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REVIEW

Multiple sclerosis and the role of immune cells

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Abstract

Multiple sclerosis (MS) is a complex disease with many different immune cells involved in its pathogenesis, and in particular T cells as the most recognized cell type. Recently, the innate immune system has also been researched for its effect on the disease. Hence, cells of the immune system play vital roles in either ameliorating or exacerbating the disease. The genetic and environmental factors, as well as the etiology and pathogenesis are of utmost importance for the development of MS. An insight into the roles play by T cells, B cells, natural killer cells, and dendritic cells in MS and the animal model experimental autoimmune encephalomyelitis, will be presented. Understanding the mechanisms of action for current therapeutic modalities should help developing new therapeutic tools to treat this disease and other autoimmune diseases.

Key words: Multiple sclerosis; Experimental autoimmune encephalomyelitis; Chemokines; Chemokine receptors; Glatiramer acetate; Central nervous system; T cells; B cells; Natural killer cells; Dendritic cells

Core tip: The role played by various immune cells in either ameliorating or exacerbating multiple sclerosis is discussed.

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INTRODUCTION

Recent developments in multiple sclerosis (MS) research have been staggering, including numerous new therapies either approved or at the brink of approval, new oral therapies and leaps made in genetics. This article will focus on the role that immune cells play in MS, and their involvement in mediating the effects of drugs used to treat relapsing-remitting (RR) MS patients.

GENERAL FEATURES OF MULTIPLE SCLEROSIS

The basic pathology of MS was recognized almost 150 years ago by Charcot who described a disease caused by sclerotic plaques in the central nervous system (CNS) of those affected[1]. There were other reports describing the pathology before this time, but failed to recognize it as a distinct disease[2]. It is reasonable to believe that MS has been prevalent also before these descriptions, as the disease often arises early in life[3]. Today, we know that MS is a chronic inflammatory, autoimmune, demyelinating and degenerative disease of the CNS[4]. Substantial discoveries have been made regarding its therapeutics, pathogenesis and genetics. Yet, there is no cure and no recognized definite cause, be it genetic, microbial or environmental. Although no definite cause of MS has yet been identified, substantial amounts of research point to a dual multifactorial influence of both genetics and environmental elements contributing to the development of the disease[5,6].

Genetic factors

The genetic component was first described after family studies were performed, with a family recurrence rate of about 20%-33%, and about 10-12 fold risk increase in first degree and three folds in second degree relatives[7,8]. For siblings or children of patients with MS, the overall risk was estimated to be 3%-5%[9], but twin studies show a higher risk (25%) if the sibling is a monozygotic twin[10,11].

The first risk allele to be identified was the human leukocyte antigen (HLA) class Ⅱ haplotype HLA-DRB 1501 in the early 1970's[12], which is also the allele with the strongest association with the disease[13]. The road to discovering additional alleles was complicated and hampered by insufficient methods[4,14]. In recent years large genome wide association studies (GWAS) using single nucleotide polymorphisms and comparing diseased to healthy individuals have made it possible to identify numerous alleles associated with MS with sufficient power and significance, most of these with low to modest MS risk association[5,15]. Researchers with the help from a huge GWAS consortium[13] identified over 50 susceptible loci, many of which are associated with the immune system, including genes encoding receptors for interleukin (IL)-2 and IL-7[15]. Other genes important for cytokine pathways include CXCR5, IL-12A, IL-12β, IL-12Rβ1 among others associated with the cytokine tumor necrosis factor α, as well as genes associated with co-stimulatory molecules such as CD80, CD86, CD37 were also described. GWAS studies have even identified risk association with genes that are important for current and new MS therapies including IL-2Rα (daclizumab) and VCAM1 (natalizumab), as well as genes related to environmental risk factor vitamin D_3[13]. Such knowledge while adding new information supports much of what is known about MS pathogenesis. Still, further research into these risk alleles is needed to improve our understanding of their associations with the disease.

Environmental factors

While family studies support a genetic association, they also show that genetics alone are not enough to develop MS, as shown by homozygous twins not both acquiring disease, even though one of them is diagnosed with MS. Other information supporting environmental effects include the geographic increase of MS with increasing latitude[16], and how individuals migrating from low-risk to high-risk areas acquire higher susceptibility if the migration occurs during childhood. Hence, the incidence of developing MS correlates with the risk in areas of childhood residence[4]. Additionally, studies have shown risk association during months of birth as spring births have higher risk of MS than autumn births[17].

Several environmental factors have been investigated. One hypothesis is that vitamin D_3 deficiency increases the risk of MS, as increased latitude is also correlated with lower blood vitamin D_3 levels. For instance, ecological studies showed the amount of exposure to sunlight was inversely correlated with the risk of MS, both by regional distribution and association with altitude, as well as by individual exposure to sunlight[18]. Sunlight is the main source of human vitamin D_3 through conversion of 7-dehydrocholesterol to previtamin D_3 in the skin, and through further metabolic steps to active hormone 1,25-Dihydroxyvitamin D_3[19]. It was also shown how vitamin D_3 intake may reduce the risk of MS because of latitude dependent deficiency, for instance in communities which consume higher amounts of vitamin D_3 rich fish[20]. These studies also point out the difficulties of such concepts, as confounding factors may be quite prevalent. There is also association with the experimental autoimmune encephalomyelitis (EAE) model, an animal model for MS, as dosing of 1,25(OH)2D_3 prevented the disease[21,22]. These effects may be induced by vitamin D_3 on the adaptive immune system not yet fully understood. Definite effects of supplementing patients with vitamin D_3 have not yet been shown, but some studies indicate that serum concentrations of vitamin D_3 may affect disease severity[19]. However, this field is quickly developing, and is being investigated for possible future prospects, for instance in preventing MS[23].

Another risk factor with strong association to MS is Epstein Barr virus (EBV) infection[24,25]. A major finding was that individuals who were seronegative for EBV had very low, almost no risk of acquiring MS when compared to seropositive individuals[24]. However, those patients who at some point infected with EBV are not necessarily at risk of developing MS[6]. Further, there was an increased risk of MS if a history of infectious mononucleosis and a temporal increase of EBV antibodies serum titers were present[26], and that MS patients were more often infected with EBV at later ages when compared to controls[27]. It has been hypothesized that EBV may mimic myelin basic protein (MBP) pathogenic antigens by presentation on HLA-DRB1*1501, hence, providing links to both environmental and genetic risk factors[28]. Although the association with MS is well investigated, the role of EBV in its pathology remains uncertain. However, EBV infection and mononucleosis as a priming or initiating factors for developing MS are seemingly likely[24]. Finally, many other factors have shown association with increased MS risk but are in need of further research to be conclusive. These include cigarette smoking, a diet rich in saturated but low in polyunsaturated fats, sex hormones, and socioeconomic status, among others[18,29]. Viruses other than EBV have also been implicated in the etiology of MS[30].

CLINICAL OBSERVATIONS

Natural history

Patients with MS show a wide variety of symptoms caused by lesions in the CNS affecting motor, sensory, visual or even autonomic functions. Lesions appear mainly in the white matter, but also appear in the grey matter. Hence, symptoms show great heterogeneity in both inter- and intra-individually ranging from slight tingling in the fingers to extreme fatigue or complete monocular loss of sight, depending on the lesion(s) localization[4]. Com-

monly, the patients present with a clinically isolated syndrome (CIS), followed by serial sub-acute relapses with varying time intervals. The number of annual relapses and time intervals between them varies among patients, but more than 1.5 relapses/year is a rare occasion[4]. Not all patients have subsequent disease activity after a single CIS, but risk of another episode is increased if white matter lesions are detected by MRI[31]. Between relapses the patients revert spontaneously to normal or near normal neurological function. This clinical subtype of MS is referred to as RR MS, and most patients are present with this form of MS[30]. The clinical course may later convert to a more progressive stage in about 65% of the patients, with fewer or no relapse activity and increasing irreversible disability. Patients have a mean age of about 40 years when they enter this stage, referred to as secondary progressive (SP) MS[32,33]. A minority of patients (about 10%-15%) may present with progressive disability and no relapsing activity, this is called primary progressive (PP) MS[34]. This subtype usually has a later onset than RRMS, at a mean age of around 40 years[33].

Patients with MS experience decades of disease and eventual progression. Due to many complications which occur alongside increased disability, the average life expectancy is reduced by about 5-10 years, and has a median time of 30 years between onset and death[35]. It remains to be seen whether current or future therapeutic methods may extend the time until irreversible disability occurs.

ROLE OF IMMUNE CELLS IN MS

The blood brain barrier

An important finding in MS lesion is the disruption of the blood brain barrier (BBB), and hence a basic understanding of this barrier is essential to understand the pathogenesis of the disease[36]. The BBB is a functional and anatomical barrier separating the blood from neurons in the CNS. It consists of both the vascular wall, CNS astrocytes covering these with glia limitans, and the perivascular space in between[37]. The BBB has several important functions vital for the brain to function properly, including maintenance of proper ionic concentrations through dynamic regulation. The BBB may be viewed as a concept rather than an actual barrier. Even though the word barrier has a certain static ring to it, the BBB is rather dynamic allowing for instance immunological surveillance[38,39]. Lesions occur when leukocytes migrate into the brain and inflammation ensues. This is a two-step process, involving an initial migration across post-capillary venules into perivascular space, and further migration through the glia limitans into the brain parenchyma[37]. The perivascular space allows normal immunosurveillance by monocytes, as this space also serves as the brains' lymphatic drainage[37]. There are at least two additional routes for leukocytes to enter the CNS, as shown by accumulation of immune cells at these sites in animal models, which include the blood-cerebrospinal fluid (CSF) barrier in the choroid plexus, and through meningeal arteries[39].

Role of T cells

The role of T cells has always been considered central in MS pathogenesis, much due to the experimental animal model EAE, but also due to the strong genetic association with HLA class Ⅱ genes[5]. EAE is a widely used animal model for MS, and is induced by immunizing mice or other rodents with myelin peptides, or by adoptively transferring myelin-reactive T-cells[6]. Hence, they cause a T cell mediated acute autoimmune reaction against myelin in the rodents CNS, with signs and symptoms that are similar to those seen in MS. The cellular pathogenesis of MS is however, more complicated than this[40]. For a long time auto-reactive $CD4^+$ T cells secreting interferon-gamma (IFN-γ), were thought to be the main mediators of the inflammation causing MS lesions[4]. Further research suggests numerous other cell types and cell subsets are also involved, with key roles assigned to T helper 17 (Th17) cells[41]. These T cells secrete the pro-inflammatory cytokines IL-17, IL-6 and are regulated by IL-23[42,43]. It is commonly believed that disease occurs when these inflammatory cells and/or other cell types become deregulated and a transition from physiological surveillance to a pathological immune response occurs[4,30].

Since EAE is considered a model of acute inflammation, it has been used to explore what cells are important for this process. It was shown in mice with EAE that $CD4^+$ Th17 cells are necessary to develop EAE[44]. Studies of lesions from MS patients confirmed an overwhelming presence of $CD4^+$ cells secreting IL-17 in active lesions. However, in MS lesions both $CD4^+$ and $CD8^+$ cells express IL-17[45]. Chemokine receptor CCR6 expressed on Th17 cells facilitates transport through the choroid plexus into the CSF and perivascular space by interacting with CCL20/MIP-3α expressed on endothelium[46]. Th17 cells may also produce GM-CSF promoted by resident antigen presenting cells (APCs) secreting IL-23, which then initiates a positive feedback loop as the same APCs are stimulated by GM-CSF[30,47,48]. Th17 cells may further increase permeability of the BBB by disrupting the endothelial tight junctions due to the secretion of IL-17 and IL-22, and through interactions with endothelium allowing further attraction of $CD4^+$ subsets as well as other immune cells. Consequently, initiating pathological cascade of inflammation, perivascular infiltrates and damage to neurons and glia cells[30,45]. One identified damaging molecule is Granzyme B secreted by the same Th17 cells[49]. However, to gain access into the parenchyma, the cells must traverse the glia limitans. This is thought to be mediated by perivascular APCs and macrophages secreting matrix metalloproteinases (MMP) 2 and MMP-9 which are gelatinases able to cleave dystroglycan, a transmembrane receptor anchoring astrocytes end feet to the parenchymal basement membrane. When mice were knocked down for MMP-2 and MMP-9, they became resistant to EAE as T-cells became trapped in perivascular space[50]. In summary, this cascade may be viewed as a stepwise model, with an initial attraction and migration of Th17 cells into the CSF and perivascular spaces, and later increased permeability of the BBB al-

Table 1 Chemokines receptor expression on T cells and T cell clones isolated from MS patient

Receptor	Main role	Ligands	$CD4^+$ subsets			PBL-74		PBL-78		CSF-25		CSF-26	
			Th1	Th2	Naive	Unact	Act	Unact	Act	Unact	Act	Unact	Act
CXC family													
CXCR1	I	CXCL6, CXCL7, CXCL8											
CXCR2	I	CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8											
CXCR3	I	CXCL9, CXCL10, CXCL11	H			H	M	H	M	H	M	H	L
CXCR4	C	CXCL12			H								
CXCR5	C	CXCL13		M									
CXCR6	I	CXCL16											
CXCR7	D	CXCL11, CXCL12											
CC family													
CCR1	I	CCL3, CCL5, CCL7, CCL8, CCL15, CCL16, CCL23	M										
CCR2	I	CCL2, CCL7, CCL8, CCL13, CCL16	H	H									
CCR3	I	CCL5 ,CCL7, CCL11, CCL13, CCL15, CCL24, CCL26, CCL28		H									
CCR4	D	CCL17, CCL22		H		H	H	H	M	H	H	L	
CCR5	I	CCL3, CCL4, CCL5, CCL8, CCL11, CCL14, CCL16	H			H	M	L		H	M		
CCR6	D	CCL20				M		M		L			
CCR7	C	CCL19, CCL21			H								
CCR8	I	CCL1, CCL16		H									
CCR9	D	CCL25,											
CCR10	I	CCL27, CCL28						L	L				
Other													
XCR1	I	XCL1, XCL2											
CX3CR1	D	CX3CL1											

Data adapted from Høglund *et al*[53], Maghazachi[90], Zlotnik *et al*[117] and Sallusto et al[118] . I: Inflammatory; C: Constitutive; D: Dual activities; Unact: Unactivated; Act: Activated; PBL: Peripheral blood lymphocytes; CSF: Cerebrospinal fluid. H: High expression; M: Medium expression; L: Low expression.

lowing additional inflammatory cells access, and later a complete disruption of the BBB with damage to the CNS occurs, resulting in active lesions and potential clinical exacerbation of the disease[25,30].

