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Bioenergetics of immune functions: fundamental and therapeutic aspects

Frank Buttgereit, Gerd-Rüdiger Burmester and Martin D. Brand

Cellular energy metabolism is an important part of the background machinery that ensures proper function of immune cells. Here, Frank Buttgereit and colleagues describe the relationship between bioenergetics and immunity and discuss current therapeutic approaches for targeting crucial processes of energy metabolism in immune cells.

Cells use considerable amounts of energy. Both the housekeeping functions and the specialized activities of immune cells depend on their energy supply. Energy in the form of ATP is needed for cation transport, macromolecule synthesis and other crucial processes involved in targeting antigens. Mechanisms that use other nucleoside triphosphates (e.g. GTP in cell signalling) ultimately use ATP as well. Without adequate energy, proper immune function would fail; this explains why processes of energy metabolism are important targets for immunotherapy.

Cellular energy metabolism

The free energy used by cells comes from respiration or glycolysis. In aerobic organisms, oxidation of fuel molecules to drive oxidative phosphorylation is the major energy source[1]. ATP is the principal immediate donor of free energy. Turnover is very high: an ATP molecule is typically consumed within a minute of its formation. The reactions of ATP production and consumption can be centred around the proton-motive force (Δp) and divided into substrate oxidation (all reactions between the oxidizable substrates and the mitochondrial redox proton pumps) and the phosphorylating system (all reactions of ATP synthesis and utilization). Alternatively, the reactions can be centred around ATP and divided into ATP-producers and ATP-consumers (Fig. 1). Oxidative phosphorylation is never fully coupled; even in their natural intracellular environment mitochondria show a significant passive permeability to protons not coupled to ATP synthesis (termed 'proton leak'). Possible functions of the leak are production of heat to maintain body temperature, the endowment of increased sensitivity of metabolic reactions to effectors, reduction of harmful free radical production and regulation of carbon flux.

Energy supply and immune functions

Effect of energy deficit on immune function

Late stages of septic and haemorrhagic shock are situations of energy deficit, in which cellular energy stores and oxygen supply become inadequate. ATP-dependent cellular functions, including those of immune cells such as lymphocytes and macrophages, become increasingly restricted. Presumably, the marked immunosuppression of patients in these clinical situations is due to reduced ability to synthesize lymphokines, decreased macrophage cytotoxicity and abnormal antigen presentation[2]. Also, energy depletion in human lymphocytes greatly inhibits expression of the membrane-bound interleukin-2-receptor (IL-2R) and release of soluble IL-2R. This is not surprising, because intracellular synthesis, transport and subsequent membrane insertion or release of receptors important for immunoregulation are known to require ATP and are sensitive to disturbances in intracellular energy levels[3]. Moreover, a change in the receptor affinity for tumour necrosis factor α (TNF-α) has been suggested to occur following the blockade of mitochondrial respiration[4].

We recently investigated the behaviour of ATP-consuming processes in thymocytes under conditions of an artificial, step-wise reduction in ATP supply with myxothiazol[5]. These processes were quantified by specifically inhibiting them and measuring the immediate changes in oxygen consumption. There is a hierarchy of energy-consuming reactions in concanavalin A (ConA)-stimulated cells: macromolecule biosynthesis (protein synthesis and RNA/DNA synthesis) is most sensitive to ATP supply, followed by sodium cycling and then by calcium cycling across the plasma membrane. These results are in accordance with previous reports that described protein synthesis to be very sensitive but ion transport to be less sensitive to ATP supply[6]. We found the mitochondrial proton leak to be least sensitive to energy supply. This hierarchy shows that if ATP supply is compromised, processes not essential for the immediate needs of the cell will be given up before those that are more critical for ionic integrity. However, this hierarchy means that when cells are deprived of their energy supply, processes of crucial importance for specific immune functions are impaired at a very early stage.

Ageing, energy deficits and immune function

There is an increasing mitochondrial deficit with ageing[7]. A reduced ATP supply could be one reason for the known decrease of protein synthesis, RNA synthesis and calcium transport in old age. These relationships might also apply to immune cells and their functions. For example, there is a dramatic impairment of T-cell functions in old age including the loss of proliferative capacity of T cells on contact with antigen, defects in IL-2 production, IL-2R expression, signal transduction and cytotoxicity, and disturbances in tyrosine kinase activity and functions of G-proteins[8]. In relation to the B-cell

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compartment, elderly individuals often produce antibodies with lower affinity, that are less protective than those of younger individuals. Moreover, qualitative and/or quantitative deviations in protein synthesis apparently occur.

