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The Immune Response Dis-Regulation and the Pathogenesis of Hematopoietic Disorders

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A mio fratello

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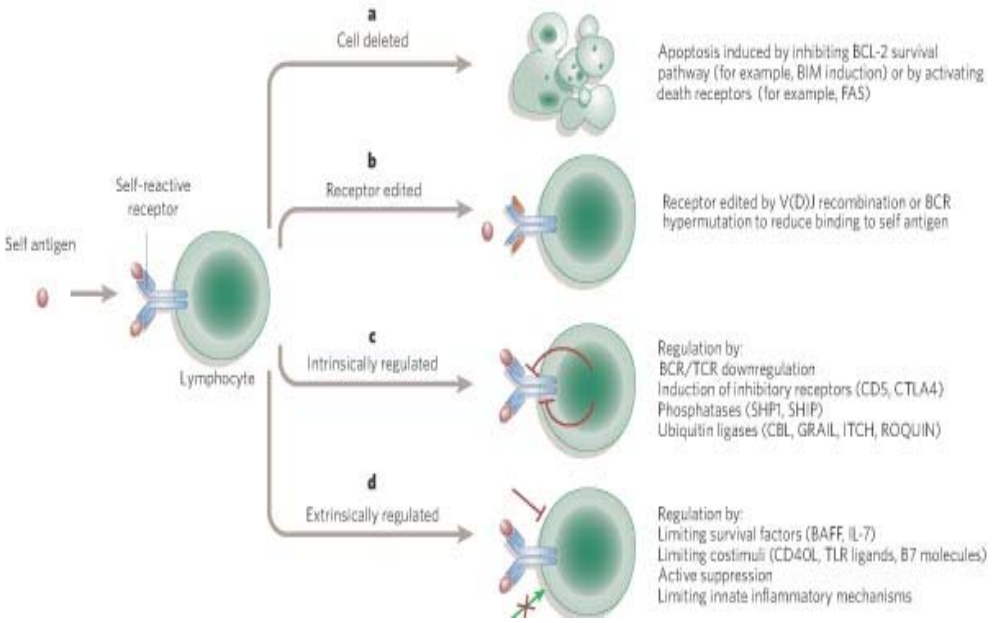
Introduction

"Our immune system is the body's sixty sense". It is faced with the daunting job of defending the organism against invading pathogens, while at the same time maintaining tolerance to body's own tissues, thereby preserving its integrity[1]. At the completion of development, T and B cells emerge from the primary lymphoid organs and enter the re-circulating pool of peripheral lymphocytes. One of the first things these naïve cells encounter in their fully mature state are antigens from various non-lymphoid organs that were thought to be restricted in their expression to a particular peripheral tissue. Several mechanisms act together to ensure self-tolerance2, including clonal deletion, anergy, ignorance and exhaustion, effector T-cell and regulatory T-cell balance, and cytokine deviation. An imbalance between pro-inflammatory and anti-inflammatory cytokines, autoreactive and inflammatory T helper 1 (Th1) cells, and regulatory T cells results in the loss of immune tolerance, the breakdown of immune homeostasis and the subsequent appearance of exacerbated inflammatory conditions and autoimmune disease[3].

Immunological tolerance

The "central lymphoid tissues", which are bone marrow for B cells and the thymus for T cells, are the site of the V(D)J recombination, the process of unique B cell receptor (BCR) and T cell receptor (TCR) genes assembly from three separate gene segments, the variable (V), diversity (D) and joining (J) genes, during B and T-cell differentiation. In addition, during each immune response, somatic hypermutation substitutes single nucleotides of BCR genes in peripheral lymphoid tissues (such as the spleen, lymph nodes and tonsils). A significant fraction of the receptors generated by both these processes bind to one or more self-components in the body by-product of a deliberately random receptor-generating process[4]. Between 20 and 50% of TCRs and BCRs generated by V(D)J recombination bind with a potentially dangerous affinity to a self antigen[5]. Since only 3–8% of the population develops an autoimmune disease[6], it is remarkable that this enormous burden of self-reactive receptors is so well regulated.

