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A step forwards in immunomodulation and immunetolerance knowledge.
HLA-G expression in co-cultures of peripheral blood mononuclear cells and stem cells
after in vitro NGAL stimulation.

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ABSTRACT

Ngal (Neutrophil Gelatinase-associated Lipocalin) è una proteina appartenente alla famiglia delle lipocaline verso cui la recente letteratura ha mostrato una notevole attenzione, soprattutto in quanto biomarcatore in alcune condizioni patologiche (danno renale acuto e cronico, patologie autoimmuni, neoplasie). Il ruolo biologico di NGAL non è però ancora del tutto compreso. Numerose sono le dimostrazioni della sua azione batteriostatica. Recenti lavori hanno inoltre evidenziato un ruolo di NGAL nella modulazione di NFκB. Nessun lavoro ha valutato il ruolo di NGAL nell'immunità umorale.

Lo scopo dello studio è quello di capire se NGAL possa esercitare un ruolo di attivazione (modulazione) della risposta T cellulare attraverso la regolazione del complesso HLA-G, un mediatore di tolleranza.

Cellule mononucleate da sangue periferico (PBMCs) sono state ottenute da 8 donatori sani dopo consenso informato e isolate tramite centrifugazione (Ficoll). PBMC sono poi state trattate con 4 concentrazioni crescenti di NGAL (da 40 a 320 ng/mL), associate o meno a ferro e analizzate con tecnica fluorimetrica ed elisa. Alle analisi eseguite NGAL stimola l'espressione di HLA-G sulle cellule T CD4⁺ con un andamento dose dipendente. L'effetto del ferro sull'espressione di HLA-G non è di univoca interpretazione. Inoltre L'aggiunta di NGAL in vitro modifica il pattern di espressione delle cellule T, aumentando la popolazione delle cellule CD4⁺ CD25⁺ FoxP3. L'utilizzo di anticorpi anti NGAL limita l'espressione di HLA-G e diminuisce significativamente la percentuale di CD4⁺ CD25⁺ FoxP3⁺. In conclusione abbiamo mostrato un coinvolgimento di NGAL nell'immunità cellulare. Valutando il ruolo di NGAL come molecola immunomodulatoria, abbiamo mostrato che NGAL gioca un ruolo chiave nell'immunotolleranza aumentando l'espressione di HLA-G e cellule T regolatorie nei donatori sani. Un possibile scenario applicativo di tale studio riguarda l'utilizzo in vivo di NGAL nell'immunomodulazione dei pazienti sottoposti a trapianto o affetti da patologie autoimmuni.

ABSTRACT

NGAL (Neutrophil Gelatinase-associated Lipocalin) is a protein of lipocalin superfamily. Recent literature focused on its biomarkers function in several pathological condition (acute and chronic kidney damage, autoimmune disease, malignancy). NGAL biological role is not well elucidated. Several are the demonstration of its bacteriostatic role. Recent papers have indeed highlight NGAL role in NFkB modulation. The aim of this study is to understand whether NGAL may exert a role in the activation (modulation) of T cell response through the regulation of HLA-G complex, a mediator of tolerance.

From 8 healthy donors we obtained peripheral blood mononuclear cells (PBMCs) and we isolated by centrifugation on a Ficoll gradient. Cells were then treated with four concentrations of NGAL (40-320 ng/ml) with or without iron. We performed flow cytometry analysis and ELISA test. NGAL increased the HLA-G expression on CD4⁺ T cells, with an increasing corresponding to the dose. Iron effect is not of unique interpretation. NGAL adiction affects regulatory T cells increasing in vitro expansion of CD4⁺ CD25⁺ FoxP3⁺ cells. Neutralizing antibody against NGAL decreased HLA-G expression and reduced significantly CD4⁺ CD25⁺ FoxP3⁺ cells percentage.

In conclusion, we provided in vitro evidence of NGAL involvement in cellular immunity. The potential role of NGAL as an immunomodulatory molecule has been evaluated: it has been shown that NGAL plays a pivotal role in the induction of immune tolerance up regulating HLA-G and T regulatory cells expression in healthy donors. As potential future scenario we highlight the in vivo role of NGAL in immunology and immunomodulation, and its possible relationship with immunosuppressive therapy efficacy, tolerance induction in transplant patients, and/or in other immunological disorders.

1. Introduction

1.1 Neutrophil Gelatinase-associated Lipocalin (NGAL)

Neutrophil gelatinase-associated lipocalin (NGAL) is a glycoprotein of 25 kDa molecular weight consisting of 198 amino acids. It owes its name to the fact that it was isolated for the first time in association with a gelatinase obtained from the supernatant of activated human neutrophils. Only later its expression was found also in other epithelial tissues including kidney, stomach, colon, trachea bone marrow, prostate, uterus [1,2].

NGAL belongs to the lipocalins superfamily, that comprises a class of proteins that are characterized by a common secondary and tertiary structural feature— called as the "lipocalin fold". It is characterized by height β -strands that form a β -barrel defining a calyx. The calyx binds and transports low molecular weight molecules, which define the biologic activity of the lipocalin. The beta sheets are connected to one another by seven short loops (L1–L7), of which the loop L1 forms a lid-like structure to close the ligand binding cavity. The difference in specific amino acids within the lipocalin fold gives rise to the wide diversity in ligands that can be bound by lipocalins (Figure 1) [3,4].

Few examples of lipocalins are retinol-binding protein that binds and transports vitamin A [5], the lipocalin α 1-microglobulin that scavenges heme [6], and nitrophorin-type lipocalins that carry heme groups complexed with nitric oxide [7].

Biological fluids contain very low levels of NGAL protein at steady state level. Serum contains approximately 20 ng/ml NGAL which is probably derived from neutrophils and from limited expression in liver, spleen and kidney [8]. Renal clearance is a major regulator of this steady state level, because circulating NGAL undergoes glomerular filtration due to its low molecular weight and positive charge. Theoretically, the kidney processes 3.4–4 mg NGAL per day ($20 \text{ ng/ml} \times 120\text{--}140 \text{ ml/min}$ glomerular filtration rate, GFR) as a result of filtration [9] followed by capture by the proximal tubule, where it was degraded to a 14 kDa fragment in lysosomes. Endocytosis of NGAL

from the apical (luminal) membrane is the most likely pathway of NGAL traffic because it appeared in the urine when the apical megalin receptor was deleted. Biacore based binding studies confirmed a direct interaction between NGAL and megalin fragments [10], consistent with capture of NGAL from the glomerular filtrate rather than from the basolateral (blood) side. Similar to serum, urine contains approximately 20 ng/ml NGAL at steady state. The origin of this protein is not clear, but some of it may derive from serum NGAL that bypasses capture in the proximal tubule (approximately 1/200 molecules). Alternatively, NGAL might derive from neutrophils or even from bladder epithelia. However, it is most likely that urinary NGAL derives from a low level of expression in the native kidney at steady state. Indeed, weak in situ RNA reactivity can be seen in the collecting ducts of some kidneys [11].

1.1.2 NGAL, the iron binding and the bacteriostatic action

In the case of NGAL, the lipophilic compound that can be tied by the lipocalin fold is represented by the siderophores, as demonstrated for the first time by Strong et al studies [12].

The estimated concentration of free iron in the body is estimated to be as low as 10^{-24} M) [13].

The exceedingly low availability of free iron is attributable to iron-binding proteins like transferrin, ferritin and

lactoferrin which form complexes with any available free iron molecules. Bacteria have developed special proteins called siderophores that have an affinity for iron (particularly ferric iron or Fe^{3+}) several times higher than that of the endogenous iron chelators. This enables siderophores to not only bind available free iron, but also extract iron from iron-binding proteins of the host [14].

Goetz et al. [15,16] cloned NGAL in Gram-bacteria and identified a bacterial compound called enterochelin bound within the protein's calyx. Enterochelin is an organic molecule that bacteria utilize to capture iron from the extracellular media.

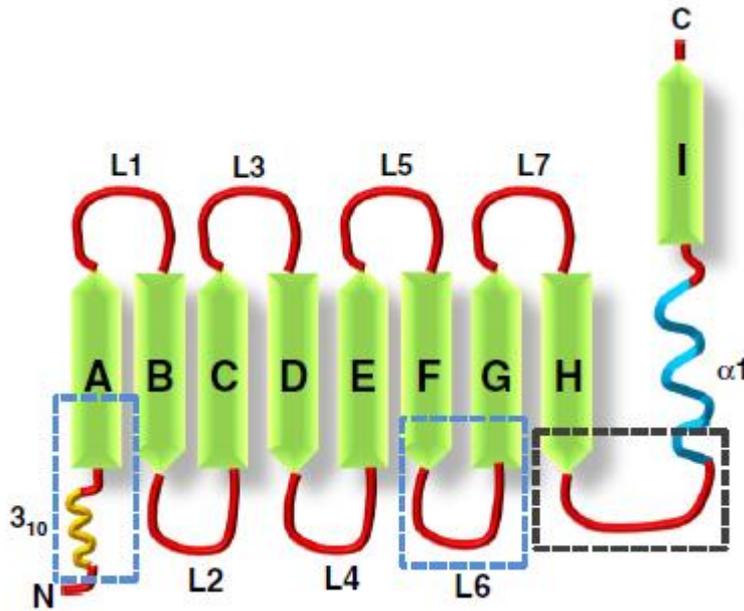


Figure 1. Schematic representation of the lipocalin fold. The characteristic feature of lipocalins is the “lipocalin fold” which comprises of an N-terminal 3–10 helix followed by eight beta sheets. The beta sheets are connected by loops (L1–L7). The portion of the lipocalin fold that are structurally conserved between different lipocalins is indicated by the blue boxed regions while the region that shows significant conservation in amino acid sequence is indicated by the black boxed region [4].