Effect of energy deficit on apoptosis and necrosis

It has recently become clear that there are two ways for immune cells to die, depending on whether there is sufficient ATP for programmed cell death (apoptosis) or only for passive degradation of cells (necrosis)[9]. Thus the type of cell death might be determined by the intensity and duration of cell damage. ATP is needed for at least two events in apoptosis: the active initiation of the end phase, which includes nuclear condensation and DNA breakdown (probably the ATP-dependent translocation of caspase into the nucleus), and the expression of membrane phosphatidylserine required for the recognition of apoptotic cells by macrophages.

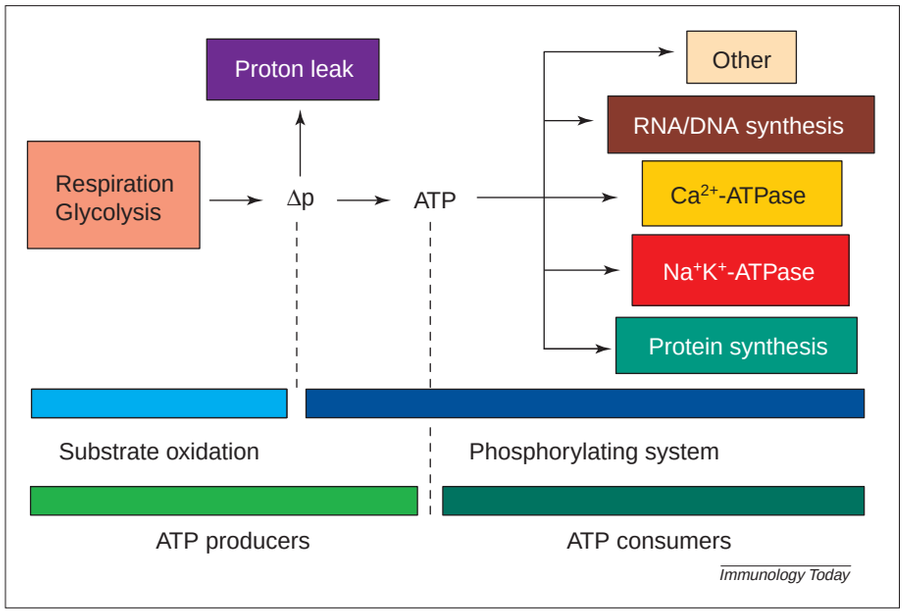

Fig. 1. *Reactions of ATP production and consumption. Respiration uses mitochondrial substrates from glycolysis and other pathways to form an electrochemical potential difference for protons across the mitochondrial inner membrane. This is known as the protonmotive force (Δp): its main component is the mitochondrial membrane potential ($\Delta\psi_m$). Δp is incompletely converted into ATP since the coupling is only between about 50 and 80%, with the remainder of the energy being lost by 'proton leak'. The term 'substrate oxidation' summarizes all reactions between the oxidizable substrates and the mitochondrial redox proton pumps, and 'phosphorylating system' includes all reactions of ATP synthesis and utilization. Alternatively, the system can be centred around ATP. The ATP-producers include glycolysis and oxidative phosphorylation; the main ATP consumers are the transport of cations and the synthesis of macromolecules.*

Housekeeping functions

Synthesis of macromolecules and ion transport are important processes for both housekeeping and specific immune functions. Housekeeping functions include maintenance of ionic integrity, volume regulation and cell growth (Fig. 2).

The pattern of energy metabolism is known in detail for lymphocytes. In quiescent rat thymocytes only 50% of the oxygen consumption has been assigned to specific processes[10]. The oxygen is mainly used by mitochondria, whereas nonmitochondrial oxygen consumption is negligible. Most of the mitochondrial oxygen consumption is used to provide ATP for protein synthesis and cation transport, but there is also a significant oxygen consumption to drive the 'proton leak'. In quiescent cells, oxygen consumption to provide ATP for RNA/DNA synthesis, ATP-dependent proteolysis and Ca^{2+}-ATPase was not measurable (Fig. 3). ConA stimulation produces a persistent 35% increase in oxygen consumption within seconds, reflecting higher ATP production (via respiration) to balance the higher ATP demand of specific activated processes. The major oxygen-consuming processes of stimulated thymocytes are mitochondrial proton leak, protein synthesis and Na^+K^+-ATPase (20% each), whereas Ca^{2+}-ATPase and RNA/DNA synthesis contribute 10% each[10] (Fig. 3). Seventy-five percent of oxygen consumption in quiescent cells and 20% in stimulated cells is used by unknown processes. A major part unaccounted for could be connected with the turnover of membrane phospholipids. For phosphatidylinositol, a 10% background hydrolysis, but a 42% hydrolysis for costimulation with antibodies to CD3 and CD28 in T cells has been reported[11].