Each lymphocyte usually produces only a single receptor out of the billions possible. Experiments have established that if this receptor is self reactive, then four cellular strategies are employed to deal with it (**Figure 1**). First, the cell displaying the "forbidden", or self-reactive, receptor can be triggered to die, as originally envisaged in Burnet's concept of clonal deletion. Second, a cell bearing a forbidden receptor can "edit" it by further V(D)J recombination or somatic hypermutation to display a different receptor that is not self reactive. Third, intrinsic biochemical and gene-expression changes can reduce the ability of the cell to be triggered by self-reactive receptors. This is generally termed clonal anergy or tuning[7]. Finally, even if the cells have evaded the three mechanisms above, collectively called "immunological ignorance", extrinsic controls can limit the danger of self-reactive receptors. These extrinsic controls limit the supply of essential growth factors, costimuli, pro-inflammatory mediators and other factors, and also include active suppression by regulatory T (Treg) cells, through a mechanism that is poorly understood.

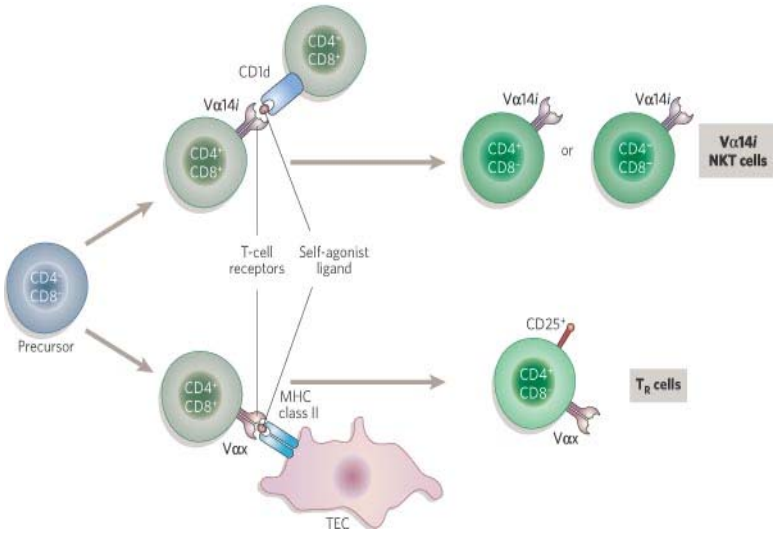


Christopher C. et al. Nature. 2005. 435, 590-597.

Figure 1. **Four cellular strategies are used to regulate self-reactive receptors at different points during B- and T-cell differentiation.** a) The cell is deleted through induction of cell death. b) The receptor is edited to one that is less self-reactive. c) Biochemical or gene-expression changes intrinsically dampen the self-reactive receptor's ability to activate the cell. d) The ability of self-reactive cells or antibody to cause autoimmunity is limited by using extrinsic suppression and by limiting essential growth factors, costimuli and inflammatory mediators.

The coexistence of autoreactive and protective T cells was revealed by the multi-organ autoimmunity observed in lymphopenic (immune-deficient) recipient mice upon adoptive transfer of naïve $CD4^+$ T cells, and by the protection from autoimmune pathology upon co-transfer of a subset of CD4+ T cells expressing interleukin (IL)-2 receptor alpha-chain (CD25)[8]. Current evidence suggests that the $CD25^+CD4^+$ T cells could be themselves self reactive (**Figure 2**), and that this property plays an essential role in the commitment to a Treg-cell lineage. An essential function for TCR signals in the development of Treg cells was suggested by the finding that TCR-transgenic mice on a recombination-activating gene-deficient background (which lack endogenous TCR rearrangements) do not develop Treg cells, whereas most TCR-transgenic mice expressing functional recombination-activating genes contain varying numbers of Treg cells[9]. Thus, self-reactivity can be beneficial as part of a dedicated cellular mechanism preventing autoimmunity.

In addition to $CD25^+CD4^+$ Treg cells, other important self-reactive T cell sub-lineages have been identified. Prominent among these are cells that express a semi-invariant T-cell receptor (TCR) specific for conserved self-ligands (**Figure 2**). These ligands, which are normally present at a low level, might be induced and serve as molecular signs of stress or infection. The best characterized such T-cell sub-lineage is the CD1d-dependent Natural Killer T cell (NKT)[10].



Kronenberg M. & Rudensky A. Nature. 2005. 435, 598-604.

