Interactions between immunity and metabolism – contributions from the metabolic profiling of parasite-rodent models

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SUMMARY

A combined interdisciplinary research strategy is even more crucial in immunology than in many other biological sciences in order to comprehend the closely linked interactions between cell proliferation, molecular signalling and gene rearrangements. Because of the multi-dimensional nature of the immune system, an abundance of different experimental approaches has developed, with a main focus on cellular and molecular mechanisms. The role of metabolism in immunity has been underexplored so far, and yet researchers have made important contributions in describing associations of immune processes and metabolic pathways, such as the central role of the L-arginine pathway in macrophage activation or the immune regulatory functions of the nucleotides. Furthermore, metabolite supplement studies, including nutritional administration and labelled substrates, have opened up new means of manipulating immune mechanisms. Metabolic profiling has introduced a reproducible platform for systemic assessment of changes at the small-molecule level within a host organism, and specific metabolic fingerprints of several parasitic infections have been characterized by ^{1}H NMR spectroscopy. The application of multivariate statistical methods to spectral data has facilitated recovery of biomarkers, such as increased acute phase protein signals, and enabled direct correlation to the relative cytokine levels, which encourages further application of metabolic profiling to explore immune regulatory systems.

Key words: Parasite-host system, metabolic profiling, immune system, acute phase protein, L-arginine, eicosanoids, nitric oxide, nucleotides.

INTRODUCTION

Immune responses to infection have classically been divided into two main functional branches based on the differentiation of the T helper (Th) cell sub populations, described by Mosmann and Coffman in 1986 for the first time (Mosmann *et al*. 1986). A Th1 milieu initiates cellular immune mechanisms, leading to direct cell and parasite killing *via* classical activation of macrophages and induction of natural killer (NK) and T cytotoxic cells (CTLs). Cellular immunity is effective in controlling viruses, intracellular bacteria and protozoan parasites such as *Trypanosoma cruzi, Leishmania* spp., *Mycobacterium* spp. and *Plasmodium* spp. (Goldsby, 2001). Although an early Th1-based immune response is effective in intracellular pathogen-defence, continuous inflammatory processes, such as the release of reactive nitrogen and oxygen species and proteases by infiltrating leukocytes, can lead to severe tissue damage (Goldsby *et al*. 2001).

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Th2 cytokines, on the other hand, lead to humoral immunity, which includes stimulation of B cells and production of antibodies for effective pathogen elimination and clearance. Helminth infections are in general successfully controlled by a clear Th2 response, disregarding helminth species or the location within the host organism, although some exceptions do exist (Loscher and Saathoff, 2008).

Two additional T cell lines, the Th17 and T regulatory cells (Tregs) have only recently been described (Belkaid *et al*. 2006; Miossec *et al*. 2009), but are crucial for appropriate understanding of inflammation and other infection-related immune mechanisms. The Th17 subset cytokines are effective against fungi and some extracellular gram-negative bacteria, and are at the same time potent mediators of tissue inflammation and organ-specific autoimmune mechanisms (Bettelli *et al*. 2007; Korn *et al*. 2007; Basso *et al*. 2009; Miossec *et al*. 2009). Th17 cells are activated by the mutual presence of the anti-inflammatory cytokine TNF-β and the pro-inflammatory IL-6. Tregs, by contrast, are regulated in an exclusive fashion, with respect to Th17 cells, solely by TNF-β, and seem to counteract the tissue damage but, at the same time, impair pathogen clearance (Belkaid *et al*. 2006; Joosten and Ottenhoff, 2008).

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The growing information on additional immune regulatory subsets confirms, once again, the complexity of the immune system and encourages application of different experimental angles in order to further expand our knowledge of the mammalian pathogen defence.

So far, immunology research has mainly focused on gene- and cell-based approaches. Relatively few studies have examined the metabolic aspect of immune regulation, although nutritional supplementation trials with a single metabolite or metabolite mixtures and also administration of labelled substrates, have emphasised the use of metabolic features for immune manipulation and optimisation of predisposition to disease. Oral supplements of n-3 polyunsaturated fatty acid, for example, have repeatedly shown an anti-inflammatory effect, and certain amino acids also seem to exert beneficial effects on pathogen defence, such as the antiviral and microbicidal effects of lysine and taurine, respectively. Other important components of the immune-metabolic interface, which have been described in detail, include L-arginine-mediated macrophage activation and the messenger function of nucleotides.

By correlating metabolic information with immune measures, we hope to be able to identify more such interactions, as we have shown in a recent study using ^{1}H nuclear magnetic resonance (NMR) spectroscopy-based metabolic profiling and a multicytokine assay (Saric *et al*. 2010), revealing positive correlation of lactate and IFN-γ in plasma upon *Plasmodium berghei* infection in mice.

PATHWAY STUDIES

Nitric oxide

Nitric oxide (NO) plays a central role in the metabolic coordination of immune networks. This is, on the one hand, due to its involvement with the L-arginine and L-kynurenine pathways (Thomas *et al*. 1994; Noel *et al*. 2004) that exert pathogen control and elimination. NO and NO-related reactive derivatives, on the other hand, are generated and used by different immune cells, such as macrophages, granulocytes and natural killer cells for direct killing, one of the most important active defence mechanisms of the innate immune system. Therefore, a variety of NO derivatives possessing microbicidal capacity are generated, including nitrogen dioxide (NO_2), nitrous acid (HNO_2) and peroxynitrite ($ONOO^-$), which is formed by the reaction of NO and O_2^-. DNA seems to be the main target of the reactive nitrogen intermediates whereby mutation and inhibition of repair mechanisms and protein synthesis are mainly affected (Bogdan *et al*. 2000). Interestingly, some pathogens possess detoxifying agents such as peroxiredoxins in *Mycobacterium tuberculosis* and *Salmonella typhimurium*, which degrades $ONOO^-$ to nitrite (Bogdan, 2001).

L-arginine pathway

One way to generate NO is by L-arginine degradation *via* inducible nitric oxide synthase (iNOS) within the L-arginine pathway (Fig. 1), whereby the pathway consists of three reactive sequences. The fate of L-arginine depends on its availability and the activity of the involved enzymes. Alternatively, L-arginine can be converted into agmatine by arginine decarboxylase (ADC), or metabolised to L-ornithine and urea *via* arginase. The two enzymes, ornithine amino transferase (OAT) and ornithine decarboxylase (ODC), then further convert L-ornithine to proline and polyamines such as putrescine, spermidine and spermine.

L-arginine itself derives from L-citrulline, which is the direct precursor and produced in the small intestinal enterocytes from proline, glutamate, and glutamine (Wu, 1998; de Jonge *et al*. 2002). Both metabolites, L-citrulline and L-arginine, were depleted during inflammatory processes (Bansal and Ochoa, 2003). L-arginine supplementation shows beneficial effects in wound healing after surgical intervention and other tissue-destructing processes (Barbul *et al*. 1990; Yan *et al*. 2007).

