# IMPACT OF IMMUNOGENETIC POLYMORPHISMS ON IMMUNE RESPONSE AND CLINICAL FEATURES IN BONE MARROW FAILURE SYNDROMES

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## Riassunto

Le cellule staminali emopoietiche sono le cellule responsabili della produzione degli elementi maturi del sangue midollare; la pancitopenia e' una condizione che caratterizza la presentazione clinica di diverse patologie, sia ematologiche che extra-ematologiche, dove viene intaccata la funzionalita' midollare. Le sindromi da insufficienza midollare di origine primaria (BMF) sono un gruppo eterogeneo di patologie caratterizzate da un disordine immunologico in grado di determinare la distruzione delle cellule staminali emopoietiche con una conseguente globale o selettiva aplasia midollare. In particolare, la distruzione su base autoimmunitaria delle cellule staminali e' alla base delle AA. Gli stessi meccanismi patogenetici sono coinvolti in emopatie clonali quali l'emoglobinuria parossistica nottura (PNH) ed alcuni tipi di sindromi mielodisplastiche (MDS). Nelle MDS, sono stati ipotizzati diversi meccanismi immunologici in grado di determinare la comparsa della citopenia: a) l'attacco immunologico alle cellule staminali potrebbe essere parte integrante delle sorveglianza anti-tumorale in risposta alle cellule displastiche midollari ed, in tal caso, essere non sufficientemente specifico tale da dare origine, come danno collaterale, ad inibizione della normale attivita' emopoietica; b) l'attacco immunologico potrebbe essere diretto contro le cellule staminali normali, come si verifica nell'AA, favorendo l' "escape" di cloni ematopoietici mutati. Questo ultimo meccanismo e' stato chiamato in causa per spiegare la patogenesi della PNH. In ogni caso numerosi dati indicano che il sistema immunitario ha un ruolo centrale nella fisiopatologia delle sindromi da insufficienza midollare; in questo studio abbiamo cercato di identificare dei fattori, geneticamente determinanti, che in qualche modo possano condizionare il funzionamento del sistema immunitario ed, in seconda battuta, lo sviluppo di una disfunzione midollare. La nostra ipotesi si e' basata sull'idea che gli antigeni leucocitari umani (HLA), i recettori inibitori delle cellule NK (KIR) cosi' come la risposta citochinica associata alle varianti genetiche delle citochine e dei loro recettori e promotori, possano influenzare la risposta immune e predisporre ad una risposta immunitaria aberrante. Tali reazioni potrebbero essere responsabili dei meccanismi patogenetici delle sindromi da insufficienza midollare di origine immuno-mediata. Per tale motivo siamo andati ad analizzare l'associazione tra BMF e numerosi fattori immunogenetici in 167 pazienti, includendo fattori quali l'antigene leucocitario umano (HLA), il genotipo dei recettori per NK immunoglobulin-like (KIR), I KIR/KIR-L mismatch, ed i polimorfismi di singoli nucleotidi per i geni del CTLA-4 (+49 A/G), CD16(−158V/F), e di alcune citochine quali: IL-1α (-889 T/C), IL-1R (-1970 C/T), IL-1RA (11100 T/C), IL-4RA (+ 190 G/A), IL-1β (-511 C/T, +3962 T/C), IL-6 (-174 C/G, nt565 G/A), IL-10 (-1082 G/A, –819 C/T, -592 C/A), IL-12 (-1188 C/A), TGF-β (codon 10 C/T, codon 25 G/C), TGF-βR2 (+358 A), INF-γ (+874 A/T), TNF-α (-308 G/A, –238 G/A), IL2 (-330 T/G, +166 (G/T), IL4 (-1098 T/G, -590 T/C, -33 T/C). Il nostro studio porta alla conclusione che la regolazione genetica dell'infiammazione e il pathway immunologico T-cell mediato possono essere coinvolti nella patogenesi delle sindromi da insufficienza midollare, rafforzando l'ipotesi che sia l' AA che la PNH sono disordini autoimmunitari organo-specifici.

## SUMMARY

Hematopietic stem cells (HSC) are responsible for the production of mature blood cells in bone marrow; peripheral pancytopenia may result from several different conditions, including hematological or extra-hematological diseases (mostly cancers) affecting the marrow function as well as primary failure of hematopoiesis. Although the clinical presentation may appear homogeneous, primary bone marrow failure syndromes are a heterogeneous group of diseases with specific pathogenic mechanisms, which share a profound impairment of the hematopoietic stem cell pool resulting in selective or global marrow aplasia. Immunomediated elimination of stem cells due to the presence of a cross-reactive antigen or autoantigen restricted to the stem cell compartment may be responsible for AA. Similar pathophysiologic mechanisms may also operate in related diseases such as paroxysmal nocturnal hemoglobinuria (PNH) or some forms of myelodisplastic syndromes (MDS). Most often these disease are characterized by an extrinsic damage of hematopoietic stem cells that affect their function. Effector mechanisms in hematopoietic inhibition may involve various pathways, including release of cytokine leading to apoptosis of hematopoietic progenitor and stem cells. In MDS, various possible mechanisms have been postulated to explain the occurrence of cytopenia due to inhibition of normal residual hematopoiesis. For example, the immune attack can be part of physiologic anti-tumor surveillance response to abnormal and/or dysplastic cells in the bone marrow. In such situation, the immune attack may be sufficiently specific and results in collateral damage with inhibition of normal hematopoiesis. Conversely, the initial immune attack may be directed against normal stem cells as in AA, resulting in selection pressure with outgrowth and escape of mutant hematopietic clones. Similar consideration apply to the evolution of glycophosphatidyl- deficient clones in PNH. A number of evidences demonstrated that the immune system plays a pivotal role in the pathophysiology of bone marrow failure syndromes. In this study we planned to look for biological factors, especially at the genetic levels, which may somehow drive the function of the immune system, possibly leading to the development of a form of bone marrow failure. Our hypothesis is that the human leukocyte antigen (HLA) and killer inhibitory receptor (KIR) background as well as the quality of the cytokine response due to genetic variants of the cytokine, cytokine receptors genes and their promoters may modulate the quality of immune response and predispose to aberrant overshooting immune reactions. Such reactions may determine the risk for immune-mediated bone marrow failure. The association of BMF with a number of immunogenetic factors was analysed in 167 patients, including human leucocyte antigen (HLA) and killer-cell immunoglobulin-like receptor (KIR) genotype, KIR/KIR-L mismatch, CTLA-4 (+49 A/G),CD16−158V/F, and cytokine single nucleotide polymorphisms including: IL-1α (-889 T/C), IL-1R (-1970 C/T), IL-1RA (11100 T/C), IL-4RA (+ 190 G/A), IL-1β (-511 C/T, +3962 T/C), IL-6 (-174 C/G, nt565 G/A), IL-10 (-1082 G/A, –819 C/T, -592 C/A), IL-12 (-1188 C/A), TGF-β (codon 10 C/T, codon 25 G/C), TGF-βR2 (+358 A), INF-γ (+874 A/T), TNF-α (-308 G/A, –238 G/A), IL2 (-330 T/G, +166 (G/T), IL4 (-1098 T/G, -590 T/C, -33 T/C). Our data suggest that genetic regulation of inflammatory and T-cell-mediated immunological pathways could be involved in the pathgenesis of bone marrow failure, reinforcing the view that both AA and PNH are organ-specific autoimmune disorders.

# Introduction

Hematopoietic stem cells (HSC) are responsible for the long-life production of mature blood cells; they physiologically work within the bone, where they interact with other cellular types and soluble factors, formally known as microenvironment, to constitute the bone marrow [1]. Hematopoiesis is a hierarchical process which starts from the multipotent HSC and proceeds through more committed and differentiated progenitors [2]; while differentiation and maturation are essential for producing mature circulating cells, self-renewal is the main feature of HSC ensuring the long-term maintenance of hematopoiesis [3]. All these functions are finely regulated by a complex network which includes cell-cell interactions between hematopoietic and stromal cells, as well as the action of several soluble cytokines, often working in a paracrine fashion within the so-called hematopoietic niches. Bone marrow is a functional tissue which may be insufficiently performing in several conditions, all clinically presenting as mono- or multi-lineage cytopenia. Bone marrow failure (BMF) may result from various extra-hematological diseases, such as malignancies, infectious diseases and nutritional deficiencies, all of them secondarily affecting the HSC function. Similarly, hematopoiesis may be impaired in various hematological diseases, such as lymphoproliferative and myeloproliferative disorders; even when the disease affects the HSC itself, such as in leukemias, the BMF is usually considered secondary to the underlying disease. By contrast, primary BMF syndromes are a heterogeneous group of hematological diseases (Tab. 1) characterized by the absence of any other disorder potentially affecting marrow function. However, while they are considered distinct disease, in some cases their patophysiology involves similar pathogenic mechanisms, which finally lead to stem cell and/or hematopoietic progenitor damage. In this work, we test the hypotesis that genetic factors may affect the functioning of the immune system, and especially the complex cytokine network which may interfere with normal hematopoiesis.

## APLASTIC ANEMIA

***Aplastic anemia*** **(AA**) is the paradigm of bone marrow insufficiency; as other BMF syndromes, it is characterized by peripheral pancytopenia [4]. The hallmark of AA is an empty or fatty marrow as evidenced by bone aspirate and biopsy, which directly demonstrates the contraction of the hematopoietic cell compartment leading to deficient hematopoiesis. According to the hierarchical model of hematopoiesis, the desert marrow in AA results from the impaired HSCs function. The alteration affecting the HSC may be different depending on the specific form of AA; indeed, even within the AA setting, different entities may be sorted, each one with specific pathophysiologic mechanisms. A first distinction has to be made between constitutional and acquired forms of AA. In the present thesis, we will not deal with constitutional forms.

***Acquired aplastic anemia.*** Acquired forms of AA are far more frequent than the constitutional ones; typically they affect young adults or elderly people, who present with peripheral pancytopenia in absence of other hematological diseases. Pancytopenia of AA patients results from the impairment of the hematopoietic

progenitor compartment, including HSC and more committed progenitors; the nature of the injury damaging hematopoiesis very often remains undetected (Fig. 1). Cytotoxic drugs and radiation are the best examples of a direct injury to HSC; although stem cells, due to their dormant nature, are more resistant to cytotoxic drugs, for most agents a dose-response relationship with the degree of stem cell damage can be established. However, iatrogenic direct injury by chemotherapy or radiation is rarely involved in marrow failure syndromes; sometimes, although exposure to a list of drugs may be documented (Tab. 1), a definitive causative relationship cannot be demonstrated. Indeed, even if a putative inciting agent directly attacks the stem cell pool, causing a permanent depletion of HSC, the clinical presentation of cytopenia may be delayed for weeks or months, appearing just when a critically low stem cell number is reached. These considerations are applicable to chemical agents as well as to a number of viruses which may infect hematopoietic progenitors; in all these conditions the damaging mechanism may also involve non-direct injury of the HSC. The indirect damage of HSC is mainly sustained by immune effector mechanisms (Fig. 1), which may be possibly triggered by viruses or by drug metabolites. An appropriate example for such a mechanism is the *hepatitis/AA syndrome*, in which AA follows with a delay of months an episode of acute hepatitis that carries all the characteristics of a viral infection. The viral agent responsible for this syndrome has not been identified; it is possible that at the time of overt cytopenia the viral infection is already cleared, AA being mediated by lymphocytes recognizing a cross-reactive antigen in the marrow. EBV-associated AA has been described; even this form seems not to be mediated by direct viral cytoxicity, rather by the immune system. Similar pathogenic mechanisms may be postulated in the majority of cases of *idiopathic AA*, possibly involving the presence of neo-antigens or cross-reactive triggering antigens which result in a breach of immune tolerance; this would generate an immune-mediated attack towards hematopoietic progenitors, leading to HSC consumption or functional impairment with subsequent pancytopenia.

Regardless the nature of injury, AA patients are characterized by a severe dysfunction of the hematopoietic stem cells; this defect has been deeply characterized on both quantitative and qualitative fashion. As those with constitutional AA, patients with acquired AA show a very low number of hematopoietic progenitors, as measured by flow cytometric CD34+ cell assessment or by in vitro colony assays [5-7]. By flow cytometry, CD34+ cells are reduced in all AA patients, and the contraction affects both committed and immature CD34+/c-kit- or CD34+CD38- progenitors [8]. Unlike in murine models, the measurement of more immature progenitor and stem cells is not easily accomplished in humans. Consequently, several surrogate in vitro stem cell assays have been developed, including long-term culture-initiating cells (LTC-IC) as well as cobblestone forming assay [9,10]. The LTC-IC assay assesses cells capable of colony formation after 5 weeks in long-term bone marrow culture; LTC-ICs share the frequency, phenotype, and kinetic properties of true stem cells [10-12]. Several studies indicate a profound deficiency in LTC-IC as well as cobblestone unit initiating cells in all patients with AA [5,9,13]. At the time of clinical presentation, the number of LTC-IC is usually at least one log below the normal level; combined with a reduction in total marrow cellularity to <10%, the stem cell number in AA is estimated to be reduced of at least two logs compared to healthy individuals [9]. Neither the LTC-IC number nor that of colony forming cells correlate with the blood counts, suggesting that in addition to the quantitative defect, a functional impairment may be present [5]; this

may also be extrapolated by the observation that the clonogenic capacity of an individual progenitor is lower than in normals. The reduced clonogenicity was demonstrated both on CD34+ cells (as number of colonies obtained from a purified CD34+ population) and on the putative stem cell LTC-IC (as number of secondary colonies assayed from LTC-IC in limiting dilution experiments) [5,9,14]. These findings suggest that the hematopoietic stem cell compartment is affected by the pathophysiologic process operating in AA, whereas mesenchymal or even more immature pluripotent stem cells are likely functionally normal. Indeed, several studies have documented that marrow cells from AA patients are able to generate in vitro perfectly functional stromal layers, as confirmed on cross-over experiments, which strongly support that stromal progenitors or early pluripotent stem cells are not affected in AA [15-17]. This is also confirmed by the clinical observation that allogeneic stem cell transplantation is a highly successful therapy for AA, even if most stromal elements remain of host origin. Serial studies were conducted to determine the number of stem cells during the course of the disease and the kinetics of decline/recovery in stem cell number; a profound defect in LTC-IC number may persist for a long time, even in patients successfully treated by immunosuppression [9,13]. In most cases, a residual numerical LTC-IC defect may be permanent, despite blood counts full recovery, while a complete reconstitution is found only in a minority of patients with sustained complete remission [9]. Nevertheless, at least a partial recovery of stem cells is possible, and a highly contracted stem cell pool can sustain a seemingly normal blood cell production, even if the compensatory capacity in response to stress conditions may be diminished.