Although our understanding of how inflammation may be initiated has increased with the advent of the Th17 hypothesis, much remains to be discovered of how T cells and other cell types are recruited into the CNS during inflammation. It is known that chemokines are important for recruiting these cells across the BBB. Chemokines are classified into subfamilies, consisting of CXC, CC, C and CX3C. While some are constitutively expressed, others are up-regulated during inflammation[51]. In MS lesions, chemokines expressed on post-capillary venule endothelial walls and bind chemokine receptors expressed on T cells, allowing extravasation. Chemokine concentration gradients in tissues allow the cells to be further guided towards the sites of inflammation[52].

We explored the role of chemokine receptors expression, which include CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR9, CCR10, CXCR1, CXCR3, CXCR4, CXCR5 and CXCR6 on four T cell clones isolated from the blood or CSF of MS patient who was treated with the drug glatiramer acetate (GA). We observed that only four chemokine receptors warranted further study. These were CCR4, CCR5, CCR6 and CXCR3[53]. The fact that all four clones from both peripheral blood and CSF expressed similar patterns of chemokine receptors could be due to migration of the same clones from blood into the CSF, plausibly using CCR5 or CCR6 for entry (Table 1). When these cells were activated, all chemokine receptors expression with the exception of CCR4 was markedly reduced. The migration pattern corresponded well with the expression of receptors observed, with reduced migration observed when receptors were reduced or no longer expressed. Similarly after activation, migration towards chemokines was less robust. These observations support the hypothesis of diminished effects of GA due to inflammation and premature activation of GA-reactive cells. As migration into the CNS is a necessary step of the bystander suppression hypothesis[24], knowledge of how these cells migrate into the CNS is important in order to possibly enhance the effectiveness of this drug.

These results could in part explain why aggressive immunosuppression followed by maintenance therapy is very effective. Such combination therapy is relatively new to MS treatment, but has been used in other autoimmune diseases. Inspired by this, researchers applied combination therapies which resulted in a better outcome for RRMS patients. Several combinations have been tested, some show more promise than others. It was for instance described how patients treated with a short induction of mitoxantrone followed by longer term GA therapy showed a reduction in new relapses compared to GA alone, as well promising results regarding gadolinium enhanced lesion load[54-57]. This suggests possible synergistic effects among these drugs. Mitoxantrone is a powerful immunosuppres-

sive drug that is approved for treatment of RRMS and secondary progressive MS (SPMS) in the United States. By suppressing inflammation induced by autoreactive T cells such as Th17 and/or Th1, this drug may pave the way for GA-reactive cell migration into the CNS upon reconstitution of the immune system. This way, the cells may mediate their bystander suppression without being impeded by an inflammatory environment. However, while initial studies of this showed important clinical efficacy, further studies are warranted in order to determine whether this treatment regimen is better suited than newer therapies such as alemtuzumab and other future immunomodulators.

The presence of inflammatory T cells in perivascular space and parenchyma triggers recruitment of more T cells, as well as B cells, dendritic cells, microglia and natural killer (NK) cells. The cytokines secreted may by themselves cause damage to the surrounding tissues[58]. Complement depositions, opsonization and local activation of microglia and macrophages causing demyelination[59], and neuronal cell death are few examples of the effects of cytokines[60]. Axonal damage is also present[61,62], and the degree of this was associated with abundance of microglia and $CD8^+$ T cells[63]. Clonally expanded $CD8^+$ T cells are present usually at the lesion edge, as well as in the perivascular infiltrates, indicating a specific antigen response and also possibly responsible for damages to neurons through cell dependent cytotoxicity[64-67]. It was also demonstrated that clonal γδ T cells are present in MS lesions[68]. When the lesion acute phase is over, removal of cellular debris starts, and simultaneously remyelination occurs within the MS plaque. A minority of the patients (about 20%) show more abundant remyelination[69].

In addition, a study showed that MS patients had a significant loss of effector function in the regulatory $CD4^+CD25^+$ subset of T cells (Treg), when compared to healthy individuals[70]. Another study showed reduced frequency of this subset in general as well as reduced expression of transcription factor FOXP3[71]. Although circulating numbers may be the same in patients and healthy individuals, the suppressive potential of Treg cells in MS patients was reduced[72]. Interestingly, these T cells were also able to produce IL-17 when stimulated with APCs in the presence of IL-2 and IL-15[73], but retained their suppressive abilities dependent on the surrounding cytokine environment[74]. Although this regulatory subset may inhibit auto reactive T cells, it is not certain where such inhibition may occur[41].

Finally, it may be that MS is not initially caused by the adaptive immune system at all, but rather as a response to an intrinsic CNS neurodegeneration, indicating that inflammation may follow initial axonal degeneration caused by a currently unknown factor. This is supported by the fact that progression of disease is mainly caused by the amount of lost neurons[30]. In addition, there seems to be a correlation between age at onset than initial clinical course on the disease progression[33].

Role of B cells

The most obvious argument for B cells being involved in MS pathogenesis is the presence of immunoglobulins such as immunoglobulin G1 in the CSF, as detected by isoelectric focusing or gel electrophoresis in as many as 95% of diagnosed patients. Although myelin reactive antibodies have been detected, their relevance is not certain[25], much like the role of antigens. In MS lesions, there have been findings of both immunoglobulin and complement which may suggest a pathogenic role[75,76]. Additionally, B lymphoid follicles, T cells and APCs were identified in the meninges of patients at later stages of MS (SPMS but not PPMS)[76,77]. B cells may contribute to the pathology through antigen presentation, cell interactions or production of immunoglobulins from plasma cells, although B cell activity may represent a response to the autoimmune reaction, rather than to a primary inducer[25].

In EAE, the antigens responsible for the disease have been described. By immunizing the animal with myelin proteins such as MBP, proteolipid protein or myelin oligodendrocyte glycoprotein (MOG), disease can be induced[78]. In humans, such common antigens remain unknown in spite of several attempts to identify them[25,30]. Several candidate antigens are being or have been investigated, but no single candidate has been targeted as the one responsible. Myelin or peptides derived from myelin were thought to be good candidates due to the similarity between EAE and MS, but the responses to these antigens have proven to be unspecific and may suggest that several antigens are involved and/or an extensive epitope spreading occurring after initiation of the disease[30,41,79]. One recently suggested antigen is αβ-crystallin, which contrary to previous candidates is not present in human myelin, but rather is detected in early active MS lesions, and patients have antibodies against it in their CSF[6]. When the gene encoding αβ-crystallin was knocked down in mice, a more intense inflammatory EAE with higher cytokine load occurred[80]. This suggests a protective role that may be disrupted by a pathogenic immune response. Another candidate not directly associated with myelin is neurofascin, expressed on neuronal axons. As antibodies have been detected in patients with MS, this may contribute to axonal damage[81].

Role of NK cells

NK cells are large granular lymphocytes that possess the ability to spontaneously lyse target cells without a prior sensitization[82]. NK cells also have immunoregulatory features, including secretion of cytokines, chemokines and cell to cell contact[83,84]. Functionally, these cells are important in immune responses to viral infections as well as controlling tumor growths[85,86]. The activities of these cells are regulated by activating and inhibitory receptors, which by intracellular integration of challenges and inhibitions determine the cell course of action[87]. NK cells recognize and are activated by cells that are in distress by detecting stress induced ligands on target cells through natural cytotoxicity receptors, such as NKp30, NKp44, or NKp46, and the C-type lectin receptor NKG2D, among others[87,88]. In healthy cells however, activating

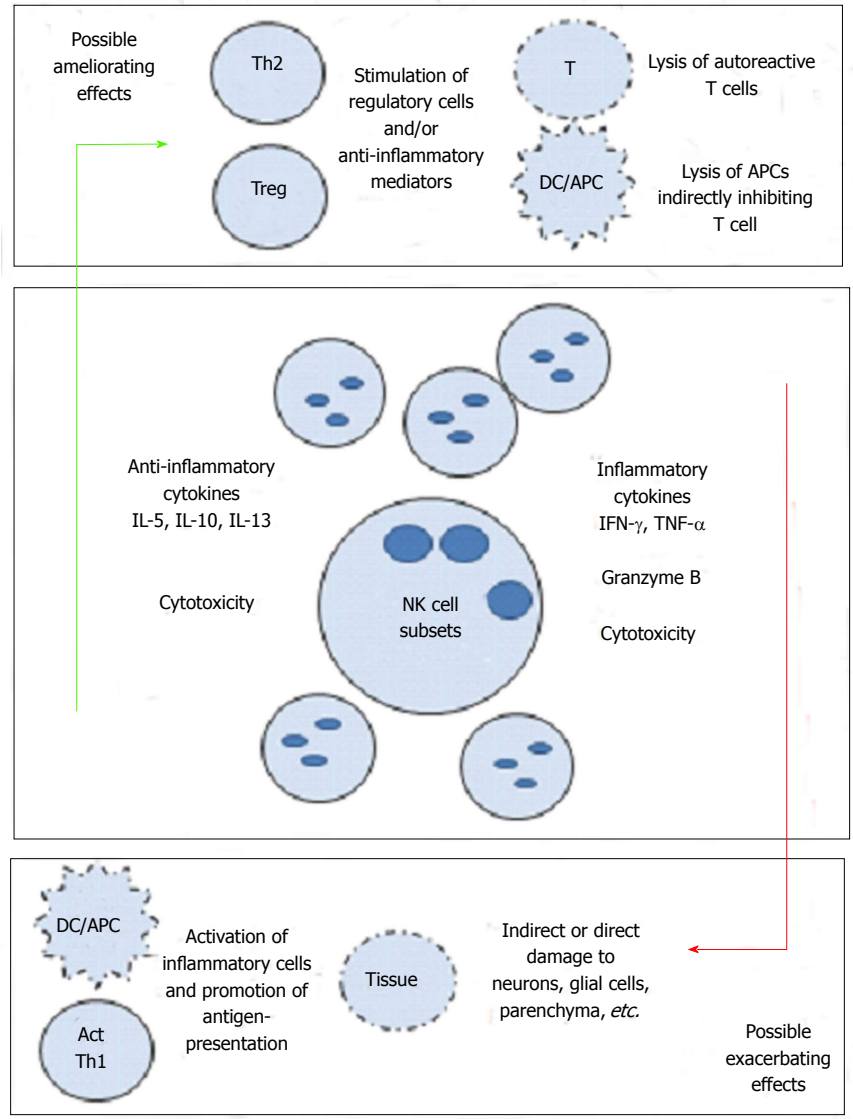

Figure 1 Natural killer cells influence multiple sclerosis pathogenesis in both protective and exacerbating ways. Lysis of either auto-reactive cells or antigen presenting cells (APCs) may protect the central nervous system (CNS) from damage. Stimulation of protective Th2 or Treg cells may also encourage an anti-inflammatory environment. On the other hand, stimulation of APCs or auto-reactive cells may have opposite effects. IL: Interleukin; TNF: Tumor necrosis factor; IFN: Interferon; NK: Natural killer.

factors are held in equilibrium by inhibiting signals. NK cells express numerous receptors that inhibit activation, including members of the killer-cell immunoglobulin-like receptor (KIR) family that interact with HLA-I molecules and CD94-NKG2A that interact with HLA-E. In the absence of these "self" ligands, NK cells are activated and consequently, kill target cells[89].

In human blood, NK cells constitute about 2% to 18% of the total circulating lymphocytes[90,91], and can be classified into subsets based on expression of surface receptors. $CD16^{+}CD56^{-}$ constitute about 85%-90% of circulating NK cells, are highly cytotoxic but produce little cytokines, while $CD16^{-}CD56^{+}$ cells are less cytotoxic but effective cytokine producers[92]. NK cells may also be classified based on their cytokine expression. Similar to Th1/Th2 subsets, these cells were divided into NK1 expressing IFN-γ, or NK2 expressing IL-5 and IL-13[93]. Further research divided these NK cells into even smaller subsets, recognized as NK22 secreting IL-22 and found in lymphoid tissues of the gastrointestinal tract[94], or NK17/NK1 cells secreting IL-17 and IFN-γ when stimulated with IL-2[95].

The role of NK cells in autoimmune diseases is being investigated, and is highly debated as both positive and negative associations have been observed[96-99]. In the EAE model, depletion of NK cells resulted in a severe relapsing EAE, and more pronounced CNS pathology[100-103]. This suggests protective effects for NK cells, especially since the depletion was associated with increased $CD4^{+}$ T cell activity and hence, may be associated with direct killing of these cells[88,104]. Also in EAE, mice deficient in the chemokine receptor CX_3CR1 had more severe EAE. This receptor is necessary for the recruitment of NK cells into the CNS, providing evidence showing that NK cell infiltrating into the CNS represents an important event in ameliorating and controlling the disease[105]. In the same mice there were increased responses from Th17 cells locally in CNS, suggesting

a plausible role for NK cells in curbing these cells[106]. Conflicting with these results, it was found that depletion of NK cells in MOG-induced EAE actually ameliorated the disease[107]. Additionally, by stimulating NK cells to produce IFN-γ, these cells may also cause inflammation in part by stimulating Th1 cell response[108,109]. These findings represent detrimental effects of NK cells eliciting inflammatory lesions and exacerbating the inflammatory response. Much of the confusion regarding conflicting findings may be attributed to failure to recognize different effects by different subsets, which should encourage further investigation into this field.

