Cellular and molecular aspects of iron and immune function

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Fe plays a critical role in the immune system and defence against infection. However, many aspects of the way in which Fe influences these processes at the molecular and cellular level are unclear. The present review summarizes the role of Fe in lymphocyte activation and proliferation, and discusses how Fe is handled by macrophages.

Iron: Lymphocyte activation and proliferation: Macrophages

Fe plays a critical role in many metabolic processes. While its major function is O_2 transport by haemoglobin, it is also a crucial cofactor in many other enzymes, whose function may be reduced if there is an inadequate supply of Fe. The immune system consists of a complex network of cells and molecules whose activities may be up regulated in highly specific ways following antigenic challenge, and it is therefore not surprising that abnormal Fe status can lead to impaired immune function.

The whole area of Fe and its relationship with immunity is complex, and has been extensively reviewed (Brock, 1993; Jurado, 1997; Walter *et al*. 1997). Moreover, many cells and molecules participate in immune reactions. Two of the key cells involved are lymphocytes and macrophages, and the present review will therefore be limited to their function in relation to Fe status, in particular to studies by the authors related to how Fe affects lymphocyte activation and proliferation, and how macrophages handle Fe.

Iron and lymphocyte function

It has been known for many years that the proliferative phase of lymphocyte activation is an Fe-requiring step, mainly because Fe is essential for enzymes such as ribonuleotide reductase, involved in DNA synthesis (Leberman *et al*. 1984). This finding corroborates a large number of clinical studies, reviewed previously (Brock, 1993), which have found reduced T-cell function *in vivo*, as manifested by impaired skin-test reactions, and reduced *in vitro* proliferation of T-cells from Fe-deficient individuals. Activated lymphocytes, like other Fe-requiring cells, normally acquire Fe from transferrin by a mechanism involving interaction with cell-surface transferrin receptors, internalization of the complex and release of Fe in an acidic endosomal compartment (Baker & Morgan, 1994). Uptake of Fe is regulated according to cellular needs by modulating transferrin receptor mRNA stability through the binding of Fe-regulatory proteins (IRP) to the 3′-untranslated region (Haile, 1999). However, although Fe deficiency can up regulate transferrin receptor expression, it can also reduce the Fe saturation of transferrin to a level where it can no longer provide optimal Fe delivery, and as a consequence proliferation is impaired (Mainou-Fowler & Brock, 1985). On the other hand, Fe overload can lead to complete saturation of transferrin and the presence of non-transferrin-bound Fe in plasma, which can also inhibit lymphocyte proliferation (Djeha & Brock, 1992).

Although cell proliferation is the most clearly established step in lymphocyte activation in which Fe plays a crucial role, other aspects of lymphocyte function may also be susceptible to modulation by Fe. For example, Fe deficiency in mice reduced protein kinase C (PKC) activity and translocation in T-cells (Kuvibidila *et al*. 1999). This finding accords with earlier work showing that Fe–transferrin up regulates expression of PKC-β (but not PKC-α) in various cell lines (Alcantara *et al*. 1994). Interestingly, non-transferrin-bound Fe could not induce PKC (Alcantara *et al*. 1991). Further evidence of a role for Fe in lymphocyte signalling pathways comes from a study showing that phosphatidyl inositol-4,5-biphosphate hydrolysis is reduced in activated spleen cells from Fe-deficient mice (Kuvibidila *et al*. 1997). However, the involvement of Fe–transferrin in lymphocyte signalling pathways may depend in part on ligand binding to the transferrin receptor rather than Fe delivery, as the transferrin receptor can associate with the T-cell receptor ζ chain, and tyrosine phosphorylation can be

Abbreviations: γIFN, γ-interferon; IRP, iron-regulatory proteins; LPS, lipopolysaccharides; PKC, protein kinase C; TNF-α, tumour necrosis factor α

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induced using antibodies to the transferrin receptor as well as by transferrin itself (Salmerón *et al*. 1995). Another Fe-sensitive step in which Fe–transferrin may play a role is in lymphocyte maturation. Blockage of transferrin-bound Fe uptake by thymocytes prevented their maturation into αβ-T-cells, but not into γδ-T-cells (Brekelmans *et al*. 1994).