The interaction of enterocalin with NGAL directly blocked the capture of iron by bacteria: NGAL delayed bacterial growth. Complexes of NGAL with enterocalin:Fe could form in the bloodstream [17] followed by their rapid clearance by macrophages in liver and spleen and by filtration and tubular destruction in the kidney. Hence, the binding of enterocalin to NGAL not only sequestered the siderophore but resulted in its clearance in the kidney. Because NGAL levels can rise 100 fold to >2000 ng/ml, as much as 300–400 mg NGAL/24 h, containing nearly 1 mg of iron can theoretically traffic to the kidney for clearance and recycling, provided that enterocalin is fully saturated and glomerular filtration is maintained [11].

But an oversupply of iron or the expression of siderophores [18] which do not recognize NGAL, rescued bacterial growth. Consistently, the deletion of NGAL accelerated the growth of enterocalin dependent bacteria, resulting in sepsis and heightened mortality [19,20].

NGAL is therefore an important role in innate immune response and is expressed in particular tissues exposed to the external environment (and therefore susceptible to infection) as the urinary or the gastrointestinal tract (figure 2). There are also many apparent differences between known urinary antimicrobial peptides and NGAL. For example, unlike other urinary antimicrobial proteins [21,22,23] the cathelicidins, the defensins [24,25], Tamm–Horsfall [26-28] and Lactoferrin [29], which are expressed constitutively, uNGAL is not likely to play a large role at steady state because it is expressed only at very low levels (20 ng/ml). In addition, while many of these proteins are modulated only to a small degree by infections (cathelicidin by 3–8 fold; THP and lactoferrin are not stimulated by infections [30-34] and the AMPs and lactoferrin remain in the low ng/ml range even after stimulation), NGAL was intensively upregulated by significant infections [35-39] as well as aseptic stimuli. Consequently the mechanisms of expression and induction apparently differ between NGAL and other antimicrobial peptides even though NGAL originates from the same cells as many of these proteins.

1.1.3 NGAL, inflammation and oxidative stress

The inflammatory response is a natural defense mechanisms used by the body to clear irritants and pathogens. NGAL has been shown to be a pro-inflammatory molecule causing some to call it a cytokine. [40] Studies conducted in pulmonary inflammation (mouse models) for example have revealed that Lcn2 (the mouse equivalent of NGAL) mRNA and protein are strongly upregulated with the exposure to pro-inflammatory stimuli [41]. A similar upregulation was also seen in the

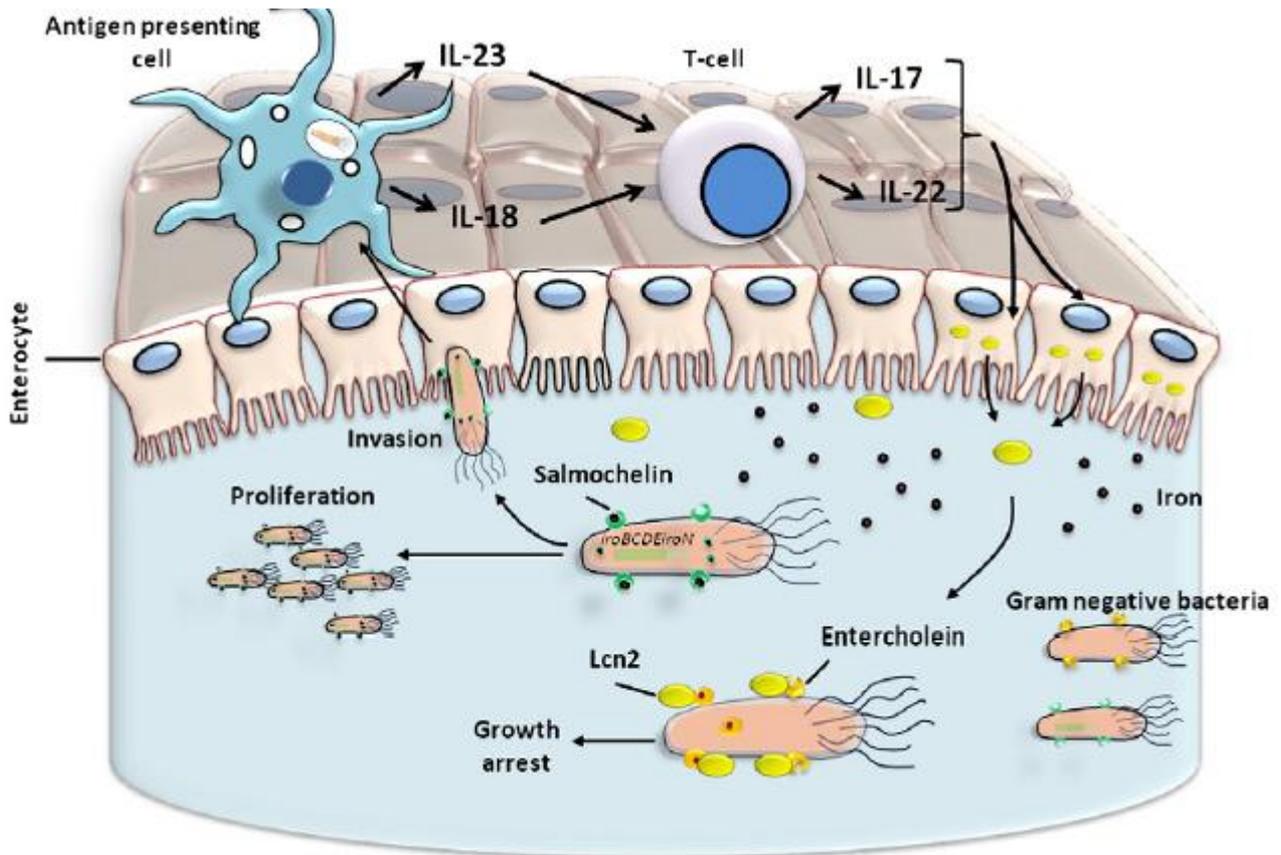


Figure 2 Exemple bacteriostatic action of NGAL. Gram negative bacteria (like *Salmonella typhimurium*) trigger an immune response characterized by the activation of antigen presenting cells (macrophages and dendritic cells) upon engulfing the bacteria. These activated cells then release cytokines like IL-18 and IL-23 that in turn activate T-lymphocytes. These activated T-cell in turn release IL-17 and IL-22. These two cytokines act on the intestinal cells and stimulate the de novo synthesis of NGAL- lipocalin 2 (Lcn2). The NGAL-Lcn2 is secreted into the intestinal lumen and binds to bacterial iron-binding proteins (siderophores) like enterochalin. Since iron is essential for the growth of bacteria, the sequestration of bacterial siderophores by NGAL-Lcn2 has a bacteriostatic effect. [4].

lungs of patients with bronchial inflammation [42]. The pro-inflammatory cytokine IL-1 β is able to induce a significant upregulation of NGAL in human lung epithelial cells [42]. This suggests a mechanism for upregulation of NGAL in pulmonary inflammation but its functional role in this process is still unclear. Several hypotheses have been proposed to explain it. One such hypothesis is that acute or chronic inflammation leads to the accumulation of granulocytes at the sites of inflammation. These granulocytes undergo apoptosis, release their granules (containing NGAL) and

thereby mediate local tissue injury. In support of its pro-inflammatory function, NGAL appears to be a chemoattractant for neutrophils [43].

The importance of NGAL as an acute phase protein was demonstrated by the observation that following an intraperitoneal injection of *E. coli*, NGAL levels are elevated in the serum and in liver tissue within 4 h, and in the spleen within 6 h. The increase in serum levels of NGAL was preceded by an increase in mRNA synthesis in the peripheral blood cells. Serum NGAL levels rose by nearly 22-fold (from 100 ng/ml to 2200 ng/ml) within 8 h following injection (of *E. coli*), reached a peak by 24 h and then gradually returned to baseline levels [44].

Oxidative stress can be defined as the imbalance between the prooxidant and antioxidant machinery in the cell [45]. Aberrant expression of NGAL in Chinese hamster ovary (CHO) and human embryonic kidney (HEK293T0) cells resulted in an upregulation of the mRNA and protein levels of the antioxidant enzymes superoxide dismutase (SOD1 and SOD2) and heme oxygenase (HO1 and HO2) together with decrease in expression of NF- κ B. Silencing of NGAL in A549 lung cancer cells had the opposite effect on HO1 and NF κ B expression but increased the expression of SOD1 and SOD2. Further, the cells ectopically expressing NGAL were more resistant to the cytotoxic effects of hydrogen peroxide in vitro [46]. These results suggest that NGAL by inducing the expression of antioxidant enzymes helps cells counteract oxidative damage. Other studies have also suggested that NGAL may have a protective effect against cellular injury mediated by reactive oxygen species (ROS) [47].

1.1.4 NGAL in kidney formation

The normal development of urinary system has three different stages: pronephros, mesonephros, and metanephros. The development of metanephros, the permanent kidney, is the result of the expression of many genes in the cells of bud ureter and the metanephric blastema that send each other messages to induce organogenesis. [Nelson, trattato di pediatria].