The functional activity of NK cells is variable and is generally lower in MS patients than in healthy individuals[110]. Periods of reduced NK cell numbers in the blood was associated with a higher relapse tendency after. The same study also found a correlation between high mean NK cell activity and total lesion load determined by MRI[111]. As reduced numbers of NK cells were thought to be mediated by migration into tissues including the CNS, this could indicate a pathological role for NK cells. However, it could also be viewed as a risk factor for new attacks due to reduced activity of NK cells. In a more recent study, researchers found a reduced number of $CD8^{low}CD56^{+}CD3^{-}CD4^{-}$ cells in untreated patients with CIS, when compared to healthy controls[112].

Along these associations, there have been speculations as to how exactly NK cells mediate their effects, be it positive or negative. One suggested pathway was interactions between NK cells and dendritic cells[97,101]. The cognate and non-cognate interactions among NK cells and DCs have been previously described. Activated NK cells are also able to kill immature (i) DCs, but mature (m) DCs are spared. How and where these cells interact are not clear, but it was hypothesized that such interactions may occur in inflamed tissues. Our work on the effects of GA on NK cells in MS patients and healthy individuals shows that GA influences this cross-talk between DCs and NK cells. Consequently, we hypothesized that this may impede antigen presentation to auto reactive T cells (Th17 or Th1), and may further reduce inflammation. We described that NK cells stimulated *in vitro* with GA became more cytotoxic towards autologous and allogeneic iDCs and mDCs[113]. Furthermore, we described how NK cells from EAE mice treated with GA were more cytotoxic than NK cells isolated from EAE mice treated with vehicle alone, which was associated with ameliorating the disease[114]. While these findings support a mechanism of action for GA on NK cells, it had to be further addressed in humans with in vivo exposure to the drug. Consequently, we examined the activities of NK cells and DCs in MS patients receiving the drug GA for one year. In this study, we compared the cytotoxicity levels before treatment with those observed after treatment. We demonstrated that NK cells isolated from GA-dosed MS patients had significantly increased cytotoxicity against K562 tumor cells. Trends of significantly increased cytotoxicity were also observed against both iDC and mDC in the same patients[115]. In summary, it seems that GA increases the cytotoxic activity of NK cells when compared to pretreatment levels. The increased cytotoxicity correlated well with the elevated expression of activating NK cells cytotoxicity receptors. Figure 1 represents our current knowledge regarding the role that NK cells might play in MS.

CONCLUSION

The evidence for a complex immunopathology has become ever clearer with the reveal of numerous genes associated with the immune system carrying risk of developing MS, novel cell subsets being discovered and shown to possibly be related to the pathogenesis, and knowledge of how disease modifying therapies (DMT) target several immune cells rather than single subsets. All of this call for continuous bedside-to-bench research into how the newer DMTs enhance, inhibit or modulate the immune system of patients resulting in a better clinical course than without such treatment. The introduction of the new oral therapeutics teriflunomide (Aubagio®), fingolimod (Gilenya®) and dimethyl fumarate (Tecfidera®), all with incomplete understandings of their mechanisms of action on the immune system, will be particularly interesting to follow in this regard.

So far, research into how immune cells act in MS patients, EAE models and *in vitro* have primarily focused on single subsets or categorically on either adaptive- or innate immune cells. Recent knowledge of NK cells may belong in a grey area between these traditional groupings challenges this concept[116]. For instance, while our own research focused on the interactions between NK and dendritic cells, similar interactions between NK and B cells would be of interest, as B cells also have antigen presenting abilities. NK cells also have the ability to modify or lyse T cells, and hence investigations into how these cells interact in MS patients or in the EAE model could improve our understanding of the regulation or dysregulation the immune systems of MS patients.

REFERENCES

1 **Charcot J**. Histologie de la sclerose en plaques. *Gazette des hopitaux* 1868; **41**: 554-555
2 **Compston A**. The 150th anniversary of the first depiction of the lesions of multiple sclerosis. *J Neurol Neurosurg Psychiatry* 1988; **51**: 1249-1252 [PMID: 3066846 DOI: 10.1136/jnnp.51.10.1249]
3 **Holmøy T**, Hestvik AL, Vartdal F. Management of progressive multifocal leukoencephalopathy associated with natalizumab--possibilities and responsibility. *Eur J Neurol* 2006; **13**: e2 [PMID: 16879280 DOI: 10.1159/000091431]
4 **Compston A**, Coles A. Multiple sclerosis. *Lancet* 2008; **372**: 1502-1517 [PMID: 18970977 DOI: 10.1016/S0140-6736(08)61620-7]
5 **Gourraud PA**, Harbo HF, Hauser SL, Baranzini SE. The genetics of multiple sclerosis: an up-to-date review. *Immunol Rev* 2012; **248**: 87-103 [PMID: 22725956 DOI: 10.1111/j.1600-065X.2012.01134]
6 **Holmøy T**, Hestvik AL. Multiple sclerosis: immunopathogenesis and controversies in defining the cause. *Curr Opin Infect Dis* 2008; **21**: 271-278 [PMID: 18448972 DOI: 10.1097/QCO.0b013e3282f88b48]
7 **Robertson NP**, Clayton D, Fraser M, Deans J, Compston DA.

Clinical concordance in sibling pairs with multiple sclerosis. *Neurology* 1996; **47**: 347-352 [PMID: 8757003 DOI: 10.1212/WNL.47.2.347]

8 **Carton H**, Vlietinck R, Debruyne J, De Keyser J, D'Hooghe MB, Loos R, Medaer R, Truyen L, Yee IM, Sadovnick AD. Risks of multiple sclerosis in relatives of patients in Flanders, Belgium. *J Neurol Neurosurg Psychiatry* 1997; **62**: 329-333 [PMID: 9120443 DOI: 10.1136/jnnp.62.4.329]

9 **Sadovnick AD**, Yee IM, Ebers GC, Risch NJ. Effect of age at onset and parental disease status on sibling risks for MS. *Neurology* 1998; **50**: 719-723 [PMID: 9521263 DOI: 10.1212/WNL.38.6.990]

10 **Willer CJ**, Dyment DA, Risch NJ, Sadovnick AD, Ebers GC. Twin concordance and sibling recurrence rates in multiple sclerosis. *Proc Natl Acad Sci USA* 2003; **100**: 12877-12882 [PMID: 14569025 DOI: 10.1073/pnas.1932604100]

11 **Mumford CJ**, Wood NW, Kellar-Wood H, Thorpe JW, Miller DH, Compston DA. The British Isles survey of multiple sclerosis in twins. *Neurology* 1994; **44**: 11-15 [PMID: 8290043 DOI: 10.1212/WNL.44.1.11]

12 **Svejgaard A**. The immunogenetics of multiple sclerosis. *Immunogenetics* 2008; **60**: 275-286 [PMID: 18461312 DOI: 10.1007/s00251-008-0295-1]

13 **Sawcer S**, Hellenthal G, Pirinen M, Spencer CC, Patsopoulos NA, Moutsianas L, Dilthey A, Su Z, Freeman C, Hunt SE, Edkins S, Gray E, Booth DR, Potter SC, Goris A, Band G, Oturai AB, Strange A, Saarela J, Bellenguez C, Fontaine B, Gillman M, Hemmer B, Gwilliam R, Zipp F, Jayakumar A, Martin R, Leslie S, Hawkins S, Giannoulatou E, D'alfonso S, Blackburn H, Martinelli Boneschi F, Liddle J, Harbo HF, Perez ML, Spurkland A, Waller MJ, Mycko MP, Ricketts M, Comabella M, Hammond N, Kockum I, McCann OT, Ban M, Whittaker P, Kemppinen A, Weston P, Hawkins C, Widaa S, Zajicek J, Dronov S, Robertson N, Bumpstead SJ, Barcellos LF, Ravindrarajah R, Abraham R, Alfredsson L, Ardlie K, Aubin C, Baker A, Baker K, Baranzini SE, Bergamaschi L, Bergamaschi R, Bernstein A, Berthele A, Boggild M, Bradfield JP, Brassat D, Broadley SA, Buck D, Butzkueven H, Capra R, Carroll WM, Cavalla P, Celius EG, Cepok S, Chiavacci R, Clerget-Darpoux F, Clysters K, Comi G, Cossburn M, Cournu-Rebeix I, Cox MB, Cozen W, Cree BA, Cross AH, Cusi D, Daly MJ, Davis E, de Bakker PI, Debouverie M, D'hooghe MB, Dixon K, Dobosi R, Dubois B, Ellinghaus D, Elovaara I, Esposito F, Fontenille C, Foote S, Franke A, Galimberti D, Ghezzi A, Glessner J, Gomez R, Gout O, Graham C, Grant SF, Guerini FR, Hakonarson H, Hall P, Hamsten A, Hartung HP, Heard RN, Heath S, Hobart J, Hoshi M, Infante-Duarte C, Ingram G, Ingram W, Islam T, Jagodic M, Kabesch M, Kermode AG, Kilpatrick TJ, Kim C, Klopp N, Koivisto K, Larsson M, Lathrop M, Lechner-Scott JS, Leone MA, Leppä V, Liljedahl U, Bomfim IL, Lincoln RR, Link J, Liu J, Lorentzen AR, Lupoli S, Macciardi F, Mack T, Marriott M, Martinelli V, Mason D, McCauley JL, Mentch F, Mero IL, Mihalova T, Montalban X, Mottershead J, Myhr KM, Naldi P, Ollier W, Page A, Palotie A, Pelletier J, Piccio L, Pickersgill T, Piehl F, Pobywajlo S, Quach HL, Ramsay PP, Reunanen M, Reynolds R, Rioux JD, Rodegher M, Roesner S, Rubio JP, Rückert IM, Salvetti M, Salvi E, Santaniello A, Schaefer CA, Schreiber S, Schulze C, Scott RJ, Sellebjerg F, Selmaj KW, Sexton D, Shen L, Simms-Acuna B, Skidmore S, Sleiman PM, Smestad C, Sørensen PS, Søndergaard HB, Stankovich J, Strange RC, Sulonen AM, Sundqvist E, Syvänen AC, Taddeo F, Taylor B, Blackwell JM, Tienari P, Bramon E, Tourbah A, Brown MA, Tronczynska E, Casas JP, Tubridy N, Corvin A, Vickery J, Jankowski J, Villoslada P, Markus HS, Wang K, Mathew CG, Wason J, Palmer CN, Wichmann HE, Plomin R, Willoughby E, Rautanen A, Winkelmann J, Wittig M, Trembath RC, Yaouanq J, Viswanathan AC, Zhang H, Wood NW, Zuvich R, Deloukas P, Langford C, Duncanson A, Oksenberg JR, Pericak-Vance MA, Haines JL, Olsson T, Hillert J, Ivinson AJ, De Jager PL, Peltonen L, Stewart GJ, Hafler DA, Hauser SL, McVean G, Donnelly P, Compston A. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature* 2011; **476**: 214-219 [PMID: 21833088 DOI: 10.1038/nature10251]

14 **Sawcer S**. Bayes factors in complex genetics. *Eur J Hum Genet* 2010; **18**: 746-750 [PMID: 20179745 DOI: 10.1038/ejhg]

15 **Hafler DA**, Compston A, Sawcer S, Lander ES, Daly MJ, De Jager PL, de Bakker PI, Gabriel SB, Mirel DB, Ivinson AJ, Pericak-Vance MA, Gregory SG, Rioux JD, McCauley JL, Haines JL, Barcellos LF, Cree B, Oksenberg JR, Hauser SL. Risk alleles for multiple sclerosis identified by a genomewide study. *N Engl J Med* 2007; **357**: 851-862 [PMID: 17660530 DOI: 10.1056/NEJMoa073493]

16 **Simpson S**, Blizzard L, Otahal P, Van der Mei I, Taylor B. Latitude is significantly associated with the prevalence of multiple sclerosis: a meta-analysis. *J Neurol Neurosurg Psychiatry* 2011; **82**: 1132-1141 [PMID: 21478203 DOI: 10.1136/jnnp]

17 **Grytten N**, Torkildsen Ø, Aarseth JH, Benjaminsen E, Celius EG, Dahl OP, Holmøy T, Løken-Amsrud K, Midgard R, Myhr KM, Risberg G, Vatne A, Kampman MT. Month of birth as a latitude-dependent risk factor for multiple sclerosis in Norway. *Mult Scler* 2013; **19**: 1028-1034 [PMID: 23257620 DOI: 10.1177/1352458512471094]

18 **Ascherio A**, Munger KL. Environmental risk factors for multiple sclerosis. Part II: Noninfectious factors. *Ann Neurol* 2007; **61**: 504-513 [PMID: 17492755 DOI: 10.1002/ana.21117]

19 **Ascherio A**, Munger KL, Simon KC. Vitamin D and multiple sclerosis. *Lancet Neurol* 2010; **9**: 599-612 [PMID: 20494325 DOI: 10.1016/S1474-4422(10)70086-7]

20 **Brustad M**, Sandanger T, Aksnes L, Lund E. Vitamin D status in a rural population of northern Norway with high fish liver consumption. *Public Health Nutr* 2004; **7**: 783-789 [PMID: 15369617 DOI: 10.1079/PHN2004605]

21 **Lemire JM**, Archer DC. 1,25-dihydroxyvitamin D3 prevents the in vivo induction of murine experimental autoimmune encephalomyelitis. *J Clin Invest* 1991; **87**: 1103-1107 [PMID: 1705564 DOI: 10.1172/JCI115072]

22 **Cantorna MT**, Hayes CE, DeLuca HF. 1,25-Dihydroxyvitamin D3 reversibly blocks the progression of relapsing encephalomyelitis, a model of multiple sclerosis. *Proc Natl Acad Sci USA* 1996; **93**: 7861-7864 [PMID: 8755567 DOI: 10.1073/pnas.93.15.7861]

