by extracellular ATP (Fig 4G). The results indicated that the two receptors are involved

Figure 2. Calcium waves in lymph node slices.

A Fresh mouse lymph nodes were cut into slices and loaded with caged-IP3 and calcium indicator Fluo-4 for ex vivo calcium wave imaging. Calcium wave rapidly formed upon uncaging of IP3 at ROI (the small, green circle) in mouse lymph node slices. Cytosolic calcium is pseudocolored with the ‘Fire’ spectrum, where basal calcium level is in blue and the increase is indicated in red and yellow colors. White lines demarcate the expansion of the calcium wave. These data are representative of six independent experiments; scale bar, 30 μm; time is indicated in seconds. See also Supplementary Video S3.

B The spread of calcium waves is represented as a function distance from the uncaged ROI and time.

C No calcium waves were detected in lymph node slices incubated in apyrase (n = 4). Related time-lapse illustration is shown in Supplementary Video S4. Basal calcium oscillation in resting mouse ex vivo lymph node slices is shown in Supplementary Video S5.
in ATP-induced calcium signaling in T cells and that when both receptors are knocked down, T cells are unresponsive to extracellular ATP.

**Functional significance of calcium waves in the lymphoid tissue**

Our data suggest that T-cell activation in lymph nodes would result in ATP release and raise of [Ca\(^{2+}\)]\(_i\), in neighboring, unstimulated T cells. Robust evidence indicates that an increase in [Ca\(^{2+}\)]\(_i\) reduces T-cell motility in tissues (Bhakta et al., 2005). Thus, we speculated that ATP-dependent calcium waves may reduce bystander cell motility to create a zone of lymphocyte swarming and clustering as observed in antigen challenged lymph nodes (Miller et al., 2002) and to favor T-cell scanning of antigen-loaded dendritic cells. Accordingly, in vitro experiments with human CD4\(^+\) T cells showed that addition of extracellular ATP significantly reduced the mean migration speed toward the chemokine CXCL12, and it disrupted the straight chemotactic migration and the final cell displacement (Fig 5A–D). The P2X receptor antagonist suramin prevented these ATP-induced effects on T-cell motility. Importantly, addition of ATP did not alter the migration velocity in the absence of extracellular calcium (Fig 5E). The projected migration tracks of the T cells from the time-lapse videos are shown in Supplementary Fig S3. In these experiments, when the ATP-induced intracellular calcium increase was buffered by the calcium chelator BAPTA, we no longer observed the slowing down of T-cell chemotactic migration velocity (Supplementary Fig S4).

Altogether, the data allow us to propose a scenario in which ATP released by antigen-triggered T cells reduces the motility of the unstimulated T cells that are in close proximity. To verify this hypothesis, the motility of T cells in LN slices was analyzed by two-photon microscopy. In these experiments, two different types of mouse T cells, expressing either a TCR specific for the OVA peptide (OT-III T cells) or wild-type T cells (WT T cells), were overlaid and allowed to penetrate into LN slices containing dendritic cells (DCs) loaded with the OVA peptide. In these conditions, only OT-II T cells can be triggered by OVA-DCs, whereas the large majority of WT T cells would remain unstimulated. As expected, OT-II T cells reduced their motility in the presence of the specific antigen, indicating establishment of contacts (Miller et al., 2004b) (Fig 6 and Supplementary Video S6). In agreement with recent findings that observed a general reduction of T-cell motility—individually of antigen specificities—in regions of LN where there are prolonged interactions between antigen-specific T cells and antigen-presenting ones (McKee et al., 2013; Salgado-Pabon et al., 2013), in the presence of the OVA peptide, also the WT T cells displayed reduced motility (Fig 6A). On the basis of the data presented above in the manuscript, we speculated that the reduced motility of the untriggered WT T cells depended on the ATP released by triggered OT-II T cells, and to prove this hypothesis, we performed the same experiments in the presence of apyrase. Notably, the velocity of WT T cells recovered to the control condition in the presence of the ATPase apyrase (Fig 6A and Supplementary Video S7), whereas OT-II T cells remained unaffected (Fig 6B).