Already at the beginning of the last century Boyden in the 1927 and later Gruenwald and Grobstein in the 1943 demonstrated that the stimulus to the conversion from mesenchymal cells in epithelial cells was given by the presence of the ureteric bud. In fact, surgical ablation of the ureteric bud prevented the appearance of new epithelium from metanephric blastema. [48].

More recent studies have shown that the gene deletion of ureteric bud obtained the same results. [49]. So it appeared clear that the ureteric bud provided the signals necessary for conversion of mesenchymal cells in epithelial cells, but the signals wasn't known.

In the late 1990s the Barasch and Sakuri research groups, to reveal the identity of these factors have developed a line of cells of the ureteric bud that served as surrogate of the embryos ureteric bud [50]. These cells expressed a number of typical ureteral bud proteins including growth factors and mesenchymal survival factors and receptors for factors produced by mesenchymal cells. In this way were isolated numerous factors each of which can stimulate the growth of metanephric mesenchyme and prevent its apoptosis (FGF and tissue inhibitors of metallo-proteinase) and in 1999 also the first factor able to convert mesenchymal cells in epithelial cells (LIF, leukemia inhibitory factor) [51].

Recently several other factors belonging to the complex network of signals able to synergistically induce mesenchymal cells into epithelial cells differentiation have been identified: FGF2, TGF β 2, etc [52]. In 2002 Yang et al purified and recognized between these proteins also NGAL. [53].

1.1.5 NGAL as a growth factor

The differentiation-inducing properties of NGAL are not limited to the embryonic kidney. Mounting evidence points toward growth factor effects of NGAL that modulate various cellular responses, such as proliferation, apoptosis, and differentiation, but this is not well understood mechanistically. [54].

In a 4T1-Ras-transformed mesenchymal tumor cell line, NGAL induces markers of epithelial cells [55]. Furthermore, in cultured collecting duct cells, NGAL is expressed downstream of hepatocyte

growth factor and promotes the organization of epithelial cells into tubular structures [56]. Antagonization of NGAL induction by expression of NGAL shRNA induces cystic structures rather than properly assembled tubules. Therefore, in addition to inducing epithelial characteristics in non epithelial cells, NGAL seems to affect the structure of established epithelia. It is interesting that glycodelin, another protein of the lipocalin family, displays effects on epithelial differentiation very similar to NGAL [57]. In vivo, NGAL protein is expressed predominantly by stimulated, growing, dysplastic, or involuting epithelial cells, pointing to a relevance of the in vitro observations in pathologic states [58].

Some of these effects, however, are enhanced when NGAL is associated with siderophores and iron, raising the possibility that in the absence of bacterial infection, endogenous molecules associate with NGAL to mediate its iron-binding properties. The cellular events differ strikingly—at least in some biologic systems depending on whether NGAL is associated with iron [59]. Cellular uptake of NGAL is followed by distribution of the protein in endosomes [60]. Different trafficking routes of endosomal NGAL have been proposed depending on the cell type and the association of NGAL with its binding partners. In kidney-derived cell lines, siderophore:iron-associated NGAL (holo-NGAL) traffics to endosomes and releases iron from the complex, which results in regulation of iron-responsive genes, such as ferritin and transferrin receptor [61]. Similarly, in the adult mouse kidney in vivo, systemically applied holo-NGAL is taken up by proximal tubule cells, where it delivers ^{55}Fe [62]. The endosomal NGAL protein core is either degraded in lysosomes [63] or recycled to the extracellular space [64]. On the basis of these observations, siderophore:iron-associated NGAL is predicted to facilitate cytoplasmic iron delivery into target cells. A recent report suggested that the situation may be different when NGAL is delivered into target cells in the absence of the siderophore:iron complex. In this setting, NGAL is proposed to scavenge intracellular iron and exit the cell via the endosomal recycling pathway [65]. Therefore, at least some of the biologic effects of NGAL may depend markedly on its association with the siderophore:iron complex. This notion is supported by different biologic

responses to NGAL depending on the ligand. For instance, siderophore: iron-associated NGAL is more effective than apo-NGAL in inducing epithelial characteristics in 4T1-Ras-transformed mesenchymal tumor cells [66].

1.1.6 NGAL and pathological kidney conditions

There is an urgent need in clinical medicine for early predictive biomarkers of both acute kidney injury (AKI) and chronic kidney disease (CKD). In both situations, early intervention can significantly improve the prognosis. However, currently available biomarkers (such as serum creatinine concentrations) are imprecise, and their delayed response has impaired our ability to institute potentially effective therapies promptly. In recent years a series of kidney disease biomarker candidates were proposed both for AKI and CKD. NGAL is widely considered one of the most promising novel biomarkers of acute and chronic kidney damage [67].

Several investigators have used molecular techniques such as cDNA microarrays and subtractive hybridizations combined with downstream proteomic analysis to identify novel pathways, biomarkers and drug targets in AKI. Supavekin et al. identified NGAL as one of the most upregulated genes in the early post-ischaemic mouse kidney [68], a finding that has now been confirmed in several other transcriptome profiling studies following ischaemic and nephrotoxic kidney injuries. Downstream proteomic studies have also revealed NGAL to be one of the earliest and most robustly induced proteins in the kidney after ischaemic or nephrotoxic AKI in animal models [69]. Importantly, NGAL protein is easily detected in the blood and urine soon after AKI in pre-clinical studies [70]. These findings have initiated a number of translational studies to evaluate NGAL as a novel biomarker in human AKI. In a cross-sectional study, subjects in the intensive care unit with established acute renal failure displayed a greater than 10-fold increase in plasma NGAL concentration and more than a 100- fold increase in urine NGAL concentration by Western blotting when compared to normal controls [71]. Both plasma and urine NGAL concentrations correlated highly with serum creatinine concentrations. Kidney biopsies in these patients showed intense

accumulation of immunoreactive NGAL in 50 % of the cortical tubules. These results identified NGAL as a widespread and sensitive response to established AKI in humans.

NGAL has also been evaluated as a biomarker of AKI in kidney transplantation. Biopsies of kidneys obtained 1 h after vascular anastomosis revealed a significant correlation between NGAL staining intensity and the subsequent development of delayed graft function [72]. In a prospective multicentre study of children and adults, urine NGAL concentrations in samples collected on the day of transplant clearly identified cadaveric kidney recipients who subsequently developed delayed graft function and dialysis requirement (which typically occurred 2-4 days later). The ROC curve for prediction of delayed graft function based on urine NGAL concentration at day 0 showed an AUC of 0.9, indicative of an excellent predictive biomarker [73]. In a retrospective study of kidney transplant patients undergoing either protocol biopsies or clinically indicated biopsies, urine NGAL concentrations were found to be significantly increased in subjects with tubulitis or other tubular pathologies [74]. Urine NGAL also tended to be increased in subjects with subclinical tubulitis ($p=0.06$), raising the possibility of NGAL representing a non-invasive screening tool for the detection of tubulointerstitial disease in the early months following kidney transplantation.

Several investigators have examined the role of NGAL as a predictive biomarker of nephrotoxicity following contrast administration [75]. In a prospective study of children undergoing elective cardiac catheterization with contrast administration, both urine and plasma NGAL predicted contrast-induced nephropathy (defined as a 50 % increase in serum creatinine from baseline concentration) within 2 h after contrast administration [76]. Using a cut-off value of 100 $\mu\text{g/L}$, the AUC for prediction of contrast nephropathy was excellent for the 2-h urine NGAL (0.92) as well as the 2-h plasma NGAL (0.91). By multivariate analysis, the 2-h NGAL concentrations in the urine and plasma were found to be powerful independent predictors of contrast nephropathy [77].

As discussed above, NGAL is a promising biomarker of AKI. In CKD, there is a growing literature suggesting that NGAL is also a marker of kidney disease and severity. In 45 subjects with CKD secondary to renal dysplasia, obstructive uropathy and glomerular and cystic diseases, plasma

NGAL concentrations were inversely associated with GFR [78]. As kidney function declined to <30 mL/min, NGAL outperformed cystatin C as a biomarker of kidney failure [79]. Another study [80] in subjects with CKD (due to chronic glomerulone-phritis) demonstrated that mean urinary NGAL concentrations were higher in CKD patients ($378.28 \pm 111.13 \mu\text{g/L}$ vs. $7.38 \pm 3.26 \mu\text{g/L}$ in controls; $p=0.01$). Furthermore, urinary NGAL concentrations were significantly correlated with serum creatinine concentrations ($r=0.588$, $p\text{-value}=0.02$), GFR ($r=-0.528$, $p\text{-value}=0.04$) and proteinuria ($r=0.294$, $p\text{-value}=0.01$) [81]. Both urine and plasma NGAL represent biomarkers of CKD severity in patients with autosomal dominant polycystic kidney disease [82]. In these subjects, urine and plasma NGAL concentrations correlated with residual GFR, and those with greater severity of cystic disease (measured as number of cysts >10) displayed the highest NGAL values [83]. Urine NGAL has also been shown to represent an early biomarker for degree of chronic tubulointerstitial injury in patients with IgA nephropathy [84].