23 **Munger KL**, Levin LI, Hollis BW, Howard NS, Ascherio A. Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis. *JAMA* 2006; **296**: 2832-2838 [PMID: 17179460 DOI: 10.1001/jama.296.23.2832]

24 **Ascherio A**, Munger KL. Environmental risk factors for multiple sclerosis. Part I: the role of infection. *Ann Neurol* 2007; **61**: 288-299 [PMID: 17444504]

25 **Hestvik AL**. The double-edged sword of autoimmunity: lessons from multiple sclerosis. *Toxins* (Basel) 2010; **2**: 856-877 [PMID: 22069614 DOI: 10.3390/toxins2040856]

26 **Ascherio A**, Munger KL, Lennette ET, Spiegelman D, Hernán MA, Olek MJ, Hankinson SE, Hunter DJ. Epstein-Barr virus antibodies and risk of multiple sclerosis: a prospective study. *JAMA* 2001; **286**: 3083-3088 [PMID: 11754673 DOI: 10.1001/jama.286.24.3083]

27 **Martyn CN**, Cruddas M, Compston DA. Symptomatic Epstein-Barr virus infection and multiple sclerosis. *J Neurol Neurosurg Psychiatry* 1993; **56**: 167-168 [PMID: 8382268 DOI: 10.1136/jnnp.56.2.167]

28 **Lang HL**, Jacobsen H, Ikemizu S, Andersson C, Harlos K, Madsen L, Hjorth P, Sondergaard L, Svejgaard A, Wucherpfennig K, Stuart DI, Bell JI, Jones EY, Fugger L. A functional and structural basis for TCR cross-reactivity in multiple sclerosis. *Nat Immunol* 2002; **3**: 940-943 [PMID: 12244309 DOI: 10.1038/ni835]

29 **Marrie RA**. Environmental risk factors in multiple sclerosis aetiology. *Lancet Neurol* 2004; **3**: 709-718 [PMID: 15556803

DOI: 10.1016/S1474-4422(04)00933-0]
30 **Dolei A**, Garson JA, Arru G, Clerici M, Germi R, Marche PN, Perron H. Multiple sclerosis-associated retrovirus and related human endogenous retrovirus-W in patients with multiple sclerosis. *J Neuroimmunol* 2014; **266**: 87-88 [PMID: 24355750 DOI: 10.1016/j.jneuroim.2013.11.009]
31 **Fisniku LK**, Brex PA, Altmann DR, Miszkiel KA, Benton CE, Lanyon R, Thompson AJ, Miller DH. Disability and T2 MRI lesions: a 20-year follow-up of patients with relapse onset of multiple sclerosis. *Brain* 2008; **131**: 808-817 [PMID: 18234696 DOI: 10.1093/brain/awm329]
32 **Weinshenker BG**. The natural history of multiple sclerosis: update 1998. *Semin Neurol* 1998; **18**: 301-307 [PMID: 9817534 DOI: 10.1055/s-2008-1040881]
33 **Confavreux C**, Vukusic S. Age at disability milestones in multiple sclerosis. *Brain* 2006; **129**: 595-605 [PMID: 16415309 DOI: 10.1093/brain/awh714]
34 **Miller DH**, Leary SM. Primary-progressive multiple sclerosis. *Lancet Neurol* 2007; **6**: 903-912 [PMID: 17884680 DOI: 10.1016/S1474-4422(07)70243-0]
35 **Brønnum-Hansen H**, Koch-Henriksen N, Stenager E. Trends in survival and cause of death in Danish patients with multiple sclerosis. *Brain* 2004; **127**: 844-850 [PMID: 14960501 DOI: 10.1093/brain/awh104]
36 **Engelhardt B**. The blood-central nervous system barriers actively control immune cell entry into the central nervous system. *Curr Pharm Des* 2008; **14**: 1555-1565 [PMID: 18673197 DOI: 10.2174/138161208784705432]
37 **Bechmann I**, Galea I, Perry VH. What is the blood-brain barrier (not)? *Trends Immunol* 2007; **28**: 5-11 [PMID: 17140851 DOI: 10.1016/j.it.2006.11.007]
38 **Kivisäkk P**, Mahad DJ, Callahan MK, Trebst C, Tucky B, Wei T, Wu L, Baekkevold ES, Lassmann H, Staugaitis SM, Campbell JJ, Ransohoff RM. Human cerebrospinal fluid central memory CD4+ T cells: evidence for trafficking through choroid plexus and meninges via P-selectin. *Proc Natl Acad Sci USA* 2003; **100**: 8389-8394 [PMID: 12829791 DOI: 10.1073/pnas.1433000100]
39 **Ransohoff RM**, Kivisäkk P, Kidd G. Three or more routes for leukocyte migration into the central nervous system. *Nat Rev Immunol* 2003; **3**: 569-581 [PMID: 12876559 DOI: 10.1038/nri1130]
40 **Steinman L**. Multiple sclerosis: a two-stage disease. *Nat Immunol* 2001; **2**: 762-764 [PMID: 11526378 DOI: 10.1038/ni0901-762]
41 **McFarland HF**, Martin R. Multiple sclerosis: a complicated picture of autoimmunity. *Nat Immunol* 2007; **8**: 913-919 [PMID: 17712344 DOI: 10.1038/ni1507]
42 **Langrish CL**, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, McClanahan T, Kastelein RA, Cua DJ. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med* 2005; **201**: 233-240 [PMID: 15657292 DOI: 10.1084/jem.20041257]
43 **Steinman L**. A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. *Nat Med* 2007; **13**: 139-145 [PMID: 17290272 DOI: 10.1038/nm0307-385a]
44 **Komiyama Y**, Nakae S, Matsuki T, Nambu A, Ishigame H, Kakuta S, Sudo K, Iwakura Y. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J Immunol* 2006; **177**: 566-573 [PMID: 16785554 DOI: 10.4049/jimmunol.177.1.566]
45 **Tzartos JS**, Friese MA, Craner MJ, Palace J, Newcombe J, Esiri MM, Fugger L. Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. *Am J Pathol* 2008; **172**: 146-155 [PMID: 18156204 DOI: 10.2353/ajpath.2008.070690]
46 **Reboldi A**, Coisne C, Baumjohann D, Benvenuto F, Bottinelli D, Lira S, Uccelli A, Lanzavecchia A, Engelhardt B, Sallusto F. C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. *Nat Immunol* 2009; **10**: 514-523 [PMID: 19305396 DOI: 10.1038/ni.1716]
47 **El-Behi M**, Ciric B, Dai H, Yan Y, Cullimore M, Safavi F, Zhang GX, Dittel BN, Rostami A. The encephalitogenicity of T(H)17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF. *Nat Immunol* 2011; **12**: 568-575 [PMID: 21516111]
48 **Codarri L**, Gyülvészi G, Tosevski V, Hesske L, Fontana A, Magnenat L, Suter T, Becher B. RORγt drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation. *Nat Immunol* 2011; **12**: 560-567 [PMID: 21516112 DOI: 10.1038/ni.2027]
49 **Kebir H**, Kreymborg K, Ifergan I, Dodelet-Devillers A, Cayrol R, Bernard M, Giuliani F, Arbour N, Becher B, Prat A. Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. *Nat Med* 2007; **13**: 1173-1175 [PMID: 17828272 DOI: 10.1038/nm1651]
50 **Agrawal S**, Anderson P, Durbeej M, van Rooijen N, Ivars F, Opdenakker G, Sorokin LM. Dystroglycan is selectively cleaved at the parenchymal basement membrane at sites of leukocyte extravasation in experimental autoimmune encephalomyelitis. *J Exp Med* 2006; **203**: 1007-1019 [PMID: 16585265 DOI: 10.1084/jem.20051342]
51 **Bruserud Ø**. The chemokine system in experimental and clinical hematology. Preface. *Curr Top Microbiol Immunol* 2010; **341**: v-vi [PMID: 20973160 DOI: 10.1007/978-3-642-12639-0]
52 **Cheng WJ**, Chen GJ. Chemokines and Chemokine Receptors in Multiple Sclerosis. *Mediat inflamm* 2014; 2014: 659206 [PMID: 24639600 DOI: 10.1155/2014/659206]
53 **Høglund RA**, Hestvik AL, Holmøy T, Maghazachi AA. Expression and functional activity of chemokine receptors in glatiramer acetate-specific T cells isolated from multiple sclerosis patient receiving the drug glatiramer acetate. *Hum Immunol* 2011; **72**: 124-134 [PMID: 20977917 DOI: 10.1016/j.humimm]
54 **Vollmer T**, Panitch H, Bar-Or A, Dunn J, Freedman MS, Gazda SK, Campagnolo D, Deutsch F, Arnold DL. Glatiramer acetate after induction therapy with mitoxantrone in relapsing multiple sclerosis. *Mult Scler* 2008; **14**: 663-670 [PMID: 18424479 DOI: 10.1177/1352458507085759]
55 **Ramtahal J**, Jacob A, Das K, Boggild M. Sequential maintenance treatment with glatiramer acetate after mitoxantrone is safe and can limit exposure to immunosuppression in very active, relapsing remitting multiple sclerosis. *J Neurol* 2006; **253**: 1160-1164 [PMID: 16990994 DOI: 10.1007/s00415-006-0178-z]
56 **Boggild M**. Immunosuppression followed by immunomodulation. *J Neurol Sci* 2009; **277** Suppl 1: S50-S54 [PMID: 19200868 DOI: 10.1016/S0022-510X(09)70014-0]
57 **Arnold DL**, Campagnolo D, Panitch H, Bar-Or A, Dunn J, Freedman MS, Gazda SK, Vollmer T. Glatiramer acetate after mitoxantrone induction improves MRI markers of lesion volume and permanent tissue injury in MS. *J Neurol* 2008; **255**: 1473-1478 [PMID: 18854910 DOI: 10.1007/s00415-008-0911]
58 **Zajicek JP**, Wing M, Scolding NJ, Compston DA. Interactions between oligodendrocytes and microglia. A major role for complement and tumour necrosis factor in oligodendrocyte adherence and killing. *Brain* 1992; **115** (Pt 6): 1611-1631 [PMID: 1486453 DOI: 10.1093/brain/115.6.1611]
59 **Prineas JW**, Graham JS. Multiple sclerosis: capping of surface immunoglobulin G on macrophages engaged in myelin breakdown. *Ann Neurol* 1981; **10**: 149-158 [PMID: 7025748 DOI: 10.1002/ana.410100205]
60 **Breij EC**, Brink BP, Veerhuis R, van den Berg C, Vloet R, Yan R, Dijkstra CD, van der Valk P, Bö L. Homogeneity of active demyelinating lesions in established multiple sclerosis. *Ann Neurol* 2008; **63**: 16-25 [PMID: 18232012 DOI: 10.1002/ana.21311]

61 **Trapp BD**, Peterson J, Ransohoff RM, Rudick R, Mörk S, Bö L. Axonal transection in the lesions of multiple sclerosis. *N Engl J Med* 1998; **338**: 278-285 [PMID: 9445407 DOI: 10.1056/NEJM199801293380502]

62 **Ferguson B**, Matyszak MK, Esiri MM, Perry VH. Axonal damage in acute multiple sclerosis lesions. *Brain* 1997; **120** (Pt 3): 393-399 [PMID: 9126051 DOI: 10.1093/brain/120.3.393]

63 **Kuhlmann T**, Lingfeld G, Bitsch A, Schuchardt J, Brück W. Acute axonal damage in multiple sclerosis is most extensive in early disease stages and decreases over time. *Brain* 2002; **125**: 2202-2212 [PMID: 12244078 DOI: 10.1093/brain/awf235]

64 **Traugott U**, Reinherz EL, Raine CS. Multiple sclerosis: distribution of T cell subsets within active chronic lesions. *Science* 1983; **219**: 308-310 [PMID: 6217550 DOI: 10.1126/science.6217550]

65 **Babbe H**, Roers A, Waisman A, Lassmann H, Goebels N, Hohlfeld R, Friese M, Schröder R, Deckert M, Schmidt S, Ravid R, Rajewsky K. Clonal expansions of CD8(+) T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction. *J Exp Med* 2000; **192**: 393-404 [PMID: 10934227 DOI: 10.1084/jem.192.3.393]

66 **Skulina C**, Schmidt S, Dornmair K, Babbe H, Roers A, Rajewsky K, Wekerle H, Hohlfeld R, Goebels N. Multiple sclerosis: brain-infiltrating CD8+ T cells persist as clonal expansions in the cerebrospinal fluid and blood. *Proc Natl Acad Sci USA* 2004; **101**: 2428-2433 [PMID: 14983026 DOI: 10.1073/pnas.0308689100]

67 **Junker A**, Ivanidze J, Malotka J, Eiglmeier I, Lassmann H, Wekerle H, Meinl E, Hohlfeld R, Dornmair K. Multiple sclerosis: T-cell receptor expression in distinct brain regions. *Brain* 2007; **130**: 2789-2799 [PMID: 17890278 DOI: 10.1093/brain/awm214]

68 **Wucherpfennig KW**, Newcombe J, Li H, Keddy C, Cuzner ML, Hafler DA. Gamma delta T-cell receptor repertoire in acute multiple sclerosis lesions. *Proc Natl Acad Sci USA* 1992; **89**: 4588-4592 [PMID: 1374907 DOI: 10.1073/pnas.89.10.4588]

69 **Patrikios P**, Stadelmann C, Kutzelnigg A, Rauschka H, Schmidbauer M, Laursen H, Sorensen PS, Brück W, Lucchinetti C, Lassmann H. Remyelination is extensive in a subset of multiple sclerosis patients. *Brain* 2006; **129**: 3165-3172 [PMID: 16921173 DOI: 10.1093/brain/awl217]

70 **Viglietta V**, Baecher-Allan C, Weiner HL, Hafler DA. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. *J Exp Med* 2004; **199**: 971-979 [PMID: 15067033 DOI: 10.1084/jem.20031579]

