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Session 2: Micronutrients and the immune system
Nutritional imbalances and infections affect the thymus: consequences on T-cell-mediated immune responses

Wilson Savino[1]* and Mireille Dardenne[2]

[1]*Laboratory on Thymus Research, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil*
[2]*Hôpital Necker, Université Paris Descartes, CNRS UMR-8147, Paris, France*

The thymus gland, where T lymphocyte development occurs, is targeted in malnutrition secondary to protein energy deficiency. There is a severe thymic atrophy, resulting from massive thymocyte apoptosis (particularly affecting the immature $CD4^+CD8^+$ cell subset) and decrease in cell proliferation. The thymic microenvironment (the non-lymphoid compartment that drives intrathymic T-cell development) is also affected in malnutrition: morphological changes in thymic epithelial cells were found, together with a decrease of thymic hormone production, as well as an increase of intrathymic contents of extracellular proteins. Profound changes in the thymus can also be seen in deficiencies of vitamins and trace elements. Taking Zn deficiency as an example, there is a substantial thymic atrophy. Importantly, marginal Zn deficiency in AIDS subjects, children with diarrhoea and elderly persons, significantly impairs the host's immunity, resulting in an increased risk of opportunistic infections and mortality; effects that are reversed by Zn supplementation. Thymic changes also occur in acute infectious diseases, including a severe thymic atrophy, mainly due to the depletion of $CD4^+CD8^+$ thymocytes, decrease in thymocyte proliferation, in parallel to densification of the epithelial network and increase in the extracellular matrix contents, with consequent disturbances in thymocyte migration and export. In conclusion, the thymus is targeted in several conditions of malnutrition as well as in acute infections. These changes are related to the impaired peripheral immune response seen in malnourished and infected individuals. Thus, strategies inducing thymus replenishment should be considered as adjuvant therapeutics to improve immunity in malnutrition and/or acute infectious diseases.
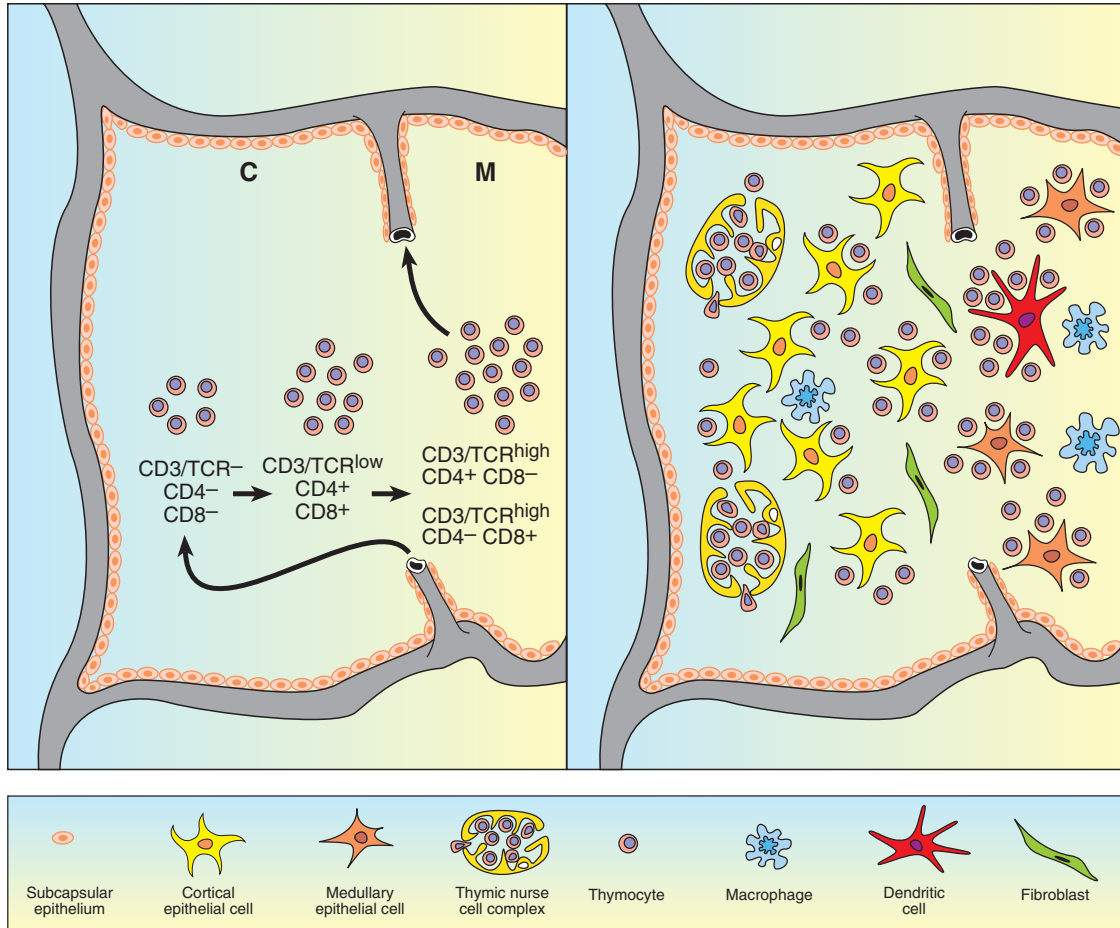
Thymus: Thymocytes: Thymic microenvironment: Extracellular matrix: Malnutrition: Infections: Chagas disease