**Discussion**

Extracellular ATP is an important modulator during inflammation and immunity, regulating several aspects of immune cell biology such as the shedding of CD21 and CD62L (L-selectin) in neutrophils (Sengstake et al., 2006; Scheuplein et al., 2009), the differentiation of Th17 cells in the lamina propria (Atarashi et al., 2008) and the generation of regulatory T cells (Schenk et al., 2011). ATP was first described to have mitogenic effect on human T lymphocytes stimulated by PHA or anti-CD3 antibodies (Baricordi et al., 1996) and, more recently, it was demonstrated that ATP is released during T-cell activation via gap junction pannexin-1 hemichannels (Schenk et al., 2008; Yip et al., 2009; Woehrle et al., 2010) and that it acts as autocrine costimulator for IL-2 production (Schenk et al., 2008; Yip et al., 2009).

Our data indicate that, in addition to these autocrine effects, ATP may act as a paracrine signaling molecule by inducing calcium waves that regulate T-cell motility during immune responses. We show that extracellular ATP released by triggered T cells is sensed by resting, bystander cells through P2X4 and P2X7 receptors, since pharmacological inhibition as well as silencing of P2X4 and
P2X7 receptors abolished ATP-induced calcium signaling in human T cells. Interestingly, during T-cell priming, P2X1 and P2X4 receptors are recruited into the immunological synapse, whereas P2X7 receptors remain diffused in the plasma membrane (Woehrle et al., 2010). This suggests that different P2X receptor subtypes have different functions during T-cell activation and that P2X7 receptors may allow T cells to sense ATP present in the microenvironment (Junger, 2011). Our data indicate that P2X1 is not expressed in memory T cells. It would be therefore interesting to analyze how ATP signaling at the immunological synapse is orchestrated in these cells lacking P2X1. As for paracrine signaling, the fact that memory T cells do not express P2X1 but are characterized by a strong ATP signaling at the immunological synapse is orchestrated in these memory T cells. It would be therefore interesting to analyze how.

Figure 4. P2X4 and P2X7 receptors are both involved in paracrine adenosine triphosphate (ATP) signaling.
A) Reverse transcriptase PCR analysis of P2X receptors expression in Jurkat and human CD4+ T cells.
B) All human CD4+ T cells (naive, central and effector memory) show calcium influx in response to paracrine ATP signals, with effector/memory T cells showing higher percentages of fluxing cells than naïve ones. Jurkat T cells demonstrated very low levels of bystander cell calcium responses (**P < 0.001).
C) Real-Time PCR analysis of P2X1, X4 and X7 in naïve, central memory, effector memory CD4+ T (four donors) and Jurkat T cells. P2X2 and P2X4 are not expressed in lymphocytes, not shown). The expression of P2X1 was barely detectable, whereas P2X4 and P2X7 are highly expressed in effector/memory cells. Jurkat cells expressed low levels of the P2X4 and P2X7 receptors.
D) Human CD4+ T cells were treated with pharmacological blockers of purinergic receptors P2X1, P2X4 or P2X7 (n = 56 cells) and subjected to single-cell IP3 uncaging using the UV laser as the experiment described in Fig. 1. NF023 (P2X1 blocker) did not affect the percentage of bystander cells showing calcium influx in the imaging frame. 5-BDBD (P2X4 blocker) reduced the percentage of responding bystander cells, although not significantly. P2X7 blockers KN62 and A438079 both significantly reduced the percentage of responding bystander cells (*P < 0.05). Inhibition of both P2X4 and P2X7 receptors significantly prevented bystander cell calcium influx (**P < 0.001).
E) Calcium influx in response to addition of extracellular ATP is prevented by A438079 and 5-BDBD.
F) To reconfirm the data, we employed siRNA to knocked down P2X4 and P2X7 in primary human CD4+ T cells. Successful knock down of mRNA expression was confirmed with real-time PCR analysis.
G) FACs analysis of calcium influx in human CD4+ T cells in response to extracellular ATP (paracrine signaling). Single knock down of P2X4 or P2X7 (dotted lines) did not alter the magnitude of calcium influx, but double knock down (gray solid line) rendered T cells unresponsive to extracellular ATP (two-way ANOVA P < 0.001).

Figure 5. Adenosine triphosphate (ATP) signaling reduces T-cell chemotactic migration.
A-D) Human CD4+ T cells were seeded onto chemotaxis chambers and bright field time-lapse images were recorded to analyze migratory responses to CXCL12 (2.5 nM). Addition of extracellular ATP (100 µM) significantly modified T-cell chemotaxis, in term of (A) migratory tracks, (B) migration speed, (C) straightness and (D) final displacement (n = 3 experiments; control 79 cells, ATP 176 cells ***P < 0.001). The P2X receptor antagonist suramin blocked the effect of ATP on migration speed and final displacement of T cells, but it only inhibited ATP effect on directionality (n = 3, 36 cells).
E) Chemotactic T-cell migration toward CXCL12 in the absence of extracellular calcium (calcium-free PBS). The addition of ATP did not alter the migration velocity in the absence of extracellular calcium. ns, not significant.
Extracellular ATP alters T-cell motility

condition. These results are in line with recent studies of T-cell without the OVA peptide, and this depended on the release of ATP down of wild-type T cells was clearly due to the simultaneous trig-

could not form conjugates with OVA-presenting DCs. The slowing also reduced their motility, although the great majority of them DCs. Interestingly, in the same slices, unstimulated wild-type T cells their velocity in lymph node slices in the presence of OVA-pulsed