1.2 IMMUNE TOLERANCE

Immune tolerance consists of two main processes, namely central and peripheral tolerance. Central tolerance takes place in the thymus where most of the self-reactive T cells are deleted at an immature stage of their development [85]. Despite negative selection, self-reactive T cells can escape thymic clonal deletion, and subsequently provoke autoimmune diseases such as type 1 diabetes (T1D), multiple sclerosis (MS), and inflammatory bowel disease (IBD) unless they are controlled by one of many peripheral mechanisms [86].

However thymic selection has been considered as an effective tolerogenic mechanism only for widely expressed self molecules. This assumption is based on the consideration that proteins with tissue-restricted expression would not be available for presentation in the thymus. Thus, tolerance to such proteins could only be achieved through mechanisms of peripheral tolerance. Peripheral tolerance mechanisms are indeed operative in extrathymic lymphoid tissues and include deletion,

anergy, ignorance and regulatory cells, and contribute to maintaining autoreactive lymphocyte under tight control.

Immunologic tolerance was first introduced in 1945 when Ray Owen observed that placental interchange resulted in red cell chimerism between dizygotic bovine twins [87]. In the follow decade Medawar, McFarlane Burnet, and colleagues elaborated upon this phenomenon of acquired immunologic tolerance with experimental models of transplantation, which awarded them the Nobel Prize in Physiology or Medicine in 1960.

Most of the work at the time involved non-self antigen exposure in immunologically immature hosts, until 1959 when Schwartz and Dameshek demonstrated a marked delay in the adult rabbit immune response to iodine-labeled injections of human serum albumin when treated with 6-mercaptopurine [88]. Their descriptions of the inhibition of immune pathways in this “drug-induced immunological tolerance” notably foreshadowed the era of pharmacologic development for tolerance induction.

The next 50 years heralded a boom in drug development and subsequent improvements in graft survival. In contrast to 1-year graft survival in 1977 of 53 and 78% for deceased and living-related donors, respectively [89], modern immunosuppression has enabled transplant recipients to enjoy very favorable graft survival. One-year rates having asymptotically approached 93–96%; therefore, short-term graft survival alone can no longer be held as the metric of success for new immunosuppressants.

Instead, as 10-year graft survival rates still trail at 47–61%, new agents must address factors leading to chronic rejection as well as the comorbidities associated with chronic immunosuppression. The decisive measure of success is for a therapy to demonstrate allospecific immunosuppression while minimizing side effects and preserving immune competence to infectious pathogens and cancer during drug administration, and permanent graft survival after its withdrawal.

The understanding of the mechanisms underlying the immune tolerance of self and non self are essential not only for organ transplant but also in the autoimmune disease and neoplastic disease.

1.2.1 TOLERANCE INDUCTION

Traditional and novel approaches to inducing tolerance in organ transplant are known:

MOLECULE-BASED APPROACHES

1) T CELL THERAPIES–DEPLETION

Early attempts at transplantation in humans were fraught with early graft failure due to a robust alloimmuneresponse mediated by activated Tcells. We have since learned that the suppression of these alloreactive Tcells permits long-term graft survival and, at times, operational tolerance [90]. *Inthe1980s*, [91] observed that some renal transplant patients undergoing total lymphoid irradiation acquired tolerance to their allografts after withdrawal of immunosuppression and demonstrated donor-specific unresponsiveness in vitro. Over 30years later, the concept of eliminating alloreactive Tcells upon induction continues to prevail, as Tcell depletion remains the most common induction therapy in the U.S [92] Steroids, calcineurininhibitors, rapamycin, and mycophenolatemofetil comprise essential components of most immunosuppressive maintenance regimens.

Induction strategies are instead represented by:

- Anti-thymocyteglobulin(ATG), the oldest depleting agent dating back to the late 1890s, has been a mainstay in induction therapy since the 1960s [93] (Due to its potency and markedly heterogeneous target antigen specificities, ATG is particularly useful in high-risk recipients as well as in preventing ischemia-reperfusion injury.
- Alemtuzumab (Campath-1H, Genzyme), a humanized monoclonal Ab to CD52 found densely distributed on T and B lymphocytes and natural killer cells [94].

2) T CELL THERAPIES–COSTIMULATION BLOCKADE

Alloreactive Tcell activation requires antigen-specific engagement of the Tcell receptor with major histocompatibility complex molecules (signal1), followed by antigen non-specific ligation of a variety of receptor–ligand combinations, or costimulation (signal2; Jenkins and Schwartz, 1987).

Blockade of costimulation effectively prevents Tcell activation and allograft rejection [95].

While costimulation blockade renders the Tcell anergic [96], the seanageric Tcells may express inducible costimulator (ICOS) and play a regulatory role [97]. In addition, costimulation blockade does not require radical ablation of the immune system by lymphocyte depletion or irradiation, thus shifting the emphasis from induction to maintenance immunosuppression [98].

Costimulatory signals of the CD28:B7(CD80/86) immunoglobulin superfamily and CD40:CD154(CD40L) tumornecrosisfactor (TNF) family are the most studied and potentially most important activating costimulation pathways. Cytotoxiclymocyteantigen-4 (CTLA-4) shares about 30% homology with CD28, and binds with 10–20-fold higher affinity than CD28 toB7 molecules on the antigen presentingcell(APC). Not only does this potently inhibit the Tcell, but also its ligation with APC B7 molecules induces indoleamine 2,3-dioxygenase expression, promoting the suppressive function sinCTLA4+ regulatoryCD4+ cells [99]. Abatacept(Orencia,Bristol-MyersSquibb) and belatacept (Nulojix,Bristol-MyersSquibb), fusion proteins composed of CTLA-4 and immoglobulinIgG1, have utilized this mechanism to confer potent inhibition of alloreactive Tcell responses.

3) B CELL THERAPIES

The role of B cells in operational tolerance has yet to be defined. On one hand ,an ITN-sponsored collaboration identified a unique B cell signature associated with 25 operationally tolerant renal transplant recipients. Not only did tolerant patients exhibit an increase in total and naïve Bcells, but also the majority of genes that were increasingly expressed were Bcell-specific ,particularly of transitional Bcells [100]. While these transitional B cells could represent a regulatory B cell population based on their increased IL-10 production as discussed by Redfieldetal. [101]., no difference in B cell subsets (total, naïve, and transitional cells) or inhibitory cytokines (IL-10 and TGFβ) was detected when compared to healthy controls [102].

1.2.2 HLA-G

While both HLA-A, -B, and -C class I molecules and class II molecules play an important role in the induction of a specific immune response by presenting peptide antigens to T cells [103], the non-classical major histocompatibility complex (MHC) class I molecule HLA-G has been identified as a key mediator in immune tolerance [104]. It is characterized by :

- 1) A tissue-restricted distribution. Whereas expression of classical HLA class I molecules is known to be ubiquitous, HLA-G was first described on extraembryonic trophoblast tissues that invade the maternal decidua during implantation of the embryo [105]. Recently, its expression was also reported in endothelial cells from first trimester placental chorionic blood vessel, in thymic epithelial cells and activated peripheral blood monocytes [106].
- 2) A limited polymorphism. Sixteen HLA-G alleles have been described to date, four of which may encode membrane-bound HLA-G proteins [107], plus one truncated soluble protein which bears the 'delC130 mutation' [108];
- 3) An alternative transcription of spliced messenger RNAs (mRNAs) that encode at least six different HLA-G isoforms, namely the HLA-G1, -G2, -G3 and -G4 membrane bound and HLA-G5 and -G6 soluble proteins [109].

The "complete" HLA-G1 molecule and its soluble counterpart HLAG5 are those that have been studied the most. They have an identical extracellular structure, which is classic HLA class I-like: a heavy chain of 3 globular domains non-covalently bound to β 2-microglobulin and a nonapeptide. The other isoforms are likely to be of simpler structure: lacking one or 2 globular domains, they are smaller, and should not bind β 2-microglobulin and present peptides. [110].

Three HLA-G receptors have been described: ILT2/CD85j/LILRB1 (ILT2), ILT4/CD85d/LILRB2 (ILT4), and KIR2DL4/CD158d (KIR2DL4) [111].

ILT2 is expressed by B cells, some T cells, some NK cells, and all monocytes/dendritic cells, [112] but ILT4 is myeloid-specific and only expressed by monocytes/dendritic cells [113]. Concerning

KIR2DL4, its expression is mainly restricted to the CD56bright subsets of NK cells [114] which constitute a minority of peripheral NK cells, but a majority of uterine NK cells. [115]. Binding to CD8 has also been reported [116]. Through these differentially expressed receptors, HLA-G can interact with B cells, T cells, NK cells, and antigen-presenting cells (APC).

Functionally, HLA-G1 inhibits the cytolytic function of uterine and peripheral blood NK cells [117] the antigen-specific cytolytic function of cytotoxic T lymphocytes [118] the alloproliferative response of CD4+ T cells [119] the proliferation of T cells and peripheral blood NK cells [120] and the maturation and function of dendritic cells [121].