71 **Venken K**, Hellings N, Thewissen M, Somers V, Hensen K, Rummens JL, Medaer R, Hupperts R, Stinissen P. Compromised CD4+ CD25(high) regulatory T-cell function in patients with relapsing-remitting multiple sclerosis is correlated with a reduced frequency of FOXP3-positive cells and reduced FOXP3 expression at the single-cell level. *Immunology* 2008; **123**: 79-89 [PMID: 17897326 DOI: 10.1111/j.1365-2567.2007.02690.x]

72 **Haas J**, Hug A, Viehöver A, Fritzsching B, Falk CS, Filser A, Vetter T, Milkova L, Korporal M, Fritz B, Storch-Hagenlocher B, Krammer PH, Suri-Payer E, Wildemann B. Reduced suppressive effect of CD4+CD25high regulatory T cells on the T cell immune response against myelin oligodendrocyte glycoprotein in patients with multiple sclerosis. *Eur J Immunol* 2005; **35**: 3343-3352 [PMID: 16206232 DOI: 10.1002/eji.200526065]

73 **Koenen HJ**, Smeets RL, Vink PM, van Rijssen E, Boots AM, Joosten I. Human CD25highFoxp3pos regulatory T cells differentiate into IL-17-producing cells. *Blood* 2008; **112**: 2340-2352 [PMID: 18617638 DOI: 10.1182/blood-2008-01-133967]

74 **Beriou G**, Costantino CM, Ashley CW, Yang L, Kuchroo VK, Baecher-Allan C, Hafler DA. IL-17-producing human peripheral regulatory T cells retain suppressive function. *Blood* 2009; **113**: 4240-4249 [PMID: 19171879 DOI: 10.1182/blood-2008-10-183251]

75 **Storch MK**, Piddlesden S, Haltia M, Iivanainen M, Morgan P, Lassmann H. Multiple sclerosis: in situ evidence for antibody- and complement-mediated demyelination. *Ann Neurol* 1998; **43**: 465-471 [PMID: 9546327 DOI: 10.1002/ana.410430409]

76 **Magliozzi R**, Howell O, Vora A, Serafini B, Nicholas R, Puopolo M, Reynolds R, Aloisi F. Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology. *Brain* 2007; **130**: 1089-1104 [PMID: 17438020 DOI: 10.1093/brain/awm038]

77 **Serafini B**, Rosicarelli B, Magliozzi R, Stigliano E, Capello E, Mancardi GL, Aloisi F. Dendritic cells in multiple sclerosis lesions: maturation stage, myelin uptake, and interaction with proliferating T cells. *J Neuropathol Exp Neurol* 2006; **65**: 124-141 [PMID: 16462204]

78 **Miller SD**, Karpus WJ, Davidson TS. Experimental autoimmune encephalomyelitis in the mouse. *Curr Protoc Immunol* 2010; **Chapter 15**: Unit 15.1 [PMID: 20143314 DOI: 10.1002/0471142735.im1501s88]

79 **McMahon EJ**, Bailey SL, Castenada CV, Waldner H, Miller SD. Epitope spreading initiates in the CNS in two mouse models of multiple sclerosis. *Nat Med* 2005; **11**: 335-339 [PMID: 15735651 DOI: 10.1186/ar3399]

80 **Ousman SS**, Tomooka BH, van Noort JM, Wawrousek EF, O'Connor KC, Hafler DA, Sobel RA, Robinson WH, Steinman L. Protective and therapeutic role for alphaB-crystallin in autoimmune demyelination. *Nature* 2007; **448**: 474-479 [PMID: 17568699 DOI: 10.1038/nature05935]

81 **Mathey EK**, Derfuss T, Storch MK, Williams KR, Hales K, Woolley DR, Al-Hayani A, Davies SN, Rasband MN, Olsson T, Moldenhauer A, Velhin S, Hohlfeld R, Meinl E, Linington C. Neurofascin as a novel target for autoantibody-mediated axonal injury. *J Exp Med* 2007; **204**: 2363-2372 [PMID: 17846150 DOI: 10.1084/jem.20071053]

82 **Herberman RB**. Natural killer cells. *Prog Clin Biol Res* 1989; **288**: 161-167 [PMID: 2470107]

83 **Maghazachi AA**. Chemokines, G proteins and natural killer cells. *Arch Immunol Ther Exp* (Warsz) 2000; **48**: 65-72 [PMID: 10807045]

84 **Maghazachi AA**. Role of chemokines in the biology of natural killer cells. *Curr Top Microbiol Immunol* 2010; **341**: 37-58 [PMID: 20369317 DOI: 10.1007/82_2010_20]

85 **Maghazachi AA**, Al-Aoukaty A. Chemokines activate natural killer cells through heterotrimeric G-proteins: implications for the treatment of AIDS and cancer. *FASEB J* 1998; **12**: 913-924 [PMID: 9707163]

86 **Yang Q**, Goding SR, Hokland ME, Basse PH. Antitumor activity of NK cells. *Immunol Res* 2006; **36**: 13-25 [PMID: 17337762]

87 **Maghazachi AA**. Insights into seven and single transmembrane-spanning domain receptors and their signaling pathways in human natural killer cells. *Pharmacol Rev* 2005; **57**: 339-357 [PMID: 16109839 DOI: 10.1124/pr.57.3.5]

88 **Kaur G**, Trowsdale J, Fugger L. Natural killer cells and their receptors in multiple sclerosis. *Brain* 2013; **136**: 2657-2676 [PMID: 22734127 DOI: 10.1093/brain/aws159]

89 **Oberg L**, Johansson S, Michaëlsson J, Tomasello E, Vivier E, Kärre K, Höglund P. Loss or mismatch of MHC class I is sufficient to trigger NK cell-mediated rejection of resting lymphocytes in vivo - role of KARAP/DAP12-dependent and -independent pathways. *Eur J Immunol* 2004; **34**: 1646-1653 [PMID: 15162434 DOI: 10.1002/eji.200424913]

90 **Maghazachi AA**. Compartmentalization of human natural killer cells. *Mol Immunol* 2005; **42**: 523-529 [PMID: 15607808 DOI: 10.1016/j.molimm.2004.07.036]

91 **Maghazachi AA**. G protein-coupled receptors in natural killer cells. *J Leukoc Biol* 2003; **74**: 16-24 [PMID: 12832438 DOI: 10.1189/jlb.0103019]

92 **Rolin J**, Maghazachi AA. Implications of chemokines, chemokine receptors, and inflammatory lipids in atherosclerosis. *J Leukoc Biol* 2014; **95**: 575-585 [PMID: 24493826]

93 **Peritt D**, Robertson S, Gri G, Showe L, Aste-Amezaga M, Trinchieri G. Differentiation of human NK cells into NK1 and NK2 subsets. *J Immunol* 1998; **161**: 5821-5824 [PMID: 9834059]

94 **Cella M**, Fuchs A, Vermi W, Facchetti F, Otero K, Lennerz JK, Doherty JM, Mills JC, Colonna M. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. *Nature* 2009; **457**: 722-725 [PMID: 18978771 DOI: 10.1038/nature07537]

95 **Pandya AD**, Al-Jaderi Z, Høglund RA, Holmøy T, Harbo HF, Norgauer J, Maghazachi AA. Identification of human NK17/NK1 cells. *PLoS One* 2011; **6**: e26780 [PMID: 22039549 DOI: 10.1371/journal.pone.0026780]

96 **Gandhi R**, Laroni A, Weiner HL. Role of the innate immune system in the pathogenesis of multiple sclerosis. *J Neuroimmunol* 2010; **221**: 7-14 [PMID: 19931190 DOI: 10.1016/j.jneuroim.2009.10.015]

97 **Maghazachi AA**. On the role of natural killer cells in neurodegenerative diseases. *Toxins* (Basel) 2013; **5**: 363-375 [PMID: 23430541 DOI: 10.3390/toxins5020363]

98 **Tian Z**, Gershwin ME, Zhang C. Regulatory NK cells in autoimmune disease. *J Autoimmun* 2012; **39**: 206-215 [PMID: 22704425 DOI: 10.1016/j.jaut.2012.05.006]

99 **Segal BM**. The role of natural killer cells in curbing neuroinflammation. *J Neuroimmunol* 2007; **191**: 2-7 [PMID: 17904646 DOI: 10.1016/j.jneuroim.2007.09.006]

100 **French AR**, Yokoyama WM. Natural killer cells and autoimmunity. *Arthritis Res Ther* 2004; **6**: 8-14 [PMID: 14979926 DOI: 10.1186/ar1342]

101 **Maghazachi AA**. Role of natural killer cells in multiple sclerosis. *ISRN Immunology* 2012; **2012**: Article ID 795075 [DOI: 10.5402/2012/795075]

102 **Zhang B**, Yamamura T, Kondo T, Fujiwara M, Tabira T. Regulation of experimental autoimmune encephalomyelitis by natural killer (NK) cells. *J Exp Med* 1997; **186**: 1677-1687 [PMID: 9362528 DOI: 10.1084/jem.186.10.1677]

103 **Matsumoto Y**, Kohyama K, Aikawa Y, Shin T, Kawazoe Y, Suzuki Y, Tanuma N. Role of natural killer cells and TCR gamma delta T cells in acute autoimmune encephalomyelitis. *Eur J Immunol* 1998; **28**: 1681-1688 [PMID: 9603475]

104 **Hao J**, Liu R, Piao W, Zhou Q, Vollmer TL, Campagnolo DI, Xiang R, La Cava A, Van Kaer L, Shi FD. Central nervous system (CNS)-resident natural killer cells suppress Th17 responses and CNS autoimmune pathology. *J Exp Med* 2010; **207**: 1907-1921 [PMID: 20696699 DOI: 10.1084/jem.20092749]

105 **Xu W**, Fazekas G, Hara H, Tabira T. Mechanism of natural killer (NK) cell regulatory role in experimental autoimmune encephalomyelitis. *J Neuroimmunol* 2005; **163**: 24-30 [PMID: 15885305 DOI: 10.1016/j.jneuroim.2005.02.011]

106 **Huang D**, Shi FD, Jung S, Pien GC, Wang J, Salazar-Mather TP, He TT, Weaver JT, Ljunggren HG, Biron CA, Littman DR, Ransohoff RM. The neuronal chemokine CX3CL1/fractalkine selectively recruits NK cells that modify experimental autoimmune encephalomyelitis within the central nervous system. *FASEB J* 2006; **20**: 896-905 [PMID: 16675847 DOI: 10.1096/fj.05-5465com]

107 **Winkler-Pickett R**, Young HA, Cherry JM, Diehl J, Wine J, Back T, Bere WE, Mason AT, Ortaldo JR. In vivo regulation of experimental autoimmune encephalomyelitis by NK cells: alteration of primary adaptive responses. *J Immunol* 2008; **180**: 4495-4506 [PMID: 18354171 DOI: 10.4049/jimmunol.180.7.4495]

108 **Vollmer TL**, Liu R, Price M, Rhodes S, La Cava A, Shi FD. Differential effects of IL-21 during initiation and progression of autoimmunity against neuroantigen. *J Immunol* 2005; **174**: 2696-2701 [PMID: 15728477 DOI: 10.4049/jimmunol.174.5.2696]

109 **Benczur M**, Petrányl GG, Pálffy G, Varga M, Tálas M, Kotsy B, Földes I, Hollán SR. Dysfunction of natural killer cells in multiple sclerosis: a possible pathogenetic factor. *Clin Exp Immunol* 1980; **39**: 657-662 [PMID: 6155232]

110 **Kastrukoff LF**, Morgan NG, Zecchini D, White R, Petkau AJ, Satoh J, Paty DW. A role for natural killer cells in the immunopathogenesis of multiple sclerosis. *J Neuroimmunol* 1998; **86**: 123-133 [PMID: 9663557 DOI: 10.1016/S0165-5728(98)00014-9]

111 **Grunebaum E**, Malatzky-Goshen E, Shoenfeld Y. Natural killer cells and autoimmunity. *Immunol Res* 1989; **8**: 292-304 [PMID: 2687403 DOI: 10.1007/BF02935514]

112 **De Jager PL**, Rossin E, Pyne S, Tamayo P, Ottoboni L, Viglietta V, Weiner M, Soler D, Izmailova E, Faron-Yowe L, O'Brien C, Freeman S, Granados S, Parker A, Roubenoff R, Mesirov JP, Khoury SJ, Hafler DA, Weiner HL. Cytometric profiling in multiple sclerosis uncovers patient population structure and a reduction of CD8low cells. *Brain* 2008; **131**: 1701-1711 [PMID: 18567923 DOI: 10.1093/brain/awn118]

113 **Sand KL**, Knudsen E, Rolin J, Al-Falahi Y, Maghazachi AA. Modulation of natural killer cell cytotoxicity and cytokine release by the drug glatiramer acetate. *Cell Mol Life Sci* 2009; **66**: 1446-1456 [PMID: 19277466 DOI: 10.1007/s00018-009-8726-1]

114 **Al-Falahi Y**, Sand KL, Knudsen E, Damaj BB, Rolin J, Maghazachi AA. Splenic natural killer cell activity in two models of experimental neurodegenerative diseases. *J Cell Mol Med* 2009; **13**: 2693-2703 [PMID: 19397784 DOI: 10.1111/j.1582-4934.2008.00640]

115 **Høglund RA**, Holmøy T, Harbo HF, Maghazachi AA. A one year follow-up study of natural killer and dendritic cells activities in multiple sclerosis patients receiving glatiramer acetate (GA). *PLoS One* 2013; **8**: e62237 [PMID: 23614042 DOI: 10.1371/journal.pone.0062237]

116 **Cooper MA**, Yokoyama WM. Memory-like responses of natural killer cells. *Immunol Rev* 2010; **235**: 297-305 [PMID: 20536571 DOI: 10.1111/j.0105-2896.2010.00891]

117 **Zlotnik A**, Yoshie O. The Chemokine Superfamily Revisited. *Immunity* 2012; **36**: 705-716 [PMID: 22633458 DOI: 10.1016/j.immuni.2012.05.008]

118 **Sallusto F**, Lanzavecchia A, Mackay CR. Chemokines and chemokine receptors in T-cell priming and Th1/Th2 mediated responses. *Immunol Today* 2008; **19**: 568-574 [PMID: 9864948 DOI: 10.1016/S0167-5699(98)01346-2]

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OBSERVATIONAL STUDY

Oxidative stress and labile plasmatic iron in anemic patients following blood therapy

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Abstract

AIM: To determine the plasmatic iron content and evaluate the oxidative stress (OS) markers in subjects receiving blood therapy.