Specific immune functions

Most of the activities of immune cells depend directly or indirectly on cellular energy supply (Fig. 2). This includes significant ATP consumption for the following specific immune functions.

Cytokinesis, migration and phagocytosis

Eukaryotic cells use ATP to transport organelles and to alter cellular morphology during cell locomotion and division. The motility of immune cells *per se* requires significant amounts of ATP for cytoskeleton rearrangement through the actomyosin system[12].

Endothelial cells also participate in the transendothelial migration of leukocytes. Leukocyte adhesion induces an increase in cytosolic calcium concentration in endothelial cells, which may signal ATP-dependent phosphorylation of myosin light chains. This appears to be the critical event in those cytoskeletal alterations that commonly accompany diminished endothelial barrier function[13]. Also involved in the regulation of adhesion and migration are small GTPases: for example, RhoA, which controls the dynamic interaction between adhesion receptors and connecting peripheral proteins of the actin-based cytoskeleton and Rac1, which induces cytoskeletal changes such as lamellipodia formation and membrane ruffling[13]. The ATP requirement for cytoskeletal processes in endothelial cells is considerable. More than 70% of total ATP-consuming processes of endothelial cells can be attributed to specific cellular processes, with actomyosin-ATPase (18%) and protein synthesis (23%) comprising the largest fraction[14].