Apart from its involvement in tissue regeneration, L-arginine exerts additional immune regulatory functions. It seems to be, for instance, essential for an effective switch from pro- to pre-B cells in the bone marrow (de Jonge *et al*. 2002), and depletion of the metabolite leads to a reduced B cell presence in the lymph nodes, spleen and other secondary lymphoid organs. Also, the T cell lineage depends on L-arginine to establish appropriate growth and signalling processes, including T cell proliferation and up-regulation of the T cell receptor CD3ζ chain (Rodriguez *et al*. 2003). The central position of L-arginine in the differential activation of macrophages in response to infection is well described (Noel *et al*. 2004).

L-arginine-mediated macrophage activation

The L-arginine pathway gives rise to two sub-forms of macrophages, i.e. classically (caMΦ) or alternatively activated macrophages (aaMΦ), whereby the differential activation process influences the resulting macrophage function. Th1 cytokines, particularly IFN-γ, induce NO and L-citrulline production from L-arginine in macrophages by up-regulating iNOS, a process that increases the effective clearance of intracellular pathogens by the subsequent secretion of pro-inflammatory cytokines such as IL-1 and IL-6 and the direct antimicrobial capacity of NO.

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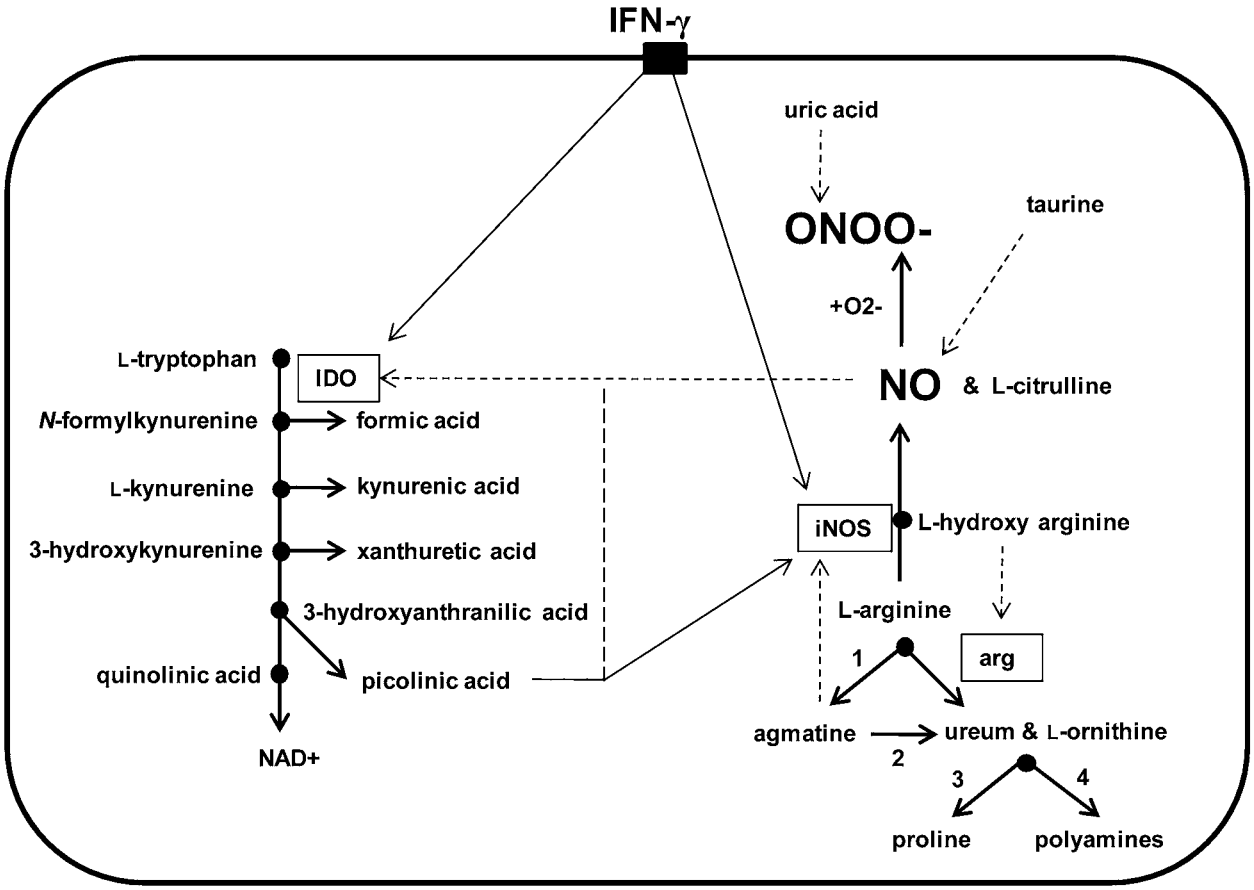

Fig. 1. Cross-regulation between L-arginine and L-kynurenine pathways within a macrophage. L-arginine is substrate for three different reactions that result in the production of either L-citrulline and NO by inducible nitric oxide synthase (iNOS), urea and L-ornithine, or agmatine, whereas L-tryptophan degrades stepwise to NAD^+. While IFN-γ stimulates expression of both key enzymes, iNOS and indoleamine 2,3-dioxygenase (IDO), picolinic acid stimulates iNOS but inhibits IDO expression. Agmatine and L-hydroxy arginine both exert negative feedback on iNOS and arginase (arg), respectively and NO is an important inhibitor of IDO. The broken arrows indicate negative regulation and the solid arrows positive regulation. Key: 1, arginine decarboxylase; 2, agmatinase; 3, ornithine amino decarboxylase; 4, ornithine decarboxylase.

Additionally the L-arginine pool is depleted from the generation of polyamines (putrescine, spermine and spermidine), *via* arginase and ODC (Fig. 1). Helminth parasites use these host-derived polyamines for growth, and *Leishmania* spp. and *T. cruzi* also seem to rely on the uptake of polyamines for intracellular processes of growth and replication in aaMΦ (Wanasen and Soong, 2008).

In contrast to caMΦ, an alternative activation pathway of macrophages is initiated by a mixture of Th2 cytokines, including IL-4 and IL-13 (Gordon, 2003). NO production is typically decreased in aaMΦ due to a down-regulation of iNOS, and the cells can perform certain immunosuppressive actions, such as the secretion of anti-inflammatory cytokines or suppressing T cell proliferation, which prevent effective parasite elimination. The anti-inflammatory functions of aaMΦ, on the other hand, can limit the tissue damage induced by the cell-mediated defence mechanisms.