Figure 2. **Recognition of self-agonist ligands in the thymus can create at least two different sublineages of self-reactive T cells.** They probably branch off from the mainstream pathway of development at the double positive stage of differentiation. Thymic TR precursors can also branch off at the CD4 single positive stage of differentiation. MHC class II+ bone-marrow derived cells may also participate in TR-cell selection. TEC, thymic epithelial cell. V□x, diverse V□regions.

Treg population is able to regulate the immune responses to autoantigens, tissue transplant, allergens and microbial pathogens[8]. Despite the first observation on Treg was published on 1995 by Sakaguchi et al.[11], a complete functional characterization of these cells is still lacking. Treg cells are able to control the effector cells in terms of clonal expansion, differentiation, cytokine profile and tissue migration during immune response. Recent in vivo observations[12] have been suggesting that Treg activity occurs at level of T cell-Dendritic Cells (DC) interaction during the antigen-priming phase. It is relevant that Treg control T cell priming in lymphoid organs but are also able to inhibit immune response in peripheral tissues[13]. Intriguingly, the removal of Treg significantly enhances NK cell-mediated bone marrow rejection in murine models.

NKT cell subset, in humans, preferentially expresses an invariant Valpha24$^+$Jalpha18$^+$Vbeta11$^+$ TCR (NKTi). NKTi are activated by alpha-galatoctosylceramide with a CD1d-restricted pattern[10]. Recent reports indicate that NKTi cells can be subdivided in CD4$^+$CD8$^-$ and CD4$^-$CD8$^-$ subsets, diverging in their ability to target CD4$^+$T cells, NK and B lymphocytes, and CD8$^+$ T cells, respectively[14]. NKTi cells produce a variety of immune-regulatory cytokines ascribing either to pro-inflammatory T helper 1 (IFN-gamma and TNF-alpha) and/or to anti-inflammatory T helper 2 (IL-10 and IL-4) profile[15]. As recently reviewed, this paradoxical behavior renders difficult to predict the functional consequences of NKTi activation in immune regulation in vivo[16]. Indeed, NKTi cells can exert anti-tumor cytotoxicity and anti-proliferative activity[17,18]. Intrigue, tumor immune surveillance by NKTi can be also detrimental[15].

Why does autoimmunity develop in about 5% of people?

Autoimmune diseases arise when the immune system turns its antimicrobial defenses upon normal components of the body such as insulin-producing pancreatic cells in Type 1 diabetes or chromatin in systemic lupus erythematosus (SLE). Immunologists identified a number of

specific genes and cellular mechanisms involved in immunological self-tolerance that, when disrupted by inherited mutations, cause autoimmune disease. Despite complete failure of individual tolerance mechanisms, these autoimmune diseases have a delayed stochastic penetrance[19].

Major Histocompatibility Complex (MHC), namely Human Leukocyte Antigens (HLA) in humans, represents a major susceptibility factors for the development of autoimmune diseases in humans. The MHC, or HLA, consists of a set of polymorphic genes encoding both class I and class II glycoproteins. The main biological role of such molecules is to bind antigenic peptides and present them to T cell scrutiny. Moreover, MHC polymorphism tends to concentrate in hypervariable regions[20], corresponding to MHC binding pockets engaging specific anchor residues of their peptide ligands. This pattern of variation in HLA molecules is different from that in most other protein-coding genes, in which allelic variation tends to occur more in introns than in exons[21].

HLA are unequivocally involved in several autoimmune diseases. However, the mechanism by which HLA genes contribute to disease development in humans is still largely unknown. One exception is Celiac Disease, whereby a mechanism through which HLA molecules contribute to the disease has now been unraveled by carefully analyzing T cells from the lesions[22]. HLA genes are crucial for antigen presentation to T lymphocytes and for activation of NK cells. Moreover, HLA genomic region contains many other genes with putative or proven immune functions.[23]