Soluble HLA-G5 or soluble HLA-G1, which is generated by proteasomal cleavage from the cell membrane, has similar functions. The other HLA-G isoforms have been less well studied, and little is known about their function except that membrane-bound HLA-G2, HLA-G3, and HLA-G4 can inhibit NK-cell and cytotoxic T lymphocyte cytolysis in vitro [122].

| Effector cell, HLA-G function | Receptors involved |
|---|--------------------------------|
| NK cells | |
| Inhibition of cytotoxic function | ILT2, KIR2DL4 |
| Indirect inhibition of cytotoxic function through stabilization of cell-surface HLA-E | CD94/NKG2A (interacts w/HLA-E) |
| Inhibition of proliferation | ILT2 |
| Up-regulation of inhibitory receptors | — |
| Apoptosis | CD8 |
| Increased proliferation and IFN γ production | KIR2DL4 |
| Increased secretion of pro-angiogenic factors | Internalized KIR2DL4 |
| Inhibition of transendothelial migration | ILT2 |
| CD8⁺ T cells | |
| Inhibition of cytotoxic function | — |
| Inhibition of proliferation | ILT2 |
| Generation of CD8 ^{low} regulatory T cells | — |
| Apoptosis | CD8 |
| CD4⁺ T cells | |
| Inhibition of alloreactivity | ILT2, ILT4 ⁵⁷ |
| Inhibition of proliferation | ILT2 |
| Up-regulation of inhibitory receptors | — |
| Generation of regulatory T cells, which include CD4 ^{low} T cells | — |
| APC | |
| Inhibition of DC maturation, antigen presentation, trafficking, and induction of regulatory T cells | ILT4 |
| Up-regulation of inhibitory receptors | — |
| PBMC: secretion of Th2 cytokines | CD160 |
| Endothelial cells: apoptosis | — |

Table 1. HLA-G functions: Modified by Carosella Blood 2008.

Because of this broad inhibitory function, capable of targeting multiple immune cell subsets, much effort has been put into determining whether HLA-G is pathologically relevant and whether it can be used as a diagnostic tool or as a therapeutic tool and/or target. Transplantation and oncology are 2 particularly clear situations. In the context of transplantation, HLA-G expression might be beneficial and promote tolerance to grafts. To date, the expression of HLA-G was studied in more than 1000 patients after heart [123] kidney (54) liver (55) and liver-kidney(55-57) transplantation, with those expressing HLA-G in the graft and/or the plasma exhibiting significantly better graft acceptance. Thus, in transplanted patients, titration of HLA-G might be used as a monitoring tool to determine and follow tolerance status, which could then be used to adjust immunosuppressive therapies. In this context, patients with high HLA-G titers could be candidates for a reduction in immunosuppressive treatment, whereas HLA-G–negative patients would have a comparatively higher risk of rejection. Furthermore, HLA-G itself might be used as therapeutic tolerogenic agent, exogenously provided to HLA-G–negative patients as complementary and/or alternative therapy [124].

In the context of oncology, studies on more than 1000 malignant lesions confirmed Carosella et al first study on melanoma [125] which showed that HLA-G transcription and protein expression may be switched on in tumor lesions and protect them from NK cytotoxicity. It was later shown that HLA-G expression by tumor lesions protected against cytotoxicity [126] correlated with malignancy in ovarian and breast carcinomas (61) as well as in melanocytic lesions [127] with unfavourable outcome in chronic lymphocytic leukemia,63 and gastric and colorectal cancers [128].

High HLA-G plasma levels were also recently observed in patients with neuroblastoma and correlated with relapse[129]. Expression of HLA-G has been evidenced in several malignant hematopoietic diseases, particularly in acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), and B-chronic lymphocytic leukemia (B-CLL).

Thus, HLA-G expression would favor tumor development by impairing antitumor immunity. Here again, HLA-G titration in peripheral blood might be used for diagnosis and/or monitoring, but in

this context, high titers of HLA-G would represent a negative factor [130]. In HLA-G–positive patients, HLA-G itself might finally constitute a therapeutic target: if expressed as a membrane-bound protein, as observed in some hematologic malignancies [131] HLA-G could be used as a tumor marker to deliver therapy. Alternatively, HLA-G could be blocked or deleted as a contributor to tumor immunosuppression and/or tumoral escape [132]. Recently, new aspects of HLA-G biology have been reported that are critical to HLA-G pathologic relevance and should help design HLA-G–based diagnosis and therapeutic strategies. Second is the demonstration that HLA-G is not only a shield against immune aggression but can also have a long-term inhibitory function through regulatory cells [133].

1.2.3 HLA-G and Regulatory T cells

Treg cells constitute 1–10% of thymic and peripheral CD4⁺ T cells in human and mice, and arise during thymic selection. They are characterized by the constitutive expression of the IL-2R α chain (CD25) and expression of the forkhead winged helix transcriptional regulator Foxp3. The importance of Foxp3 has been demonstrated by natural mutations of the *foxp3* gene that result in a loss of Treg cell function and the development of severe autoimmune diseases [134]. Treg cell population can be divided into the naturally occurring Foxp3⁺ Treg population, generated in the thymus and any one of many inducible Treg cell populations that are derived in the periphery from CD4⁺Foxp3[–] precursors upon activation in presence of differentiating signals like TGF- β and IL-10 [135].

The functional hallmark of CD4⁺CD25⁺ Treg cells is their remarkable capacity to suppress T effector/memory (Teff/mem) cell activation both in vitro and in vivo. Recent reviews have updated the idea that Tregs can regulate self-reactive T cells to maintain peripheral tolerance (non-autoimmune recognition of organ antigens) [136]. Impaired capacity or removal of Tregs allows

unrestrained proliferative responses of pathogenic, autoreactive T cells. Adoptive transfer of Treg cells reduces the pathology of experimentally induced autoimmune diseases such as gastritis, insulin-dependent diabetes mellitus, and colitis [137] whereas depletion of CD4⁺CD25⁺ Treg cells results in the development of systemic autoimmune diseases [138]. Both the suppressive capacity [139] as well as the frequency of Treg cells [140] are diminished in patients with autoimmune diseases such as psoriasis and pemphigus vulgaris.

So it was immediately recognized the therapeutic potential of these cells, not only in autoimmune disease, but also in certain infections and tumors. The peripheral blood of epithelial cancer patients has elevated circulating regulatory T cells, and numerous mouse models have shown that manipulation of this cell population can increase or decrease immune-mediated tumor rejection [141]. Their tolerogenic effect also has been hypothesized to underlie the persistence of certain viral infections such as hepatitis C [142].

Particular interest in their ability to determine patient tolerance to non-self antigens was augmented by the discovery that antigen-specific CD4⁺ regulatory T cells were increased in mice, which tolerated allografted tissues long-term [143]. A number of human studies have since shown that a high number of circulating TRegs in kidney and liver transplant patients is correlated with the stability of graft acceptance [144]. As such, considerable excitement about the clinical usage of TRegs in organ transplantation has been drawn up in the past decade.

Foxp3 is considered to be a “master regulator” of the Treg lineage, and mutations or absence of the gene lead to a fatal, autoimmune lymphoproliferative disease in both mice and humans. [145]. The activation of Foxp3 itself is mediated by both acetylation and phosphorylation events [146] and the protein acts in complex with other transcription factors, including the nuclear factor of activated T cells (NFAT), to control gene expression [147]. The interaction of Foxp3 with other transcription factors, such as NFAT, serves to sequester these factors and thereby down-modulate expression of genes involved in T-cell activation and effector functions [148]. Although Foxp3 is a unique marker of murine Treg cells, its expression in human CD4⁺ T cells is not restricted to Treg cells. [149].

Regulatory T cells employ a variety of effector mechanisms to suppress immune responses [150] through both contact dependent mechanisms as well as the secretion of soluble factors. Several specific mechanisms have been described, including the inhibition of IL-2 secretion; release of inhibitory cytokines; perforin- or granzyme- dependent cytolysis of APCs or responder T cells; synthesis of immunosuppressive adenosine; and down-regulation of APC function via co-stimulation with cytotoxic T-lymphocyte antigen 4 (CTLA-4, Figure 1).

Several studies showed that HLA-G, which is expressed by target cells, engages inhibitory receptors on effector cells, resulting in a transient block in their functions, and so acts as a shield against immune aggression [151]. However, it is now clear that HLA-G-related regulatory cells exist and that some of these can have a long-lasting inhibitory effect on immune responses. HLA-G-induced regulatory T cells were first observed after stimulation of T cells with HLA-G1-expressing APCs [152]. These regulatory T cells arise during an antigenic stimulation, do not respond to stimulation, and can block the alloreactivity of autologous T cells in vitro. Such regulatory T cells can be generated by membrane-bound HLA-G [153] or soluble HLAG5 [154]. and were detected in vivo after transplantation [155].

2.OBJECTIVES:

As we have seen NGAL is involved in many physiological and pathological process such as iron delivery, bacteriostatic action, inflammation, oxidative stress and the cells growth and differentiation in embrional period, after tissues damage and in malignancy. The physiopathological link between this different action is still not well elucidated. It is not well elucidated yet if it participate in to the establishment of processes useful to counterbalance a condition of “aggression” (bacterial attack, infection disease, ischemic injury, apoptosis and necrosis), or if it’s activation promote, in the same conditions, regeneration process that may conduce to tissue repair or to the restoring physiological conditions.

To take a step forward in the knowledge of this complex question we purpose to investigate the role of NGAL in immune response modulation.

In particular, we evaluated:

- 1) NGAL effect on membrane-bound (mHLA-G) and soluble (sHLA-G) forms of HLA-G expression of PBMNCs (in vitro model of healthy subjects)
- 2) Relevance of iron presence on NGAL effects on HLA-G expression of PBMNCs
- 3) NGAL effect of on T regulatory cells expression.