L-kynurenine pathway

The L-arginine pathway is strongly interconnected with the L-kynurenine pathway (Fig. 1), in which L-tryptophan is degraded stepwise to NAD^+ (Sanni *et al*. 1998; King and Thomas, 2007). Indoleamine 2,3-dioxygenase (IDO), the key enzyme, initiates the conversion of L-tryptophan to a set of neuroactive intermediates, including kynurenic acid, quinolinic acid, and 3-hydroxyanthranilic acid. IFN-γ is, among other pro-inflammatory mediators, the most important inducer of IDO synthesis but, at the same time, it stimulates the L-arginine pathway and NO generation. NO, the key component of the L-arginine pathway is, in turn, able to inactivate directly IDO by binding to the haeme complex of the enzyme (Thomas *et al*. 2007); hence it acts as a direct inhibitor of the L-tryptophan degradation process. This cross-pathway regulation has been confirmed by higher activity of IDO in response to NOS inhibitors, but

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IDO-expression is suppressed in murine macrophages after NO-supplementation (Thomas *et al*. 1994; Alberati-Giani *et al*. 1997; Thomas and Stocker, 1999). Picolinic acid, an intermediate of the L-kynurenine pathway, substantially augments the IFN-γ mediated activation of iNOS (Melillo *et al*. 1994), but suppresses IDO activity (Alberati-Giani *et al*. 1997).

Activating the L-kynurenine pathway and subsequent IDO-mediated L-tryptophan depletion can, on one hand, cause growth inhibition in certain microorganisms that depend on exogenous tryptophan, such as *Toxoplasma gondii, Chlamydia pneumonia* and certain bacteria, including *Mycobacterium* spp. (Mellor and Munn, 2004). On the other hand, a low tryptophan level can also impair T cell proliferation and hence induce immune suppression (Munn *et al*. 1999). A possible cell cycle arrest as a response to tryptophan depletion has been proposed as the main mechanism for the inhibiting effect on T cell proliferation and the concomitant increased sensitivity to apoptosis.

The intermediates of the L-kynurenine pathway and their role within immune regulation have been described previously (Grohmann *et al*. 2003; Moffett and Namboodiri, 2003).

METABOLITE SUPPLEMENTATION STUDIES

The eicosanoic cascade

Arachidonic acid is an n-6 polyunsaturated fatty acid (n-6 PUFA) that gives rise to an entire cascade of patent pro-inflammatory intra-cellular signalling molecules also called eicosanoids (Wong *et al*. 2000) (Fig. 2A). Several different sub-structures and functions have been identified, including prostaglandin E_2, which induces vasodilatation, fever and pain and increases the production of the pro-inflammatory cytokine IL-6 (Bagga *et al*. 2003; Calder, 2008, 2009). Also, the 4-series leukotrienes exert pro-inflammatory functions including chemotactic mediation of leukocytes, induction of release of lysosomal enzymes and reactive oxygen species by granulocytes, and increased production of TNF, IL-1 and IL-6 (Calder, 2009).

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), two other important inflammation-related fatty acids, are n-3 PUFA-precursors for a panel of eicosanoic mediators (Fig. 2B), which are less potent compared to their n-6 PUFA-derived analogues. LTB_5, for instance, which is EPA-derived, is significantly less effective in inducing neutrophil chemotaxis than LTB_4, which derives from n-6 PUFA arachidonic acid. The recently discovered E- and D-series resolvins, which are EPA- and DHA-derived respectively, have several anti-inflammatory functions, including inhibition of migration and chemotaxis of leucocytes (Serhan *et al*. 2000, 2002, 2004; Seki *et al*. 2009).

Some n-6 PUFA eicosanoids can act in an anti-inflammatory manner (Levy *et al*. 2001; Vachier *et al*. 2002) and *vice versa*, PGE_2 and PGE_3 showed similarly effective inhibition of TNF-α and IL-1β (Dooper *et al*. 2002; Miles *et al*. 2002). Orally administering EPA and DHA in fish oil has shown beneficial effects in many chronic inflammatory conditions such as colitis (Calder, 2008), and the overall counter-inflammatory effect of a higher n-3/n-6 PUFA-ratio has been proven in various other nutritional studies (Eritsland *et al*. 1996; Troseid *et al*. 2009). The interaction at the mediator level is not clear yet, but human (Caughey *et al*. 1996; Belluzzi *et al*. 2000; Cleland and James, 2000; Cleland *et al*. 2006; Troseid *et al*. 2009) and animal studies (Serhan *et al*. 2000; Arita *et al*. 2005; Hudert *et al*. 2006) have revealed that the beneficial immunosuppressive effects of such DHA and EPA supplements resulted mainly as a response to the competitive incorporation into inflammatory active cells, which minimises inflammation.

Amino acids

Most of the amino acids described previously relate to innate and adaptive immunity, which both depend on the availability of a constant pool of amino acids to synthesis the micro- and macromolecules necessary for an appropriate immune defence, such as immunoglobulins, acute phase proteins, reactive nitrogen species, histamine, major histocompatibility complex (MHC) and T cell receptors (Li *et al*. 2007).

While some amino acids represent direct or indirect energy substrates for immune cells, such as glutamine for cells of the immune system in general (Wu *et al*. 1991), or alanine, which is converted into glucose in the liver and acts as an important substrate for leucocytes (Newsholme and Newsholme, 1989), others have regulatory functions within immune-related pathways. Lysine, for instance, acts as a direct antagonist to arginine, as both amino acids compete for the same transporter for cellular uptake, and excessive lysine minimises viral potency during a *Herpes simplex* infection, which might be due to restricted availability of polyamines for growth and replication of the pathogen (Griffith *et al*. 1978). Moreover, phenylalanine and taurine can exert an indirect function by modifying the L-arginine pathway. The former seems to play an important role in synthesising an essential NOS-cofactor, tetrahydrobiopterin (Shi *et al*. 2004; Li *et al*. 2007), whereas the latter inhibits the expression of iNOS in macrophages (Wu and Meininger, 2002). Additionally, taurine can act as an effective antioxidant (Fang *et al*. 2002). Its chlorinated form (i.e. taurine chloramine), has potent microbicidal