A qualitative defect of HSC is suggested by the reduced clonogenic potential, as already mentioned above [5]; however, this does not necessarily imply that the dysfunction is intrinsic to the HSC as in constitutional AA. Clearly, the hematopoietic recovery following successful immunosuppression demonstrates that some stem cells must have been spared from the pathologic process; impaired primary and secondary clonogenic capacity concerns a majority of HSCs, which likely are most damaged, but not all individual HSCs. Indeed, it is well known that CD34+ cells from AA patients show a high proportion of apoptotic cells [18-20]; it is possible that a few HSC resisting to the apoptotic stimuli in vivo may have normal functional properties. Several decades of laboratory experiments have suggested that the nature of the stem cell damage in AA involves activation of general immune effector mechanisms (Fig. 1) [21-23]. The stem cell damage can be due to direct cell-mediated killing by cytotoxic lymphocytes (CTL) as well as by cytokine-transduced inhibition; the latter is documented by excess production of type I cytokines, especially interferon-γ and tumor necrosis factor-$\alpha$ [24-26]. Additionally, Fas-ligand or tumor necrosis factor-derived inhibitory ligand (TRAIL) appear to play an important role as effector cytokines in the hematopoietic inhibition in AA [18,24,25,27]. Such mechanisms may not be restricted to the primary target only, but may also attack innocent bystander cells; ultimately, these factors result in apoptosis of all existing cells. Fas, IFN-$\gamma$ and TNF-$\alpha$ modulate the expression of their receptors through feedback mechanisms; in this way they may enhance each other action [26,28]. Furthermore, the effects of individual factors may be additive or synergistic [28]; chronic exposure to these cytokines in vivo may be more damaging compared with in vitro models, which challenge the acute effects of high cytokine concentrations [62]. Apoptosis is the main key mechanism of HSC damage. The apoptotic machinery may be constitutively activated in differentiated

cells, which may need appropriate signals to survive; in contrast, in stem cells apoptosis likely has to be induced by specific stimuli. Indeed, the abundance of trophic signals argues against the lack of survival signals (e.g. growth factors) as a mechanism of apoptosis for HSCs [30]. Increased apoptotic rate within the CD34+ cell population from AA patients has been demonstrated [18-20]; in addition, CD34+ cells from AA show increased expression of Fas [19,20,28,31], which may be induced by IFN-$\gamma$ and TNF-$\alpha$ in an aplastic marrow [18,25,32,33]. Both cytokines can up-modulate Fas expression even in normal CD34+ cells [28,34]; CD34+ cells derived from AA patients appear to undergo apoptosis in response to Fas at a higher rate compared with normal CD34+ cells [19,20,27,31]. Additional pathways of apoptosis may involve nitric oxide or oxygen radical secretion, similarly to what observed in FA; the production of such factors in hematopoietic progenitors may be triggered by classical pro-apoptotic stimuli, such as inhibitory cytokines, leading to apoptosis in a paracrine fashion [35,36].

A more accurate description of HSC in AA has become possible with the oligonucleotide microarray technology, which allows quantitation of the expression levels of a large number of genes. Recently, the gene expression profile in healthy human CD34+ stem/progenitor cells has been reported; the same technology has been applied to assess gene expression in CD34+ marrow cells from AA patients [37]. The study documented that the expression of several genes implicated in apoptosis and cell death was markedly increased in AA CD34+ cells, as well as that of genes involved in the negative control of cell proliferation. Examples of up-regulated genes were the death receptors *Fas, DR3*, *DR5, TNFRII*, and *TRAIL*. In contrast, genes promoting cell cycle progress showed a lower expression compared to CD34+ cells from healthy individuals, possibly explaining the inability of the residual stem cells to compensate the progenitor pool contraction. As anticipated from other evidences of heightened immune activity, several cytokine/chemokine signal transducer genes, stress response genes, and defense/immune response genes were up-regulated. In summary, the transcriptome analysis of HSC in AA is consistent with the presence of stressed, dying and immunologically activated target cells rather than of an intrinsically abnormal population. This support the hypothesis of an organ-specific immune attack on hematopoietic stem/progenitor cells, possibly T-cell mediated.

The presence in the circulation and in marrow of activated cytotoxic lymphocytes (CTLs), which inhibit hematopoiesis in vitro, has been reported in AA patients [38,39]; furthermore, we and others have described abnormalities of T cells and of the TCR repertoire [40-43], suggestive of an oligoclonal T cell response. For some patients, CD4+ and CD8+ T cell clones which responded to and destroyed autologous hematopoietic progenitors were characterized after in vitro immortalization [38,43]. More recently, we have identified dominant T cell clonotypes by sequencing the CDR3 of the TCR-β chain [44]; cells bearing this clonotype were found expanded in vivo, likely as a result of an antigen-driven dominant immune response, suggesting their pivotal pathophysiologic role. Indeed, these pathogenic T cell clones appeared to correlate with disease activity, and showed potent cytotoxicity directed against autologous marrow progenitor cells. The observed homology among various patient-specific clonotypes may suggest a semi-public immune response against common epitopes; however, the antigen(s) driving the immune attack on the HSC are still unknown, as well as the possible primary abnormalities of HSC leading to the breach of immune self-tolerance [44].

Other qualitative abnormalities of HSCs and their progeny in AA have been described; the most worthy is increased telomere shortening. In true stem cells, self-renewal does not result in telomere shortening, due to the activity of telomerase [45-47]; however, telomerase activity decreases upon commitment and differentiation [48], thus telomere length of the progeny reflects the number of doublings of the committed and more mature progenitors. Short telomeres have been reported in constitutional AA, as mentioned; even patients affected by acquired AA show telomere shortening, as measured by various methods [47,49,50]. Apparently paradoxically, patients with chronic moderate AA showed telomeres shorter than those of patients with a more severe disease; actually, in acute severe cytopenia, telomere shortening may not be evident due to a more extended block of stem cell cycling [47,49,50]. Upon recovery, due to recruitment of new stem cells, telomeres of the progeny may provide longer measurements again; however, if the stem cell number operating at a given time is small, in order to maintain a normal circulating cell number more divisions are needed and telomere shortening may be more pronounced. Proper function of the telomerase complex requires the presence of an intact RNA primer (TERC), as well as of the functional protein (TERT). DKC, a mutation in the *TERC* gene has been occasionally reported in patients with acquired AA, harboring very short telomeres; however, this defect seems restricted to patients with positive family history, and to peculiar clinic presentation (early onset, chronicity, lack of response to immunosuppression). Thus, these cases likely represent uncommon presentation of a form of constitutional aplasia. More recently, AA patients with a mutation within the *TERT* gene have been described. As patients with *TERC* mutation do, subjects with abnormal *TERT* show increased telomere shortening and low telomerase activity; this suggests that heterozygous mutations in the TERT gene impair telomerase activity by haploinsufficiency, and may be risk factors for marrow failure, possibly explaining some cases of familial acquired AA. On the other side, telomere shortening evidenced in typical acquired forms of AA is likely to be considered an epiphenomenon linked to the proliferative stress of surviving progenitors rather than the expression of primary lesions of the HSC, as for DKC. Of note, once a critical telomere length is reached, chromosomes may become unstable; such a mechanism could be one of the explanations for the possible clonal evolution from AA into myelodysplasia [51,52] or even acute leukemia. The acquisition of stem cell damage and the expansion of the dysplastic clone may be the result of a clonal escape; an alternative explanation is that the depletion of normal stem cells may facilitate the recruitment of a preexisting defective (under normal circumstances quiescent) stem cell (oligoclonality theory). A different susceptibility to the depletion mechanisms between normal and possible mutated stem cells has also been hypothesized. Finally, it has been hypothesized that exogenous G-CSF may interfere with some of these processes [53,54].

## PAROXYSMAL NOCTURNAL HEMOGLOBINURIA

***Paroxysmal nocturnal hemoglobinuria*** **(PNH)** is a marrow failure syndrome closely embedded with AA; PNH is a clonal stem cell disorder arising by an acquired somatic mutation in the *phosphatidylinositol glycan class A* (*PIG-A*) gene [55-57]. Affected progeny cells are incapable of synthesizing the glycosylphosphatidylinositol (GPI)-anchor, and present the typical phenotype lacking from their surface all GPI-anchored proteins (GPI-AP). The abnormality

affects all hematopoietic lineages; a mutation occurred in a single HSC may sustain hematopoiesis even lifelong. It is well known that PNH red cells have an intrinsic susceptibility to complement-mediated hemolysis, due to the lack from their surface of the GPI-AP complement inactivator CD59 [55]; this leads to the intravascular hemolysis typical of PNH patients. However, other cardinal features of PNH, namely propensity to venous thrombosis and bone marrow failure, remain unclear, as well as the reasons for the PNH clone expansion [55,58]. Thus, PNH is a true disease of the HSC, as marked by the *PIG-A* mutation, but the HSC defect does not explain entirely the phenotype. In fact, a series of observations suggest that the *PIG-A* mutation is necessary but not sufficient to cause PNH.

(i) The existence of a few (10-50 cells per million) circulating PNH granulocyte may be demonstrated also in healthy individuals by flow cytometry, and confirmed by a nested PCR technique identifying the specific *PIG-A* mutation [59,60].

(ii) Lymphocytes with the PNH phenotype appeared in lymphoma patients during treatment with alemtuzumab (an antibody that recognizes a GPI-anchored protein) and disappeared at treatment interruption [61].

(iii) Several murine knock-out models were developed to recreate the disease, but the expansion of the aberrant clone seen in PNH patients could not be reproduced [62-64]. Even in experiments employing embryonic stem cells, the PNH clone did not overcome normal hematopoiesis, suggesting that the PIG-A mutation itself did not confer any intrinsic proliferative advantage to PNH HSC [63-64]. This has been confirmed by several in vitro data using PNH and wild-type hemopoietic progenitors obtained by PNH patients [55].

(iv) PNH hematopoiesis may be in some cases oligo- rather than monoclonal, as initially supported by differential susceptibility to complement lysis [65,66] , then by flow cytometry [67] and finally confirmed by *PIG-A* sequencing [68]; this observation raised the question whether the expansion of more clones carrying the same functional defect, but molecularly heterogeneous, is compatible with a random process.

On these bases, the hypothesis of a dual pathophysiology for PNH has been developed, which is also known as the “relative advantage” [69] or the “escape” theory [70]. According to this theory, a mutation in the *PIG-A* gene might be a fairly common phenomenon, which has no biological consequences, because the mutated cell has no chance of expanding in presence of a vast majority of normal cells. However, the presence of external conditions may alter this equilibrium, creating an environment permissive for the expansion of PNH clone(s). The nature of such external trigger may be inferred from the close clinical association between PNH and AA. An antigen-driven immune response specifically targeting the marrow tissue may be postulated; if the target on HSC membrane is a GPI-linked molecule, PNH HSC will escape this injury while normal HSC are killed. Several evidences of immune derangement in PNH have been produced; as for AA, oligoclonality of the T cell pool has been reported [71], and immunodominant pathogenic CTL clones may be detected in most PNH patients [41,44,72]. In rare cases, such expansion may be extremely large, phenotypically resembling a subclinical LGL proliferation [73]. It has been recently described that these effector T cells express in excess the activating isoforms of inhibiting superfamily receptors, which elicit a powerful cytolitic activity [74]. However, once again the most striking evidence comes from gene expression profiling: when HSC from PNH patients were separated according to the presence on surface of the GPI-AP, distinct patterns of gene expression were identified. Phenotypically normal (GPI-AP positive) CD34+ cells harbored diffuse

abnormalities of their transcriptome, with over-expression of genes involved in apoptosis and immune activity, paralleling the findings seen in CD34+ cells of AA patients. By contrast, phenotypically pathologic PNH CD34+ (GPI-AP negative) showed a gene expression profiling closer to that obtained in CD34+ cells from healthy individuals [75]. This finding strongly supports the presence of an immune attack to the hematopoietic stem/progenitor cells, which spares the PNH cells. The "escape" of PNH cells may be interpreted in various manners. Contradictory data have been produced on a putative differential sensitivity to inhibitory stimuli between normal and PNH cells; susceptibility to apoptosis has been reported increased, normal or decreased in different models. Recently, it has been shown that human cell lines carrying the *PIG-A* mutation are less susceptible to NK-mediated killing compared to their normal counterpart [76]. In a more sophisticated model, GPI-deficient cells showed impairment in inducing primary and secondary stimulation of both antigen-specific and alloreactive T cells, providing experimental support for the hypothesis that the PNH clone could inefficiently interact with the immune system [77]. However, the actual mechanisms causing the escape are still elusive. They may include the absence of specific GPI-APs directly targeted by effector immune cells, or a protection due to the absence of important molecules involved in cell-cell interaction (e.g., accessory molecules). Alternatively, it may be hypothesized a broader impaired sensitivity to common effector mechanisms, which may be due to the lack of GPI-APs or to non specific structural changes of the raft structure in the outer surface.