It has been a long time since scientists noticed that in the context of the malnutrition-related immunodeficiency, the thymus undergoes a variety of alterations, comprising, among others, a consistent severe atrophy (reviewed in[1]), leading to the notion that the thymus can be considered as a barometer of malnutrition[2]. Interestingly, such a thymic atrophy pattern can also be found in a variety of infectious diseases[3]. Importantly, these two pathological situations likely cause profound alterations in the host's immune system, in part as a consequence of targeting the thymus.

Thus, the impact of malnutrition plus infection is a relevant issue in health sciences including public health, since in many countries malnutrition frequently parallels infections. Herein, we will review the similarities concerning the changes seen in the thymus of individuals suffering from malnutrition and/or infectious diseases. Yet, before discussing these specific data, it seemed useful to provide a general background of the normal thymus structure and function, comprising both the thymic microenvironment and the process of intrathymic T-cell differentiation.

Fig. 1. Normal intrathymic T-cell differentiation and the thymic microenvironment. In the left panel, we show a simplified view of normal thymocyte differentiation. Bone marrow-derived precursors enter the thymus and migrate from the cortico-medullary junction to the subcapsular cortical region of the thymic lobules. These immature cells do not express the CD3/T-cell receptor (TCR) complex, neither CD4 or CD8 molecules, being referred as CD4$^-$CD8$^-$ double negative. As shown in the right panel, as developing thymocytes progress in differentiation, they interact with microenvironmental cells, such as cortical thymic epithelial cells and fibroblasts localized in the cortex. At this stage, thymocytes start to express the TCR/CD3 complex and the molecules CD4 and CD8, thus becoming TCR^{low}/CD3^{low}CD4$^+$CD8$^+$ double-positive for these molecules. These cells are submitted to the processes of positive and negative selection, as a consequence of the interaction with the thymic microenvironmental cells through MHC/peptide–TCR interactions. Cortical epithelial cells are involved in positive selection, whereas both dendritic cells and epithelial cells can drive negation selection. Negatively selected cells die by apoptosis (most of them being phagocytized by macrophages) and positively selected thymocytes progress in differentiation, migrating through the medulla, ultimately becoming mature TCR^{high}/CD3^{high}CD4$^+$CD8$^-$ or TCR^{high}/CD3^{high}CD8$^+$CD4$^-$ single-positive T lymphocytes, which are the cells that normally leave the organ. Based on Savino and Dardenne[66].

The thymic microenvironment and its role in T-cell differentiation

The thymus is a primary lymphoid organ, in which bone marrow-derived T-cell precursors undergo differentiation, ultimately leading to the migration of positively selected thymocytes to the T-cell-dependent areas of peripheral lymphoid organs (see Fig. 1). Such a process involves a sequential expression of various proteins and rearrangements of the T-cell receptor (TCR) genes. Most immature thymocytes express neither the TCR complex nor the CD4 or CD8 accessory molecules; being called double-negative thymocytes, and representing 5% total thymocytes. As maturation progresses thymocytes acquire the membrane expression of the CD4 and CD8 markers, generating the CD4$^+$CD8$^+$ double-positive cells, which comprise 80% of the whole population. In this stage, TCR genes are rearranged, and productive rearrangements yield the membrane expression of the TCR (complexed with the CD3) in low densities (TCR^{low}). Thymocytes that do not undergo a productive TCR gene rearrangement die by apoptosis, whereas those expressing productive TCR interact with peptides presented by molecules of the MHC, expressed on microenvironmental cells. This interaction determines the positive and negative selection events, crucial for normal thymocyte differentiation. Negative selection results in apoptosis-mediated cell death. Positively selected thymocytes progress to the mature

TCR^{high}CD4$^+$CD8$^-$ or TCR^{high}CD4$^-$CD8$^+$ single positive stage, comprising 15% thymocytes that ultimately leave the organ to form the large majority of the peripheral T-cell repertoire (reviewed in[4]).