3. MATERIALS AND METHODS

3.1 PBMCs isolation and cell culture

In order to obtain cells for cell cultures, peripheral blood mononuclear cells (PBMCs) were obtained from 8 healthy donors after informed consent. Blood samples of 30 ml were taken in EDTA anticoagulant tubes. PBMCs were isolated from whole blood by Ficoll gradient (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). and resuspended in RPMI medium (Lonza, Basel, Switzerland) with 10% fetal bovine serum (FBS) (Lonza, Basel, Switzerland), 100 U/mL penicillin and 100 U/mL streptomycin (Sigma-Aldrich, St Louis, MO, USA). PBMCs activation was performed by the addition of phytohaemagglutinin, PHA (Sigma Aldrich, St. Louis, MO) 5 µg/ml in cell culture.

3.2 NGAL in vitro effect on HLA-G expression in PBMCs

In order to assess the in vitro effect of NGAL on HLA-G expression, PBMCs treatment was performed by the addition of NGAL (R&D Systems, Minneapolis, USA) conjugated with Enterobactin:Iron (ECM Microcollection, Tuebingen, Germany). Considering NGAL concentration used in Gwira's study [156] we added to PBMC cell cultures 40 ng/ml of NGAL. Cells were incubated at 37° C and 5% CO₂ for 72 hours. The intracytoplasmic expression of HLA-G was evaluated through the use of the monoclonal antibody MEMG/9-FITC (Abcam, Cambridge, UK) by cytometric analysis.

To assess the expression of the following markers on PBMCs flow cytometric analysis was made: CD3-PC5, CD4-PE and CD8-FITC (BD).

3.3 NGAL in vitro effect on HLA-G expression in PBMCs after treatment with scalar doses of NGAL:Enterobactin:Iron (40-320 ng/ml)

In order to understand whether the effect of NGAL on expression of HLA-g in PBMNs was dose-dependent, PBMCs were treated with increasing concentrations of NGAL:Enterobactin:Iron (40 ng/ml, 80 ng/ml, 160 ng/ml and 320 ng/ml). Cells were incubated at 37° C and 5% CO₂ for 72 hours.

The intracytoplasmic expression of mHLA-G was evaluated through the use of the monoclonal antibody MEMG/9-FITC (Abcam, Cambridge, UK).

3.4 NGAL:Enterobactin Iron Free effect on HLA-G expression.

In order to determinate if NGAL in vitro effect on HLA-G expression in PBMCs cultere was influenced by iron presence, we used NGAL 40 ng/ml conjugated with Enterobactin Iron free. Cells were incubated at 37° C and 5% CO₂ for 72 hours. Intracytoplasmic expression of HLA-G was evaluated.

3.5 NGAL:Enterobactin:Iron effect on HLA-G expression following anti-NGAL antibody in vitro administration.

In order to evaluate if HLA-G expression depended directly by NGAL activity, Anti-NGAL Monoclonal Antibody (200 µg; 1 mg/ml) (Thermo Scientific, Waltham, MA, USA) was used . To evaluate NGAL activity inhibition we used 25 µl/ml of anti-NGAL (50 ng/ml) and 50 µl/ml of anti-NGAL (100 ng/ml) to inhibit NGAL 40 ng/ml and 80 ng/ml respectively.

3.6 Indirect evaluation of NGAL role on HLA-G expression after treatment with Enterobactin:Iron.

PBMCs were treated only with Enterobactin:Iron (ECM Microcollection, Tuebingen, Germany) 160 ng/ml to evaluate if HLA-G expression is directly modulated by NGAL.

3.7 Soluble HLA-G evaluation through ELISA after NGAL:Enterobactin:Iron treatment.

We evaluated sHLA-G after treatment of PBMCs with NGAL:Enterobactin:Iron 40-320 ng/ml and with NGAL:Enterobactin 40 ng/ml through sHLA-G ELISA (BioVendor).

3.8 NGAL effect on T regulatory cells.

T reg Detection Kit, CD4/CD25/FoxP3-PE (Miltenyi Biotech) was used to evaluate T regulatory cells expression and to verify if NGAL is able to influence T regulatory cells expression after treatment with NGAL 40-320 ng/ml. In order to evaluate if CD4⁺ FoxP3⁺ and CD4⁺ CD25⁺ FoxP3⁺ expression depended directly by NGAL activity, anti-NGAL Monoclonal Antibody (200 µg; 1 mg/ml) (Thermo Scientific, Waltham, MA, USA). To evaluate NGAL activity inhibition we used 25 µl/ml of anti-NGAL (50 ng/ml) and 50 µl/ml of anti-NGAL (100 ng/ml) to inhibit NGAL 40 ng/ml and 80 ng/ml respectively.

In order to understand whether the effect of NGAL on CD4⁺ FoxP3⁺ and CD4⁺ CD25⁺ FoxP3⁺ expression was dose-dependent and influenced by iron presence, PBMCs were treated with increasing concentrations of NGAL:Enterobactin:Iron (40 ng/ml, 80 ng/ml, 160 ng/ml and 320 ng/ml) and NGAL:Enterobactin (40 ng/ml). Cells were incubated at 37° C and 5% CO₂ for 72 hours.

The intracytoplasmic expression of HLA-G was evaluated through the use of the monoclonal antibody MEMG/9-FITC (Abcam, Cambridge, UK) by flow cytometry. The expression of CD4 and FoxP3 were evaluated through the use of antibodies anti-CD4 and anti-FoxP3 respectively.

4. RESULTS

4.1 NGAL in vitro effect on HLA-G expression in PBMCs

We analyzed in our research intracytoplasmic HLA-G expression on healthy donors PBMCs cultures. We treated PBMCs from two healthy donors with NGAL to consider HLA-G expression

in the cytoplasm. After 72 hours NGAL: enterocalin:iron incubation, We observed an increase in HLA-G expression on CD4+. This condition is not observed for CD8+. The detection of HLA-G expression with flow cytometry, was observed in not activated cells and also in cells activated with phytohaemagglutinin (PHA).

Analyzing withflow cytometry PBMC cells from healthy donors, CD4+, we notice that after incubation with NGAL: enterocalin: iron, there is an increase in the cells expressing HLA-G percentage compared to control cells. (The data regarding two of the healthy donors are presented in figure 3.

4.2 NGAL in vitro effect on HLA-G expression in PBMCs after treatment with scalar doses of NGAL:Enterobactin:Iron

In the light of the results so far exposed, we continued our research trying different NGAL concentrations to understand if CD4+ and CD8+ cells HLAG expression was dose dependent.

We then verified that increasing dose of NGAL:Enterobactin:Iron (40 ng/ml, 80 ng/ml, 160 ng/ml and 320 ng/ml) caused an increasing HLA-G expression on CD4+ T cells using as control PBMCs and activated (PBMCs trated with PHA). Figure 4.

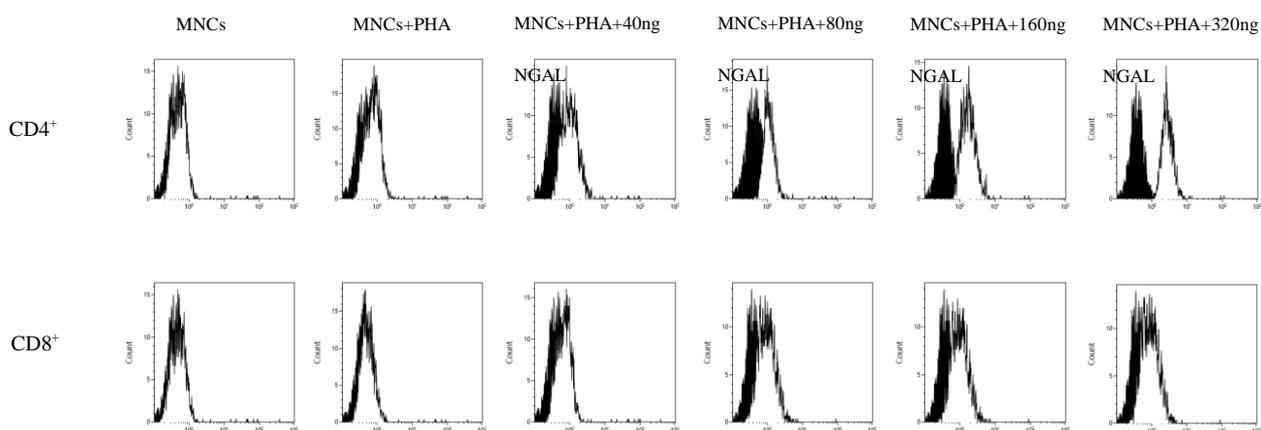


Fig. 4 HLA-G expression on CD4+ T cell: a) control PBMCs; b) PBMCs activated with PHA; c) PBMCs activated with PHA, treated with NGAL 40 ng/ml; d) PBMCs activated with PHA, treated

with NGAL 80 ng/ml; e) PBMCs activated with PHA, treated with NGAL 160 ng/ml; f) PBMCs activated with PHA, treated with NGAL 320 ng/ml; isotype control in black, PBMCs at different conditions in white.

The increase was proportional to NGAL increasing concentration: in fact for subject 4 we found a 7.3% of cell expressing HLAG in PBMCs cells, 32.8% of cell expressing HLAG in PBMCs cells activated with PHA, 43.8% of cell expressing HLAG with NGAL 40 ng/mL, 45.8% of cell expressing HLAG with NGAL 80 ng/mL, 61.8% of cell expressing HLAG with NGAL 160 ng/mL, 67.6% of cell expressing HLAG with NGAL 320 ng/mL; in subject 5 the trend was similar: 2.8% of cell expressing HLAG in CD4+, 34.5% of cell expressing HLAG in CD4+ cells activated with PHA, 44.1% of cell expressing HLAG with NGAL 40 ng/mL, 54.6% of cell expressing HLAG with NGAL 80 ng/mL, 63.8% of cell expressing HLAG with NGAL 160 ng/mL, 71.9% of cell expressing HLAG with NGAL 320 ng/mL (figure 4)..