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exerts autocrine effects that may potentiate activation, but also bystander signaling.

that in distinct T-cell subpopulations P2X1 may also play a role in bystander signaling.

We propose that ATP released by T cells during priming not only exerts autocrine effects that may potentiate activation, but also paracrine effects on the other lymphocytes present in the LN micro-

environment. Indeed, in agreement with previous studies (Miller et al., 2004a,b), we observed that OVA-specific T cells slowed down their velocity in lymph node slices in the presence of OVA-pulsed DCs. Interestingly, in the same slices, unstimulated wild-type T cells also reduced their motility, although the great majority of them could not form conjugates with OVA-presenting DCs. The slowing down of wild-type T cells was clearly due to the simultaneous trig-

erring of OT-II T cells, as demonstrated by the control experiments without the OVA peptide, and this depended on the release of ATP in the LN microenvironment, as demonstrated by the apyrase condition. These results are in line with recent studies of T-cell

motility in the LN microenvironment that have demonstrated a significant drop in the velocity of polyclonal T cells during antigenic stimulation of TCR-specific cells (McKee et al., 2013; Salgado-Pabon et al., 2013). The reduced motility of T lymphocytes in a tissue where antigenic recognition is occurring may be strategic for a better scanning of resident DCs and, in this perspective, our data provide an additional and novel mechanism to the emerging concept that the extracellular ATP is an ‘alert signal’ for the immune system. We have recently demonstrated the existence of a delicate equilibrium between adhesive and chemotactic forces operating in lymph nodes during T-cell priming, allowing enough motility for T-cell repertoire scanning while ensuring the formation of long-lasting conjugates, once a cognate T-APC pair is formed (Viola et al., 2006; Kallikourdis et al., 2013). Variations in this equilibrium, such as those due to an hyperfunctional mutant CXCR4, leads to impaired stability of the immunological synapse and consequently contribute to an aberrant adaptive immune response (Kallikourdis et al., 2013). ATP may thus alter this equilib-

and S7.

Adenosine triphosphate (ATP) signaling inhibits bystander cell motility during T-cell activation in LNs.

A, B Multiphoton analyses of T-cell motility in LN slices. Mouse LN slices containing either OVA-pulsed or unpulsed DCs were overlaid with murine CD4+ T cells with different antigen specificity (OVA or wild-type). The graph shows the motility of T cells from WT mice (WT T bystander cells) (A) or from OT-II mice (triggered T cells) (B). Control condition was LN slices containing unpulsed DC with both OT-II and WT T cells (nDC). The migration speed of bystander WT T cells (A) decreased significantly when OT-II T cells were stimulated by pulsed DCs (OVA-DC), but migration speed returned to control conditions with the addition of apyrase (OVA-DC + apy) or pre-treatment of WT T cells with suramin, confirming that ATP signaling modulated the motility of bystander T cells within the LN microenvironment. Interestingly, the motility of OT-II T cells was not affected by apyrase or suramin (B). *P < 0.05, **P < 0.01; each dot represents one cell in the LN slice, n = 4 independent experiments; representative graphs from three experiments are shown here; ns, not significant. Related time-lapse recordings are shown in Supplementary Videos S6, S7 and S8.

Using an approach similar to the one described in our study (two-photon microscopy in thymus slices), it was demonstrated that Ca2+ signaling during positive selection inhibits motility and prolongs interactions with stromal cells, reinforcing the interaction that is essential during differentiation from double-positive thymocytes to mature T cells (Bhakta & Lewis, 2005; Cahalan, 2005). In this study, we confirm the role of Ca2+ in tuning lymphocyte motility in tissues and propose that ATP is responsible for modulation of motility in unstimulated T cells in a reactive lymph node. Thus, the ‘stop’ signal mediated by Ca2+ influx involves not only cells that have already found their antigenic partners, but also lymphocytes that may be potentially triggered within the tissue.