Thymocyte differentiation occurs as cells migrate within the thymic lobules: TCR$^-$CD4$^-$CD8$^-$ and TCR$^+$CD4$^+$CD8$^+$ cells are cortically located, whereas mature TCR$^+$CD4$^+$CD8$^-$ and TCR$^+$CD4$^-$CD8$^+$ thymocytes are found in the medulla. Along with this journey, thymocytes interact with various components of the thymic microenvironment, a three-dimensional network formed of thymic epithelial cells (TEC), macrophages, dendritic cells, fibroblasts and extracellular matrix (ECM) components. In addition to the key interaction, involving the TCR/peptide-MHC, in the context of CD8 or CD4 molecules, the thymic microenvironment influences thymocyte maturation via adhesion molecules and ECM; interactions that are relevant to thymocyte migration[5,6]. Moreover, microenvironmental cells modulate thymocyte differentiation by soluble polypeptides, comprising: (a) cytokines, such as IL-1, IL-3, IL-6, IL-7, IL-8 and stem cell factor; (b) chemokines and (c) thymic hormones, including thymulin, thymopoietin and thymosin-α1[5–8].

Thymocyte development is altered in protein–energy malnutrition

As stated earlier, one of the most conspicuous changes in malnutrition is thymic atrophy, which is essentially due to a massive thymocyte death, particularly affecting the immature stage of CD4$^+$CD8$^+$ cells[1]. Yet, it should be pointed out that, in addition to the increase in thymocyte death seen in thymuses from malnourished individuals, there is an intrathymic decrease in cell proliferation, as ascertained by the very low numbers of cells labelled with proliferating cell nuclear antigen, a marker for cell proliferation[9]. Thus, protein–energy malnutrition-related thymocyte depletion results from enhanced thymocyte death plus decreased thymocyte proliferation. It should be pointed out that the major change in the thymic lymphoid compartment is also observed in human subjects suffering from malnutrition: a severe thymic atrophy with cortical thymocyte depletion is a consistent finding in necropsies of malnourished subjects[1,10]. Indeed, by means of echography, atrophy of the organ was also observed *in vivo* in malnourished children, as compared to age-matched healthy individuals[11]. Importantly, a study conducted in Guinea-Bissau showed that the reduction in thymus size at birth correlated with infant mortality[12]. Fortunately, malnutrition-induced thymic atrophy seems to be reversible if appropriate diet is provided, as it has been demonstrated in a longitudinal study carried out with Bolivian severely malnourished children who were treated for nutrition rehabilitation, and that has been accompanied by ultrasound scanning. In these malnourished children, there was a severe reduction in the thymus volume, paralleled by abnormally high proportions of circulating immature T lymphocytes and lower proportions of mature T cells. Two months after beginning the diet rehabilitation, the thymic area of these children was recovered[13].

Thymocyte depletion associated with deficiencies in vitamins and trace elements: the zinc-deficiency paradigm

In addition to protein-related malnutrition, trace element as well as vitamin deficiencies result in thymic atrophy, with cortical thymocyte depletion[14–17]. In Fe-deficient mice, a decrease in mitogen-induced proliferative response of thymocytes has been demonstrated[14].

Many of the effects of protein–energy malnutrition on the immune function may be a result of associated alterations in metabolism of metals or vitamins, which in turn affect directly the immune system[18]. The abnormal incidence of various infections and the existence of lymphopenia and lymphoid organ atrophy in malnourished children have been repeatedly demonstrated and evidence points to Zn insufficiency in this type of immunodeficiency[19].

Zn plays a major role in cell division, differentiation, apoptosis and gene transcription, and strongly influences the immune system, affecting primarily T cells[20]. The studies of the effects of severe Zn deficiency in experimental animals and human subjects showed substantial thymic atrophy as well as accelerated lymphopenia, leading to the reduction in cell- and antibody-mediated responses, thus influencing the susceptibility to infectious diseases[21–23].