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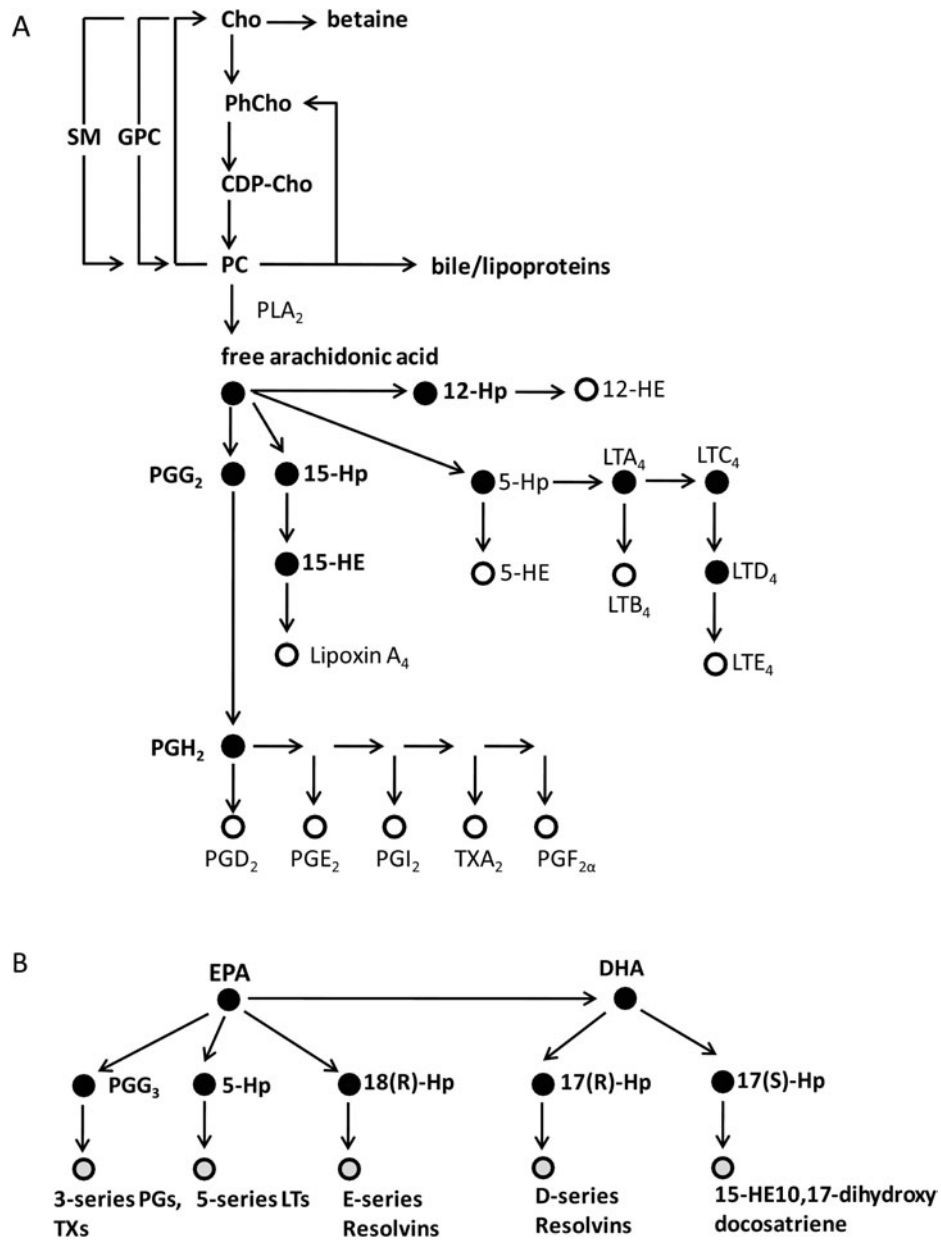

Fig. 2. (A) Link between the choline pathway and arachidonic acid cascade. The choline-containing intermediates can be metabolised to phosphorylcholine, which represents an important precursor for n-6 PUFA arachidonic acid and the subsequent production of pro-inflammatory mediators. (B) Alternative cascade of mediators derived from n-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which have certain anti-inflammatory functions and which can be competitively built into leukocytes. Adapted from Zeisel *et al*. (2003); Li and Vance (2008) and Calder (2008). Key: CDP-Cho, CDP-choline; Cho, choline; GPC, glycerophosphocholine; HE, hydroxyeicosatetraenoic acid; Hp, hydroperoxyeicosatetraenoic acid; LT, leukotriene; PC, phosphatidylcholine; PG, prostaglandin; PhCho, phosphocholine; PL, phospholipase; SM, sphingomyelin; TX, thromboxane.

(Schuller-Levis and Park, 2004) and anti-inflammatory capacity, suppressing pro-inflammatory cytokine and prostaglandin E_2 expression (Weiss *et al*. 1982; Chorazy *et al*. 2002).

The sulphur-containing proteogenic amino acids take a special position with regard to immune modulation, as they are essential for the synthesis of important immune regulatory molecules, such as glutathione (GSH) and the acute phase proteins which contain a high proportion of cysteine and methionine (Grimble and Grimble, 1998; Li *et al*. 2007). Whereas the main role of the acute phase proteins is the control of tissue inflammation, GSH acts as scavenger of reactive oxygen intermediates. Peterson and colleagues showed that it influences Th subgroup differentiation, whereby depleted GSH leads to suppression of Th1 cytokines, a subsequent shift towards a Th2 response and, moreover, to antibody production in non-responding mice (Peterson *et al*. 1998). Sulphur-containing amino acids are in higher demand during states of disease or injury, due to the production of acute phase proteins, and a

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competitive situation arises over the fate of cysteine. Studies on sulphur amino acid intake in rats by Hunter and colleagues showed that low abundance leads to relatively higher protein production, which might in turn affect the physiological control of oxidative damage by the depleted GSH levels (Hunter and Grimble, 1994, 1997).

Nucleotides

The role of nucleotides in the immune system has been assessed from different angles. For instance, nutritional supplementation showed relevant positive effects on mammalian health. Although *de novo* synthesis of nucleotides and the salvage pathway seem to produce sufficient quantities of nucleotides for basic physiological needs, certain pathological states, such as infection, surgery or growth processes, induce a higher demand which can be covered by externally provided nucleotides. Generally, nucleotide-enhanced diets show beneficial effects in various states of challenged immunity. Kulkarni and colleagues found that RNA and uracil supplementation increased T cell proliferation, and RNA in the diet has been directly linked to relatively higher levels of IL-2. Further studies showed the immunosuppressive consequences of nucleotide-free diets, resulting in increased susceptibility of cardio allografts in mice, and also indicated a role for nucleotides in T cell differentiation (Kulkarni *et al*. 1994). The main reason for impaired T cell differentiation and proliferation induced by depletion of purines and pyrimidines may be cell cycle arrest, impairing cell transit from G to S phase, a process which is mediated by IL-2 and IL-3 (Bender *et al*. 1986; Rudolph *et al*. 1990; Jyonouchi, 1994). Studies on infection-rodent models confirmed the positive influence of nucleotides on pathogen defence. When mice on a nucleotide-free diet were compared to mice fed with RNA and uracil supplements, the latter showed a markedly improved defence to *Candida albicans* (Fanslow *et al*. 1988) and *Staphylococcus aureus* (Kulkarni *et al*. 1986*a*, *b*). Similarly, Carver and colleagues assessed spleen cells from mice after they were fed on a nucleotide-free diet and a five-nucleotide formula and found a generally lower activity of macrophages and natural killer cells in the tissue of the nucleotide-deprived animals (Carver *et al*. 1990). Navarro and colleagues performed a more detailed assessment of supplemented nucleotides, whereby Balb/c mice were fed on single-nucleotide diets. Whereas AMP, GMP and UMP had a positive effect on the IgG response, IMP and CMP did not introduce any measurable changes (Navarro *et al*. 1996).