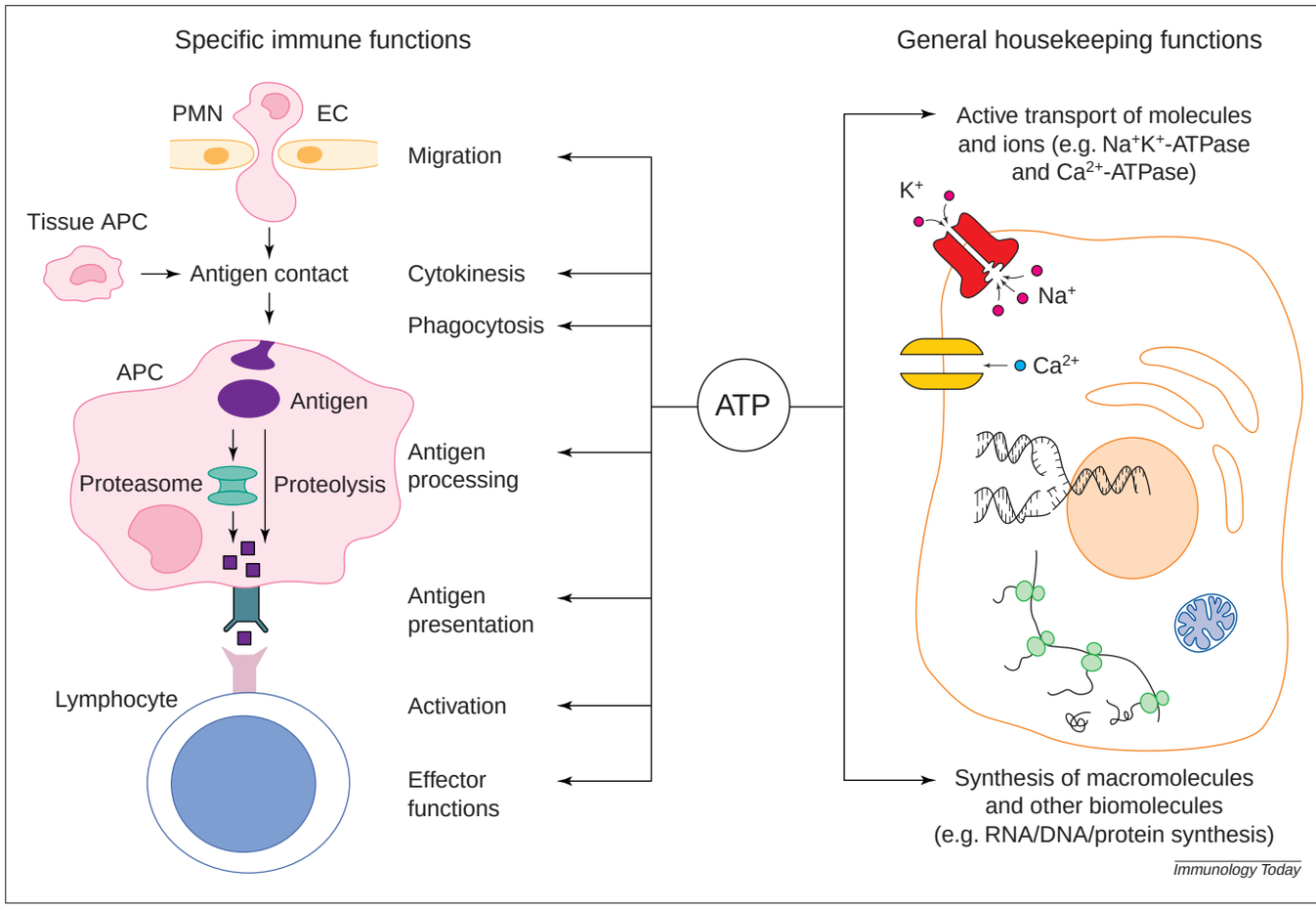

Fig. 2. *Important energy consuming functions of immune cells. Most of the activities of immune cells depend directly or indirectly on cellular energy supply. This includes ATP consumption for specific immune functions such as motor functions (migration, cytokinesis, phagocytosis), antigen processing and presentation, activation functions (signalling, initiation, maintenance) and effector functions (synthesis of antibodies, cytotoxicity, regulatory functions). The main housekeeping functions that use significant amounts of ATP are processes of ion transport and macromolecule synthesis. Abbreviations: APC, antigen-presenting cell; EC, endothelial cell; PMN, polymorphonuclear leukocyte.*

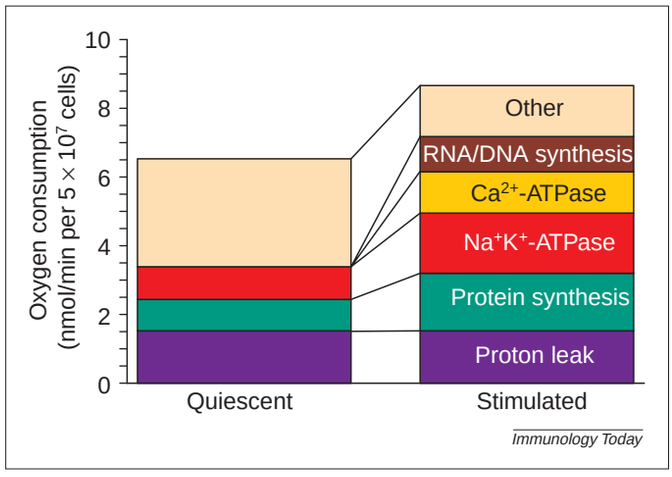

Fig. 3. *ATP-consuming processes of quiescent and ConA-stimulated thymocytes. In quiescent lymphocytes ATP is mainly used to drive protein synthesis, cation transport and other (unidentified) processes. In contrast to stimulated cells, ATP use for RNA/DNA synthesis, ATP-dependent proteolysis and Ca^{2+}-ATPase is not measurable. However, this comes into play a few seconds after mitogenic activation. The oxygen consumption to drive the mitochondrial proton leak is about the same under both conditions.*

Antigen processing and antigen presentation

There are several important steps of antigen processing and antigen presentation that are energy dependent. Proteasomes are involved in the degradation of cytoplasmic and viral proteins and produce antigenic peptides that bind to major histocompatibility complex (MHC) class I molecules. Substrate proteins are covalently linked to polyubiquitin in an ATP-requiring process, then deubiquinated and unfolded. The principal targeting signal in the degradation pathway is a homopolymeric, K48-linked polyubiquitin chain, which is recognized by a specific factor(s) in the 19S regulatory complex of the 26S proteasome, while the substrate is degraded by the 20S catalytic complex. Activity is realized only upon assembly of the 20S and 19S complexes. The assembly reaction and the degradation of ubiquitin conjugates are both ATP-dependent. The lumen of the 20S proteasome is too small to be reached by native proteins. The 19S regulator may recognize ubiquitinated substrate proteins in an ATP-dependent manner, and dissociate and unfold them before threading them into the 20S core where they are degraded[15].

Peptide products are transported from the cytoplasm into the lumen of the endoplasmic reticulum via an active ATP-dependent process mediated by the TAP (transporter associated with antigen processing) complex. TAP comprises two units, TAP1 and TAP2, both

ATP-binding cassette transporters. Peptide binding to TAP precedes ATP binding, and ATP hydrolysis is required to release and translocate peptides. In the ER, peptides interact with nascent class I molecules and β_2-microglobulin to form a stable complex that is then released for exocytosis to the cell surface where cytotoxic T lymphocytes examine it for peptides derived from foreign proteins[16].