METHODS: Thirty-nine individuals with unspecified anemia receiving blood transfusions and 15 healthy subjects were included in the study. Anemic subjects were divided into three subgrouP: (1) those that received up to five blood transfusions (n = 14); (2) those that received from five to ten transfusions (n = 11); and (3) those that received more than ten transfusions (n = 14). Blood samples were collected by venous arm puncture and stored in tubes containing heparin. The plasma and cells were separated by centrifugation and subsequently used for analyses. Statistical analyses were performed using Kruskal-Wallis analysis of variance followed by Dunn's multiple comparison tests when appropriate.

RESULTS: The eletrophoretic hemoglobin profiles of the subjects included in this study indicated that no patients presented with hemoglobinopathy. Labile plasmatic iron, ferritin, protein carbonyl, thiobarbituric acid-reactive substances (TBARS) and dichlorofluorescein diacetate oxidation were significantly higher ($P < 0.05$), whereas total thiol levels were significantly lower ($P < 0.05$) in transfused subjects compared to controls. Additionally, the activity of catalase, superoxide dismutase and glutathione peroxidase were significantly lower in the transfused subjects ($P < 0.05$). Antioxidant enzyme activities and total thiol levels were positively correlated ($P < 0.05$), and negatively correlated with the levels of protein carbonyl and TBARS ($P < 0.05$). In contrast, protein carbonyl and TBARS were positively correlated ($P < 0.05$). Altogether, these data confirm the involvement of OS in patients following therapy with repeated blood transfusions.

CONCLUSION: Our data reveal that changes in OS markers are correlated with levels of labile plasmatic iron and ferritin and the number of transfusions.

Key words: Antioxidant enzymes; Labile iron content;

Oxidative stress; Polytransfused subjects

Core tip: Here, the readers will find important information regarding iron accumulation and its correlation with oxidative damage markers in anemic subjects following blood therapy. This research, regarding iron accumulation and its associated toxicology is remarkable because the mechanism(s) involved in its mode of action are not fully understood. Thus, our data are extremely important for research concerning the involvement of iron overload on the development of human diseases.

Fernandes MS, Rissi TT, Zuravski L, Mezzomo J, Vargas CR, Folmer V, Soares FAA, Manfredini V, Ahmed M, Puntel RL. Oxidative stress and labile plasmatic iron in anemic patients following blood therapy. *World J Exp Med* 2014; 4(3): 38-45 Available from: URL: http://www.wjgnet.com/2220-315X/full/v4/i3/38.htm DOI: http://dx.doi.org/10.5493/wjem.v4.i3.38

INTRODUCTION

Iron is an essential element of cells that participates in various cellular processes due to its ability to accept and donate electrons, interconverting between Fe^{3+} and Fe^{2+} forms[1]. However, this redox property renders iron potentially toxic in biologic systems. The labile plasmatic iron (LPI) component of non-transferrin-bound iron is redox-active, chelatable and capable of permeating into organs to induce tissue iron overload[2]. Thus, LPI is an accessible diagnostic marker of iron overload and cell toxicity[2]. Moreover, LPI can participate in the Fenton reaction and generate a large amount of reactive oxygen species (ROS)[3]. To prevent ROS overproduction, circulating and intracellular free iron are tightly regulated by binding to transferrin, ferritin and other proteins[4,5]. However, the iron balance can be disrupted in some situations, such as with chronic anemia, repeated blood transfusions, and following increased gastrointestinal absorption, which lead to iron overload[6]. Therefore, subjects undergoing repeated blood transfusions are at risk of iron-associated toxicity[7].

Elevated tissue iron can overwhelm protective mechanisms and lead to an increase in iron complexes with small molecules, such as nucleotides and citrate, in the serum of transfusion patients and also within cytoplasm and organelles[8,9]. Furthermore, repeated blood transfusions increase the levels of iron available to generate catalytically active complexes, free radicals and oxidative damage[9]. Thus, LPI promotes free radical formation that culminates in the oxidation of biomolecules. Accordingly, iron overload in humans and in experimental animals is associated with oxidative stress (OS)[10]. Indeed, it is known that an imbalance in the oxidant/antioxidant status of the cell is associated with OS, leading to important cellular macromolecule modifications and cell damage[11]. The cell injury observed in patients with iron overload is attributed to OS[12]. Hence, the oxidation reactions result in the formation of lipid peroxides and protein carbonyls, damaged deoxyribonucleic acid bases, and mitochondrial dysfunction[13]. Additionally, individuals with an iron overload demonstrate impaired antioxidant defenses[6]. Accordingly, the long-term consequence of chronic iron overload is organ injury, which could contribute to the initiation and development of several metabolic disorders, such as endocrinopathies, diabetes mellitus, cirrhosis, hypogonadism and heart failure[14].

In general, oxidative damage of biomolecules can be counteracted by enzymatic as well as non-enzymatic defenses. Indeed, humans have several biologic mechanisms to defend against intracellular OS. One of the most important mechanisms involves the actions of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx)[15]. In spite of a well-developed antioxidant defense system, cells can still be oxidatively damaged under some pathologic conditions[11].

Data concerning labile iron accumulation in anemic subjects receiving repeated blood transfusions and the association with oxidative damage markers are scarce in the literature. We hypothesize that OS correlates with LPI in anemic patients following therapy with repeated blood transfusions. In this study, we evaluated OS markers and the activity of enzymatic antioxidant defenses in the blood of patients receiving repeated transfusions and in control subjects (not transfused). Additionally, we determined the LPI and ferritin levels in these subjects and correlated both parameters with other evaluated markers.

MATERIALS AND METHODS

Chemicals

1,1,3,3-tetramethoxypropane, 2-thiobarbituric acid, sodium dodecyl sulfate, 5,5'-dithiobis-(2-nitrobenzoic acid), trichloroacetic acid, 2',7'-dichlorodihydrofluorescein diacetate (DCHF-DA), and 2,4-dinitrophenylhydrazine, were purchased from Sigma (St. Louis, MO, United States). The kit for iron determination was obtained from Bio-Systems Corp. (Beloit, WI, United States), kits for measuring SOD (RANSOD) and GPx (RANSEL) were purchased from Randox Laboratories Ltf. (Crumlin, United Kingdom), and the Total Protein kit for protein determination was obtained from BioClin (Delft, Netherlands). All the other chemicals were commercial products of the highest purity grade available.

Subjects

This study was approved by the Ethics Committee in Research of Universidade Federal do Pampa. Altogether, 39 individuals with unspecified anemia receiving blood transfusions and 15 healthy subjects (blood donors) from the Banco de Sangue do Municipio de Uruguaiana were included in the study. Since most of our patients were male, the female patients were excluded from this study. Thus, both anemic and control healthy individuals were male. Anemic individuals were included in the study if they were diagnosed according to the International

Classification of Diseases (anemia unspecified, ICD 10: D64.9), and were not diagnosed with other diseases, such as cancer, renal failure, hepatic disease, blood loss or others. Additionally, anemic patients had received blood therapy during the year prior to collection (*i.e.*, no more than 12 mo from the first transfusion until sample collection). Additionally, it is important to mention here that the sample collection was done before a new transfusion, namely clinical screening. The anemic subjects were divided into three subgrouP: (1) those that received less than five blood transfusions (n = 14); (2) those that received from five to ten blood transfusions (n = 11); and (3) those that received more than ten blood transfusions (n = 14).

Sample collection

Blood from controls and anemic subjects was collected by venous arm puncture and stored in tubes containing heparin. The plasma and cells were separated by centrifugation at 1500 r/min for 10 min and were subsequently used for biochemical analyses. All biochemical assays were done in duplicate or triplicate, depending on availability of samples.

Analysis of hemoglobin

The electrophoretic analysis of hemoglobin was performed using a Minicap system (Sebia, Norcross, France) according to the manufacturer's instructions, and controls were run with each test. The Minicap system uses the principle of capillary electrophoresis in free solution. Charged molecules are separated by their electrophoretic mobility in an alkaline buffer with a specific pH. Separation also occurs according to the electrolyte pH and electro-osmotic flow. Electropherograms were expressed with zones divided from Z1 to Z15 based on standardizing the location of hemoglobin as previously described[16].

Measurement of LPI

LPI refers to non-heme bound, non-ferritin bound and non-transferrin-bound iron (*i.e.*, free iron) according to the previously validated convention[17]. The LPI content was determined by its reactivity with ferrozine, in the presence of the denaturant sodium dodecyl sulfate and the reducing agents ascorbate and sodium metabisulphite, as previously described[18,19]. The results are expressed as μg/dL.

Ferritin

Ferritin content was determined as described by Bernard and Lauwerys[20]. Serum ferritin causes agglutination of latex particles coated with anti-human ferritin that is proportional to the concentration of ferritin and can be measured by turbidimetry. The results are expressed as μg/L ferritin.

Protein carbonyl determination

Protein carbonyl content, which is indicative of oxidation, was determined as described by Levine *et al*[21]. Plasma samples were added to 0.2 mL of 10% trichloroacetic acid and placed on ice for 5 min. After centrifugation (5 min), samples were incubated for 90 min at 37 ℃ with 1 mL of 10 mmol/L 2,4-dinitrophenylhydrazine in 2 mol/L HCl. Finally, proteins were dissolved in 6 mol/L guanidine and interference was removed after washing with ethanol-ethyl acetate 1:1 (v/v). The extent of the damage was estimated by reading absorbance at 370 nm. The results are expressed as nmol carbonyl/mg protein.

Determination of thiobarbituric acid reactive substances levels

Levels of thiobarbituric acid reactive substances (TBARS) in plasma were determined using the method described by Ohkawa *et al*[22]. In brief, samples were incubated in acidic medium containing 0.45% sodium dodecyl sulfate and 0.6% thiobarbituric acid at 100°C for 60 min. After centrifugation, the reaction product was determined at 532 nm using a 1,1,3,3-tetramethoxypropaneas standard and the results are expressed as nmol malondialdehyde/mg protein.

Total thiol determination

Plasmatic total thiol was determined as described by Ellman *et al*[23]. The colorimetric assay was carried out in 1 mol/L phosphate buffer (pH 7.4) and calculated against a standard curve constructed with glutathione. Total thiol content is expressed as nmol total thiol/mg protein.

Determination of DCHF-DA oxidation

The determination of intracellular oxidant production was based on the cleavage of DCHF-DA to DCHF, which fluoresces when oxidized by ROS according to previously described methods[24]. The plasma sample was diluted (1:10) in 10 mmol/L Tris-HCl buffer. Then, 50 μL of diluted plasma was incubated with 10 μmol/L DCHF-DA at 37 ℃ for 20 min. The fluorescence emission at 520 nm was measured using a Perkin-Elmer spectrofluorometer with an excitation wavelength of 488 nm and an emission wavelength of 520 nm. The results are expressed as arbitrary fluorescence units.

CAT activity

CAT activity was measured by the method previously described[25]. Packed erythrocytes were hemolyzed by adding 100 volumes of distilled water, then 20 μL of this hemolyzed sample was added to a cuvette and the reaction was started by the addition of 100 μL of freshly prepared 300 mmol/L H_2O_2 in phosphate buffer (50 mmol/L, pH 7.0) to give a final volume of 1 mL. The rate of H_2O_2 decomposition was measured by a spectrophotometer at 240 nm for a duration of 2 min. The CAT activity is expressed as UI/mg protein.

SOD activity

SOD activity was measured in erythrocytes using a RANSOD kit, which uses xanthine and xanthine

Table 1 Subject characteristics

Characteristics	Controls (n = 15)	Transfusions		
		< 5 (n = 14)	5-10 (n = 11)	> 10 (n = 14)
Age (yr)	40.1 (20–50)	62.8 (24–92)	64.8 (49–84)	57.5 (24–74)
Number of transfusions	0 (0)	3.20 (2–4)	7.17 (5–9)	18.78 (14–26)
Hemoglobin (g/dL)	13.8 ± 0.5	7.5 ± 2.1	6.75 ± 0.5	4.9 ± 0.9
Labile iron content (μg/dL)	108.9 ± 13.8	149.2 ± 45.1	216.2 ± 68.3[a]	366.9 ± 68.5[a]
Ferritin (μg/L)	219.6 ± 18.2	190.3 ± 11.7	221.2 ± 16.1	277.5 ± 27.5[a]

Values are presented as median (range), or mean ± standard deviation; [a]P < 0.05 *vs* control.

oxidase to produce superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazol chloride to form formazan red. The SOD activity was measured by the degree of inhibition of this reaction at 505 nm and is expressed as UI/mg protein.

GPx activity

GPx activity was determined in erythrocytes using the RANSEL kit according to the method previously described[26]. The GPx activity is expressed as UI/mg protein.

Protein determination

The protein content was determined by the biuret method using the Total Protein kit with bovine serum albumin as a standard. The copper ions in an alkaline medium (biuret reagent) react with peptide, producing a purple color, whose intensity is proportional to the concentration of proteins in the samples being measured in a spectrophotometer at 545 nm.

Statistical analysis

All results are reported as median (range) and presented as box-plot graphics for the different grouP of patients. Hemoglobin, LPI and ferritin levels are presented as mean ± SD deviation. A Shapiro-Wilk test was performed to assess the normality of data distributions, and Kruskal-Wallis analysis of variance followed by Dunn's multiple comparison tests were used when appropriate. Spearman's correlational analyses were also performed between variables. For all analyses, we used a GraphPad Prism 5.0 software, and a $P < 0.05$ was considered significant.