Fig. 1

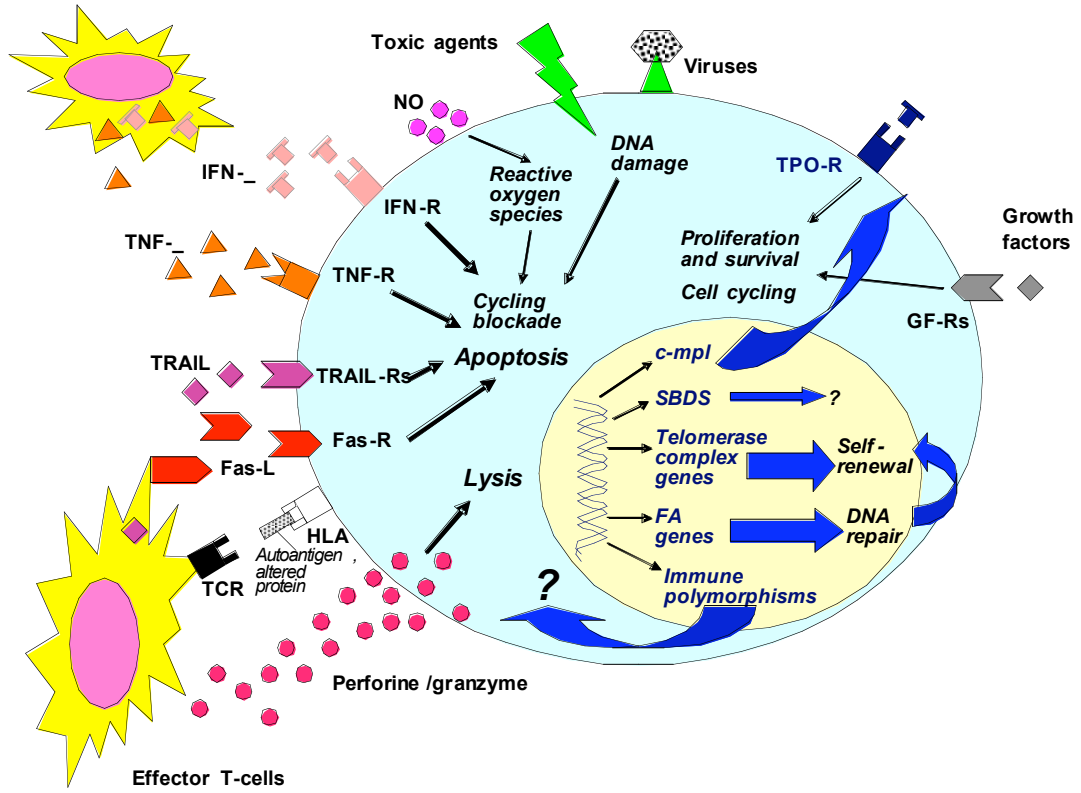
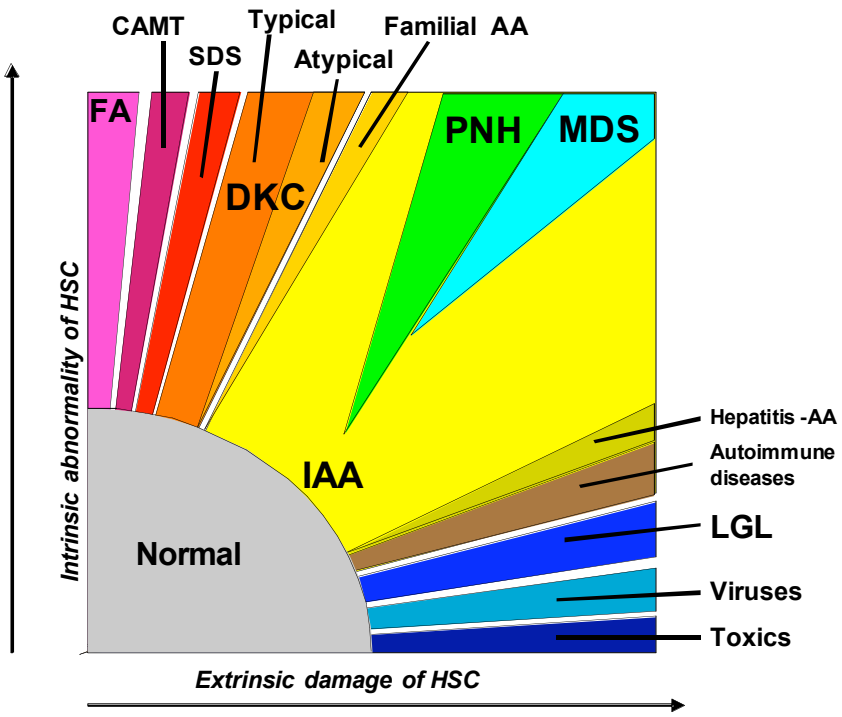

Toxic agents
Viruses
NO
IFN-_
Reactive oxygen species
DNA damage
TPO-R
IFN-R
Growth factors
TNF-_
Proliferation and survival
TNF-R
Cell cycling
Cycling blockade
GF-Rs
TRAIL
Apoptosis
c-mpl
TRAIL-Rs
SBDS
?
Fas-R
Telomerase complex genes
Self-renewal
Fas-L
Lysis
FA genes
DNA repair
HLA
Autoantigen , altered protein
TCR
Immune polymorphisms
?
Perforine /granzyme
Effector T-cells

Fig. 2


CAMT
SDS
Typical
Atypical
Familial AA
FA
DKC
PNH
MDS
IAA
Normal
Hepatitis -AA
Autoimmune diseases
LGL
Viruses
Toxics
Intrinsic abnormality of HSC
Extrinsic damage of HSC

## GENETIC BACKGROUND AFFECTING IMMUNE SYSTEM FUNCTIONING

Immunogenetic background resulting from predisposing complex traits can influence the quality of the immune response and shape certain clinical features of BMF. Examples of such immunogenetic factors are human leucocyte antigen (HLA) and killer-cell immunoglobulin-like receptor (KIR) genotypes, and cytokine and cytokine receptor gene single nucleotide polymorphisms (SNP). Several studies have provided evidence that some HLA alleles are associated with a constitutive predisposition to an exaggerated immune response. Moreover, the interaction between HLA class I molecules and killer cell immunoglobulin-like receptor (iKIR) is essential for human NK cell function. The interaction of inhibitory or stimulatory KIR variants with their matching KIR-ligands (KIR-L) modulates the immune response by suppressing or activating cytotoxicity. For example, KIR3DL2 recognizes HLA-A3 and HLA11 allotypes. The KIR3DL1 requires the presence of the Bw4+ epitope for an effective binding. The KIR2DL1 recognizes HLA-Cw 2/4/5/6/15 allotype, termed C1, based on the lysine amino acid residues present at position 80, while KIR2DL2 and 2DL3 recognize the remaining HLA-C allotype termed C2 that carry an asparagine at position 80. In recent years, many single-nucleotide polymorphisms (SNPs) have been detected by gene sequencing, particularly within the promoter regions of these genes. Several of these SNPs may be associated with differential levels of gene transcription and consequently to the susceptibility to develop an autoimmune disease.

For example, an association has been reported between SNPs located in the promoter region of Interleukin-10 (IL10) and increased risk of acute GvHD [78} and with a particular phenotype in Juvenile Rheumatoid Arthritis [79]. Tumor necrosis factor receptor polymorphisms and SNPs in the TNF-$\alpha$ gene promoters have been involved in the pathogenesis of several autoimmune disease, including RA [80] and MS [81]. In bone marrow failure the role of SNPs has also been investigated; in patients with AA and PNH a higher frequency of high secretor variants of proinflammatory cytokine like TNF-$\alpha$ and IFN-$\gamma$ has been reported [82-83]. Other polymorphisms of immunomodulatory receptors can also have similar functional consequences [84]. Cytotoxic T lymphocyte associated protein -4 (CTLA-4), expressed mainly on activated T cells, is a potent downregulator of T cell response. Presence of some genetic variants in exon 1 region can be related with reduced expression of CTLA-4 and influenced immune response. Association between this SNP and clinical outcome after allo-HSCT [85] and some diseases such as MS [86] and diabetes mellitus type 1 [87] have been described. CD16, also know as Fc$\gamma$ receptor III, plays a central role in NK cell activation, and his pholymorphisms may change the affinity for NK cells. The presence of SNP in the extracellular domain 2 can modify the normal receptor function and increase autoimmune disease susceptibility.

We hypothesize that the quality of the immune response and some clinical features of MDS can be associated with the prevalence of specific immunogenetic factors.

**Killer cell immunoglobuline-like receptor**

Natural killer (NK) cells are regulated in part by inhibitory receptors that recognize MHC class I molecules [88-89] on normal cells. In human, inhibitory receptors that recognize MHC class I molecules (HLA-A, HLA-B, HLA-C) belong to the KIR (immunoglobuline-like receptor family). Evidence is emerging from disease-association studies that KIR receptors can play beneficial roles in viral infections, such as HIV and HCV, but may also predispose to autoimmune diseases. Two main families of receptors on NK cells that recognize HLA class I antigen have been discovered: killer cell immunoglobuline-like receptors (KIR) and the CD94/NKG2 receptor system. The KIR genes are highly polymorphic [90-91], and the receptors have complex clonal expression patterns on NK cells. Although structurally different, KIR and CD94/NKG2 receptors families have both activatory and inhibitory receptors for HLA class I antigen. These receptors collaborate to monitor and to respond to changes in HLA class I antigen on cells of the body. Because of the complexities inherent in these NK cell receptors and the complex nature of HLA ligand, it was a considerable challenge to understand how these receptors work on NK cells to enable an immune response. There are 16 different KIR genes [92-94] that are encoded on chromosome 19q13.4. These receptors have high similarity and most of the anti-KIR antibodies recognize more than one gene product. KIRs contain either two or three immunoglobuline –like domains with either long (DL) or short (DS) cytoplasmic tails. Long-tailed receptors carry one or two immunoreceptor tyrosine-based inhibition motifs (ITIMs), which contribute to inhibitory signaling [fig.3]. Short tailed receptors have a lysine residue in their transmembrane domain which is required for pairing with the immunoreceptor tyrosine-based activation motifs (ITAM). Of the 16 genes, 7 encode for the inhibitory KIR receptors (3DL1-3, 2DL1-3, 2DL5), 6 genes encode for the activating KIR receptors (3DS1, 2DS1-5), one gene encodes for KIR2DL4 receptors with both inhibitory and activating function, and two genes are pseudogenes which do not encode a functional KIR receptor. Five of the inhibitor KIR receptors (3DL1, 3DL2, 2DL1-3) recognize distinct motif on the HLA class I molecules which are ubiquitously expressed [90-95] on the surface of normal cells. The KIR2DL1 recognizes HLA-Cw2/4/5/6/15 allotypes (termed C2 group) which carry a lysine at amino acid position 80. KIR2DL2 and 2DL3 recognize the remaining of the HLA-C allotypes (Cw1/3/7/8-termed C1 group) which carry an asparagine at position 80. KIR3DL1 binds to the Bw4 epitope which is conserved on a third of HLA-B alleles. KIR3DL2 recognizes HLA-A3 and A11 allotypes. The KIR2DL4 receptor binds to the trophoblast-specific nonclassical class I molecules HLA-G and induces rapid interferon-$\gamma$ production which promotes vascularization of the maternal deciduas during early pregnancy [fig.4]. The interaction of inhibitory receptors with HLA class I ligands trigger signals that turn off NK cells. Therfore, by expressing HLA-A, B, and C molecules, normals cells are protected from NK cell lysis. Downregulation of HLA class I expression due to tumor transformation or viral infection relieves the inhibitory influence of NK cells, permitting NK cells to lyse these unhealthy target cells, a phenomenon first described as the “missing-self” hypotesis [fig. 5]. Futhermore, NK cells can also directly recognize unhealthy cells through their cell surface-activating receptors, which can enhance lysis by NK cells.