Presentation of antigen by class II molecules occurs after antigen has been taken up by antigen-presenting cells, processed intracellularly into peptides that can bind to class II molecules, and returned to the cell surface as class II–peptide complexes. A compartment with lysosomal properties (endosomes, lysosomes) is important in the class II antigen pathway. Processing of intracellular antigens in the endocytic vesicles is regulated by changes in endosomal acidity caused by an ATP-dependent proton pump that modulates protease activity and protein unfolding[17]. A functionally intact H^+-ATPase that ensures endosomal acidification is important: cells defective in endosomal acidification have a reduced capacity to process foreign antigens, and agents that raise endosomal pH, such as ammonium chloride and chloroquine, inhibit presentation of peptides to B cells by MHC class II (Ref. 17).

Table 1. Therapeutically relevant effects of drugs on cellular energy metabolism (selection)

Drug	Used in the treatment of	Bioenergetic effect(s)	Refs
Anthralin (dithranol)	Psoriasis	Uncoupler of oxidative phosphorylation; loss of energy supply in keratinocytes	41
Chloroquine (antimalarial; disease-modifying antirheumatic drug)	Rheumatoid arthritis, SLE, malaria	Decrease in the activities of mitochondrial inner membrane enzymes; inhibition of mitochondrial respiration, thereby impairing availability and utilization of energy	42
Auranofin (oral gold compound; disease-modifying antirheumatic drug)	Rheumatoid arthritis	Decrease in glycolytic activity and depletion of intracellular ATP	43
Indomethacin (and other NSAIDs)	Rheumatic diseases	Uncoupling of oxidative phosphorylation; seems to be one cause of NSAID-induced gastrointestinal damage	44
Amiodarone (antiarrhythmic drug)	Cardiac arrhythmia	Direct toxic effect on mitochondria; alterations in ATP synthesis in human lymphocytes; seems to be one cause for life-threatening toxicities, including hepatotoxicity and pulmonary toxicity	45
Gliquidone and glibenclamide (hypoglycaemic sulphonylurea)	Diabetes mellitus	Partial uncoupling effect on mitochondrial respiration; direct inhibition of ATPase	46

Abbreviations: NSAID, nonsteroidal anti-inflammatory drug; SLE, systemic lupus erythematosus.

Activation (signalling, initiation, maintenance)

Lymphocyte activation includes energy-requiring events such as phospholipid turnover, ionic signals, alterations in cytoskeleton and gene transcription and the final increase of macromolecule synthesis. For T cells, the stimulus-induced opening or closing of ion channels is most important bioenergetically, since this leads to activation of energy-consuming ion pumps to restore ion gradients.

The Na^+K^+-ATPase uses ATP to pump sodium out of and potassium into the cell. When lymphocytes are stimulated to proliferate there is a rapid (within three minutes) two- to threefold increase in the ATPase rate of the Na^+K^+-ATPase. This increase lasts for three hours and is followed by an increase in the number of Na^+K^+-ATPase pumps in the membrane[18]. We have estimated that the oxygen (energy) demand of this enzyme in thymocytes doubles three minutes after mitogen addition[10] (Fig. 3).

Similar findings have been made for the Ca^{2+}-ATPase (Ref. 19). No significant oxygen consumption can be assigned to this enzyme in quiescent cells, but after ConA-stimulation it is responsible for about 15% of total oxygen consumption[10] (Fig. 3). This demonstrates that pumping of calcium across the plasma membrane requires significant amounts of ATP only in the activated state.

Surprisingly, a significant increase in ATP use by macromolecule synthesis appears immediately after ConA stimulation. Oxygen consumption for protein synthesis rises from 1.0 to 1.7 nmol/min/5×10^7 and oxygen consumption for RNA/DNA synthesis rises from immeasureable to 1.0 nmol/min per 5×10^7 cells within three minutes[10] (Fig. 3). It is well known that mitogenic stimulation is followed by increased macromolecule synthesis, but the reported effects appear much later than those described here. There appears to be an immediate ATP demand by precursor processes (e.g. transport of amino acids across the plasma membrane) that are directly linked to the basic steps of transcription, translation and replication.

Effector functions (synthesis of antibodies, cytotoxicity, regulatory functions)

In terms of effector functions, synthesis of antibodies and cytotoxicity are probably the most important consumers of energy. We have already discussed protein synthesis and will concentrate here on the ATP requirements for cytotoxicity.