RESULTS

Patient characteristics are presented in Table 1. Electrophoretic analyses indicated that all subjects had normal hemoglobin profiles (data not shown). As expected, LPI and ferritin levels were higher in the transfused subjects. Specifically, subjects receiving five or more transfusions had significantly higher LPI levels ($P < 0.05$), and patients receiving more than ten transfusions had significantly higher ferritin levels ($P < 0.05$) compared to controls. Additionally, we found that the number of transfusions was significantly correlated with LPI and ferritin levels ($P < 0.05$) (Table 2).

The OS markers TBARS (Figure 1A), protein carbonyl (Figure 1B) and DCFH-DA oxidation (Figure 1C) were all significantly higher in transfused subjects compared to the control group ($P < 0.05$). However, total thiol levels were significantly lower in subjects receiving more than ten transfusions compared to controls ($P < 0.05$) (Figure 1D).

The activity CAT and GPx were significantly lower in subjects receiving five or more transfusions compared to controls ($P < 0.05$) (Figure 2A and C). SOD activity was significantly lower in subjects receiving more than ten transfusions compared with controls ($P < 0.05$) (Figure 2B). Furthermore, significant negative correlations were observed between the number of transfusions and the activity of these antioxidant enzymes ($P < 0.05$) (Table 2).

Additional correlations were found between LPI levels and OS markers ($P < 0.05$), with the exception of DCDH-DA oxidation (Table 2). LPI and ferritin were negatively correlated with antioxidant enzyme activities and total thiol, and positively correlated with carbonyl and TBARS levels (all $P < 0.05$). Indeed, it was found that antioxidant enzyme activities were positively correlated with total thiol levels, and negatively correlated with the levels of protein carbonyl and TBARS ($P < 0.05$). In contrast, protein carbonyl and TBARS levels were positively correlated ($P < 0.05$).

DISCUSSION

Our data are in accordance with a previous study showing that the increase in LPI content could lead to an increase in ROS generation, and consequently an increase in oxidative damage[12]. Additionally, based on data concerning hemoglobin profile, we discarded hemoglobin disorders in these individuals. These data are extremely important to avoid misinterpretations, as it was previously shown that any imbalance between α and β chains of hemoglobin (α or β-thalassemia, respectively) plays a crucial role in OS[27]. Besides, there are data linking the observed levels of the various biomarkers evaluated in this study to health outcomes, such as in renal failure[28] and breast cancer[29].

Taking into account our results and those previously found, it is plausible to assume that under blood transfusion therapy, the excess of labile (catalytically active) iron must generate free radicals *via* Fenton chemistry, resulting in oxidative damage to biomolecules *in vivo*[30]. Our assumption is further supported by a previous report showing that iron-catalyzed ROS generation leads to an increase in the genomic instability in hematopoietic progenitor cells[31]. Moreover, it was shown in animal models that iron overload causes liver damage *via* both oxidative and nitrosative mechanisms[32]. Indeed, we assume that under repeated blood transfusions, the iron content increases to values that overwhelm the protective mechanisms, leading to an increase in the amount of iron available to form complexes with small molecules, the

Table 2 Spearman's correlations between biochemical and oxidative markers in polytransfused subjects

	LPI	Ferritin	GPx	SOD	CAT	TBARS	DCHF-DA oxidation	Carbonyl	Total thiol
Transfusion number	0.8569[b]	0.7991[b]	-0.8796[b]	-0.7103[b]	-0.8143[b]	0.5114[b]	0.0111	0.5793[b]	-0.5555[b]
Total thiol	-0.4151[b]	-0.3354[b]	0.4830[b]	0.4849[b]	0.5401[b]	-0.2790[b]	0.0106	-0.1164	-
Carbonyl	0.5583[b]	0.5122[b]	-0.5613[b]	-0.3713[b]	-0.4862[b]	0.5208[b]	-0.0627	-	-
DCHF-DA oxidation	-0.1291	-0.0049	0.0370	-0.0446	-0.0401	-0.2293[a]	-	-	-
TBARS	0.4984[b]	0.4144[b]	-0.4638[b]	-0.3113[b]	-0.3457[b]	-	-	-	-
CAT	-0.7266[b]	-0.5944[b]	0.8945[b]	0.7251[b]	-	-	-	-	-
SOD	-0.6085[b]	-0.5744[b]	0.7443[b]	-	-	-	-	-	-
GPx	-0.7973[b]	-0.7144[b]	-	-	-	-	-	-	-
Ferritin	0.9112[b]	-	-	-	-	-	-	-	-

[a]$P < 0.05$, [b]$P < 0.001$ *vs* DCHF-DA oxidation. CAT: Catalase; DCHF-DA: 2',7'-dichlorodihydrofluorescein diacetate; GPx: Glutathione peroxidase; LPI: Labile plasmatic iron; SOD: Superoxide dismutase; TBARS: Thiobarbituric acid-reactive substances.

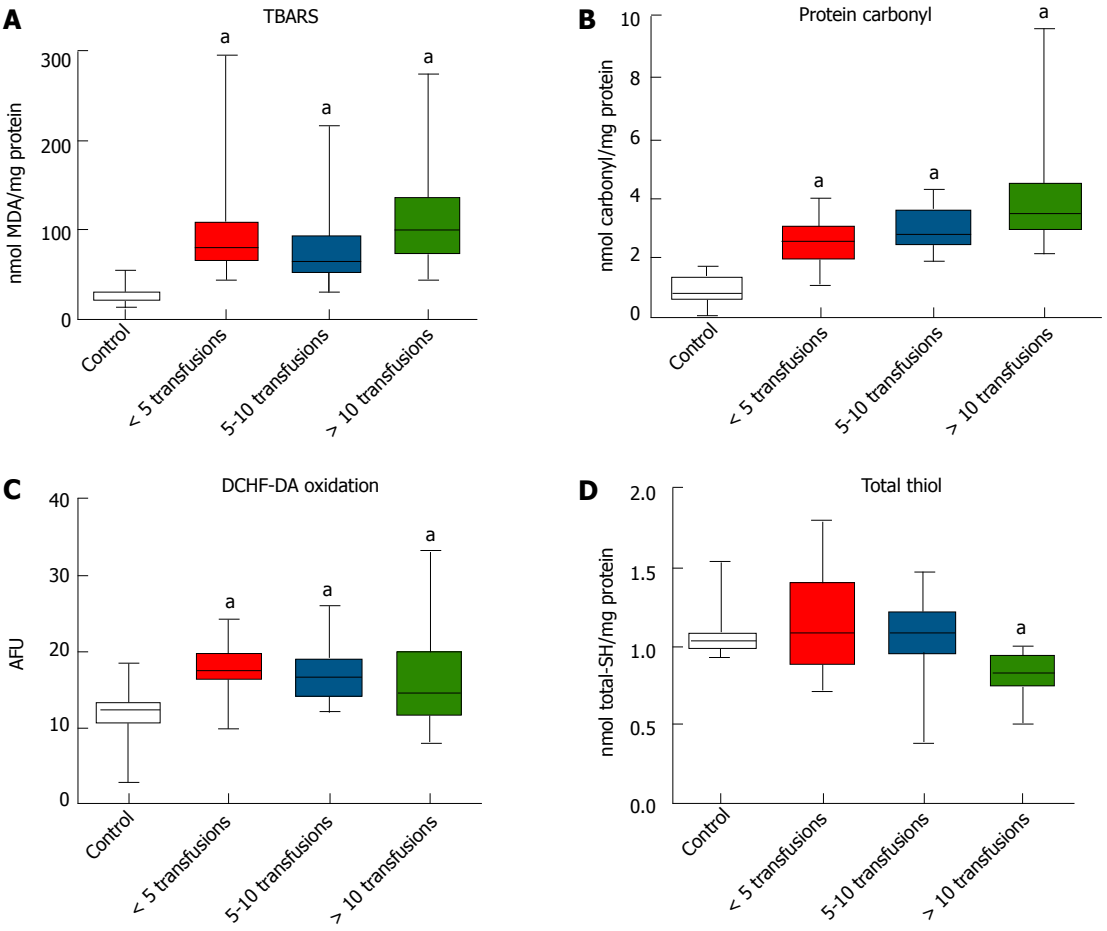

Figure 1 Oxidative stress markers in transfusion patients. A: Thiobarbituric acid-reactive substances (TBARS); B: Protein carbonyl; C: 2',7'-dichlorodihydrofluorescein diacetate (DCHF-DA) oxidation; and D: Total thiol levels in controls (n = 15), those receiving < 5 transfusions (n = 14), 5-10 transfusions (n = 11), and > 10 transfusions (n = 14); [a]$P < 0.05$ *vs* controls.

"catalytically active iron complexes". Thus, we assume that the ROS generated are responsible for the oxidation of DCHF-DA found in the transfused subjects, which is supported by a previous report showing that overload with iron (ferric nitrilotriacetate) leads to an increase in DCHF-DA oxidation in cultured rat hepatocytes[33].

Interestingly, we found some changes in the OS parameters even in the absence of significant iron accumulation, suggesting that alterations in OS markers could precede iron accumulation in patients following blood therapy. Accordingly, it seems logical that the differences in other parameters, such as hemoglobin and ferritin levels, could potentially contribute to the different oxidative state among patients. Thus, it is difficult to affirm that iron alone is the primary factor responsible for these differences. This point is extremely relevant and deserves further attention in future investigations.

The results of this study also show that levels of

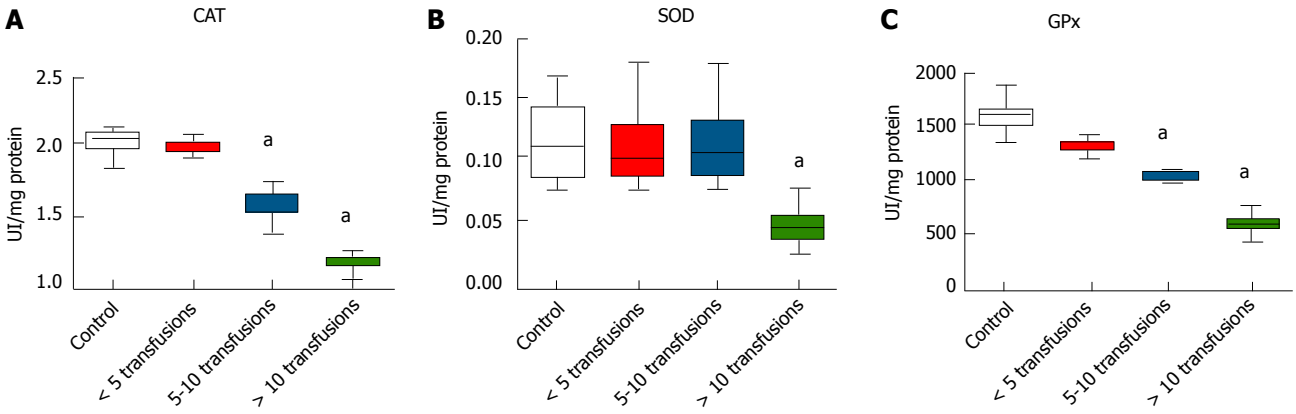

Figure 2 Antioxidant enzymes in transfusion patients. A: Catalase (CAT) activity; B: Superoxide dismutase (SOD) activity; and C: Glutathione peroxidase (GPx) activity in controls (n = 15), those receiving < 5 transfusions (n = 14), 5-10 transfusions (n = 11), and > 10 transfusions (n = 14); $^{a}P < 0.05$ *vs* controls.

TBARS significantly increased in subjects receiving blood transfusions, which was positively correlated to LPI content, ferritin content and the number of transfusions. These findings are in accordance to previous reports showing that the levels of lipid peroxidation products were increased in β-thalassaemic patients receiving blood transfusions[19] and in subjects with hepatic iron overload[6]. Moreover, we found a significant increase in the protein carbonyl in the subjects receiving repeated blood transfusions, which was correlated with LPI content. Additionally, our data are in accordance to a previous paper showing a significant increase in the protein carbonyl content associated with iron overload[33].

A significant reduction in total thiol levels was found in the subjects receiving repeated blood transfusions, which is consistent with a report showing a decrease in thiol content in the liver of rats treated with iron[34]. Albeit not completely understood, we believe that thiols are oxidized (consumed/used) in these subjects due to OS status following iron overload. Another possibility is that the iron could react non-enzymatically with thiols in plasma to generate ROS, which directly leads to reduction of antioxidant capacity in plasma and the increased susceptibility of blood components to oxidation[35]. Thus, this thiol-dependent free radical generation by iron overload might be a potential contributing factor for the changes in the oxidative markers reported here. Our assumptions are supported by a study showing that oxygen radicals can be produced by iron-catalyzed auto-oxidation of cysteine or glutathione[36]. Therefore, the generated ROS (either by Fenton chemistry or by iron-catalyzed auto-oxidation of thiols) may be responsible for the oxidation of other biomolecules reported here, such as lipids and proteins.

The results of the present study demonstrate a marked decrease in antioxidant enzyme activity in the subjects with iron overload, which is consistent with previous reports[30,33]. Moreover, enzyme activities were negatively correlated with LPI, TBARS and protein carbonyl levels. In line with this, we presume that the decrease in the enzymatic activity of antioxidants further contributed to the OS condition. Indeed, Chakraborty *et al*[19] showed that the decrease in antioxidant enzymes strongly contributes to an increase in OS markers (TBARS, protein carbonyl and ROS). Although our data do not support this supposition, we hypothesize that a decrease in the antioxidant enzymes reported here could, at least in part, be due to a decrease in their expression. Indeed, it was previously shown that both CAT and GPx were downregulated under OS conditions in human cells[37]. However, the mechanisms regulating the expression of antioxidant enzymes under iron overload remain to be explored in more detail.