Early observations showed that mice maintained on a Zn-deficient diet develop a progressive thymic involution: after 4 weeks the thymus retains only 25% of its original size and at 6 weeks only a few thymocytes remain in the organ[24]. Such changes are observed mostly in the thymic cortex, with a severe loss of CD4$^+$CD8$^+$ thymocytes, and can be reversed by Zn supplementation[25,26]. Moreover, marginal Zn deficiency, in the early post-natal period, also results in substantial reduction in thymic size[27].

The mechanism(s) of heightened apoptosis in Zn deficiency mice remain(s) to be precisely determined. However, glucocorticoid hormones seem to be involved, since Zn deficiency yields a chronic stimulation of corticosterone production[28], and adrenalectomy prevents thymic atrophy secondary to Zn deficiency.

These studies raise concern about the impact of intrathymic cell death in human subjects who are deficient in Zn due to suboptimal diet or chronic disease[29]. In this respect, nutritional supplementation should be considered in chronically ill patients, with compromised immune defence, as reported in AIDS patients[30,31]. Zn supplementation resulted in a significant increase in CD4$^+$ T cells and a decreased mortality. This notion can also be applied in Chagasic patients, since they exhibit a decrease in serum Zn concentrations[32]; the same being observed in a variety of haemopoietic organs of infected rats[33]. Accordingly, the severity of experimental Chagas disease is much higher in Zn-deficient mice[34].

Acute infections induce thymic atrophy

Severe thymic atrophy is also a common feature in acute infections, reflecting the massive depletion of CD4$^+$CD8$^+$ cortical thymocytes (Table 1). This has been

Table 1. Thymic atrophy in human subjects and experimental infectious diseases (modified from[3])

Type of infectious agent	Disease	Infectious agent	Cortical atrophy (histology)	CD4$^+$CD8$^+$ thymocyte depletion	Human subjects data	Animal data
Viruses	AIDS	HIV/SIV	+	+	+	+
	Rabies	Rabies virus	+	+	ND	+
	Measles	Measles virus	+	+	+	+
	Hepatitis	Hepatitis virus (A59)	ND	+	ND	+
	Ebola infection	Ebola virus	+	ND	ND	+
Bacteria	Tularemia	*Francisella tularensis*	+	+	ND	+
	Listeriosis	*Listeria monocytogenes*	+	+	ND	+
	Syphilis	*Trepanema pallidum*	+	ND	+	
Protozoa	Chagas disease	*Trypanosoma cruzi*	+	+	+	+
	Malaria	*Plasmodium chabaudi; Plasmodium berghei*	+	+	ND	+
Fungi	Paracocciodosis	*Plasmodium brasiliensis*	+	ND	ND	+
	Histoplasmosis	*Histoplasma capsulatum*	+	+	ND	+
	Neosporosis	*Neospora caninum*	+	ND	ND	+
Helminths	Schistosomiasis	*Schistosoma mansoni*	+	+	ND	+
	Trichinosis	*Trichinella spiralis*	+	ND	ND	+

SIV, Simian immunodeficiency virus; ND, not determined.

shown in a variety of infections, such as AIDS, rabies, malaria, Chagas disease and schistosomiasis, among others (reviewed in[3]). In some cases, thymocyte loss is so severe that the cortical region of thymic lobules virtually disappears, as a consequence of the CD4$^+$CD8$^+$ thymocyte death. Also, similar to what is seen in malnutrition, proliferative response of thymocytes from acutely infected individuals is reduced: we found a significant decrease in both concanavalin A- and anti-CD3-driven proliferative responses in murine Chagas disease. Interestingly, this was paralleled by a decrease in the intrathymic production of IL-2, a major T-cell proliferation cytokine[35].