Table 2. Dose-dependent bioenergetic effects of glucocorticoids

	Prednisolone equivalent [mol/l]	Mechanisms	Onset of action	Affected processes of energy metabolism
Module I	$>10^{-12}$	Genomic effects mediated by cytosolic receptors that alter expression of specific genes	After at least 30 min	Inhibition of leukocyte access to inflammatory sites; interference with different functions of leukocytes, endothelial cells and fibroblasts; suppression of the production and effects of humoral factors involved in the inflammatory response; induction of apoptosis
Module II	$>10^{-9}$	Additional specific nongenomic effects, assumed to be mediated by steroid-selective membrane receptors	Seconds to 1–2 min	Interference with second messenger systems [e.g. inositol(1,4,5)-trisphosphate, Ca^{2+}, protein kinase C]; effects on transmembrane current
Module III	$>10^{-4}$	Additional unspecific nongenomic effects, assumed to be mediated by physicochemical membrane actions	Within seconds	Inhibition of cation transport across the plasma membrane; no effect on protein synthesis; reduction in ATP production by partial inhibition of the reactions of substrate oxidation and by increasing proton permeability with consequent partial uncoupling of oxidative phosphorylation; induction of apoptosis (?)

Perforin- and Fas-based killing pathways are two major mechanisms of cytotoxic T lymphocyte (CTL)-mediated cytotoxicity[20]. Inhibitors of vacuolar type H^+-ATPase inhibit perforin-based cytotoxic activity, mostly due to accelerated degradation of perforin by an increase in the pH of lytic granules. Acidic pH is essential to maintain not only quantity but also quality of perforin in the lytic granules. This mechanism of ATP consumption to facilitate indirectly a specific immune process is similar to that discussed above for endosomal acidification.

Extracellular ATP (ATP_e) is important in the immune system and may influence cytotoxicity. Large amounts of ATP are released from activated platelets, endothelial cells, antigen-stimulated T cells and other cells following hypoxia, stress and tissue damage. The biological activities of ATP_e are multiple and include mitogenic stimulation and induction of cell death. ATP_e activates specific purinergic receptors[21], especially the P2Z purinoreceptor in cells of the macrophage lineage, resulting in increased membrane permeability due to pore formation, and the induction of cell death. Therefore, the P2Z receptor might mediate apoptotic elimination of macrophages infected by microorganisms. Indeed, exposure of bacille Calmette–Guérin (BCG)-infected human macrophages to ATP_e both initiates macrophage cell death and kills intracellular bacteria[22]. Therefore, a main function of ATP_e, if released by injured or dying cells during the inflammatory response, is to activate the P2Z receptor, which then sets in motion an irreversible intracellular death machinery[23]. ATP_e is also involved in cell signalling, as shown by the induced release of interleukin 1β (IL-1β) or the activation of NF-κB (Ref. 21). Furthermore, ATP_e might play a role in modulating the lytic interaction between CTLs and their target cells by mediating the phosphorylation of extracellular proteins on T cells through the action of ectoprotein kinases[24].

Therapeutic targeting of energy metabolism in immune cells

Processes of energy metabolism are important targets for immunotherapy. Examples of drugs with therapeutically relevant bioenergetic effects but not discussed here are listed in Table 1.

Glucocorticoids

Glucocorticoids are important anti-inflammatory and immunosuppressive drugs with three distinct effects: genomic, specific nongenomic and unspecific nongenomic[25] (Table 2). Genomic effects are mediated by cytosolic receptors that alter the expression of specific genes after at least 30 min (Ref. 26). Specific nongenomic effects occur within a few minutes and are mediated by steroid-selective membrane receptors. Unspecific nongenomic effects occur within seconds, but only at high glucocorticoid dosages, and seem to result from direct interactions with biological membranes. Unspecific nongenomic effects are important bioenergetically. Methylprednisolone (MP) and other glucocorticoids inhibit calcium and sodium cycling across the plasma membrane and decrease intracellular free calcium concentrations, but have little effect on protein synthesis[27–29]. The inhibition of cation cycling is direct and not caused by ATP depletion, even though MP reduces ATP availability by inhibiting the reactions of substrate oxidation and by increasing mitochondrial proton leak[27,28]. These glucocorticoid effects occur *in vitro* at concentrations (around 10^{-4} mol l^{-1}) that might be experienced temporarily by immune cells during bolus infusion therapy, intra-articular or topical administration. Thus, MP could diminish or prevent the acute immune response by interfering with processes such as the rise in intracellular Ca^{2+} concentration, which are

essential for lymphocyte activation. These observations provide one possible explanation for the clinical experience that generally only high doses of glucocorticoids are successful in acute exacerbations of immunologically mediated diseases. The immediate effects produced by high doses may be additive to the effects mediated by nuclear receptors[25].