In addition, while it seems likely that non-transferrin-bound Fe in the form of citrate or other chelates cannot support lymphocyte activation, there may be other forms, apart from Fe–transferrin, that can do so. Lymphocytes may be able to use intracellularly stored Fe (Golding & Young, 1995). In addition, haem-haemopexin could substitute for Fe–transferrin in permitting proliferation of lymphoblastoid T-cells, with PKC activation again being implicated as a possible mechanism (Smith *et al*. 1997). It is suggested that this mechanism of Fe uptake might be important at sites of injury or inflammation.

Iron and macrophages

Macrophages play a key role in Fe metabolism. They are responsible for catabolism of effete erythrocytes taken up by the liver and spleen, and the release of Fe to the circulation for subsequent binding by transferrin. The Fe is then transported to the erythroid marrow, where it is reincorporated into haemoglobin in erythroid precursors. Despite this key role in Fe metabolism, the way in which macrophages handle Fe remains the least-understood step in Fe recirculation. Furthermore, the macrophage may hold the key to understanding some disorders of Fe metabolism, such as the anaemia of chronic disease.

Although most cells acquire Fe for metabolic use from transferrin via the transferrin-receptor endocytotic route, macrophages involved in erythrocyte catabolism take up large amounts of Fe via phagocytosis of effete erythrocytes. This process, while still incompletely understood, is clearly not regulated by cellular Fe status in the same way as uptake from transferrin. Moreover, the intracellular routing of Fe is likely to be different; Fe acquired from transferrin is released from the carrier molecule (i.e. transferrin) in the early endosome, from which it is transported, probably via Nramp2/DCT1, to the cytoplasm and thence to the mitochondria for metabolic use (Fleming & Andrews, 1998). In contrast effete erythrocytes are broken down in a phagolysosome, where Fe must first be released from haemoglobin by haem oxygenase (Kleber *et al*. 1978). Subsequent steps are unclear, although we have previously shown that Fe acquired from transferrin and Fe taken up into phagosomes appear to follow different pathways within the cell (Oria *et al*. 1988).

In our laboratory we have investigated the effect of various mediators on Fe handling by macrophages, in order to gain some insight into how macrophages handle Fe and how this process is modified during inflammatory disease.

For this purpose, we devised a method of loading the cells with radiolabelled transferrin–antitransferrin immune complexes (Esparza & Brock, 1981). This method has the advantage of delivering Fe to the macrophage by a phagocytic route, while at the same time allowing high specific activity of radiolabelling (impossible with labelled erythrocytes) and bypassing the need for haem oxygenase to liberate Fe from its carrier. Using this model we found that inflammatory macrophages released Fe more slowly than resting macrophages, thus supporting early *in vivo* work which pointed to a 'reticulo-endothelial blockade' of Fe recirculation in inflammation (Freireich *et al*. 1957; Konijn & Hershko, 1977).

We also investigated whether a similar effect could be induced by cytokines implicated in the inflammatory response. We found that tumour necrosis factor α (TNF-α), but not interleukin 1α or lipopolysaccharide (LPS), when added to mouse peritoneal macrophages that had ingested Fe–transferrin–antitransferrin immune complexes, reduced Fe release (Alvarez-Hernández *et al*. 1989). Injection of TNF-α into mice reduced serum Fe, and peritoneal macrophages from these animals incubated with Fe–transferrin–antitransferrin immune complexes showed increased uptake and degradation of the complexes without a corresponding increase in Fe release, which would have the overall effect of increasing Fe retention. Interleukin 1α had no significant effect on serum Fe, and while it also increased the rate of immune complex degradation by macrophages *in vitro*, this process was not accompanied by a significantly greater uptake of complexes.

These studies show that impairment of macrophage Fe release by inflammatory stimuli can be directly demonstrated *in vitro*, and that the pro-inflammatory cytokine TNF-α appears to play a role in this process, and may do so by a direct effect on macrophage Fe metabolism. This finding is in agreement with other studies indicating an up regulation of ferritin by TNF-α (Miller *et al*. 1991), which would cause the cell to store more Fe and release less, as originally proposed by Konijn & Hershko (1977).

It is now known that the activity of IRP, mentioned earlier, is crucial to maintenance of Fe homeostasis in most cells (Haile, 1999), due to its ability to simultaneously and inversely regulate Fe uptake via the transferrin receptor and Fe storage by ferritin according to cellular Fe levels. However, macrophages do not conform completely to the IRP paradigm (Testa *et al*. 1991), and it is possible that other factors, notably γ-interferon (γIFN; Byrd & Horwitz, 1990), can affect macrophage Fe homeostasis. Since it is known that NO can activate IRP1 independently of Fe status (Weiss *et al*. 1993; Drapier *et al*. 1993), it seemed likely that this mediator, whose synthesis is greatly increased when macrophages are stimulated with γIFN, might play a key role, and that modulation of transferrin-bound Fe uptake by macrophages might occur during inflammation.