Thymocyte depletion seen in malnutrition and acute infections is partially under hormonal control

It is now well established that the physiology of the thymus (including both lymphoid and microenvironmental compartments) is influenced by a variety of hormones and neuropeptides[7]. It has been shown that glucocorticoid-circulating levels are increased in protein-malnourished mice, as compared to age-matched controls. Additionally, implanted corticosterone-containing pellets, able to generate glucocorticoid serum levels equivalent to those found in malnourished mice, were sufficient to yield thymocyte depletion[36]. As discussed later, leptin also seems to be involved. It has been shown that human subjects and rodents lacking proper leptin production or expressing defective leptin receptors, bear a certain degree of immunodeficiency characterized by reduced T-cell proliferative response to various mitogens, impaired production of IL-4 and inappropriate antibody production after immunization[37–39]. Interestingly, leptin/leptin receptor-deficient animals exhibit an atrophy of lymphoid tissues, particularly the thymus, and such a defect can be reversed by the reposition of the hormone[40]. Leptin was also able to

prevent starvation-induced thymic atrophy[40,41], strongly suggesting that this hormone is one mediator of malnutrition-induced thymic atrophy. It is thus conceivable that in malnutritional states, the imbalance in the production of leptin (which is decreased) and glucocorticoid hormones (which are increased) is at least partially responsible for thymocyte depletion and consequent atrophy of the organ, as we previously proposed[42,43].

The precise mechanisms responsible for the thymic atrophy seen in acute infections are not completely elucidated, and may vary in distinct diseases. But similar to malnutrition, one major pathway is related to the rise in glucocorticoid hormone levels in the blood, a classical effect comprised within the stress response of the organism to the infection. In fact, glucocorticoid serum levels are enhanced in *Trypanosoma cruzi*-infected mice[44,45], and, as discussed later, are likely involved, at least partially, in the *T. cruzi*-induced thymic atrophy[46]. Thymocyte depletion seen in rabies virus-infected mice[47] can be prevented by adrenalectomy prior to infection. In murine Chagas disease, adrenalectomy alone did not prevent *T. cruzi*-induced cortical thymocyte depletion[44]. Nevertheless, more recently it was demonstrated that a complete functional inhibition of glucocorticoid receptors by *in vivo* injection of RU-486, did prevent thymocyte depletion following acute *T. cruzi* infection[48]. Whether leptin levels are down-regulated in acutely infected levels remains to be determined and represents an interesting open field of investigation.

The thymic microenvironment is altered in malnutrition and acute infections

In addition to the lymphoid compartment, the thymic microenvironment is affected in various malnutritional and infectious conditions. Morphological changes in the thymic

epithelium from protein-malnourished mice include the decrease in the volume of the epithelial tissue in the cortex and in the medulla of thymuses from malnourished mice, as compared to well-nourished control animals[49]. By contrast, an increase of intracytoplasmic accumulations of large, circular, homogeneously electron-dense profiles, rich in free and esterified cholesterol was reported in both cortical and medullary TEC of malnourished animals[50]. Unfortunately, no data were reported concerning TEC death in this experimental model.

In *T. cruzi* acutely infected mice, we demonstrated changes in the expression of medullary and cortical-specific markers, as compared to controls, together with a general shrinkage of the thymic epithelial network[51].

Conceptually, these findings tell us that the thymic epithelium is morphologically altered in malnutrition and infection. As seen later, functional changes of the thymic epithelium are also seen in both these pathological conditions.

Decreased thymic endocrine function in malnourished and acutely infected individuals

One functional parameter that has been largely evaluated in malnutritional conditions is the thymic hormone production by TEC. It was initially found that that protein-malnourished mice exhibited abnormally low levels of circulating thymulin[1,49], and that such a decrease was also observed in protein-malnourished rats and human subjects[52]. Interestingly, even in human subjects protein malnutrition secondary to *anorexia nervosa*, low thymulin serum levels were reported[53]. Furthermore, decreased thymulin serum levels were reported in mice submitted to diets designed to trigger deficiency in Zn, Fe, or vitamins[1,14,54]. At least regarding Zn deficiency, similar results were found in human subjects[55].

It is noteworthy that the decrease in thymic hormone serum levels seen in malnutrition is not restricted to thymulin, since it was recently reported with regard to thymopoietin production[56]. In this study, the authors further demonstrated that prenatal undernutrition was significantly associated with reduced thymopoietin production in interaction with the duration of exclusive breast-feeding. These findings provide support for the importance of fetal and early infant programming of thymic function, and long-term implications for the immune system, and consequently adult disease risk.

In severe infection conditions, thymic endocrine function is also affected. We observed in *T. cruzi*-infected mice a transient decrease in the serum levels of the thymic hormone thymulin[51]. In HIV infection, we and others showed a consistent and long-term diminution of thymulin secretion, in terms of both serum levels and intrathymic contents of the hormone[57–59].