The sensitivity of apoptosis in activated human peripheral blood T cells to high doses of glucocorticoids might contribute to their immunosuppressive efficacy[30]. Migita *et al.* (1997) investigated high-dose (1 g) MP infusion *in vivo* on induction of apoptosis in peripheral blood T cells in patients with severe autoimmune diseases[31]. Induction was more significant in $CD4^+$ than in $CD8^+$ T cells and was an important contributor to the immunosuppression observed after high-dose therapy. It is unclear whether these effects are mediated nongenomically, or if apoptosis results from changed gene expression. Mechanisms discussed are the repression of genes necessary for cell survival by attenuating AP-1 (FOS/JUN) transcription factor activity or the expression of genes involved in carrying out the death programme. Two genes encoding an ATP-gated cation channel, termed the purinergic receptor or $P2X_1$ receptor, and an inositol(1,4,5)-trisphosphate receptor may be involved in glucocorticoid-induced apoptosis via calcium-mediated mechanisms[32]. $P2X_1$ receptors were upregulated in thymocytes during glucocorticoid-induced apoptosis. Furthermore, ATP_e enhanced glucocorticoid-induced apoptosis and antagonists of ATP substantially reduced it, suggesting that glucocorticoid-induced apoptosis is dependent on $P2X_1$ receptor activation by extracellular ATP_e (Ref. 33).

Another major issue is the relationship between glucocorticoid-induced apoptosis and mitochondrial function. Glucocorticoids and other apoptosis inducers decrease $\Delta\psi_m$ (mitochondrial membrane potential) and consequently reduce ATP availability. This occurs very early in apoptosis, before DNA fragmentation, and is associated with alteration in mitochondrial structure and function[34]. Complete $\Delta\psi_m$ disruption appears to be a specific marker of apoptosis because it is found only in cells that are condemned to death. The mechanisms through which glucocorticoids collapse $\Delta\psi_m$ are not clear, but might involve the mitochondrial permeability transition. Apoptosis induction can be inhibited by blocking mRNA or protein synthesis[34], implicating genomic glucocorticoid mechanisms. However, nongenomic glucocorticoid effects on ATP production in mitochondria at high doses may also be involved: high dose methylprednisolone significantly lowered thymocyte $\Delta\psi_m$ from 144 to 124 mV within 25 min[27]. This is probably too fast to be mediated by genomic effects.

Lazaroids

21-Aminosteroids (lazaroids), acting as free radical scavengers and as membrane stabilizers, are beneficial in various pathological conditions. In the injured CNS they mimic the high-dose neuroprotective pharmacology of the glucocorticoid MP. Despite structural analogies to MP, lazaroids lack glucocorticoid activities[35] and exert their tissue-protective effects independently of glucocorticoid-receptor binding. Other studies have shown that lazaroids exert anti-inflammatory effects by attenuating the production of cytokines, suppressing the expression of adhesion molecules and inhibiting transendothelial neutrophil infiltration and activation[36]. The clinical relevance of these lazaroid effects is still unclear.

Cyclosporine A and FK506

The immunosuppressants cyclosporine A (CsA) and FK506 have been in clinical use for several years. Although structurally unrelated, they both potently suppress T-cell activation by reducing transcription of IL-2. Recent work describes inhibition of energy metabolism by these compounds. A major part of the effects has been ascribed to inhibition of glycolysis in both resting and mitogen-stimulated lymphocytes[37]. FK506 and CsA markedly depressed heat output and ConA-induced lactate production. Both compounds inhibit mitochondrial respiration in other cell types and perhaps in lymphocytes. Bearing in mind the discussion above on mitochondrial function and induction of apoptosis, a recent report that FK506 induces apoptosis in mitogen-activated human peripheral blood mononuclear cells is intriguing[38].

Channel blockers

Channel blockers represent the major therapeutic agents for stroke, epilepsy and arrhythmia, and are being considered as excellent pharmaceutical targets for modulating immune system functions. Several groups are currently trying to develop immunosuppressive drugs targeted to K^+ channels. The activity of ion channels can be modulated directly by phosphorylation or other post-translational modifications, or by altered expression. One example is the peptide antagonist of the voltage-gated K^+ channel Kv1.3, margatoxin, that was found to be significantly more potent than FK506 as an injectable immunosuppressant[39]. Another example is charybdotoxin, which blocks Ca^{2+}-activated (K_{Ca}) channels. K_{Ca} channels are considered to be particularly attractive as a target for immune suppression *in vivo*[40]. In contrast to K^+ channels, for Ca^{2+} channels no peptide toxins or sub-micromolar blockers exist, although we have used nonspecific inhibition by La^{3+} ions to quantify ATP consumption by Ca^{2+}-ATPase (see above). Further development of channel blockers as immunomodulatory agents should broaden the drug spectrum for therapeutic intervention.