It has been proposed that γIFN, produced during T-cell or natural killer cell activation, up regulates NO production, thus activating IRP1, which would in turn lead to increased transferrin receptor expression, and thus to an abnormally increased Fe uptake by macrophages (Weiss *et al*. 1995). While this theory is plausible, and the IRP-activating ability of NO is clearly established, there was little or no data about whether NO actually affected Fe uptake (as opposed to IRP activation). We have, therefore, investigated the role of NO and γIFN on macrophage Fe uptake and release *in vivo* (Mulero & Brock, 1999).

Using the murine macrophage J774 cell line, it was found that stimulation with γIFN and LPS caused, as expected, a marked increase in NO production, but surprisingly caused a

decrease rather than an increase in Fe uptake from transferrin, despite the previously-reported ability of NO to inactivate IRP1. It seemed possible that this decrease might be due to increased release of Fe, an activity previously noted when macrophages are activated to produce NO (Drapier *et al*. 1991). However, NO did not cause an increase in Fe release from the macrophages, so the reduction in uptake was presumably a direct effect of NO and/or γIFN and LPS on Fe uptake. This finding contrasted with the ability of NO to activate IRP1 and the fact that we had previously shown that exogenous NO up regulated transferrin receptor mRNA in erythroleukaemia cells (Oria *et al*. 1995).

Fluorescence-activated cell sorter analysis revealed that γIFN alone caused a marked decrease in transferrin receptor expression, even in the absence of LPS or NO production, indicating that the decrease in Fe uptake seen in the presence of γIFN and LPS appears to be primarily due to an NO-independent down regulation of transferrin receptor expression by γIFN. The apparent contradiction between this finding and the previously reported up regulation of IRP1 activity by NO was resolved when it was found that although IRP1 was indeed up regulated, activity of IRP2 was decreased by γIFN (Bouton *et al*. 1998), and that transferrin receptor expression is primarily determined by IRP2 rather than IRP1 activity (Kim & Ponka, 2000).

Overall, it appears that γIFN and LPS stimulation of mouse J774 macrophages decreases Fe uptake from transferrin. The effect of γIFN and LPS may, therefore, be to ameliorate rather than promote the hypoferraemia induced by pro-inflammatory cytokines such as TNF-α.

The work described earlier on the effect of NO on macrophage Fe uptake and release was focused towards Fe uptake from transferrin. However, as mentioned earlier, the major source of Fe uptake by macrophages is probably phagocytosis of effete erythrocytes rather than transferrin, and Fe acquired by a phagocytic route may be handled differently from transferrin-bound Fe. It was important, therefore, to know whether NO also affected macrophage handling of Fe acquired by a phagocytic route. We (JH Brock and V Mulero, unpublished results) have, therefore, recently studied the ability of macrophages from iNOS-knockout mice (Wei *et al*. 1995) to take up and release Fe delivered as transferrin–antitransferrin immune complexes. NO had no effect on the uptake of immune complexes by bone-marrow-derived macrophages, but, unlike the situation with Fe taken up from transferrin, release of Fe was clearly enhanced. The mechanism of enhancement remains to be established; it may involve accelerated release of Fe from the carrier within the phagosome, or enhanced Fe release from the cell itself. However, the overall effect is the same as with Fe taken up from transferrin, i.e. NO appears to ameliorate rather than potentiate Fe sequestration in macrophages, and is thus unlikely to promote the hypoferraemia of inflammation.

Clearly, the effect of cytokines and NO on macrophage Fe handling is complex. While pro-inflammatory cytokines such as TNF-α and interleukins 1 and 6 may provoke hypoferraemia, perhaps via up regulation of ferritin, γIFN and NO appear to work in the opposite direction. It might be speculated that Fe sequestration is of benefit during the early acute stages of infectious disease in order to reduce Fe availability to micro-organisms, but that the subsequent development of an adaptive immune response requires Fe to be 'unlocked' in order to allow proper immune function; for example, to ensure optimal lymphocyte activation and proliferation.

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