Fig. 3

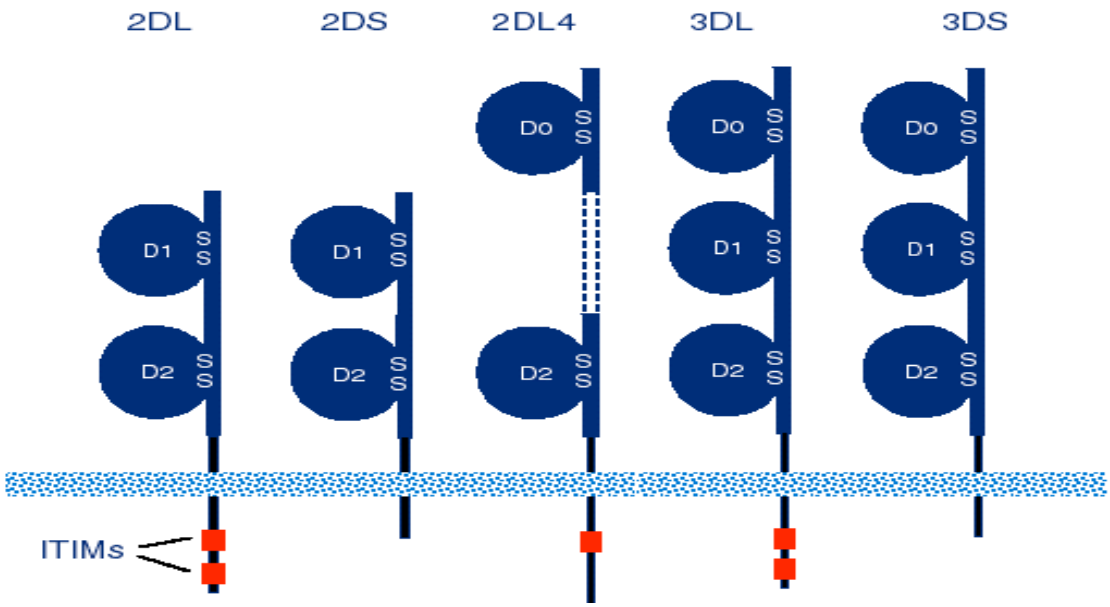


Fig. 4

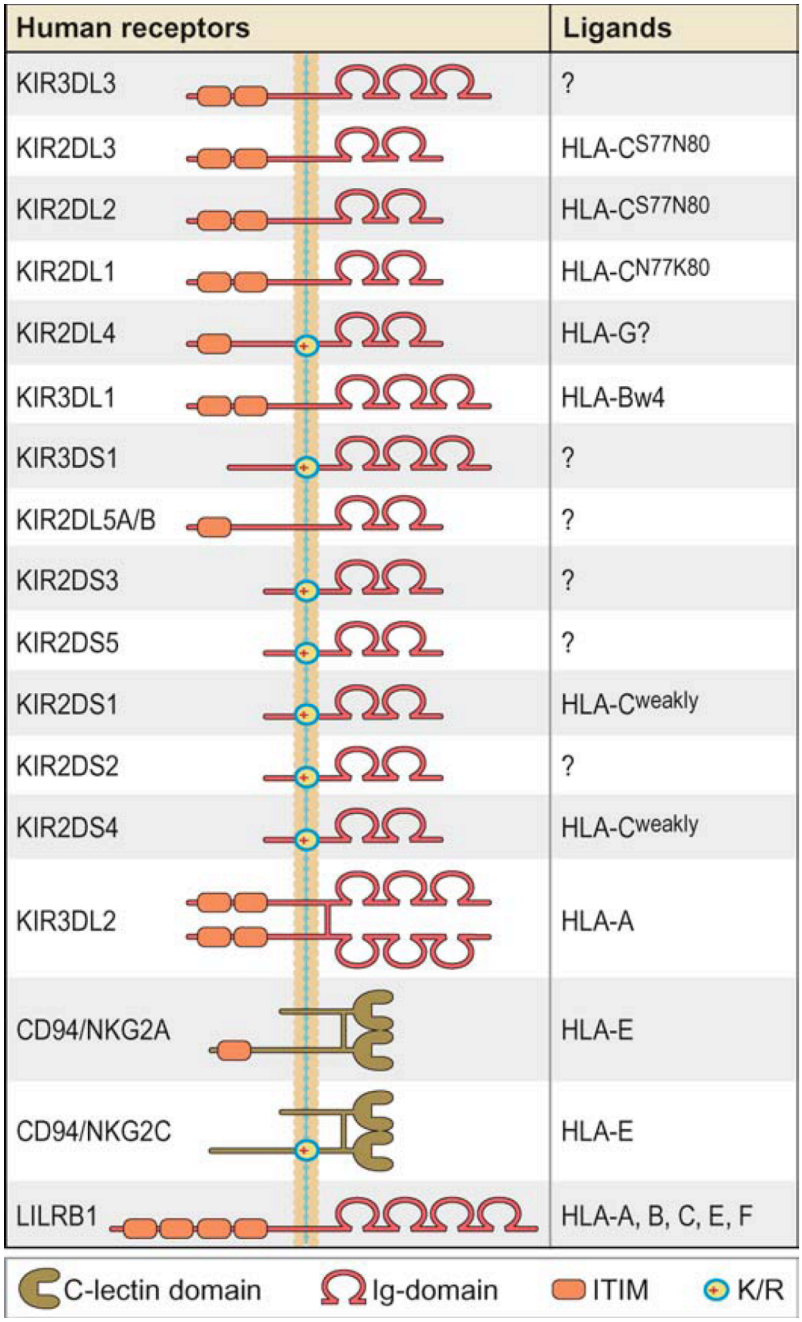
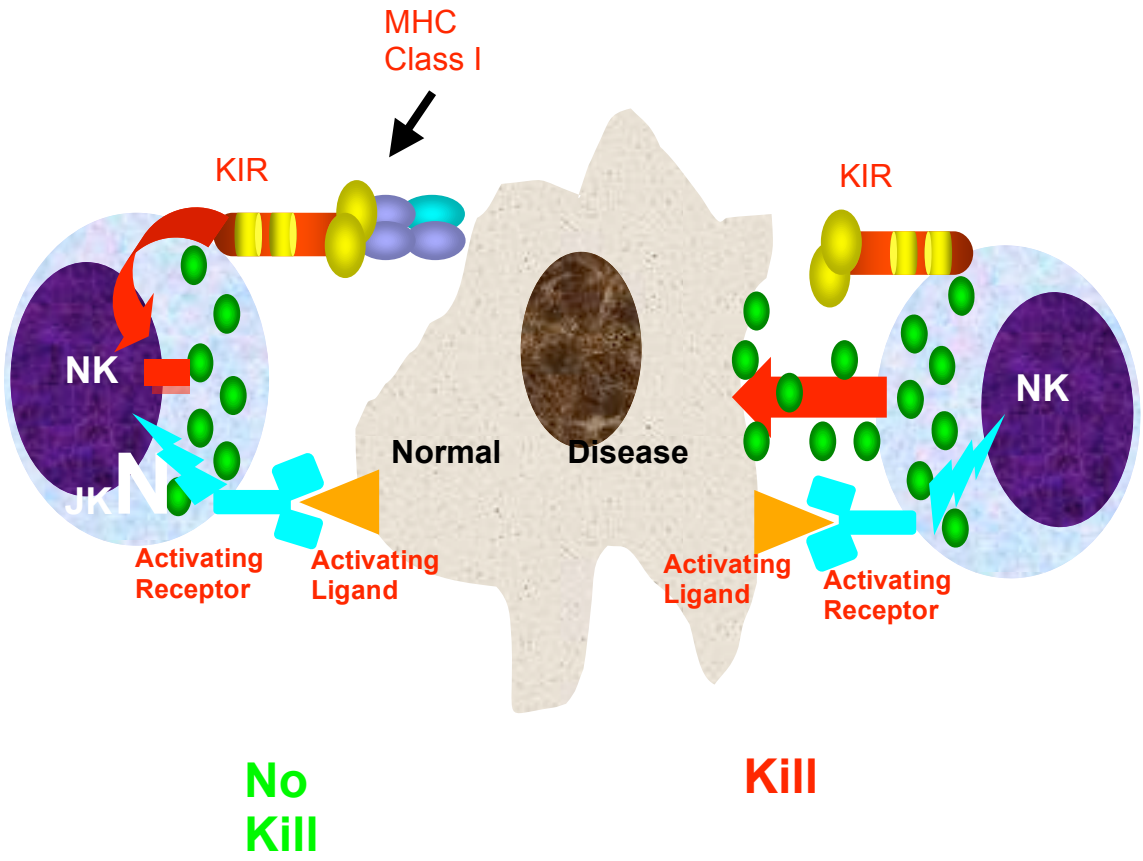

Human receptors
Ligands
KIR3DL3
?
KIR2DL3
HLA-C S77N80
KIR2DL2
HLA-C S77N80
KIR2DL1
HLA-C N77K80
KIR2DL4
HLA-G?
KIR3DL1
HLA-Bw4
KIR3DS1
?
KIR2DL5A/B
?
KIR2DS3
?
KIR2DS5
?
KIR2DS1
HLA-C weakly
KIR2DS2
?
KIR2DS4
HLA-C weakly
KIR3DL2
HLA-A
CD94/NKG2A
HLA-E
CD94/NKG2C
HLA-E
LILRB1
HLA-A, B, C, E, F
C-lectin domain
Ig-domain
ITIM
K/R

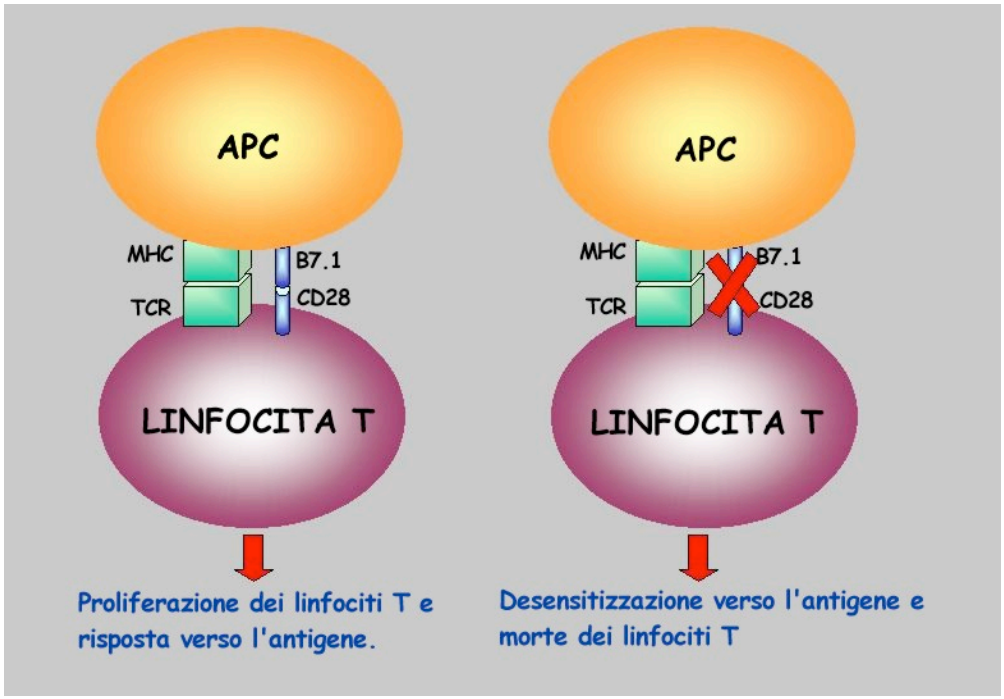
Fig. 5


MHC
Class I
KIR
KIR
NK
NK
Normal
Disease
Activating
Receptor
Activating
Ligand
Activating
Ligand
Activating
Receptor
No
Kill
Kill

## CYTOTOXIC T LYMPHOCYTE-ASSOCIATED MOLECULE-4 (CTLA-4)

Naïve T cells require two signals to proliferate and differentiate. Signal one is antigen-specific and is generated by interaction of the T cell receptor (TCR) with an antigen peptide presented on the MHC molecule. Signal two, essential in T cells for a functional response, is transduced most commonly through interaction of CD28 on the T cell with B7 [96-98] on the antigen presenting cell [fig.6]. Expression of cytotoxic T lymphocyte-associated molecule-4 (CTLA-4) is subsequently up-regulated on T cells activated in this manner. CTLA-4 is a negative regulator of T-cell activation. Mice deficient in CTLA-4 develop a lymphoproliferative disorder resulting in premature death. The CTLA-4 gene in humans is located on chromosome 2q33 and is a prime candidate autoimmunity gene, as mutations within this gene lead to alterations in function that could have profound effects on the immune system. Expression of CTLA-4 appears to be selectively induced in certain pathological conditions; there are evidences that cell-surface expression of CTLA-4 protein is influenced by common polymorphisms in promoter and first-exon sequences. Defective CTLA-4 expression could results in failure to terminate T cell activation, leading to an inappropriate and prolonged T-cell response.

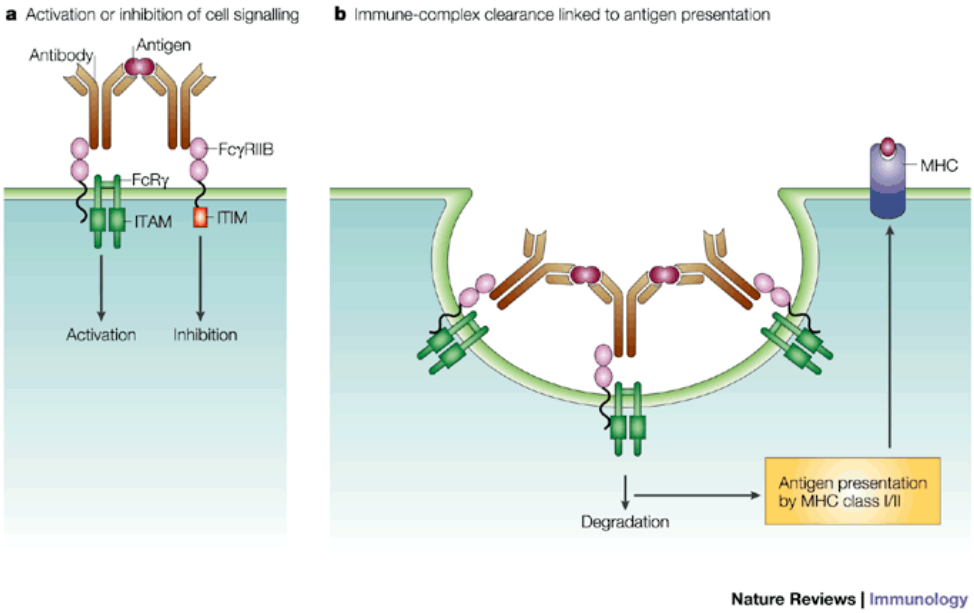
Fig. 6


APC
MHC
TCR
B7.1
CD28
LINFOCITA T
Proliferazione dei linfociti T e risposta verso l'antigene.
APC
MHC
TCR
B7.1
CD28
LINFOCITA T
Desensitizzazione verso l'antigene e morte dei linfociti T

**Fcγ receptor IIIa (CD16)**

Receptor for IgG (FCγR) are important regulatory molecules of inflammatory responses [fig.7]. The human leukocyte FcγR family consists of three major classes: CD64 (FcγRIA, IB and IC); CD32 (FcγRIIA, IIB and IIC); and CD16 (FcγRIIIA and IIIB) all mapped to the long arm of chromosome 1. The heterogeneity of FcγR has functional consequences, as reflected in their specific cell distribution patterns and initiation of specific signaling pathways [99-101]. Functional polymorphisms of three FcγR subclasses, further increase molecular heterogeneity, and cause inter-individual differences in the efficacy of FcγR function. These differences may contribute to susceptibility or disease course of auto-immune and infectious diseases. FcγRIIIA is expressed on macrophages and a subset of monocytes, NK-cells and γδ-T-cells. Functional polimorphysms in this gene may determine the level of receptor interaction with IgG1, IgG3 and IgG4, as well as the efficiency of IgG-induced effector function.

Fig. 7

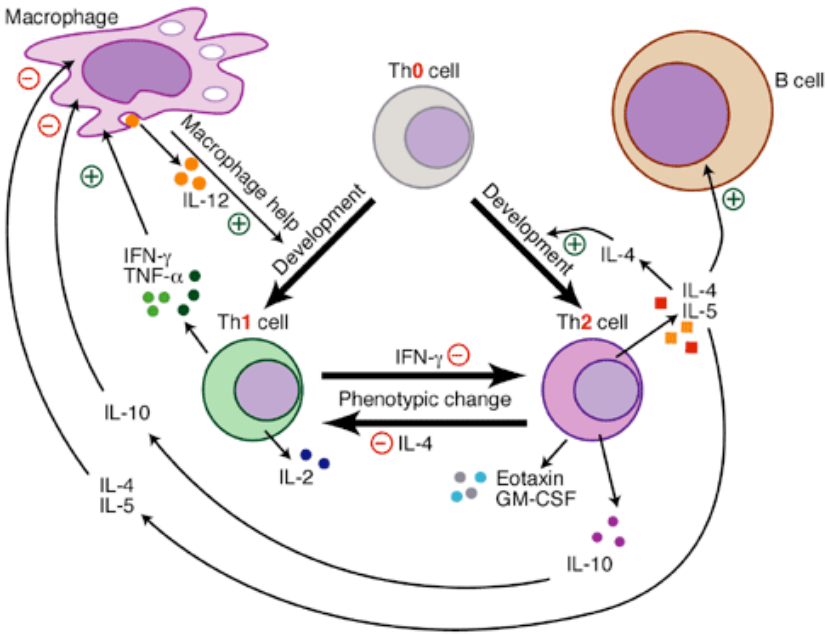

a Activation or inhibition of cell signalling
Antibody
Antigen
FcγRIIB
FcRγ
ITAM
ITIM
Activation
Inhibition
b Immune-complex clearance linked to antigen presentation
MHC
Antigen presentation by MHC class I/II
Degradation
Nature Reviews | Immunology

## CYTOKINE AND CYTOKINE GENES

Proinflammatory cytokines [102,104] and their related receptors and inhibitors (intereleukin-1 {IL-1, IL-1r,IL-1ra, IL-2, IL-6}, tumor necrosis factor $\alpha$ {TNF-$\alpha$}, and IL-10) have been implicated in a number of immunological diseases [fig.8]. An initial insult provokes an inflammatory response and release of IL-1 and TNF-$\alpha$. The subsequent cascade of cytokine production initiates tissue damage. TNF-$\alpha$ mediates a variety of functions [105,106] and exerts markedly diverse effects on the immune system, which include regulation of MHC class II molecules dependent on cell differentiation [107-108] and promotion of self-tolerance in the early induction phase of some diseases. The gene encoding the proinflammatory cytokine TNF-$\alpha$ is located within the MHC locus on chromosome 6. IL-10 is a potent immunosuppressant [109-110] produced by monocytes, macrophages, T cells, B cells, dendritic cells, mast cells and eosinophilis. As a T-cell helper type 2 (Th2) cell-derived cytokine, IL-10 has been shown [103] to inhibit the secretion of Th1 cell-derived cytokines (TNF-$\alpha$, IL-1$\alpha$, IL-1$\beta$, IL-6, IL-12, and IFN-$\gamma$), limit the inflammatory responses and regulate the differentiation and proliferation of several immune cells such as T cells, B cells, Nk cells and antigen-presenting cells. IL-10 also downregulates the expression of major histocompatibility complex (MHC) and costimulatory molecules; by these mechanisms it may attenuate alloreactive T-cell responses. The gene encoding IL-10 has been identified on chromosome 1q31-32. IFN-$\gamma$, a lymphokine produced by activated T cells [111,114], is an important regulatory molecule of the immune system and has been implicated [115] in the pathogenesis of multiple sclerosis, diabetes mellitus, and other autoimmune disease.