Bone marrow failure, hematopoietic clonal expansion and autoimmunity

Bone marrow (BM) failure syndromes are very illustrative with regard to pathophysiology of the stem cell compartment, its physiologic and pathophysiologic regulation as well as mechanisms of clonal evolution[24]. Stem cell impairment may be primary or secondary when due to systemic diseases or iatrogenic causes. A quantitative defect of stem cells has been documented in Aplastic Anemia (AA), Paroxysmal Nocturnal Hemoglobinuria (PNH) and Myelodysplastic Syndromes (MDS) as measured by flow cytometry of $CD34^+$ cells, colony-forming cells as well as

long-term culture initiating cells, the most immature in vitro equivalents of hematopoietic stem cells[25,26]. In most cases of BM failure an immune-mediated attack against haemopoietic progenitors and/or mesenchimal elements has been hypothesized . Although AA is the prototype of primary hematopoietic stem cell failure[27,28]depletion or functional deficiency of stem cells also occurs in MDS and PNH. A great number of evidence point towards an immune-mediated inhibition of hematopoiesis in these pathologies at specific differentiation stages, but the efficacy of immune-suppressive strategies targeting T cells support the hypothesis of the involvement of T cells in the pathophysiology of a great number of bone marrow failure syndromes. Clearly, some forms of immune cytopenias, such as those seen with SLE, and autoimmune neutropenia, are mediated by antibodies likely directed against early or more mature hematopoietic progenitor cells. Theoretically, even if T cells are mostly responsible for the damage to the progenitor and stem cell compartment, one could speculate that the cellular immune response will be accompanied by a corresponding antibody production[29]. Depletion of stem cell compartment could be due to an autoimmune attack by T lymphocytes and to the damage exerted by inhibitory cytokines, products of activated immune cells. Effector mechanisms in hematopoietic inhibition may involve various pathways, including release of inhibitory cytokines leading to apoptosis of hematopoietic progenitor and stem cells. The specificity of such a mechanism may be difficult to reconcile with a sometimes very narrow spectrum of hematopoietic inhibition as seen in single lineage cytopenias. Direct perforin/granzyme-mediated killing by cytotoxic effector cells may be another mechanism by which specific targeting of stem cells or progenitors can be explained. Clearly, the distribution of target antigens may determine the killing spectrum. Terminal differentiated citotoxic lymphocytes (CTL) are likely the most efficient effector cells and so far there is little evidence that the hematopoietic inhibition can be mediated by natural killer cells. It is possible that a CTL population lacking CD28 and expressing CD57 contains most of the pathogenic clones and can serve as a source of T cells for molecular TCR analysis[29].

The possibility that these processes could be of critical relevance to favor the emergence of hemopoietic clones, able to escape immune aggression via different mechanisms, is a consistent work hypothesis.

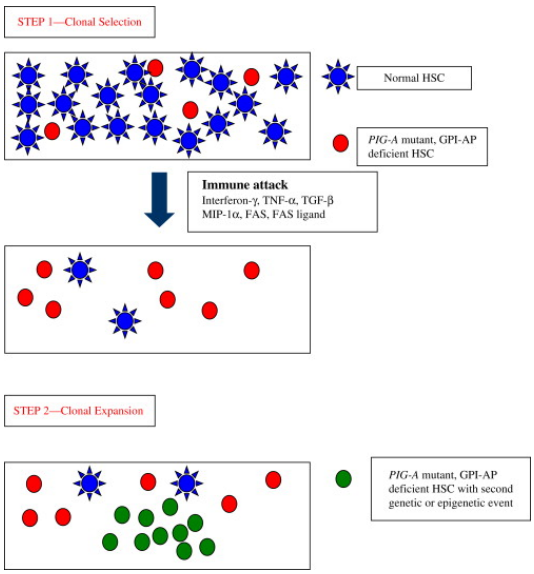
Several theories can be put forward to explain the initial steps in the evolution of clonal stem cell diseases such as MDS. Contraction of the stem cell compartment may result in a 'benign clonality' of hematopoiesis. For example, in AA blood cells may be clonal or oligoclonal as a result of very few stem cells contributing to blood production; polyclonal hematopoiesis is restored upon successful therapy. Observation of the disappearance of clonal abnormalities following successful immunosuppressive therapy and restoration of polyclonal hematopoiesis also supports the notion that clonal outgrowth may be a result of decreased numbers of operative normal hematopoietic clones. In contrast, acquisition of a genetic defect by an individual stem cell resulting in clonal expansion may be the primary event leading to the gradual dis-replacement of normal hematopoiesis[24].