Increased extracellular matrix in the thymus of malnourished children

In addition to the abnormalities seen in TEC, the thymus from malnourished children presents a further microenvironmental alteration, namely, an increase in the deposition of ECM proteins. We studied by histological, ultrastructural and immunohistochemical means, thymuses obtained in necropsies from malnourished children. There is a consistent increase in the intralobular ECM-containing network, which could be ascertained histologically by the dense reticulin staining and immunohistochemically by the higher contents of fibronectin, laminin and type IV collagen. Importantly, the enhancement of thymic ECM in malnourished individuals positively correlated with the degree of thymocyte depletion[10]. This correlation may represent a cause–effect relationship in which the contact of thymocytes with abnormally high amounts of thymic ECM triggers and/or enhances programmed cell death. However, this notion is still hypothetical, demanding experimental demonstration. Interestingly, similar changes in thymic ECM were observed in glucocorticoid-hormone-treated mice and TEC cultures[7], leading to the hypothesis that the enhanced ECM deposition seen in malnutrition may be also related to high levels of serum glucocorticoid hormones. Such an alteration was also seen in acute infections, as exemplified by experimental Chagas disease[51,60]. In this infection model, changes in ECM were accompanied by alterations in the migratory response of thymocytes, with an abnormal export of CD4+C8+ immature thymocytes, some of them having bypassed the normal selective selection process[60–62]. Whether similar cell migration abnormalities exist in malnourished subjects is to be investigated.

Changes in the patterns of thymocyte migratory responses in acute infections

In addition to the thymocyte depletion seen in several infectious diseases, changes in the migratory responses have also been observed. As mentioned earlier, thymocyte depletion parallels *T. cruzi*-induced alterations of the thymic microenvironment, comprising phenotypic changes and functional changes in the TEC network, with an enhancement in the deposition of cell migration-related molecules such the ECM proteins, fibronectin and laminin, as well as the chemokines CXCL12 and CCL21[60,61]. These changes promote increased migratory responses to the corresponding ligands, and are likely related to the abnormal release of double-positive cells from the thymus into the periphery, resulting in more than 15-fold increase in double-positive cell numbers in subcutaneous lymph nodes. In this vein, it is noteworthy that double-positive cells seen in peripheral lymphoid organs express high densities of ECM and chemokine receptors[60,61]. Among these abnormally released double-positive cells in the periphery, we found lymphocytes expressing potentially autoreactive TCR, which are normally deleted in the thymus of uninfected mice. This suggests that during the infection, immature T lymphocytes escape from the thymic central tolerance process and migrate to the lymph nodes where they eventually differentiate into mature CD4+ or CD8+ cells[3,63].

In a second murine model of parasitic diseases, the thymus was evaluated in mice acutely infected with *Plasmodium berghei*. Again there is a thymic atrophy, with loss of cortical-medullary limits and the intrathymic presence

of parasites[64]. We also analysed the thymic expression of ECM ligands and receptors, as well as chemokines and their respective receptors. An increased expression of ECM components was observed in the thymus from infected mice, in parallel to a down-regulation of fibronectin and laminin receptor surface expression in thymocytes from these animals. Moreover, in the thymus from infected mice, we found increased contents of CXCL12 and CXCR4 and decreased expression of CCL25 and CCR9. An altered thymocyte migration towards ECM elements and chemokines was seen when the thymus from infected mice were analysed. The evaluation of *ex vivo* migration patterns of CD4/CD8-defined thymocyte subpopulations revealed that double-negative and CD4$^+$ and CD8$^+$ single-positive cells from *P. berghei*-infected mice have higher migratory responses, as compared to controls. Interestingly, increased numbers of these T-cell subpopulations were found in the spleen of infected mice, suggesting an abnormal export of T lymphocytes from the thymus of mice undergoing acute malaria infection[65].

Conclusions

The various issues discussed earlier clearly show that the thymus is a constant target organ in malnutrition as well as in acute infections, being severely affected in both lymphoid and microenvironmental compartments, and resulting in abnormal intrathymic T-cell death, proliferation and migration. These changes likely have consequences, leading to the impaired peripheral immune responses, seen in malnourished and infected individuals. Thus, strategies able to promote thymus replenishment should be considered when designing adjuvant therapeutic approaches, in both malnutrition and acute infectious diseases.

Acknowledgements

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