Given this complexity of regulation of the immune system and all the possible players involved, we decided to study all genetically determined factors in a large cohort of BMF patients, including cases of AA, PNH and MDS. Specific polymorphisms affecting all these pathways were found associated with specific diseases which have an immune derangement in their pathophysiology. In our work we investigated the hypothesis that similar immunogenetic predisposition may be found in all or specific subtypes of BMFs.

Fig. 8


Macrophage
Th0 cell
B cell
Macrophage help
IL-12
Development
Development
IL-4
IFN-γ
TNF-α
IL-4
IL-5
Th1 cell
Th2 cell
IFN-γ
Phenotypic change
IL-4
IL-10
IL-2
Eotaxin
GM-CSF
IL-4
IL-5
IL-10

# MATERIALS AND METHODS

## Patients

Informed consent was obtained for peripheral blood collection according to the protocols approved by the Institutional Review Board of the Cleveland Clinic Foundation from 129 patients with MDS, and 77 patients with AA [tab.1 and 2]. MDS was diagnosed according to the WHO criteria. Twenty-one patients had refractory anemia (RACMD), 37 refractory anemia with ringed sideroblasts (RARS/RCMD-S) and 44 refractory anemia with blast excess (RAEB) OR secondary AML (sAML). Among the 77 patients with BMF 44 had a typical AA, 19 had a co-presence of a small PNH clone and 14 had typical PNH. The hypoplastic features (n=10) were supported in all cases by bone marrow histological evaluation. The control group comprised 60 internal healthy controls and a large historical control cohort.

**Table 1. BMF PATIENTS CHARACTERISTICS**

| | | N |
|---|---|---|
| **Diagnosis** | AA | 44 |
| | AA/PNH | 19 |
| | PNH | 14 |
| **DR 15** | POS | 30 |
| | NEG | 36 |
| | NA | 11 |
| **Thrombosis** | Yes | 15 |
| | Not | 62 |
| **Treatment** | Yes | 46 |
| | Not | 31 |
| **Response** | Yes | 30 |
| | Not | 16 |

**Table 2. MDS PATIENTS DIAGNOSIS**

| | |
|---|---|
| RA/RCMD= | 21 |
| RARS/RCMD= | 37 |
| RAEB/sAML= | 44 |
| CMML= | 11 |
| 5q- = | 3 |
| MDS/MPD-U= | 13 |

**Table 3. MDS PATIENTS CYTOGENETIC**

| | |
|---|---|
| NORMAL KARYOTYPE | 21 |
| COMPLEX KARYTOTYPE | 37 |
| TRISOMY 8 | 44 |

## DNA extraction

DNA was extracted from whole blood using the PureGene system (Gentra, Minneapolis, MN, USA). Samples were re-suspended in reduced Tris-EDTA buffer and the concentration was measured using a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

## HLA and KIR typing

HLA class I and II typing was performed by PCR-sequence specific primers (PCR-SSP) (Allogen Laboratories, Cleveland, OH, USA). KIR genotyping was performed using PCR-SSP to identify the presence or absence of KIR genes (Dynal Biotech; Invitrogen, Carlsbad, CA, USA). Locus-specific primer sets were utilised to amplify 1·5 *μ*g of genomic DNA for each sample. Genomic DNA was mixed with KIR PCR reaction buffer containing dNTPs and *Taq* DNA Polymerase, then applied to a 96-well tray containing 5 *μ*l optimised primer solution for thermal cycling. (Detailed description of this SSP amplification based method was reported by Gomez-Lozano & Vilches, 2002; Hsu *et al*, 2002; Marsh *et al*, 2003. Following amplification, PCR products were loaded on a 2% agarose gel containing 8 *μ*l ethidium bromide. Upon completion of electrophoresis, the gel was photographed and interpreted. A KIR profile for each patient and control subject was determined by detecting the presence or absence of specifically amplified KIR products in each of 21 lanes containing individual allele-specific KIR primers. The KIR genes, *2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 3DL1, 3DL2, 3DL3, 3DS1* and pseudogenes *2DP1* and *3DP1* were studied [fig.9].

Fig. 9

| KIR genotype | Allele Specificity |
|---|---|
| 2DL1 | 001/002 |
| 2DL2 | 001-005 |
| 2DL3 | 001-006 |
| 2DS1 | 001-004 |
| 2DS2 | 001/005 |
| 2DS3 | 002/004/007 |
| 2DS4 | 003/006 |
| 2DS4 | 00101/00102 |
| 2DS5 | 001-005 |
| 3DL1 | 00101-0012 |
| 3DS1 | 010-01301 |

**KIR and KIR-L assignment**

Ligands for *KIR3DL2, 3DL1, 2DL1, 2DS1, 2DL2, 2DL3* and *2DS2* are known [116]. For the purpose of this study, KIR mismatch was defined as the presence of a specific KIR gene and the lack of its corresponding HLA ligand according to the method proposed by Parham (2005). For example, *KIR3DL2* recognizes the A3 and/or A11 allotypes. Consequently, lack of HLA-A3 or HLA-A11 combined with the presence of *KIR3DL2* can be considered a *KIR3DL2* mismatch. Similarly, *KIR3DL1* interacts with the Bw4 epitope; presence of two Bw6 epitopes combined with *KIR3DL1* expression would result in *KIR3DL1* mismatch.

*KIR2DL2*, *2DL3*, and thus *2DS2* and *2DS3* all interact with group 1 HLA-C molecules, which have a Lys residue in position 80 (http://www.dorak.info/hla/c1c2.html). HLA-Cw 1, 3, 7, 8, 12, 13, 14 and 16 alleles make up HLA-C1. The presence of any of these three KIR genes and the absence of a group 1 HLA-C allele will result in a mismatch. Similarly, *KIR2DL1* and *2DS1* interact with group 2 HLA-C molecules, those having an Asn residue in position 80. The presence of any of the HLA-Cw alleles, Cw2, 4, 5, 6, 15, 17 and 18 would silence *KIR2DL1* and *2DS1*. For example, a patient whose HLA profile shows HLA Cw3, Cw7 would show KIR ligand HLA C1/C1 constellation; Cw2, Cw 4 would translate into KIR ligand C2/C2 constellation, both making a theoretical KIR/KIR-L mismatch possible.

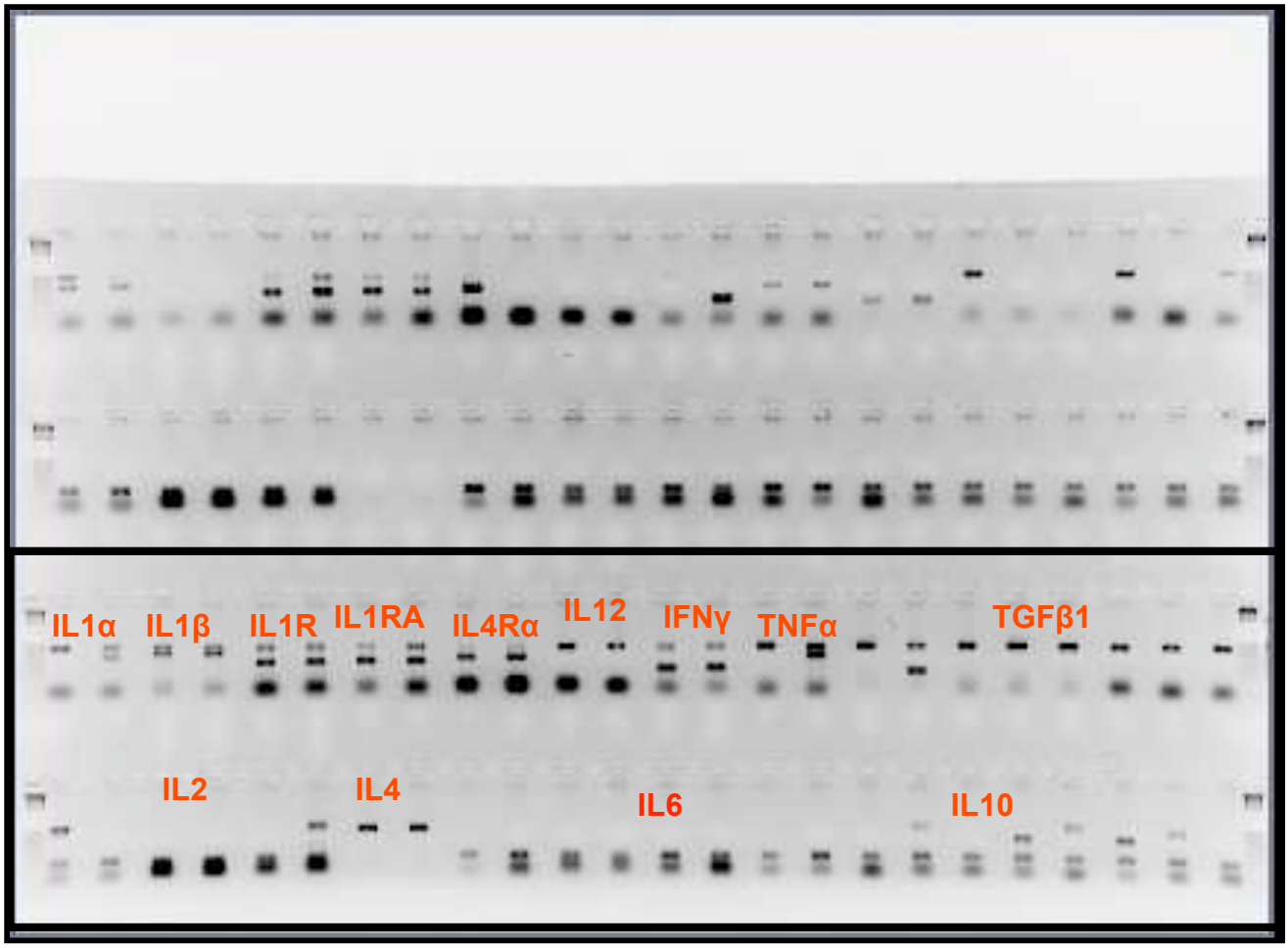
**Cytokine genotyping**

An assay designed for the detection of SNP in 13 different cytokine genes was used to create a cytokine SNP profile of MDS patients (Dynal Biotech; Invitrogen, Carlsbad, CA, USA). Sequence-specific oligonucleotide primers for amplification of specific alleles from IL1a, IL1b, IL1R, IL1RA, IL4Ra, IL12, IFNγ, TGFb, TNFa, IL2, IL4, IL6, and IL10 were used [fig.10]. Primer pairs for the amplification of a target sequence are provided for a total of 48 PCR reactions for sample. Following the PCR, amplified DNA fragments were electrophoresed on a 2% agarose gel with 8 *μ*l ethidium bromide. Positive reactions for a specific allele were discerned by the presence of a band between the larger internal control band and the smaller primer dimer band [fig.11].

Fig. 10

| Cytokine genotype | Allele Specificity |
|---|---|
| IL1α | -899 T/C |
| IL1β | -511 C/T, +3962 T/C |
| IL1R | Pst1 1970 T/C |
| Il1RA | Mspa1 11100 T/C |
| IL4Rα | +1902 G/A |
| IL12 | -1188 C/A |
| IFNγ | +874 A/T |
| TGFβ1 | Codon 10 C/T, codon 25 G/C |
| IL2 | -330 T/G, +166 G/T |
| IL4 | -1098 T/G, -590 T/C, -33T/C |
| TNFα | -308 G/A, -238 A/G |
| IL6 | -174 G/C, NT565 G/A |
| IL10 | -1082 G/A, -819 C/T, -592 C/A |
| CTLA-4 | +49 A/G |
| FcγIIIR | nt 559 G/T |
| CD45 | +77 C/G, +138 A/G |

Fig.11


IL1α
IL1β
IL1R
IL1RA
IL4Rα
IL12
IFNγ
TNFα
TGFβ1
IL2
IL4
IL6
IL10

**CTLA4 PCR and sequencing**

Exon I of the CTLA-4 gene was amplified from total genomic DNA using sense primer 5'-*catcgtcattgtagctaagc*-3' and antisense primer 5'-*tactcaagccgattagc*-5' in a total volume of 25 *µ*l. After initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 30 s, 57°C for 35 s and 72°C for 40 s were performed. PCR products were purified and 3 *µ*l were used in a sequencing reaction with 1 *µ*l of BigDye (Applied Biosystems, Foster City, CA, USA) 1 *µ*l of 3 *µ*mol/l primer in a total volume of 10 *µ*l; products were purified and run on ABI 7500 Sequencer as described previously [117].