The mechanism by which increases in [Ca2+]i inhibit lymphocyte motility is not entirely understood. In Dictyostelium discoideum, increases in [Ca2+]i induce phosphorylation of myosin heavy chain II A at a site (Thr1939) that disrupts myosin bundling, thus affecting the cell’s traction force (Jacobelli et al., 2004). Although this may be relevant for mouse cells (Jacobelli et al., 2004), the human isoform contains an alanine substitution at this site (Buxton & Adelstein, 2000), suggesting that Ca2+ signaling inhibits motility through

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additional mechanisms. Increase in [Ca\(^{2+}\)]\(_i\) is known to trap mitochondria at specific subcellular sites of high Ca\(^{2+}\) microdomains (Rizzuto et al., 2004; Yi et al., 2004). We have previously shown that accumulation of mitochondria at the uropode of migrating cells is required to sustain phosphorylation of the myosin light chain (MLC), a key step in high-speed moving cells (Campello et al., 2006). Trapping of mitochondria is therefore a possible alternative mechanism responsible for motility arrest upon increases in [Ca\(^{2+}\)]\(_i\) in lymphocytes.

In addition to our data, recent evidence strengthens the concept of ATP as a key player in the inflammatory microenvironment, regulating the release of cytokines and chemokines (Rayah et al., 2012) and triggering the activation of the Nrlp3 inflammasome (Iyer et al., 2009). Interestingly, in tumors, ATP appears to be crucial for infiltration, differentiation and maintenance of myeloid cells in the tumor upon chemotheraphy (Chen et al., 2006; Ma et al., 2013). Our data propose a new role for ATP in immunity through the paracrine regulation of lymphocyte motility during antigen recognition. In future studies, it would be important to evaluate the consequences of these findings in the context of anti-tumor immunity.

Materials and Methods

Cells

Human peripheral CD4\(^+\) cells were isolated from whole human blood collected by Desio Hospital, Milan, in accordance to institutional guidelines with patient consent for research purposes. The CD4\(^+\) lymphocytes were isolated using RosetteSep CD4\(^+\) Enrichment Cocktail (Stemcell) and cultured in RPMI supplemented with L-glutamine, non-essential amino acids, sodium pyruvate and 10% FBS. Cells were used for experiments 1 or 2 days after isolation. The purity of the CD4\(^+\) was confirmed with FACS staining and analysis. The Jurkat clone E6.1 was maintained in the same medium.

Mice

Wild-type C57BL/6j (H-2\(^b\)) and OT-II TCR transgenic mice were purchased from Charles River Laboratories (Italy). Mice were kept in regular light and dark cycles, with unrestricted access to water and food. Mice were kept under Italian national and EU directives (2010/63/EU) for animal research with protocols approved by institute Ethical Committee and the Italian Ministry of Health (162/2011-B).

Cell sorting

Total peripheral CD4\(^+\) lymphocytes were sorted with BD FACsAria cell sorter to T naive (CD45RO\(^-\)CD45RA\(^-\)CD62L\(^+\)), T central memory (CD45RO\(^+\)CD45RA\(^-\)CD62L\(^+\)) and T effector memory (CD45RO\(^+\)CD45RA\(^-\)CD62L\(^-\)) subpopulations (Sallusto et al., 2004). Briefly, total CD4\(^+\) T cells were stained with conjugated antibodies against CD4, CD45RO, CD45RA and CD62L (BD Biosciences) in PBS containing 1% of FBS for 30 min in 4°C. The stained cells were washed twice and sorted straight away.

Confocal calcium imaging

The cell membrane permeable ester of caged-IP3-PM (2 \(\mu\)M, SiChem, Bremen, Germany) was loaded into total CD4\(^+\) T cells, or for some experiments in CD4\(^+\) subpopulations or Jurkat T cells, simultaneously with calcium indicators Fluo-4-AM (3.5 \(\mu\)M) and FuraRed-AM (6 \(\mu\)M, Molecular Probes) for 30 min at 37°C (Li et al., 1998). After washing, the loaded T cells were seeded on coverslips coated with poly-L-lysine (Sigma-Aldrich) and allowed to settle for 10 min in imaging buffer (RPMI without phenol red with 1% FBS). An Olympus Fluoview FV1000 laser scanning confocal microscope fitted with two scanning lasers was used to acquire time-lapse images of calcium change. The SIM UV laser (405 nm, 45% power, 80 ms pulse) was used to release active IP3 within a region of interest (ROI), and time-lapse images were acquired every 3 s for 3 or 5 min in a 37°C heated imaging chamber. The images were acquired with a 60× immersion objective with numerical aperture 1.35 (Plan-Apochromat, Olympus). Fluo-4 and FuraRed fluorophores were excited simultaneously with a 488-nm laser, and the emissions were collected with 500- to 550-nm and 620- to 680-nm band-pass filters. Differential interference contrast (Nomarski technique) was also used. In some experiments ATPase apyrase (5 U/ml, Sigma-Aldrich) was added to the imaging buffer. Additional control experiments using heat-inactivated apyrase (10 min in 100°C; De Miranda et al., 2002), PBS vehicle or T cells loaded with calcium indicators alone were performed. To confirm the role of P2X receptors, experiments were performed on T cells pre-incubated with suramin (45-min pre-incubation, 100 \(\mu\)M). Fluorescent intensities of Fluo-4 and FuraRed were used for ratiometric calcium-level calculations in NIH ImageJ (n = 6 independent experiments).