Conclusions

Like all living cells, immunocompetent cells require sufficient energy to maintain cellular integrity and basal metabolism. Most of their specific immune functions directly or indirectly use ATP or other high-energy nucleoside triphosphates. This energy supply is crucial for processes such as motor functions, antigen processing and presentation, and effector functions. Therefore, these processes are important targets for immunotherapy. By highlighting the effects of drugs on the energy metabolism of immune cells we suggest a more complete way of analysing and describing therapeutic strategies to modulate immune functions. Ion channels in the immune

system may provide especially promising targets for future immunotherapy.

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letters

Cytokine profile data

The Th1/Th2 paradigm is now well established. In the mouse, a $CD4^+$ cell that makes interleukin 4 (IL-4) and not interferon γ (IFN-γ) is termed Th2 and a cell that makes IFN-γ and not IL-4 is termed Th1 (Ref. 1). This nomenclature has been extended to other cell types such as $CD8^+$ cytotoxic T cells (Tc1, Tc2)[2] and natural killer cells (NK1, NK2)[3]. These clear cut cell types, defined by their cytokine profile, define the function of the cell.

In humans this distinction between cell types is less clear cut because, depending on the stimulus, cells can make both IFN-γ and IL-4. Therefore, many researchers use a ratio of IFN-γ:IL-4 to define the phenotype of the cell. Although using a ratio would define the function of the cell, it does not take into account the quantity of cytokine produced or the strength of the signal that was used to stimulate cytokine production.

For example, stimulation of a $CD4^+$ cell might produce 1 unit of IFN-γ and 2 units of IL-4. The IFN-γ:IL-4 ratio would be 0.5 and the cell would be defined as Th2. If the strength of the signal was increased so that the cell made 2 units of IFN-γ and 4 units of IL-4, the cell would still have a ratio of 0.5 and thus be defined as Th2. If the cell was stimulated with an antigen that drives a Th1-type response and made 4 units of IFN-γ and 2 units of IL-4, the ratio would be 2 and the cell would be defined as Th1. In this latter case, the Th1 cells (defined by ratio) make a lot of proinflammatory cytokine but produce the same level of IL-4 as the Th2 cell in the first example .

In vivo cytokines have an effect locally (unless they become neutralized), therefore the quantity of cytokine made is important. I propose that a more physiologically relevant ratio to use when stimulating cells *in vitro* would be the cell phenotype defining cytokine (CPDC), in this example IFN-γ or IL-4, divided by a cytokine that is made by every T cell.

IL-3 is an example of a cytokine that could be used. Using a ratio such as this to look at skewing of T-cell cytokine profiles would first, not introduce artefacts due to signal strength (by normalizing against a control cytokine) and second, provide information on the role of the CPDC. This type of analysis is similar to using actin as an internal control when looking at mRNA made by cells. Molecular biologists take actin mRNA (that is always made by live cells) and standardize their analysis on this. Perhaps cytokine biologists should use similar internal controls?

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Reply to Cohen

The problem identified by Cohen is real – the definition of Th1 and Th2 responses is difficult and various definitions are used by different researchers. Cohen points out that both the ratio and amount of cytokines are important, and suggests standardizing against a cytokine expressed at constant levels by all T cells.

Unfortunately, interleukin 3 (IL-3) is not a good marker for general T-cell activation, as the level of IL-3 synthesized by various T cells can vary widely, and higher levels of IL-3 can be made in Th2 responses than in Th1 responses. In fact, I do not know of any cytokine that is produced at the same level by all T cells and until one is identified, there is no universal normalizing cytokine.

The problem is actually very similar for the normalization of mRNA levels. Although Cohen correctly points out that actin is often used as a standard, this is problematic because actin is not expressed at constant levels – for example, in T cells, actin expression is increased very strongly by concanavalin A (ConA) activation. Other 'housekeeping' genes have also been used, but the problem is similar to the difficulty with cytokines – there may not be a gene that is expressed at constant levels in all cells.

A related problem is that the cytokine patterns of an overall response do not always fit the basic cytokine patterns defined by Th2 or Th1 clones. Interferon γ (IFN-γ) and IL-4 are the most commonly used defining cytokines, as mentioned by Cohen, but this is probably due to a combination of

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