**Fc*γ*RIIIa – 158V/F genotyping**

Allele-specific amplification of the Fc*γ*RIIIa gene (*FCGR3A*) was performed as previously described with minor modifications [118]. Briefly, the PCR reaction was optimised using 50 ng of template DNA, 10 pmol of valine-specific or phenylalanine-specific primers, 2·5 mmol/l dNTPs, 2·5 mmol/l $MgCl_2$ and 1 U *Taq* polymerase diluted in PCR buffer (Invitrogen) to a final volume of 25*µ*l. For PCR amplification, an initial denaturation step of 5 min at 95°C was followed by 35 cycles (94°C for 30 s, 60°C for 30 s, 72°C for 30 s) and final extension at 72°C for 8 min. Samples were run in pairs using either *G* allele-specific (*5'-ctgaagacacatt-tttactcccaac-3'*) or *T* allele-specific (*5'-ctgaagacacatttttactcccaaa-3'*) reverse primers combined with an *FcγRIIIa*-specific forward primer (*5'-tccaaaagccacactcaaagac-3'*). The PCR products of 73 base pairs in the *T* or *G* allele-specific reaction were separated on 2·5% agarose gels and visualised under ultra-violet light using BioRad ChemiDoc XRS. Subjects were classified as heterozygous (*VF*), or homozygous (*VV* or *FF*). Ambiguous results were resolved by nucleotide sequencing.

## RESULTS

*Distribution of HLA KIR-L and KIR genotypes*

We looked for significant differences in the frequency of individual HLA alleles in patients in comparison with the normal population. We noted that in AA as well as in MDS patients positive for both A3 and A11 alleles were decreased compared to normals (0% vs 9%, p= .01). Analysis of the HLA-B haplotypes Bw4 and Bw6, and of the HLA-C haplotype C1 and C2, did not reveal any significant deviation in the KIR ligand distributions between patients and control groups (table 3 and 4). We also determined the KIR genotype defined as the absence or presence of some KIR genes in patients and controls. For this analysis we performed an SSP PCR for the full panel of KIR genes. The frequency of the KIR genes present in the BMF population was compared to controls. KIR genotype of MDS patients did not significantly differ from healthy controls. However, when patients with AA were compared to controls, we found a decreased frequency of the inhibitory KIR2DL3 (68% vs 88%, p=.0002). Similar differences (table 5 and 6) in the KIRs frequency were found between controls and MDS patients with associated cytopenia (63% vs 88%, p=.02) who also showed increased frequency of the activating KIR2DS5 compared to controls (66% vs 26%, p=.01).

Table 3. HLA DISTRIBUTION IN 91 AA PATIENTS AND IN 126 NORMAL CONTROLS

| HLA-A | AA | % | controls | % | p |
|---|---|---|---|---|---|
| 3/11 +/+ | 0 | 0 | 11 | 9.1 | 0.01 |
| 3/11 +/- | 40 | 44 | 46 | 36.4 | |
| 3/11 -/- | 51 | 56 | 69 | 54.5 | |
| HLA-B | | | | | |
| Bw4/Bw4 | 9 | 10 | 18 | 14.3 | 0.5 |
| Bw4/Bw6 | 47 | 51 | 57 | 45.2 | |
| Bw6/Bw6 | 35 | 38 | 51 | 40.5 | |
| HLA-C | | | | | |
| C1/C1 | 41 | 45 | 42 | 33.3 | 0.1 |
| C1/C2 | 34 | 38 | 54 | 42.9 | |
| C2/C2 | 15 | 17 | 30 | 23.8 | |

Table 4. HLA DISTRIBUTION IN 56 PATIENTS WITH MDS AND 126 NORMAL CONTROLS

| HLA-A | MDS | % | controls | % | p |
|---|---|---|---|---|---|
| 3/11 +/+ | 0 | 0 | 11 | 9.1 | 0.008 |
| 3/11 +/- | 13 | 24 | 46 | 36.4 | |
| 3/11 -/- | 43 | 75 | 69 | 54.5 | |
| HLA-B | | | | | |
| Bw4/Bw4 | 14 | 25 | 18 | 14.3 | 0.5 |
| Bw4/Bw6 | 29 | 52 | 57 | 45.2 | |
| Bw6/Bw6 | 13 | 23 | 51 | 40.5 | |
| HLA-C | | | | | |
| C1/C1 | 25 | 43 | 42 | 33.3 | 0.3 |
| C1/C2 | 17 | 31 | 54 | 42.9 | |
| C2/C2 | 14 | 26 | 30 | 23.8 | |

Table 5. KIR GENOTYPE DISTRIBUTION IN AA PATIENTS COMPARED TO NORMAL CONTROLS

| KIR genotype | AA % (n=51) | CTRL n | CTRL % | KIR phenotype | P value |
|---|---|---|---|---|---|
| 2DL1 | 81 | 281 | 94.6 | inhibitory | 0.001 |
| 2DL2 | 53 | 157 | 52.9 | inhibitory | 1 |
| 2DL3 | 68 | 262 | 88.5 | inhibitory | 0.0002 |
| 2DS1 | 29 | 104 | 35 | activating | 0.5 |
| 2DS2 | 53 | 142 | 48 | activating | 0.5 |
| 2DS3 | 33 | 72 | 24.2 | activating | 0.2 |
| 2DS4 | 92 | 285 | 96 | activating | 0.2 |
| 2DS5 | 29 | 76 | 26 | activating | 0.7 |
| 3DL1 | 90 | 287 | 96.6 | inhibitory | 0.004 |
| 3DS1 | 47 | 101 | 34 | activating | 0.1 |

Table 6. KIR GENOTYPE DISTRIBUTION IN PATIENTS WITH MDS COMPARED TO NORMAL CONTROLS

| KIR genotype | MDS % (n=85) | CTRL n | CTRL % | KIR phenotype | P value |
|---|---|---|---|---|---|
| 2DL1 | 96.9 | 281 | 94.6 | inhibitory | 0.4 |
| 2DL2 | 56 | 157 | 52.9 | inhibitory | 0.6 |
| 2DL3 | 87 | 262 | 88.5 | inhibitory | 0.7 |
| 2DS1 | 49 | 104 | 35 | activating | 0.06 |
| 2DS2 | 53 | 142 | 48 | activating | 0.4 |
| 2DS3 | 28 | 72 | 24.2 | activating | 0.5 |
| 2DS4 | 91 | 285 | 96 | activating | 0.09 |
| 2DS5 | 34 | 76 | 26 | activating | 0.3 |
| 3DL1 | 95 | 287 | 96.6 | inhibitory | 0.6 |
| 3DS1 | 38 | 101 | 34 | activating | 0.5 |

*KIR LIGAND GENOTYPE DISTRIBUTION*

In the next step of experiments, we studied the frequency of KIR mismatch in BMF. With this analysis we detected that in the AA population there was a decreased frequency of the 2DS1/C1 mismatch compared to the normal controls ( 14% vs 44%, p=.003) (table 7). Moreover, in the MDS population we found a decreased frequency of the 2DS2/C2 mismatch and of the 3DL1/Bw4 mismatch compared to controls (17% vs 44%, p=.01 and 18% vs 47%, p=.0003, respectively) (table 8). Interestingly, when we performed the same analysis grouping MDS patients according to the IPSS score (table 9) based on the grade of the dysplasia, we found that patients with high risk MDS had increased frequency of the 2DL3/C1 mismatch compared to the low risk MDS population (43% vs 12%, p=.006)

Table. 7 KIR- *KIR LIGAND MISMATCH IN AA PATIENTS COMPARED TO NORMAL CONTROLS*

| KIR-HLA Mismatch | function | Mismatched control n % | Mismatched AA n % | P value |
|---|---|---|---|---|
| 2DL1/C2 | inhibitory | 31/85 (36) | 28/51 (55) | 0,3 |
| 2DL2/C1 | inhibitory | 6/53 (11) | 2/51 (4) | 0,1 |
| 2DL3/C1 | inhibitory | 13/79 (16) | 4/51 (8) | 0,1 |
| 2DS1/C2 | activating | 11/25 (44) | 7/51 (14) | 0,003 |
| 2DS2/C1 | activating | 5/52 (10) | 1/51 (2) | 0,3 |
| 3DL1/BW4 | inhibitory | 43/92 (46.7) | 20/51 (39) | 0,3 |
| 3DL2/A3 or A11 | inhibitory | 54/93 (58) | 28/51 (55) | 0,7 |

Table. 8 KIR- *KIR LIGAND MISMATCH IN MDS PATIENTS*

| KIR-HLA Mismatch | function | Mismatched control n % | Mismatched patients n % | P value |
|---|---|---|---|---|
| 2DL1/C2 | inhibitory | 31/85 (36) | 22/57 (38) | 0,7 |
| 2DL2/C1 | inhibitory | 6/53 (11) | 7/57 (12) | 0,8 |
| 2DL3/C1 | inhibitory | 13/79 (16) | 14/57 (24) | 0,2 |
| 2DS1/C2 | activating | 11/25 (44) | 10/57 (17) | 0,01 |
| 2DS2/C1 | activating | 5/52 (10) | 7/57 (12) | 0,6 |
| 3DL1/BW4 | inhibitory | 43/92 (46.7) | 10/57 (18) | 0,0003 |
| 3DL2/A3 or A11 | inhibitory | 54/93 (58) | 42/57 (74) | 0,05 |

Table. 9 KIR- *KIR LIGAND MISMATCH IN MDS SUBGROUPS*

| KIR-HLA Mismatch | function | Low risk Tot % | High risk Tot % |
|---|---|---|---|
| 2DL1/C2 | inhibitory | 13 (38) | 9 (39) |
| P value | | 0.8 | 0.8 |
| 2DL2/C1 | inhibitory | 3 (9) | 4 (17) |
| P value | | 0.7 | 0.4 |
| 2DL3/C1 | inhibitory | 4 (12) | 10 (43) |
| P value | | 0.5 | 0.006 |
| 2DS1/C2 | activating | 6 (17) | 4 (17) |
| P value | | 0.02 | 0.04 |
| 2DS2/C1 | activating | 3 (9) | 4 (17) |
| P value | | 0.9 | 0.3 |
| 3DL1/BW4 | inhibitory | 6 (18) | 4 (17) |
| P value | | 0.3 | 0.1 |
| 3DL2/A3 or A11 | inhibitory | 24 (70) | 18 (78) |
| P value | | 0.1 | 0.07 |

*Cytokine and cytokine receptor SNP*

No significant differences in the MDS cohort compared to control for SNPs in IL1a, IL1b, IL1R, IL1RA, IL4Ra, IL12, IFNγ, IL2, IL4 and IL6 were found. However, when we examined the frequency of the TGF-β genotype, the MDS chort showed a higher rate of the TT codon 10 variant (59% vs 32% in controls, p=.002) and higher rate of the GG codon 25 variant (71% vs 35% in controls, p=.0001). Since we already know that the combination of TT at codon 10 and GG at codon 25 is consistent with a "high secretor phenotype", we also decided to analyze the secretor phenotype and found that in MDS patients there was a higher incidence of the high secretor phenotype compared to controls (58% vs 31%, p=.004) [fig.12]. Heightened immune response could result in similar hematopoietic suppression as observed in AA, and lead to hypoplasia. Consequently, we subdivided MDS patients according to marrow cellularity and found that patient with the hypoplastic variant of MDS (n=10) were characterized by a higher prevalence of IL10 -819 T/T and –592 A/A phenotypes (40% vs 12% p=.03), which are functionally associated with a lower secretion [fig.13]. This phenotype may be related with less blocking activity on the synthesis of inflammatory cytokines. An analogous observation was also made for the T/T genotype of TGF-β in patients with hypocellular MDS [fig.14]. In addition, looking to subgroup analysis, we found a higher incidence of the AG/AG haplotype for the TNF-α gene in high risk MDS [fig.15], consistent with high secretory phenotype (13% vs 1% p=.02). When we analyzed the AA cohort, in contrast to a few smaller studies, no association was found for SNPs in IL-4Rα, IL-12, IL-1β, IL-2, IL-1α, and TNF- α. However, like in the MDS cohort, when we examined the frequency of TGF-β genotypes, an increased frequency of GG variant on codon 25 (61% vs 35% in controls, p=.03), consistent with an high secretory phenotype, was found [fig.16]. This difference was even more significant for patients with typical PNH. In addition, we found a lower incidence of TT genotypes for the IL1RA gene (33% vs 62% p=.02). According with the data previously published we confirm that the frequency of the hypersecretory genotype T/T of the INF-γ was markedly over represented in the AA cohort compared to normal controls (28% vs 10%, p=.02) [fig.17]. When we compared the subgroup of the AA cohort based on the presence or not of a PNH clone, we interestingly found that the presence of a PNH clone seems to be related with the T/T genotype of IFN-γ phenotype (35% vs 14% p=.01).