The humoral branch of the immune system seems largely unaffected by a restriction of purines and pyrimidines, with the exception of T cell-dependent antigen presentation which resulted in relatively increased production of antibodies *in vitro* (Jyonouchi, 1994).

STUDIES ON NUCLEOTIDE-SIGNALLING

Assessment of the role of nucleotides within the immune regulatory network extends far beyond nutritional studies. On one hand, in-depth studies on the interaction between parasite and vector secretome and host nucleotide composition have generated the overall conclusion that the nucleotide-degrading enzymes not only of haematophagous arthropods but also of parasitic worms induce an anti-inflammatory milieu in the mammalian host. Such a modified nucleotide homeostasis, in turn, maintains the feeding source and prevents detection by the host, by inhibiting platelet aggregation, mast cell degranulation, chemotaxis and pain signalling (Di Virgilio *et al*. 2001; Woulfe *et al*. 2001; Cook and McCleskey, 2002; Gounaris, 2002; Ribeiro and Francischetti, 2003; Gounaris and Selkirk, 2005).

On the other hand, the specific role of inosine, adenosine and some of the phosphorylated derivatives, including ADP and ATP, have been assessed with regard to tissue and cell damage. Such damage raises the levels of nucleotides in the extracellular space, where they act as messengers of pro- and anti-inflammatory processes. Adenosine, for instance, can inhibit the secretion of pro-inflammatory cytokines, such as IL-12 and TNF-α (Hasko and Cronstein, 2004), or mediate chemotaxis of neutrophils and eosinophils, mast cell degranulation and pain signalling (McCloskey *et al*. 1999; Tilley *et al*. 2000; Linden, 2001; Hasko and Cronstein, 2004; Gounaris and Selkirk, 2005). Many inflammation-related functions related to adenosine and inosine are shared, and it is difficult to obtain a clear picture of the differential role they play. However, inosine appears to contribute slightly more to host health compared to adenosine, as it seems to suppress a broader range of pro-inflammatory cytokines (Liaudet *et al*. 2002; Hasko and Cronstein, 2004; Hasko *et al*. 2004). Additionally, it has been shown to reduce nitrosative stress (Liaudet *et al*. 2002) and support axonal growth (Benowitz *et al*. 1999; Chen *et al*. 2002).

METABOLIC PROFILING

Metabolic profiling based on ^{1}H NMR spectroscopy is an efficient and reproducible method for detecting biochemical variation between different biological conditions based on the generation of low molecular weight molecular profiles. Combined with multivariate statistical modelling methods, this approach can be applied to a large variety of biomedical fields, including parasitic infections, nutrition and toxicology studies (Nicholson *et al*. 1999; Li *et al*. 2008;

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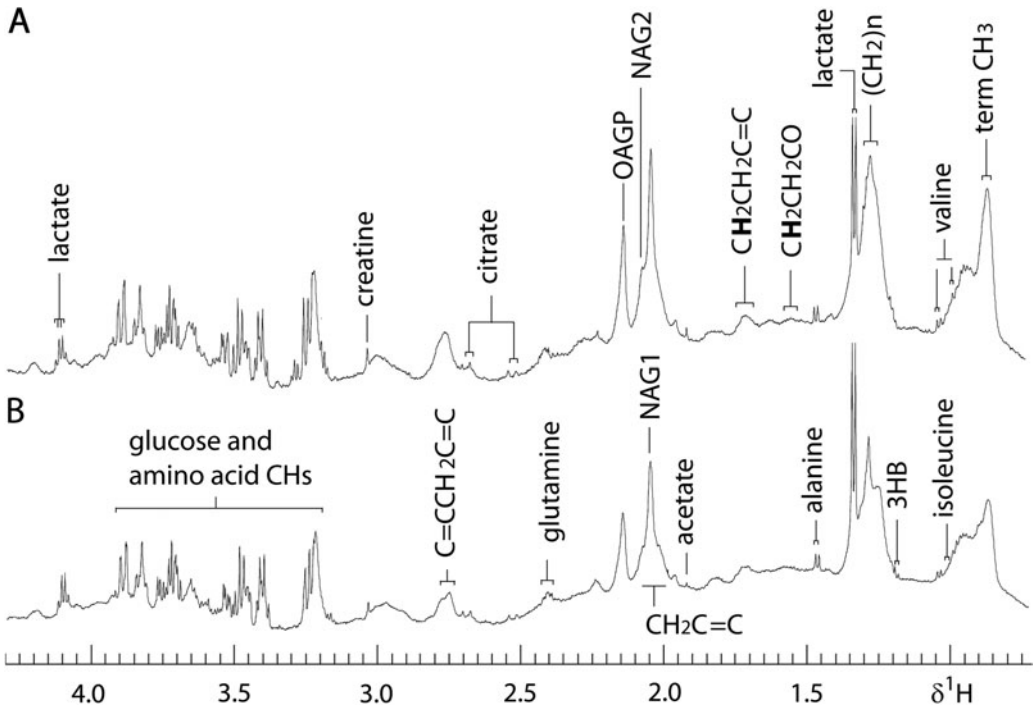

Fig. 3. Aliphatic region of a ^{1}H NMR plasma spectrum obtained from a non-infected control female Wistar rat (A) and a gender and age-matched rat, infected with *F. hepatica*. Twenty-two days post-infection, (B) showing the relative differences in the *N* and *O*-acetyl glycoprotein resonance intensities. Key: 3HB, D-3-hydroxybutyrate; NAG1, *N*-acetyl glycoprotein fragment 1; NAG2, *N*-acetyl glycoprotein fragment 2; OAG, *O*-acetyl glycoprotein fragment.

Saric *et al*. 2008*b*; Wang *et al*. 2004, 2008; Holmes *et al*. 2008). The application of multivariate statistical methods has enabled the recovery of disease-specific candidate biomarkers, whereby the screening of biofluids over disease progress and the addition of tissue metabolic information allowed to measure the temporal stability of the biomarkers and to place them into a systems context. Statistical algorithms have been adapted to integrate ^{1}H NMR-derived data across various biological compartments or to correlate it with different metabolic datasets, such as derived from ^{19}F NMR (Keun *et al*. 2008) or mass spectrometry (Crockford *et al*. 2006), in order to maximise metabolite information. Furthermore, association of the metabolic intensities with other physiological measures were identified, including gut microbial composition (Yap *et al*. 2008), or relative plasma cytokine levels (Saric *et al*. 2010).

Immunological relevance of identified infection biomarkers

A total of eight parasite-rodent models have been assessed so far *via* ^{1}H NMR-based metabolic profiling, including trematode infections, i.e. *Schistosoma mansoni*-mouse (Wang *et al*. 2004; Li, 2009), *S. japonicum*-hamster (Wang *et al*. 2006), *Echinostoma caproni*-mouse (Saric *et al*. 2008*b*, 2009), and *Fasciola hepatica*-rat (unpublished data), nematode worms *Trichinella spiralis*-mouse (Martin *et al*. 2006) and *Necator americanus*-hamster (Wang *et al*. 2009), and two protozoan models, namely *Plasmodium berghei*-mouse (Li *et al*. 2008), and *Trypanosoma brucei brucei*-mouse (Wang *et al*. 2008).