The situation is different when we go to analyze CD8 + cells : in fact these cells do not exhibit a significant increase of the expression of HLA-G if treated with increasing concentrations of NGAL. These data demonstrate that HLA-G expression mediated by NGAL shows a different trend among activated CD4+ and activated CD8+ T cells. In fact we found 6.5% of cell expressing HLAG in CD8+ cells, 26.8% of cell expressing HLAG in CD8+ activated with PHA, 25% of cell expressing HLAG with NGAL 40 ng/mL, 32.6% of cell expressing HLAG with NGAL 80 ng/mL, 33.3% of cell expressing HLAG with NGAL 160 ng/mL, 35% of cell expressing HLAG with NGAL 320 ng/mL in subject 4 and 3.7% of cell expressing HLAG in CD8+cells, 19.5% of cell expressing HLAG in CD8+ activated with PHA, 24.9% of cell expressing HLAG with NGAL 40 ng/mL, 33.5% of cell expressing HLAG with NGAL 80 ng/mL, 34.2% of cell expressing HLAG with NGAL 160 ng/mL, 34.5% of cell expressing HLAG with NGAL 320 ng/mL in subject 5.

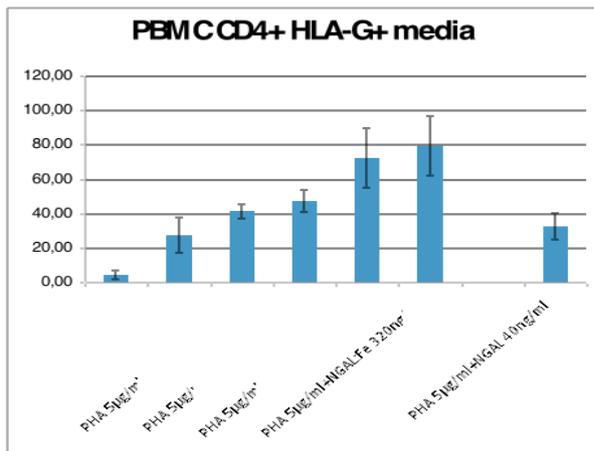


Fig. 4 HLA-G expression as fluorescence intensity percentage on CD4+ T cells in control and treated groups after treatment with NGAL (40-320 ng/ml).

4.3 NGAL:Enterobactin Iron Free effect on HLA-G expression.

We also used the complex NGAL:enterocalin iron free to verify if cells response to NGAL was iron dependent.

In PBMCs activated with PHA and treated with NGAL:Enterobactin Iron-free 40 ng/ml (g) an increase in HLA-G expression was observed, also if not of the same concentration of NGAL:Enterobactin:Iron complex. (Fig.xx). This was true both for CD4+ and for CD8+:36.7% and 31.3 % for subject 4 and 37.9% and 17.2 % for subject 5 respectively.

4.4 NGAL:Enterobactin:Iron effect on HLA-G expression following anti-NGAL antibody in vitro administration.

To asses NGAL action inhibition we used anti-NGAL Monoclonal Antibody (200 µg; 1 mg/ml) (Thermo Scientific). We found the following values of fluorescence positivity :: 1.7% in PBMCs, 13.6% in PBMCs+PHA, 28.4 % in PBMCs+PHA+NGAL:Fe 40 ng/ml, 22.2% in PBMCs+PHA+NGAL:Fe 40 ng/ml + anti-NGAL, 39.9% in PBMCs+PHA+NGAL:Fe 80 ng/ml and 18.5 % PBMCs+PHA+NGAL:Fe 80 ng/ml + anti-NGAL.

We can also observe that the monoclonal antibody action was dose-dependent with an inhibitory response more accentuated for 100 ng/mL addiction that for 50 ng/mL addiction (Figure 5 and 6).

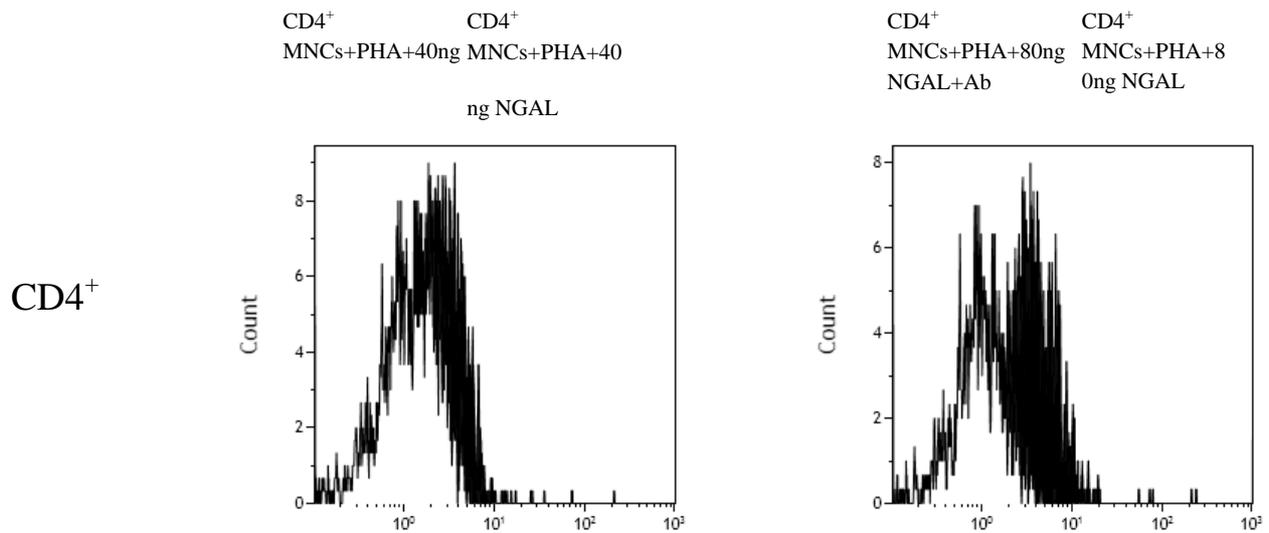


Fig. 5 HLA-G expression on PHA activated PBMCs after treatment with NGAL 40 and 80 ng/ml (black histogram) and incubation with an anti-Ngal antibody (white histogram).

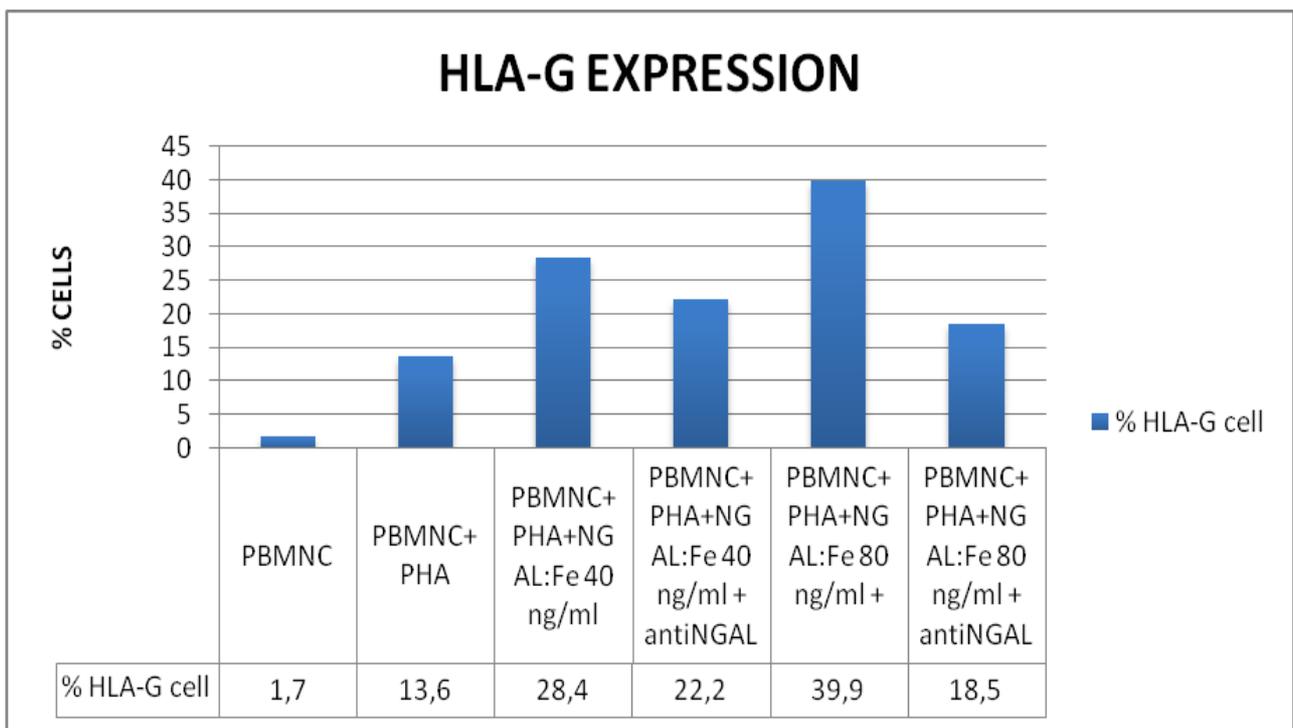


Fig. 6 HLA-G expression on PBMCs PHA activated after stimulation with NGAL 40 and 80 ng/ml and incubation with an anti-Ngal antibody.