Our data confirms the involvement of OS in patients following therapy with repeated blood transfusions. Additionally, we found that the changes in the OS markers are correlated with iron content, ferritin and the number of transfusions. Thus, iron chelators that efficiently decrease the levels of labile iron are candidates to counteract the iron-induced ROS generation[38]. However, more studies are necessary to better understand the mechanism(s) associated with iron-induced oxidative changes, to minimize the side effects associated to blood transfusion therapy, and to provide some clinical benefits. As antioxidant supplementation is not entirely safe and may cause unfavorable effects to different patients, more discussion on its potential benefits is warranted[39].

In conclusion, our data confirm the involvement of OS and its correlation with LPI and ferritin in unspecified anemic patients following therapy with repeated blood transfusions. However, we found some alterations of OS markers even in the absence of significant iron accumulation, which encourages us to further explore the changes in the OS parameters that occur before iron overload in subjects receiving blood therapy.

COMMENTS

Background

Iron is an essential element that participates in several metabolic activities of cells. However, in excess, iron can be a cause of oxidative stress (OS) in subjects undergoing blood transfusion therapy. Despite this, the relationship between plasmatic iron content, OS markers and the activity of antioxidant enzymes in anemic subjects receiving repeated blood transfusions remains to be better characterized.

Research frontiers

Blood therapy has been used in medical practice to treat anemic patients. However, the increase in the iron level in patients following blood therapy must be considered. Thus, the purpose of this research was to better understand the changes associated with OS markers in patients undergoing blood therapy in order to prevent iron-supported oxidative damage in anemic subjects.

Innovations and breakthroughs

Previous data have shown that blood therapy is associated with iron overload, and consequently, with oxidative changes in various tissues. However, efficient therapies to prevent the side effects associated with repeated blood transfusions are not known. Thus, elucidative studies regarding the plasmatic oxidative changes associated with iron overload are necessary. Here, the authors found that anemic subjects undergoing transfusions show increased levels of plasmatic labile iron, protein carbonyl, thiobarbituric acid reactive substances, and 2',7'-dichlorodihydrofluorescein diacetate oxidation, as well as decreased total thiol levels. Additionally, the activities of superoxide dismutase, catalase, and glutathione peroxidase were significantly lower in the transfused subjects. Significant correlations were found between the number of transfusions, plasmatic iron content, OS markers and the activity of the antioxidant enzymes.

Applications

The results of this study suggest that antioxidants could be associated with blood therapy. Additionally, iron chelators that efficiently decrease the levels of labile iron could be used to counteract the iron-induced generation of reactive oxygen species. However, more studies are necessary to better understand the mechanism(s) associated with iron-induced oxidative changes in order to minimize the side effects associated with blood transfusion therapy and to provide clinical benefits.

Peer review

This is a study that contains important information regarding iron accumulation in anemic subjects receiving repeated blood transfusions and its correlation with the plasmatic oxidative damage markers in these subjects.

REFERENCES

1 **Premkumar K**, Min K, Alkan Z, Hawkes WC, Ebeler S, Bowlus CL. The potentiating and protective effects of ascorbate on oxidative stress depend upon the concentration of dietary iron fed C3H mice. *J Nutr Biochem* 2007; **18**: 272-278 [PMID: 16860981 DOI: 10.1016/j.jnutbio.2006.05.004]

2 **Cabantchik ZI**, Breuer W, Zanninelli G, Cianciulli P. LPI-labile plasma iron in iron overload. *Best Pract Res Clin Haematol* 2005; **18**: 277-287 [PMID: 15737890 DOI: 10.1016/j.beha.2004.10.003]

3 **Orino K**, Lehman L, Tsuji Y, Ayaki H, Torti SV, Torti FM. Ferritin and the response to oxidative stress. *Biochem J* 2001; **357**: 241-247 [PMID: 11415455]

4 **Young IS**, Woodside JV. Antioxidants in health and disease. *J Clin Pathol* 2001; **54**: 176-186 [PMID: 11253127 DOI: 10.1136/jcp.54.3.176]

5 **Hershko C**, Graham G, Bates GW, Rachmilewitz EA. Non-specific serum iron in thalassaemia: an abnormal serum iron fraction of potential toxicity. *Br J Haematol* 1978; **40**: 255-263 [PMID: 708645 DOI: 10.1111/j.1365-2141.1978.tb03662.x]

6 **Walter PB**, Fung EB, Killilea DW, Jiang Q, Hudes M, Madden J, Porter J, Evans P, Vichinsky E, Harmatz P. Oxidative stress and inflammation in iron-overloaded patients with beta-thalassaemia or sickle cell disease. *Br J Haematol* 2006; **135**: 254-263 [PMID: 17010049 DOI: 10.1111/j.1365-2141.2006.06277.x]

7 **Lambing A**, Kachalsky E, Mueller ML. The dangers of iron overload: bring in the iron police. *J Am Acad Nurse Pract* 2012; **24**: 175-183 [PMID: 22486832 DOI: 10.1111/j.1745-7599.2011.00680.x]

8 **Zanninelli G**, Loréal O, Brissot P, Konijn AM, Slotki IN, Hider RC, Ioav Cabantchik Z. The labile iron pool of hepatocytes in chronic and acute iron overload and chelator-induced iron deprivation. *J Hepatol* 2002; **36**: 39-46 [PMID: 11804662 DOI: 10.1016/S0168-8278(01)00222-7]

9 **Gutteridge JM**, Rowley DA, Griffiths E, Halliwell B. Low-molecular-weight iron complexes and oxygen radical reactions in idiopathic haemochromatosis. *Clin Sci* (Lond) 1985; **68**: 463-467 [PMID: 2578915]

10 **Sinha S**, Saxena R. Effect of iron on lipid peroxidation, and enzymatic and non-enzymatic antioxidants and bacoside-A content in medicinal plant Bacopa monnieri L. *Chemosphere* 2006; **62**: 1340-1350 [PMID: 16219336 DOI: 10.1016/j.chemosphere.2005.07.030]

11 **Domanski AV**, LaPhina EA, Zavodnik IB. Oxidative processes induced by tert-butyl hydroperoxide in human red blood cells: chemiluminescence studies. *Biochemistry* (Mosc) 2005; **70**: 761-769 [PMID: 16097939 DOI: 10.1007/s10541-005-0181-5]

12 **Gattermann N**, Rachmilewitz EA. Iron overload in MDS-pathophysiology, diagnosis, and complications. *Ann Hematol* 2011; **90**: 1-10 [PMID: 20938663 DOI: 10.1007/s00277-010-1091-1]

13 **Welch KD**, Davis TZ, Van Eden ME, Aust SD. Deleterious iron-mediated oxidation of biomolecules. *Free Radic Biol Med* 2002; **32**: 577-583 [PMID: 11909692 DOI: 10.1016/S0891-5849(02)00760-8]

14 **Cunningham MJ**, Macklin EA, Neufeld EJ, Cohen AR. Complications of beta-thalassemia major in North America. *Blood* 2004; **104**: 34-39 [PMID: 14988152]

15 **Scott MD**. H2O2 injury in beta thalassemic erythrocytes: protective role of catalase and the prooxidant effects of GSH. *Free Radic Biol Med* 2006; **40**: 1264-1272 [PMID: 16545695 DOI: 10.1016/j.freeradbiomed.2005.11.017]

16 **Kim JE**, Kim BR, Woo KS, Kim JM, Park JI, Han JY. Comparison of capillary electrophoresis with cellulose acetate electrophoresis for the screening of hemoglobinopathies. *Korean J Lab Med* 2011; **31**: 238-243 [PMID: 22016676 DOI: 10.3343/kjlm.2011.31.4.238]

17 **Kuross SA**, Hebbel RP. Nonheme iron in sickle erythrocyte membranes: association with phospholipids and potential role in lipid peroxidation. *Blood* 1988; **72**: 1278-1285 [PMID: 3167208]

18 **Repka T**, Shalev O, Reddy R, Yuan J, Abrahamov A, Rachmilewitz EA, Low P, Hebbel RP. Nonrandom association of free iron with membranes of sickle and beta-thalassemic erythrocytes. *Blood* 1993; **82**: 3204-3210 [PMID: 8219209]

19 **Chakraborty D**, Bhattacharyya M. Antioxidant defense status of red blood cells of patients with beta-thalassemia and Ebeta-thalassemia. *Clin Chim Acta* 2001; **305**: 123-129 [PMID: 11249931 DOI: 10.1016/S0009-8981(00)00428-9]

20 **Bernard A**, Lauwerys R. Turbidimetric latex immunoassay for serum ferritin. *J Immunol Methods* 1984; **71**: 141-147 [PMID: 6736656 DOI: 10.1016/0022-1759(84)90060-7]

21 **Levine RL**, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahn BW, Shaltiel S, Stadtman ER. Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol* 1990; **186**: 464-478 [PMID: 1978225 DOI: 10.1016/0076-6879(90)86141-H]

22 **Ohkawa H**, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979; **95**: 351-358 [PMID: 36810 DOI: 10.1016/0003-2697(79)90738-3]

23 **Ellman G**, Lysko H. A precise method for the determination of whole blood and plasma sulfhydryl grouP. *Anal Biochem* 1979; **93**: 98-102 [PMID: 434474 DOI: 10.1016/S0003-2697(79)80122-0]

24 **Karja NW**, Kikuchi K, Fahrudin M, Ozawa M, Somfai T, Ohnuma K, Noguchi J, Kaneko H, Nagai T. Development to the blastocyst stage, the oxidative state, and the quality of early developmental stage of porcine embryos cultured in alteration of glucose concentrations in vitro under different oxygen tensions. *Reprod Biol Endocrinol* 2006; **4**: 54 [PMID: 17087833 DOI: 10.1186/1477-7827-4-54]

25 **Aebi H**. Catalase in vitro. *Methods Enzymol* 1984; **105**: 121-126 [PMID: 6727660 DOI: 10.1016/S0076-6879(84)05016-3]

26 **Paglia DE**, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Clin Med* 1967; **70**: 158-169 [PMID: 6066618]

27 **Manca L**, Masala B. Disorders of the synthesis of human fetal hemoglobin. *IUBMB Life* 2008; **60**: 94-111 [PMID: 18379999 DOI: 10.1002/iub.4]

28 **Bartnicki P**, Fijałkowski P, Majczyk M, Błaszczyk J, Banach M, Rysz J. Effect of methoxy polyethylene glycol-epoetin beta on oxidative stress in predialysis patients with chronic kidney disease. *Med Sci Monit* 2013; **19**: 954-959 [PMID: 24201565 DOI: 10.12659/MSM.884024]

29 **Panis C**, Herrera AC, Victorino VJ, Campos FC, Freitas LF, De Rossi T, Colado Simão AN, Cecchini AL, Cecchini R. Oxidative stress and hematological profiles of advanced breast cancer patients subjected to paclitaxel or doxorubicin chemotherapy. *Breast Cancer Res Treat* 2012; **133**: 89-97 [PMID: 21811816 DOI: 10.1007/s10549-011-1693-x]

30 **Zhang Y**, Huang Y, Deng X, Xu Y, Gao Z, Li H. Iron overload-induced rat liver injury: Involvement of protein tyrosine nitration and the effect of baicalin. *Eur J Pharmacol* 2012; **680**: 95-101 [PMID: 22306240 DOI: 10.1016/j.ejphar.2012.01.010]

31 **Naka K**, Muraguchi T, Hoshii T, Hirao A. Regulation of reactive oxygen species and genomic stability in hematopoietic stem cells. *Antioxid Redox Signal* 2008; **10**: 1883-1894 [PMID: 18627347 DOI: 10.1089/ars.2008.2114]

32 **Toyokuni S**. Iron as a target of chemoprevention for longevity in humans. *Free Radic Res* 2011; **45**: 906-917 [PMID: 21615276 DOI: 10.3109/10715762.2011.564170]

33 **Ye SF**, Hou ZQ, Zhang QQ. Protective effects of Phellinus linteus extract against iron overload-mediated oxidative stress in cultured rat hepatocytes. *Phytother Res* 2007; **21**: 948-953 [PMID: 17602436 DOI: 10.1002/ptr.2182]

34 **Devi SL**, Anuradha CV. Oxidative and nitrosative stress in experimental rat liver fibrosis: Protective effect of taurine. *Environ Toxicol Pharmacol* 2010; **29**: 104-110 [PMID: 21787590 DOI: 10.1016/j.etap.2009.11.005]

35 **Chung KY**, Lee SJ, Chung SM, Lee MY, Bae ON, Chung JH. Generation of free radical by interaction of iron with thiols in human plasma and its possible significance. *Thromb Res* 2005; **116**: 157-164 [PMID: 15907531 DOI: 10.1016/j.thromres.2004.11.021]

36 **Dabbagh AJ**, Mannion T, Lynch SM, Frei B. The effect of iron overload on rat plasma and liver oxidant status in vivo. *Biochem J* 1994; **300** (Pt 3): 799-803 [PMID: 8010963]

37 **Yoshida H**, Sasaki K, Hirowatari Y, Kurosawa H, Sato N, Furutani N, Tada N. Increased serum iron may contribute to enhanced oxidation of low-density lipoprotein in smokers in part through changes in lipoxygenase and catalase. *Clin Chim Acta* 2004; **345**: 161-170 [PMID: 15193991 DOI: 10.1016/j.cccn.2004.03.018]

38 **Thephinlap C**, Ounjaijean S, Khansuwan U, Fucharoen S, Porter JB, Srichairatanakool S. Epigallocatechin-3-gallate and epicatechin-3-gallate from green tea decrease plasma non-transferrin bound iron and erythrocyte oxidative stress. *Med Chem* 2007; **3**: 289-296 [PMID: 17504202 DOI: 10.2174/157340607780620608]

39 **Arruda MM**, Mecabo G, Rodrigues CA, Matsuda SS, Rabelo IB, Figueiredo MS. Antioxidant vitamins C and E supplementation increases markers of haemolysis in sickle cell anaemia patients: a randomized, double-blind, placebo-controlled trial. *Br J Haematol* 2013; **160**: 688-700 [PMID: 23278176 DOI: 10.1111/bjh.12185]

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7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in

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Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc*. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
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