Although the metabolic signature of each infection is parasite-specific, particular groups of metabolites and macromolecules are a recurring theme across the models, including biomarkers of energy metabolism (e.g. creatine and tricarboxylic acid cycle intermediates), gut microbial co-metabolites (hippurate, *p*-cresol glucuronide, trimethylamine, etc.), osmolytes (e.g. betaine, *myo*-inositol, taurine), components of lipid metabolism (choline, phosphocholine, glycerophosphocholine and lipoproteins) and signals from *N*- and *O*-acetyl glycoprotein fragments, which are reflective of inflammatory processes.

Choline, phosphocholine (PC) and glycerophosphocholine (GPC) are frequently observed in plasma and tissues by ^{1}H NMR spectroscopy, and altered relative concentrations can be indicative of a change in lipid degradation processes. The intestinal fluke *E. caproni*, for instance, induced a depletion of GPC and choline, with a subsequent increase of lipoprotein fractions in plasma, as a consequence of an increased degradation of biological membranes (Saric *et al*. 2008*b*). On the other hand, muscular hypertrophy has been described as a consequence of a *T. spiralis*-infection where choline and phosphocholine have been found at relatively increased levels, whereas glycerophosphocholine (GPS) and the lipoprotein fractions in plasma were depleted.

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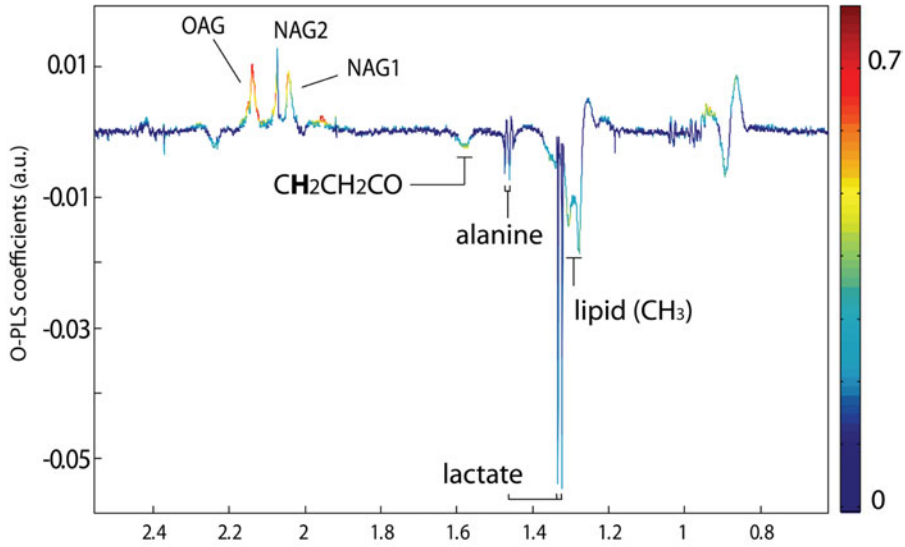

Fig. 4. O-PLS-DA analysis between *F. hepatica*-infected rats and uninfected control animals at Day 22 post-infection (n = 10), when differences between glycoprotein levels were found to be most significant. Upwards-pointing peaks illustrate positive correlation with the infection, and *vice versa*. The elevated levels in the infected animal reflect the higher production of acute phase proteins as a response to inflammation. Key: NAG1, *N*-acetyl glycoprotein fragment 1; NAG2, *N*-acetyl glycoprotein fragment 2; OAG, *O*-acetyl glycoprotein fragment.

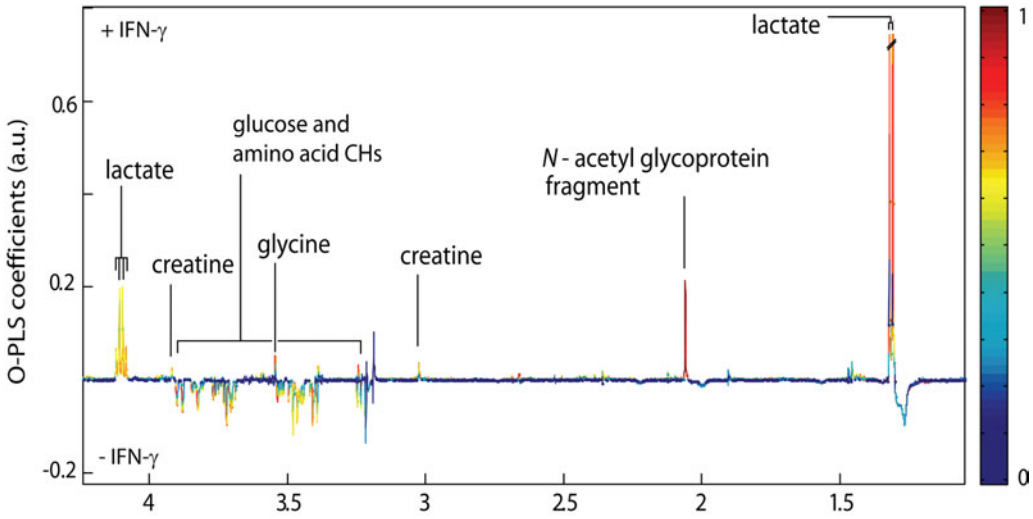

Fig. 5. O-PLS correlation plot of relative IFN-γ levels and individually matched ^{1}H NMR spectral plasma data obtained from mice pre-infection, and animals infected with *P. berghei*, at days 2 and 4 post infection (at each time-point n = 5). The colour scale indicates the significance of the correlation, i.e., red peaks illustrate highest correlation between cytokine and metabolite levels. Upwards-pointing peaks show positive correlation, whereas downwards-pointing peaks indicate anti-correlation.

Choline can be generated *de novo via* the CDP-pathway or taken up with the diet. After intestinal translocation, choline can either be oxidised to betaine in an irreversible reaction and act as a methyl-donor or osmolite in liver and kidney (Zeisel *et al*. 2003) (Fig. 2A), or it can function as a second messenger in the central nervous system *via* acetylcholine. Alternatively, choline phosphorylates to GPC or PC, the two major storage forms in the cytosol. PC then further converts to CDP-choline which forms, together with diaglycerol, phosphatidylcholine, the main phosholipid component of mammalian cell-membranes (Li and Vance, 2008). Apart from its membrane-related functions of general maintenance and signalling, phosphatidylcholine represents a major source for the generation of arachidonic acid, which is one of three immunologically relevant main fatty acids described in depth so far (Fig. 2AB).