ATP quantification

The amount of ATP released by CD4\(^+\) T cells after UV photolysis of caged-IP3 and stimulation with anti-CD3 (2 \(\mu\)g/ml) and anti-CD28 (1 \(\mu\)g/ml) antibodies was quantified using ATP Bioluminescence Assay Kit HS II (Roche). 10\(^6\) CD4\(^+\) T cells were loaded with caged-IP3, resuspended in 100 \(\mu\)l HBSS and plated in a 96-well plate (n = 7 donors). Caged-IP3 was released by photolysis (100 ms, DAPI filter) using an epi-fluorescence microscope (Olympus IX81 CellIR microscope, UPLFLN 4× objective numerical aperture 0.13), 10\(^6\) unmanipulated, resting T cells were placed in Eppendorf tubes and stimulated with anti-CD3 and anti-CD28 antibodies (n = 4 donors). The plate/samples were kept in 37°C for 10 min, after which the samples were collected and spun down for 5 min in a 4°C microfuge. For each sample, 50 \(\mu\)l of supernatant was collected and transferred into a black microplate for ATP quantification. Controls were T cells that were not loaded with caged-IP3 and that underwent the same flash photolysis or unstimulated T cells. ATP measurement was carried out using manufacturer’s protocol. Bioluminescence signals were detected using the Synergy H4 microplate reader (BioTek). Data were exported and analyzed with Prism 4 (GraphPad).

RNA and Real-Time PCR

Peripheral CD4\(^+\) lymphocytes and Jurkat T cells were centrifuged and washed once with PBS without calcium or magnesium (Biowhitaker, Lonza). The pellets were used for RNA isolation with
calcium waves in C57BL/6J murine LNs despite the known DNA polymorphism in the p2x7 gene that reduces receptor function. LN slides loaded with Fluo-4 only (no caged-IP3) were negative controls for calcium wave.

**Identification of purinergic receptors**

To distinguish the involvement of P2X or P2Y families of receptors, the same confocal calcium imaging experiment using human CD4⁺ T cells was repeated but using phosphate-buffered saline with or without calcium or magnesium (Biowhittaker, Lonza) as the imaging buffer. Pharmacological purinergic receptor blockers NF023 (10 μM), 5-BDBD (100 μM), KN62 (1 μM) and A438073 (5 μM) were used to identify the receptors involved in paracrine ATP signaling (TOCRIS, Bristol, UK). Alternatively, two pairs of siRNAs to P2X4 and P2X7 were used to knockdown gene expression (s9955, s9956 and s9959, s9960, respectively, Applied Biosciences), and a scrambled siRNA was included as control. Primary CD4⁺ lymphocytes were transfected with 3 μg of siRNA using the Amaxa Human T Cell Nucleofector kit (Lonza). Expression knockdown was confirmed with real-time PCR in duplicates. CD4⁺ T cells pre-treated with purinergic receptors blockers for 40 min prior to ROI uncaging and time-lapse imaging as described previously (n = 4). Calcium influx in response to extracellular ATP (100 μM) or soluble anti-CD3 (2 μg/ml, OKT3 clone) and anti-CD28 (1 μg/ml) antibodies (eBiosciences) was also analyzed with FAC Canto flow cytometry (BD Biosciences). CD4⁺ T cells were loaded with calcium indicators Fluo-4-AM and FuraRed-AM as described previously (Conte et al., 2010). Basal intracellular calcium level was acquired for 45 or 60 s before addition of extracellular ATP or the antibodies. Samples were acquired for a further 3 or 5 min, and ionomycin (1 μg/ml, Sigma) was added at the end as positive control for maximum calcium influx. Data were analyzed with FlowJo Software version 7.6.4 (Tree Star, Inc.) and normalized to the mean basal intracellular calcium level.