4.5 Indirect evaluation of NGAL role on HLA-G expression after treatment with Enterobactin:Iron.

Evaluation of Enterobactin:Iron 160 ng/ml effect on HLA-G expression shows that Enterobactin:Iron without NGAL had none effect on HLA-G expression. (figure 7).

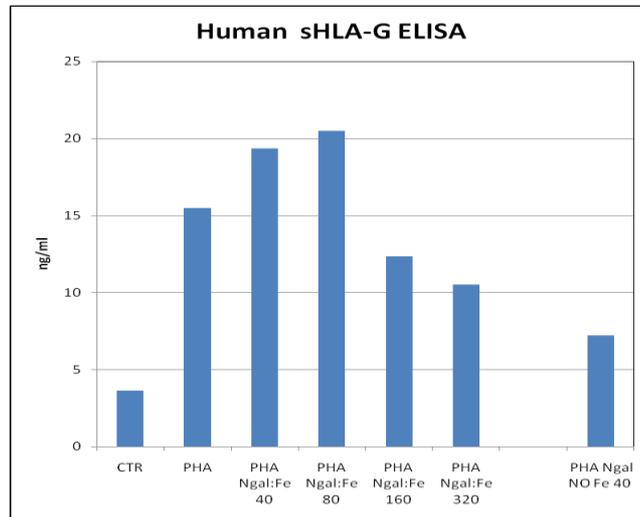


Figure 7: sHLA-G values.

4.6 Soluble HLA-G evaluation through ELISA after NGAL:Enterobactin:Iron treatment.

Soluble HLA-G evaluation through ELISA after NGAL:Enterobactin:Iron treatment shows increasing HLA-G secretion dose dependent up to NGAL:Enterobactin:Iron 80 ng/ml. In samples treated with NGAL:Enterobactin:Iron 160 and 320 ng/ml secretion of soluble HLA-G decreases. Stimulation with NGAL:Enterobactin 40 ng/ml induces HLA-G secretion only higher than the control.

4.7 NGAL effect on T regulatory cells

We wanted also to evaluate NGAL action on CD4⁺ CD25⁺ Foxp3⁺ (regulatory t cells, Tregs) to evaluate another important aspect of immune tolerance induction.

So we analyzed CD4⁺ FoxP3⁺ and CD4⁺ CD25⁺ FoxP3⁺ concentration before and after NGAL addition at increasing dose and with or without iron presence (40 ng/ml with or without iron, 80 ng/ml, 160 ng/ml, 320 ng/ml). All data about CD4⁺FoxP3⁺ cells and CD4⁺/CD25⁺/FoxP3⁺ cells are reported in (figure 8).

We observed an increase in CD4⁺ FoxP3⁺ and CD4⁺ CD25⁺ FoxP3⁺ expression after NGAL addition. This raise was dose and iron and dependent.

Indeed the percentage of Tregs increased with growing NGAL doses getting a plateau with NGAL:Enterochelin:Iron 320 ng/ml. The lack of iron did not increased Tregs cells expression.

Comparing CD4+ and CD25+ cells FoxP3 expression confirmed a dose-dependent raise for the treatment with NGAL (40-80-160-320ng/ml). The iron-free NGAL treatment presented less increased expression of Tregs.

We then verified NGAL direct activity in Tregs expression adding NGAL antibody and we observed that the presence of increasing anti-NGAL antibody determinate a significant reduction of Tregs percentage (both CD4+/FoxP3+ and CD4+/CD25+/FoxP3+ .

Finally we evaluated NGAL effect on Tregs expression after Enterobactin:Fe 160 ng/ml treatment and we found that Enterobactin:Fe had none effect on CD4+/FoxP3+ and CD4+/CD25+/FoxP3+ cells expression

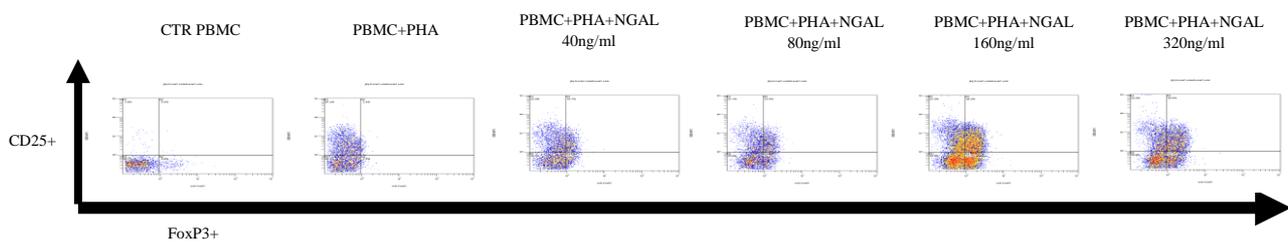


Fig.8 Percentage CD4⁺/CD25⁺/FoxP3⁺ cells after treatment of PBMCs with NGAL (40 ng/ml with or without iron, 80 ng/ml, 160 ng/ml, 320 ng/ml).

5. Discussion and Conclusions

Nowadays NGAL is emerging as a mediator of various physiological and pathological conditions. Indeed in literature is widely documented as NGAL is involved in the transport and metabolism of iron, as well as its bacteriostatic activity is well known. The presence of NGAL molecule is also important as growth factor and in the differentiation of immature kidney. In the last years researchers' attention was focused on the role of NGAL as a biomarker of many diseases including acute and chronic kidney damage, kidney transplantation, autoimmune and neoplastic disorders. (Chakraborty)

Despite numerous studies that have investigated the physiological and pathophysiological role of NGAL, this is not entirely elucidated. The use as biomarkers in many pathological conditions shows that the molecule is characterized by a low specificity for pathology: this leads us to think that there is a biological effect of the molecule that is common to these different pathological conditions [157].

In the light of pathologies involved, the molecule seems to be a response to different pathological conditions that are united by inflammation and oxidative stress. [158].

With regard to the involvement of NGAL in immunity, only one study in the literature verifies the involvement in cellular one [159], while none analyzes the role of the molecule in the humoral one. In recent years, the interaction between NGAL and NF- κ B (nuclear factor- κ B) has been studied. The expression of NGAL mRNA and protein is up-regulated in an NF- κ B dependent manner in rat and human vascular smooth muscle cells (SMCs) in response to IL-1 β stimulation [160].

Recent evidence showed that there is also a possible interaction between NF- κ B and NGAL gene requiring I κ B- ζ for its induction: the coexpression of I κ B- ζ and the NF- κ B subunits synergistically activates the transcription of NGAL genes [161]. The transcription factor NF- κ B plays a key role in

the innate and adaptive immune systems. The interaction between NGAL and NF- κ B and the involvement of NF- κ B in innate and adaptive immune system suggest a possible role of NGAL in immune tolerance.

Our study on healthy controls had the aim to individuate the possible involvement of NGAL in the processes of immune response, focusing on the hypothesis that NGAL could promote immune tolerance.

By flow cytometry analysis we demonstrated that NGAL can increase the expression of mHLA-G in PBMCs cell cultures, particularly for CD4⁺ T lymphocytes. It is known as the role of HLA-g is not only important for the maternal-fetal tolerance. He plays a significant role in adult life and especially in some pathological conditions of Autoimmunity, cancer and tolerance in organ transplantation [162]. As a factor able of activating the expression of HLA-G, NGAL may play the role of mediator in acute and chronic processes with a similar anti-inflammatory meaning [163].

We also have shown that increasing NGAL concentration raises in vitro level of mHLA-G. So we can postulate that NGAL increasing is not a random process, but it may express a biological meaning in various pathological conditions and be modulated in favor of biological necessity. We can also indirectly hypothesize that a decreased capability of NGAL production could be translated in a lower reaction to inflammation and aging conditions.

The iron absence seems to have a role in NGAL action, leaving the expression of mHLA-G unaltered. The iron deficiency may be negative not only for characterizing systemic inflammation states by affecting the anemia conditions, but also inducing aging process and reducing tissue renewal [164].

Use of NGAL:Ent:Fe in the presence anti-NGAL antibody is not able to modify the expression of mHLA-G, confirming the action of NGAL molecule on mHLA-G activation.

Using of increasing concentration of NGAL conjugated with iron induces an activation of Tregs cells. The trend of activation seems to depend from NGAL dosing.

NGAL role in FoxP3+ cells activation must be considered with extreme attention in the light of Tregs function in autoimmune and neoplastic disease[165], and in the graft tolerance in solid organ transplantation. Several authors indeed proposed Tregs cell therapy for graft tolerance induction.

In conclusion we believe NGAL is an extremely interesting molecule and its biological role is still far from being fully clarified. If clinically most study in recent literature have focused on its role of biomarker of kidney , neoplastic and inflammatory diseases we believe that other clinical interest scenarios are possible.

These include the deep understanding of the role of NGAL in humoral immunity may lead not only to the understanding of concerning physiopathological mechanisms but also a molecule used for therapeutic purposes in many conditions under which the regulation of immune processes can play a supportive role.

6. REFERENCES

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