Further disruptions amongst the choline-containing intermediates are found in the *T. b. brucei*-mouse model. This protozoan parasite breaks down and

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Table 1. Rodent metabolites commonly recovered by ^{1}H NMR spectroscopy with established connection to immune regulatory networks

Metabolite	Chemical moiety	Chemical shift in ppm and multiplicity	Reference to immune-regulatory function
adenosine	14-CH, 8-CH, 1-CH, 2-CH, 3-CH, 4-CH, half 5-CH, half 5-CH	8·32(s), 8·22(s), 6·05(d), 4·79(dd), 4·44(dd), 4·30(dt), 3·93(dd), 3·85(dd)	Hasko and Cronstein, 2004; Tilley *et al*. 2000
adenosine 5′-diphosphate	14-CH, 8-CH, 1-CH, 2-CH, 3-CH, 4-CH, half 5-CH, half 5-CH	8·52(s), 8·23(s), 6·14(d), 4·77(dd), 4·63(dd), 4·40(m), 4·24(m)	Woulfe *et al*. 2001
adenosine 5′-triphosphate	14-CH, 8-CH, 1-CH, 2-CH, 3-CH, 4-CH, half 5-CH, half 5-CH	8·51(s), 8·22(s), 6·12(d), 4·60(dd), 4·40(dt), 4·25(m)	Cook and McCleskey, 2002
alanine	α-CH, β-CH_3	3·81(q), 1·48(d)	Li *et al*. 2007
arginine	α-CH, β-CH_2, γ-CH_2, δ-CH_2	3·76(t), 1·89(m), 1·59(m), 3·17(t)	Noel *et al*. 2004; Bogdan, 2001
aspartate	α-CH, half β-CH_2, half β-CH_2	3·92(m), 2·70(m),2·81(m)	Li *et al*. 2007
choline	3xCH_3, α-CH_2, β-CH_2	3·21(s), 4·07(m), 3·52(m)	Li and Vance, 2008; Wong *et al*. 2000
formate	CH	8·45(s)	King and Thomas, 2007
glutamate	α-CH, β-CH_2, γ-CH_2	3·78(m), 2·06(m), 2·36(m)	Li *et al*. 2007
glutamine	α-CH, β-CH_2, γ-CH_2	3·78(m), 2·15(m), 2·46(m)	Li *et al*. 2007
glycerophosphocholine	3xCH_3, half α-CH_2, half α-CH_2, half β-CH_2 , half β-CH_2 , γ-CH_2	3·23(s), 4·32(t), 3·6(dd), 3·68(t), 3·89(m), 3·72(dd)	Li and Vance, 2008; Wong *et al*. 2000
glycine	CH_2	3·55(s)	Li *et al*. 2007
inosine	14-CH, 8-CH, 1-CH, 2-CH, 3-CH, 4-CH, half 5-CH, half 5-CH	8·35(s), 8·23(s), 6·10(d), 4·75(dd), 4·45(dd), 4·30(dt), 3·90(dd), 3·84(dd)	Hasko *et al*. 2004; Liaudet *et al*. 2002
isoleucine	α-CH, β-CH, half γ-CH_2, half γ-CH_2, δ-CH_3 , β-CH_3,	3·68(d), 1·93(m), 1·25(m),1·47(m), 0·99(d), 1·02(d)	Li *et al*. 2007
leucine	α-CH, β-CH_2, γ-CH, δ-CH_3, δ-CH_3	3·72(t), 1·63(m), 1·69(m), 0·91(d), 0·94(d)	Li *et al*. 2007
lipoprotein fractions	CH_3, (CH_2)n, β-CH_2CH_2CO, $CH_2C=C$, CH_2CO, $C=CCH_2C=C$, $CH=CH$	0·84(t), 1·25(m), 1·57(m), 1·97–2·00(m), 2·23(m), 2·69–2·72(m), 5·23–5·29(m)	Li and Vance, 2008; Wong *et al*. 2000
lysine	α-CH, β-CH_2, γ-CH_2, δ-CH_2, ε-CH_2	3·77(t), 1·92(m), 1·73(m), 1·47(m), 3·05(t)	Li *et al*. 2007
methionine	α-CH, β-CH_2, γ-CH_2, CH_3	3·87(m), 2·10(m), 2·65(dd), 2·15(s)	Grimble and Grimble, 1998; Li *et al*. 2007
N-acetyl glycoprotein	CH_3, CH_3	2·04(s), 2·14(s)	Grootveld *et al*. 1993
O-acetyl glycoprotein	CH_3	2·07(s)	Grootveld *et al*. 1993
phenylalanine	2,6-CH, 3,5-CH, 4-CH, half β-CH_2, half β-CH_2 , α-CH	7·44(m), 7·39(m), 7·33(m), 3·17(dd), 3·30(dd), 3·99(dd)	Li *et al*. 2007
phosphocholine	CH_3, α-CH_2, β-CH_2	3·24(s), 3·60(m), 4·18(m)	Li and Vance, 2008; Wong *et al*. 2000
proline	α-CH, half β-CH_2, half β-CH_2, γ-CH_2, δ-CH_2	4·15(dd), 2·05(m),2·38(m), 2·00(m), 3·39(m)	Li *et al*. 2007
putrescine	CH, CH_2NH	1·79(m), 3·08(m)	Noel *et al*. 2004; Bogdan, 2001
taurine	CH_2N, CH_2S	3·27(t), 3·43(t)	Li *et al*. 2007; Schuller-Levis and Park, 2004
threonine	α-CH, β-CH, γ-CH_3	3·60(d), 4·26(m), 1·33(d)	Li *et al*. 2007
tryptophan	4-CH, 7-CH, 2-CH, 5-CH, 6-CH, α-CH, half β-CH_2, half β-CH_2	7·79(d), 7·56(d), 7·34(s), 7·29(t), 7·21(t), 4·06(dd), 3·49(dd),3·31(dd)	King and Thomas, 2007; Sanni *et al*. 1998
tyrosine	2,6-CH, 3,5-CH, CH_2, α-CH	7·23(d), 6·91(d), 2·93(t), 3·25(t)	Li *et al*. 2007
uracil	5-CH, 6-CH	5·81(d), 7·59(d)	Fanslow *et al*. 1988; Kulkarni *et al*. 1994
valine	α-CH, β-CH, γ-CH_3, γ'-CH_3	3·62(d), 2·28(m), 0·98(d), 1·03(d)	Li *et al*. 2007

The chemical moieties represent the proton groups, whereby each group corresponds to a ^{1}H NMR signal at a determined chemical shift region. The direct electron environment of each proton group induces different splitting patterns or multiplicities: s, singlet; d, doublet; dd, doublet of doublets; dt, doublet of triplets; t, triplet; m, multiplet; q, quadruplet.