Fig. 12

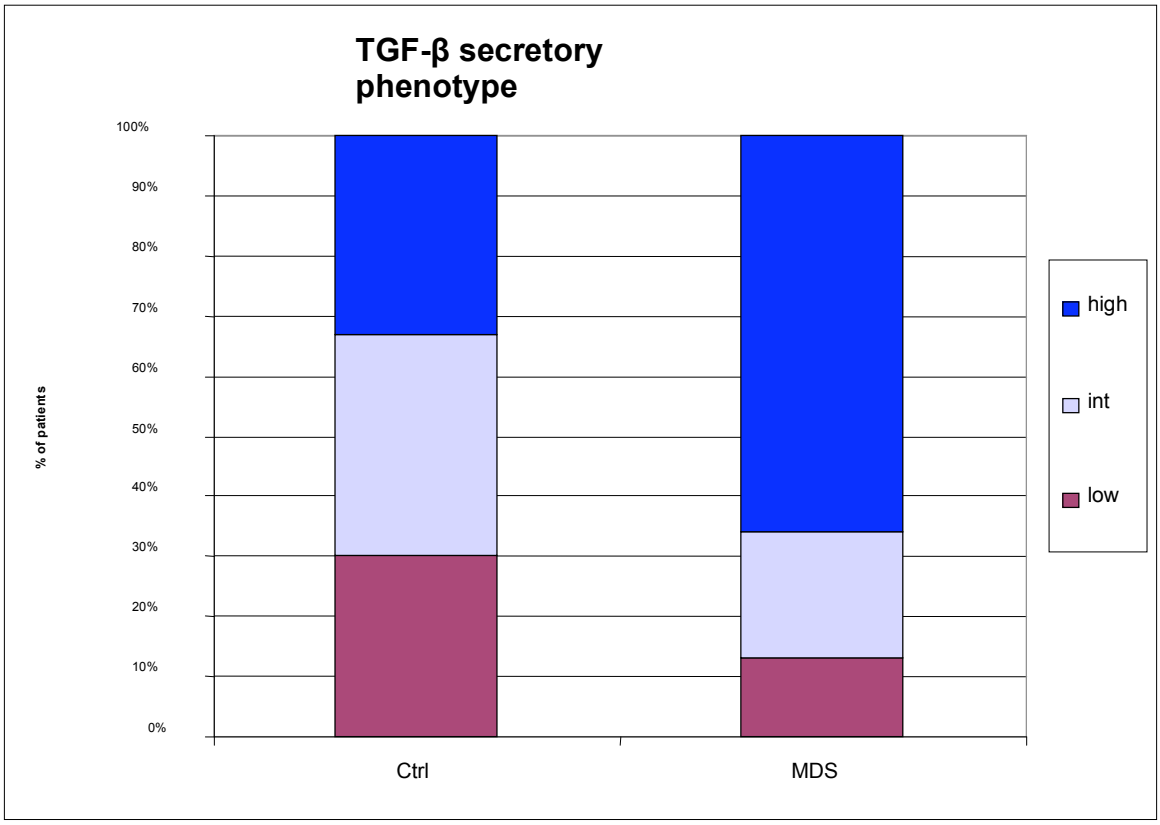

TGF-β secretory phenotype
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% of patients
Ctrl
MDS
high
int
low

Fig. 13

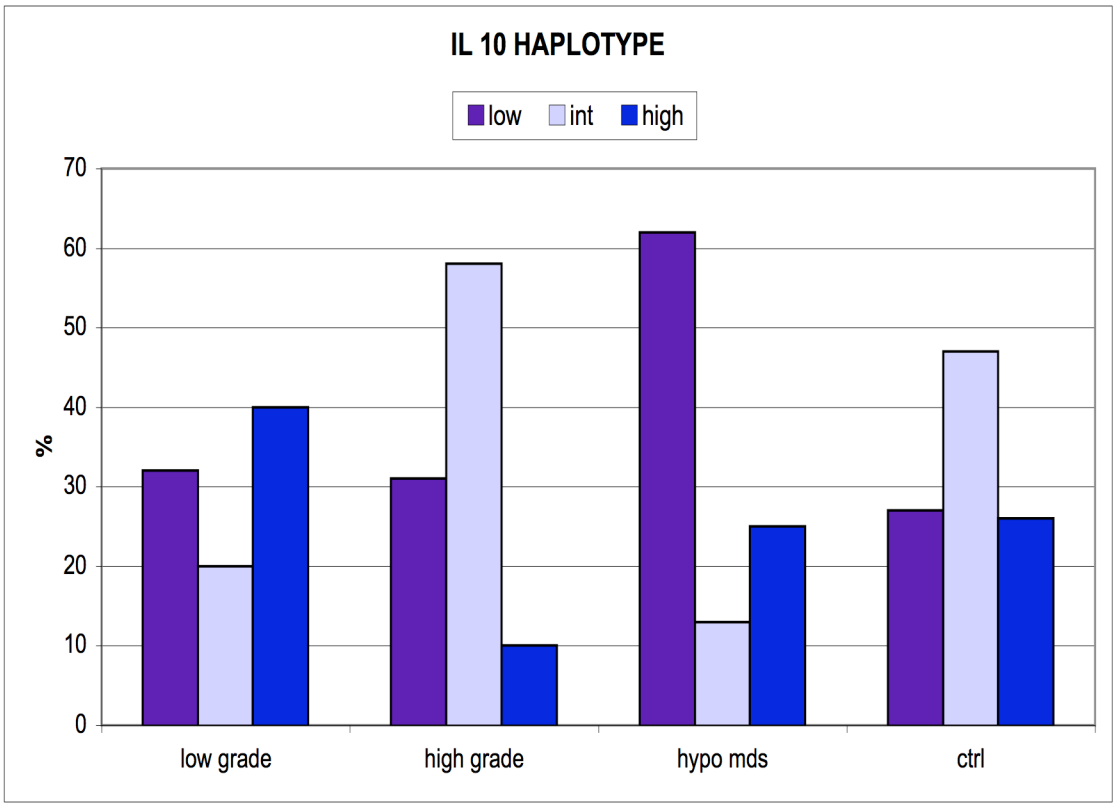

IL 10 HAPLOTYPE
low
int
high
70
60
50
40
30
20
10
0
%
low grade
high grade
hypo mds
ctrl

Fig.14

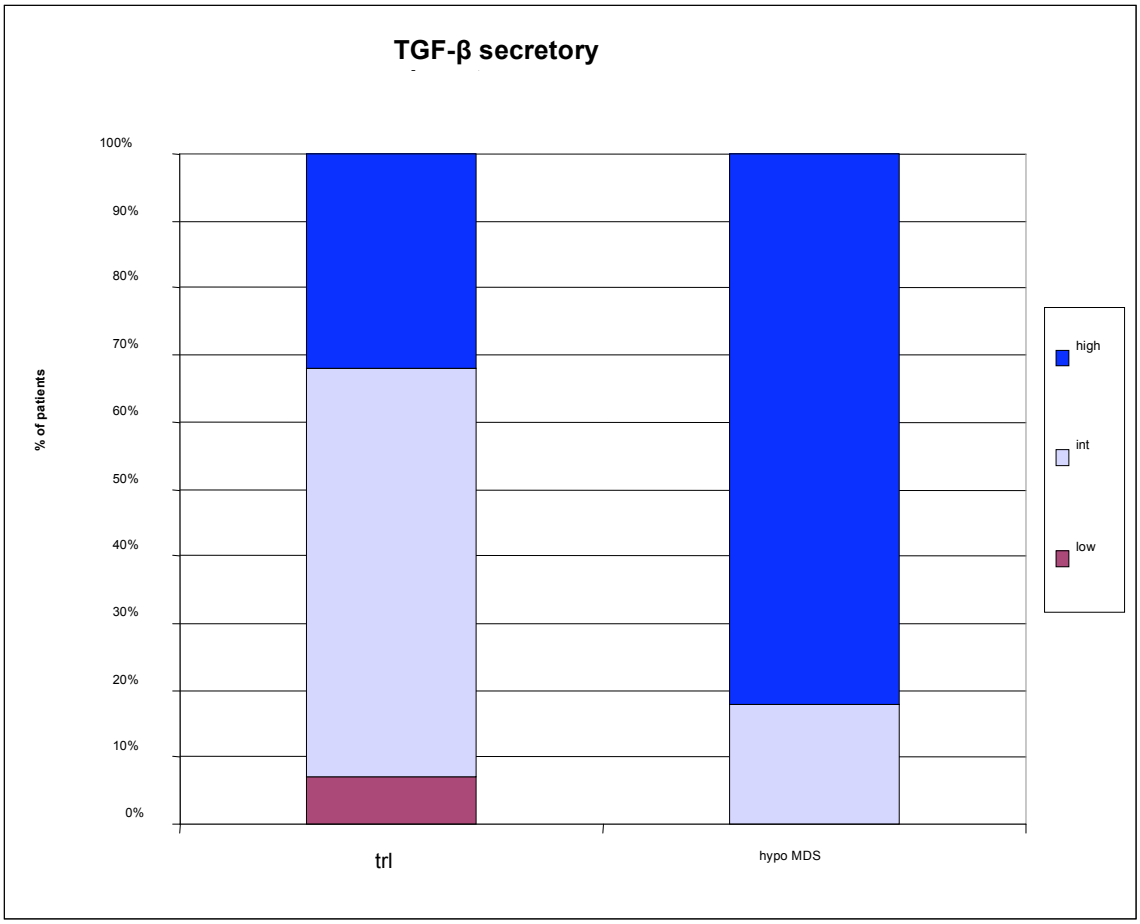

TGF-β secretory
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% of patients
high
int
low
trl
hypo MDS