**Ex vivo lymph node (LN) preparation and calcium wave imaging**

Fresh inguinal LNs were collected from 10-week-old adult C57BL/6J mice, embedded in low-melt agarose (Sigma) and cut with a vibrotome to 300-μm slices (Asperti-Boursin et al., 2007; Salmon et al., 2011). The slices then were loaded with calcium indicator Fluo-4 (7 μM, Molecular Probes) and caged-IP3 (3 μM, SiChem, Bremen, Germany) in RPMI containing 1% FBS and 0.2% pluronic acid (Sigma) in a 37°C incubator for 45 min. The slices were rinsed gently in fresh RPMI without phenol red (Lonza) and imaged on the confocal microscope with heated imaging chamber (Olympus Fluoview FV1000, n = 5 independent experiments). A ROI was selected for uncaging IP3 with the SIM laser (80% laser power, 80 ms pulse), and time-lapse images of Fluo-4 intensity changes were acquired over a period of 3 min at 3 s per frame with a 40× numerical aperture 1.30 objective (excitation 488 nm laser and emission 500- to 550-nm band-pass filter). Apyrase (5 U/ml, Sigma) was added to the imaging buffer in some of the experiments. We have observed calcium waves in C57BL/6J murine LNs despite the known DNA polypurine migration in lymph node slices (OT-II, WT)

Wild-type C57BL/6 and OT-II transgenic mice were obtained from Charles River Laboratories (Calco, Italy). OT-II transgenic mice express a TCRαβ transgene that is specific for the OVA peptide 323–339 (TCR-OVA). Wild-type CD4⁺ lymphocytes do not express TCRs that significantly recognize the OVA peptide and are therefore used as bystander T cells. Ex vivo T-cell activation visualization using multiphoton microscopy has been described elsewhere (Asperti-Boursin et al., 2007; Kallikourdis et al., 2013). Briefly, dendritic cells were matured from bone marrow (Dal Secco et al., 2009). The day before the experiment, DCs were labeled with CellTracker Orange CMTMR (5 μM, Molecular Probes, Invitrogen) and pulsed for 2 h with OVA323-339 peptide (10 μg/ml, Anaspec) or PBS vehicle. The DCs were injected intraperitoneally near the inguinal LNs with lipopolysaccharide (40 ng/mouse) in PBS, at a concentration of 4 million cells per mouse. On the day of the experiment, lymph nodes containing labeled DCs were cut into slices as described previously for the calcium wave experiments. CD4⁺ T cells from OT-II and wild-type mice were negatively selected from LNs using the mouse CD4⁺ T-cell isolation kit II (MACS Miltenyl Biotec) and labeled with CellTracker Blue CMAC (40 μM, SiChem, Bremen, Germany) in RPMI containing 1% FBS and 0.2% pluronic acid (Sigma) in a 37°C incubator. Cell interactions within a tissue volume 300 × 300 × 50 μm at 5 μm z-steps was visualized using a two-photon microscope (LaVision TrimScope) fitted with a 20× water immersion objective (Olympus XPLMPFL, numerical aperture 1.0). The slices were imaged in oxygenated phenol red free RPMI supplemented with l-glutamine, pen-strep and HEPES. In some experiments, apyrase 2 U/ml was added to the running buffer. In the suramin condition, WT T cells were pre-incubated with 100 μM suramin (irreversible) for 1 h and washed before overlaying onto the slices. Time-lapse images were acquired every 30 s for 30 min and exported to Imaris (version 7.3.2, Bitplane) for analysis (n = 6).
Statistical analysis

Data analysis was performed using Prism 4 (GraphPad, USA). Student’s t-test or ANOVA followed by Bonferroni post hoc test were used to compare groups against the control condition. Wilcoxon matched pairs test was used to compare the calcium response in T cells over the course of time-lapse video in control or presence of apyrase. Statistical significance was taken at P < 0.05; n value represents the number of independent experiments.

Supplementary information for this article is available online: http://emboj.embopress.org

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Author contributions

CMW performed the majority of the experiments and prepared the figures; FA and CP prepared samples for the multiphoton experiment; CMW, AS and AV wrote the paper. CMW and AV conceived the experiments. AV provided the paper. CMW and AV conceived the experiments. AV provided

Conflict of interest

The authors declare that they have no conflict of interest.

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1363