scavenges host lipids for integration into the parasite coat. Increased free choline and a relative decrease in phosphocholine and lipoprotein fractions in plasma have been described (Wang *et al*. 2008). In the same *T. b. brucei* model, acetylated plasma glycoproteins increased, which has subsequently been shown to be a common response to several parasite infections, including *T. spiralis* (Martin *et al*. 2006) and *F. hepatica* (unpublished data). Fig. 3A and B demonstrate the relatively lower levels of the *N* and *O*-acetyl glycoprotein fragments in the ^{1}H NMR plasma spectra obtained from an uninfected control rat (A) compared to an age- and gender-matched rat 22 days after infection with the liver fluke *F. hepatic* (B). Fig. 4 further illustrates how infection increases the relative levels of acetylated glycoproteins, whereby the colour scale indicates the strength of the correlation between the metabolites and the classes (i.e. infected and uninfected), reaching from no correlation (blue) to high correlation (red). The orthogonal projection to latent structure discriminant analysis (O-PLS-DA) applied, is the multivariate method of choice for biomarker recovery. By relating a descriptor matrix *X* (e.g. spectral information) to a response matrix *Y* (e.g., class information) class-related separation is maximised which allows improved interpretation of the class-differentiating variables (Cloarec *et al*. 2005).

Acetyl glycoproteins include acute phase proteins (e.g. α1 acid glycoprotein, haptoglobin, trasferrin, α-1 antitrypsin) whose plasma levels can change substantially with tissue damage, and which are secreted by hepatocytes to exert their mostly anti-inflammatory properties (Goldsby *et al*. 2001). The ^{1}H NMR structure of the glycoprotein components has been described and assessed in detail in human and rat plasma by Bell and colleagues (Bell *et al*. 1987*a*) and Grootveld and colleagues (Grootveld *et al*. 1993). α1 acid glycoprotein has been found to have the largest contribution to the main observed glycoprotein-related signals: three broad singlets at 2·04, 2·08, and 2·14 in the ^{1}H NMR spectra of rat plasma.

A recent co-assessment of ^{1}H NMR spectral data and the relative cytokine levels in a *Plasmodium berghei* infection in a murine host showed a direct correlation between IFN-γ and several plasma metabolites. Positive correlation was found with lactate and creatine applying analysis, whereas α and β glucose were found to be anti-correlated with relative concentrations of IFN-γ (Fig. 5).

Metabolic coverage of ^{1}H NMR spectroscopy

A large variety of physiological compartments has been profiled by ^{1}H NMR. Urine has been most extensively characterized across species (Bollard *et al*. 2005), disease conditions (Lenz *et al*. 2005; Salek *et al*. 2007; Williams *et al*. 2009), nutritional effects (Wang *et al*. 2005*b*, 2007), and geographical exposure (Holmes *et al*. 2008), information which is strongly reflected in the metabolic composition of the urine. The majority of metabolites found in urine are micromolecules and reflect energy metabolism (e.g. creatine, succinate, lactate, citrate, etc.), gastrointestinal function (e.g. hippurate, methylamines, and *p*-cresol glucuronide), amino and ketoacids, such as taurine, 2-ketoisocaproate, and detoxification products of hepatic amino acid degradation (i.e. urea) and nucleotide decomposition (i.e. uric acid).

Intense assessment of plasma *via* ^{1}H NMR spectroscopy has shown, in contrast to urine, a higher intra-individual stability due to the physiological importance of maintaining blood homeostasis. Plasma metabolic profiles contain, in contrast to urine, signals from a variety of macromolecular structures that are transported in the blood, such as glycoproteins and mobile lipid fractions. The components of lipid metabolism that provide a major contribution to the ^{1}H NMR composition of plasma include further choline, PC and GPC but amino acid and ketoacid resonances are also visible (Bell *et al*. 1987*a*, *b*; Foxall *et al*. 1993; Nicholson *et al*. 1995).

Faecal extracts have only recently been assessed (Saric *et al*. 2008*a*, *b*), and showed the highest degree of intra- and inter-individual variation. This may, at least partially, be explained by the complex composition of the gut microbiota, which contributes substantially to the faecal mass. The major components derived from gut microbial co-metabolism are bile acids and the fermentation products of complex non-digestible carbohydrates, such as cellulose and starches. The short-chain fatty acids butyrate, acetate, and propionate are typically found in the ^{1}H NMR spectra of faecal water extracts (Cummings, 1981; Guarner and Malagelada, 2003; Wong *et al*. 2006).

The metabolic profiling of tissue compartments has become a standard method to assess systemic changes. It is either performed by magic angle spinning (MAS), a non-destructive semi-solid method, or conventional ^{1}H NMR on tissue extracts, comparing liver (Bollard *et al*. 2000, 2009), spleen (Saric *et al*. 2009), kidney (Garrod *et al*. 1999), brain (Tsang *et al*. 2005), heart (Bollard *et al*. 2003) and different intestinal compartments (i.e. ileum, jejunum and colon) (Wang *et al*. 2005*a*; Marchesi *et al*. 2007). The main groups of metabolites which can be visualised by ^{1}H NMR amongst the majority of the compartments are amino acids and components of the lipid metabolism, including choline-derivatives, nucleotides, muscle degradation products (e.g. creatine, creatinine) and sugars (e.g. glucose). Brain metabolite composition is distinguished from other tissues by the presence of γ-aminobutyric acid (GABA) and *N*-acetyl aspartate (Holmes *et al*. 2006; Tsang *et al*. 2006, 2009), whereas the kidney contains the largest variety of osmolytically active molecules, including

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sorbitol, taurine, *myo*-inositol, GPC and betaine (Garrod *et al*. 1999; Waters *et al*. 2000). Table 1 shows metabolites which are commonly identified in rodent tissues and biofluids using ^{1}H NMR spectroscopy, and which have shown cross-regulatory interaction with the immune system.

CONCLUSION

Studies on the immune-metabolic interface have contributed to a better understanding of immune-regulatory events and helped to further disentangle the complex network of inter-gene, cell and metabolite signalling. Whereas metabolite-based nutrition studies have opened a novel way of manipulating immune outcome and pre-disposition to disease in the host, some pathogens have offered insight into the strategic use of metabolic features for nutritional profit or for circumventing the host-defence. However, despite these exciting findings, immune-metabolic interactions remain an under-explored domain which bears important information.

Metabolic profiling provides a tool for generating a systemic metabolic description of parasite-induced temporal changes in the mammalian host and as found here, a potential new application in re-addressing the dynamic immune-processes on parasite infection. The need for metabolic resources for building immune active components, the messenger function of certain metabolites and metabolite classes, and the intimate relationship between parasite and mammalian defence mechanism, make it likely that immune regulatory events will be reflected in the metabolism.

Initial correlation studies exploring novel links between relative cytokine levels and ^{1}H NMR plasma profiles point out the trend for future research, whereby the addition of more sensitive metabolic profiling tools, such as mass spectrometry, would be of great benefit, as certain pathways (e.g. L-kynurenine-intermediates) or metabolite classes (e.g. nucleotides and lipids) could be described in more detail.

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