Fig.15

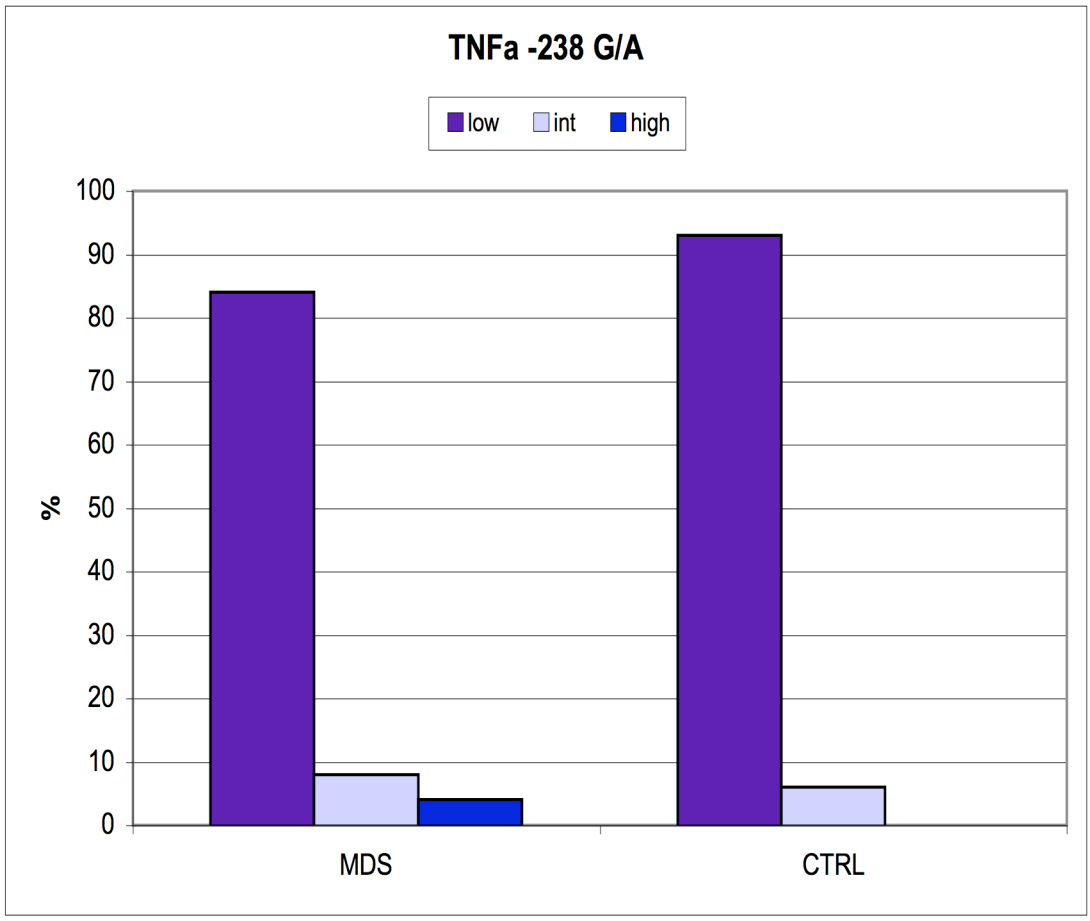


Fig. 16

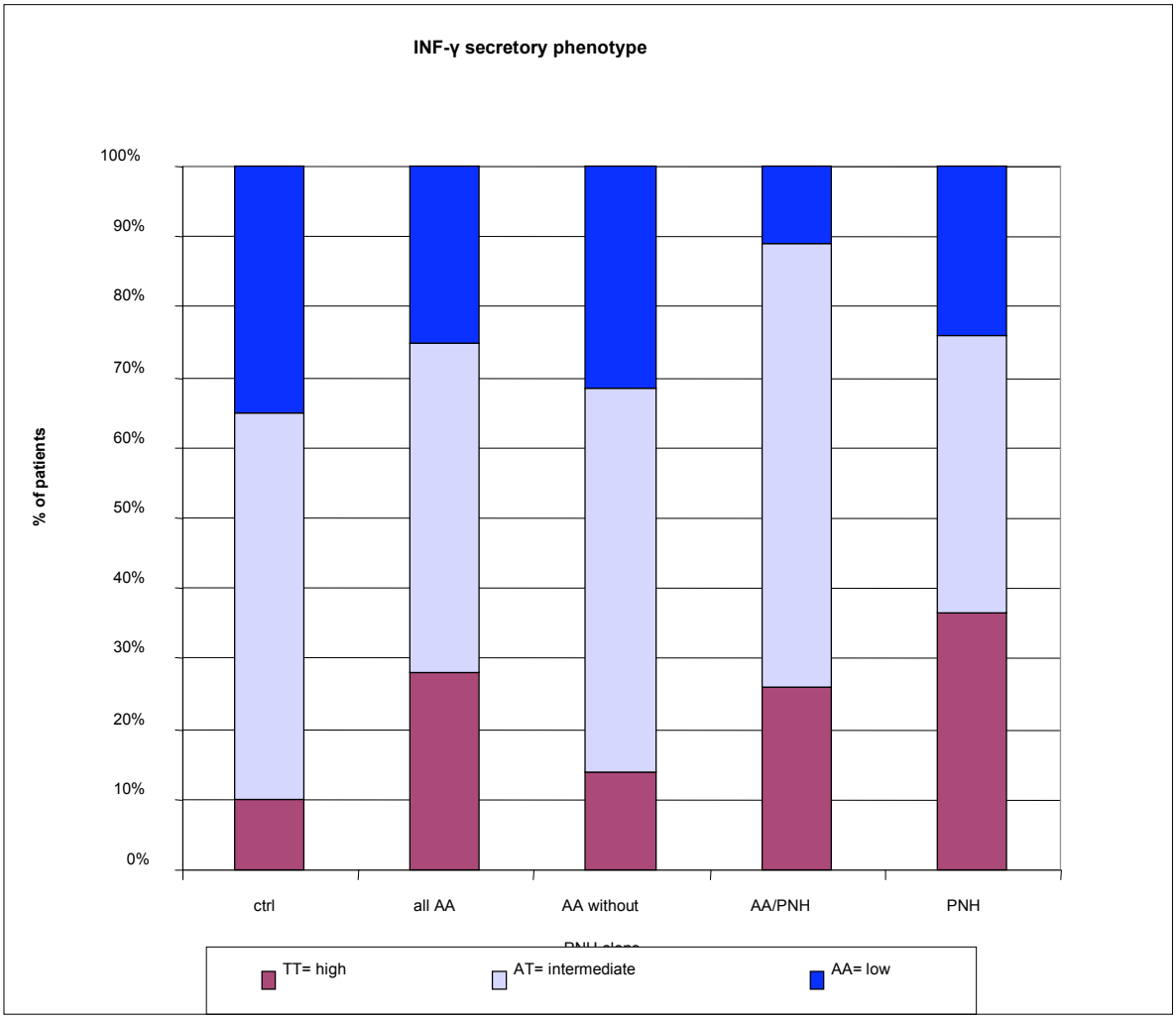
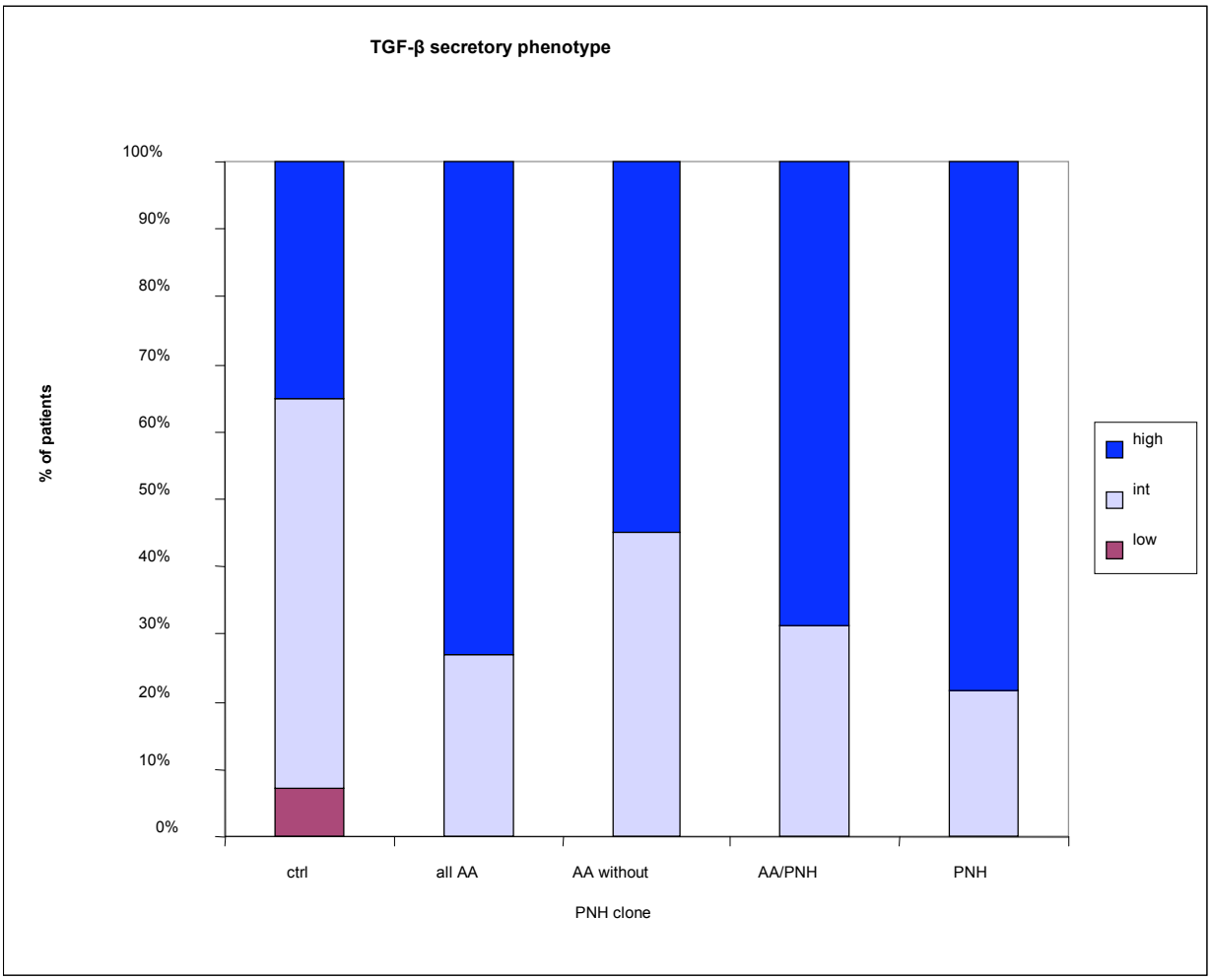

INF-γ secretory phenotype
% of patients
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
ctrl
all AA
AA without
AA/PNH
PNH
TT= high
AT= intermediate
AA= low

Fig. 17


TGF-β secretory phenotype
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% of patients
ctrl
all AA
AA without
AA/PNH
PNH
PNH clone
high
int
low

# DISCUSSION

Because a number of HLA class I alleles has been identified as ligand of KIR, it is possible to examine the frequency of ligand classes in various diseases. When we compared patients with AA, PNH and MDS with controls for the distribution of KIR-L groups, we found that in AA as well as in the MDS chort patients positive for both the A3 and A11 alleles were very low compared to normals. KIR genotyping (defined as absence or presence of specific KIR genes in patiens versus controls) revealed a significantly decreased frequency of the inhibitory KIR2DL3 in the AA population compared to normals; the fact that this inhibitor KIR is underrepresented in AA may lead to increased intensity or derangement of the immune response. In the next step of experiments, we studied the frequency of KIR miscmatch, defining as mismatch the presence of a specif KIR and the lack of the corresponding HLA ligands. Alteration of these interactions may influence KIR's activity and so be responsible for predisposition to some disease. We found that in AA patients there was decreased frequency of the 2DS1/C1 mismatch compared to normal controls, while in MDS patients we found decreased frequency of the 2DS2/C2 mismatch compared to controls. The decreased frequency of 2DS1 and 2DS2 mismatch may result in reduced silencing of cytotoxic activity. In addition we found that patients with high risk MDS had an increased frequency of the 2DL3/C1 mismatch compared to the low risk group. Increased 2DL3 and decreased 2DS2 mismatch both result in enhancment of cytotoxicity towards target cells,with marked imbalance between TCR-activated cell lysis and KIR inhibition of cytotoxic immune response. Futher studies are needed to assess whether these alteration in KIR and KIR-L occurring at genetic level actually translate into functional consequences, possibly resulting in increased disease susceptibility. In addition to HLA and KIR backgroud, other immunogenetic factors may play a role in the pathogenesis of BMF. For example, various cytokines involved in inflammation and apoptosis pathways could play a role in the intricate relationship between genetic determinants of immune response and BMF clinical course. We have empirically selected a number of cytokine and cytokine promoters and receptors polymorphisms that have been described to play a role in various autoimmune disease.We found that in MDS patients there was a higher frequency of the high secretor phenotype of TGF-$\beta$ compared to controls. In addition, looking to subgroup analysis, we found that in high risk MDS there was a higher frequency of the secretory phenotype of TNF-$\alpha$, compatible with increased inflammatory activity. This difference was even more significant for patients with typical PNH. Compared with normal, the AA cohort showed a incrased frequency of the hypersecretory phenotype of INF-$\gamma$. We also found that the presence of a PNH clone was related with a high secretory phenotype of IFN-$\gamma$. This finding support that the presence of a PNH clone may be a marker of immunomediated pathophysiology. The inhibitory ability of lymphocytes form AA patients has been explored intensively, and over-expression of Th1 cytokines, leading to bone marrow damage, has been reported [119,120]. Moreover, TGF-$\beta$ is a well-known inhibitory factor in human models, and was found to be increased in PNH clone that emerged after Campath-1H therapy [121]. In summry our data, showing a genotypic profile associated wit high TGF-$\beta$ and IFN-$\gamma$ prodution in patients with bone marrow failure, are consistent with these findings, and suggest that genetic regulation of inflammatory and T-cell- mediated immunological pathways could be involved in the pathogenesis of bone marrow

failure, reinforcing the view that both AA and PNH are organ-specific autoimmune disorders.

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31. **Impact of genetic pholymorphisms on immune response and clinical features in MDS**
B Serio, Jankowska A, G Ramsingh, Z Nearman, H Szpurka, R Tiu, C O'Keefe, M Sekere, J Maciejewski.
*The American Society of Hematology 48th Annual Meeting, Orlando, 2006. poster*

32. **Analysis of Immunogenetic Factors in Myelodysplastic Syndromes (MDS) Reveals Potential Pathogenic Role Cytokine Genotypes such as TGF-β.**
B Serio, R Tiu, A Jankowska, Z Nearman, C O'Keefe, M A Sekeres and J Maciejewski .
*The American Society of Hematology 49th Annual Meeting, Atlanta,2007.poster*

33. **Decreased Numbers of Tregs in Aplastic Anemia is Detected by Immunohistochemistry and Flow Cytometry**.
B Serio, Z Peerwani, R Tiu, J Powers, E Hsi, and J Maciejewski.
*The American Society of Hematology 49th Annual Meeting, Atlanta, 2007. poster*

34**. Immunogenetic Analysis Reveals the Association of INF-γ (+874 A/T) Hypersecretor Genotype in AA and a Low Frequency of KIR-2DL3/C1 Mismatch in Responders to**

**Immunosuppression.**
B Serio, G Ramsingh, R Tiu, A M Risitano, M A Sekeres, and J Maciejewski.
*The American Society of Hematology 49th Annual Meeting, Atlanta, 2007. poster*

35. **Non Synonymous SNP-Array-Based Disease Association Analysis in Aplastic Anemia and Paroxysmal Nocturnal Hemoglobinuria**.
B Serio, A D Viny, R Tiu, B Przychodzen, A Lichtin, C O'Keefe, J Maciejewski.
*The American Society of Hematology 49th Annual Meeting, Atlanta, 2007. poster*
36. **Impact of Cytokine Gene Polymorphisms in Aplastic Anemia (AA)**
B Serio, AM Risitano, G Ramsingh, R Tiu, A Viny, B Rotoli, C Selleri and J Maciejewski.
*41th Italian Society of Hematology, Bologna, Italy, 2007. Oral comunication*
37. **Impact of Cytokine Gene Polymorphisms in Myelodysplastic Syndromes (MDS)**
B Serio, AM Risitano, A Jankowska, Z Nearman, C O'Keefe, B Rotoli, C Selleri and J Maciejewski.
*41th Italian Society of Hematology, Bologna, Italy, 2007. Oral comunication*
38. **Circulating T regulatory cells (treg) are decreased in patients with Aplastic Anemia (AA)**
B Serio, AM Risitano, J Powers, Z McIver, B Rotoli, C Selleri and J J Maciejewski.
*41th Italian Society of Hematology, Bologna, Italy, 2007. Oral comunication*
39. **Double negative tregs are reduced in allo-transplanted patients developing graft-versus-host disease**.
B Serio, Z McIver, A M Risitano, C Selleri and J Maciejeski.
*34th Annual Meeting of the European Group for Blood and Marrow Transplantation , Florence, 2008. Oral comunication*
40. **Permissive Conditions for Evolution of PNH clones are Characterized by Overproduction of IFN-g by Clonal CD4 and CD8 T cells, Fas-L by CTLs, and Promoted by Immunogenetic Background.**
M Clemente, H Cazzolli, A Jankowska, B Serio, S Nyland, M Wlodarski, PK Burnette, R Paquette, J Maciejewski
*The American Society of Hematology 50th Annual Meeting, San Francisco, 2008. poster*

# APPENDICE

## Elenco pubblicazioni allegate

1) "**Double-negative regulatory T cells induce allotolerance when expanded after allogeneic haematopoietic stem cell transplantation"**.
McIver Z, Serio B, Dunbar A, O'Keefe CL, Powers J, Wlodarski M, Jin T, Sobecks R, Bolwell B, Maciejewski JP.
Br J Haematol. 2008 Apr;141(2):170-8. Epub 2008 Mar 3.

2) **"Hemochromatosis-associated gene mutations in patients with myelodysplastic syndromes with refractory anemia with ringed sideroblasts".**
Nearman ZP, Szpurka H, Serio B, Warshawksy I, Theil K, Lichtin A, Sekeres MA, Maciejewski JP.
Am J Hematol. 2007 Dec;82(12):1076-9.

3) **"High serum leptin in patients with chronic graft-versus-host disease after hematopoietic stem cell transplantation".**
Tauchmanovà L, Matarese G, Carella C, De Rosa G, Serio B, Ricci P, Lombardi G, Rotoli B, Colao A, Selleri C.
Transplantation. 2004 Nov 15;78(9):1376-83

4) "**Short-term zoledronic acid treatment increases bone mineral density and marrow clonogenic fibroblast progenitors after allogeneic stem cell transplantation".**
Tauchmanovà L, Ricci P, Serio B, Lombardi G, Colao A, Rotoli B, Selleri C.
J Clin Endocrinol Metab. 2005 Feb;90(2):627-34. Epub 2004 Nov 16.

5**) "Involvement of the urokinase-type plasminogen activator receptor in hematopoietic stem cell mobilization".**
Selleri C, Montuori N, Ricci P, Visconte V, Carriero MV, Sidenius N, Serio B, Blasi F, Rotoli B, Rossi G, Ragno P.
Blood. 2005 Mar 1;105(5):2198-205. Epub 2004 Oct 19.

6**) "Late relapse of acute promyelocytic leukemia treated with all- trans retinoic acid and chemotherapy: report of two cases"**
Ferrara F, Selleri C, Mele G, Serio B, Palmieri S, Pocali B, Pane F, Rotoli B.
Ann Hematol. 2004 Jul;83(7):484-6. Epub 2004 Jan 17.

7) "**Avascular necrosis in long-term survivors after allogeneic or autologous stem cell transplantation: a single center experience and a review".**
Tauchmanovà L, De Rosa G, Serio B, Fazioli F, Mainolfi C, Lombardi G, Colao A, Salvatore M, Rotoli B, Selleri C.
Cancer. 2003 May 15;97(10):2453-61.

**8) "Involvement of nitric oxide in farnesyltransferase inhibitor-mediated apoptosis in chronic myeloid leukemia cells".**
Selleri C, Maciejewski JP, Montuori N, Ricci P, Visconte V, Serio B, Luciano L, Rotoli B.
Blood. 2003 Aug 15;102(4):1490-8. Epub 2003 Apr 24.

9) "**Long-lasting bone damage detected by dual-energy x-ray absorptiometry, phalangeal osteosonogrammetry, and in vitro growth of marrow stromal cells after allogeneic stem cell transplantation".**
Tauchmanovà L, Serio B, Del Puente A, Risitano AM, Esposito A, De Rosa G, Lombardi G, Colao A, Rotoli B, Selleri C.
*Journal of Clinical Endocrinology § Metabolism* 87, 5058-5065, 2002.