Paroxysmal Nocturnal Hemoglobinuria

PNH is a hematological syndrome characterized by the emergence of a hematopoietic progenitor bearing somatic mutations in the phosphatidylinositolglycan-A (PIG-A) gene. The protein encoded by this gene is essential for the synthesis of the glycosylphosphatidylinositol (GPI) anchor[30,31]. Given that the PIG-A mutation in PNH patients occurs in a hematopoietic stem cell, a defective clonal hematopoieis, together with a residual polyclonal hematopoieis, develops through several lineages and accounts for the mixed (GPI^+ and GPI^-) phenotype, commonly present in peripheral blood of PNH patients[32,33]. From the clinical point of view, a triad of hemolytic anemia, venous thrombosis and blood cytopenias characterizes PNH. The most dramatic consequences are seen in red blood cells, where two of GPI-linked proteins, CD55 (decay accelerating factor, DAF), and CD59 (membrane inhibitor of reactive lysis, MIRL), are responsible for controlling the activity of plasma complement[34]. The Coombs-negative, intravascular hemolysis (and the resultant hemoglobinuria), that are the clinical hallmarks of classic PNH, are attributable to deficiency of CD55 and CD59 because peripheral

blood erythrocytes derived from the mutant clone lack the capacity to restrict cell-surface activation of the alternative pathway of complement[35].

Clinical data have indicated for a long time a close link between PNH and aplastic anemia, and there is much circumstantial evidence implicating an autoimmune mechanism for the development of aplastic anemia[36]. By extrapolation, this could apply to PNH as well. Infact, it has been hypothesized that the expansion of the PIG-A negative clone is the consequence of a somatic cell selection resulting from the presence of autoreactive T-cells directed against GPI-anchored proteins in the context of MHC and MHC-like molecule on the surface of hematopoietic stem cell[37,38]. Consistently, a T-cell receptor Vβ-chain skewing has been described in PNH, suggesting a T-cell mediated process leading to suppression of hematopoietic function[39]. Recent evidence points to a pathogenic role of specific subsets of cytotoxic cells, particularly T[40], NK and NKT cells[41,42]. In PNH patients, in vitro assays for BM colony-forming cells have shown that PNH cells are less sensitive to interferon-gamma (IFNγ) and to TNFα[43]. New relevant data have emerged from RNA microarray analysis of $CD34^+$ hematopoietic progenitors: amongst these, GPI^+ cells, compared with GPI^- cells, had an increased expression of pro-apoptotic genes[44]. In addition, when normal $CD34^+$ cells were exposed to IFN- γ this produced changes in the gene expression profile similar to those seen in GPI^+ cells from PNH patients[45], suggesting that the latter individuals were suffering from exposure to IFN-γ (**Figure 3**).



C.J. Parker.Experimental Hematology . 2007. 35, 523–533

Figure 3. **Model of two-step hypothesis of PNH pathophysiology**. Upper panel: Hematopoietic stems cells and primitive progenitors with mutant PIGA are present in normal marrow, but they are not apparent because no selection pressure has been applied and they have no intrinsic growth/survival advantage. Middle panel: In the setting of immune-mediated bone marrow injury, PIGA mutant cells are selected because they have a growth/survival advantage based on GPI-AP-deficient phenotype. Additional PIGA mutant HSC are produced as a consequence of this process because the mutational frequency of the gene is enhanced by stress erythropoiesis. Lower panel: Clonal expansion is the result of genetic or epigenetic events that activate genes that work in concert with mutant PIGA, GPI-AP deficiency to enhance further the proliferative advantage of the mutant cells. Under these conditions, PNH has the characteristics of a benign clonal myelopathy.

Myelodysplastic Syndrome

Myelodysplastic Syndromes (MDS) are clonal disorders characterized by an ineffective hematopoiesis followed by frequent development of Acute Myeloid Leukemia (AML). Cytopenia, accompanied by a bone marrow, generally hyper-cellular, exhibiting dysplastic changes, represents the hallmark of MDS[46]. Etiological factors of MDS are largely unknown; most cases are idiopathic (de novo MDS). The onset of idiopathic MDS depends on a complex sequence of events; several factors, such as antineoplastic alkylating agents, ionizing radiation, and benzene, were shown to have a clear association and etiological factors for secondary MDS. A great number of evidence proposed immune-suppression of progenitor cells growth with accelerated rate of apoptotic cell death. In this context, the emergent MDS clones acquire resistance to apoptosis induction, allowing them to proliferate without undergoing immune-selection. Successive, progressive genetic changes have been suggested to account for the leukemic transformation of MDS cells[47]. (**Figure 4**)

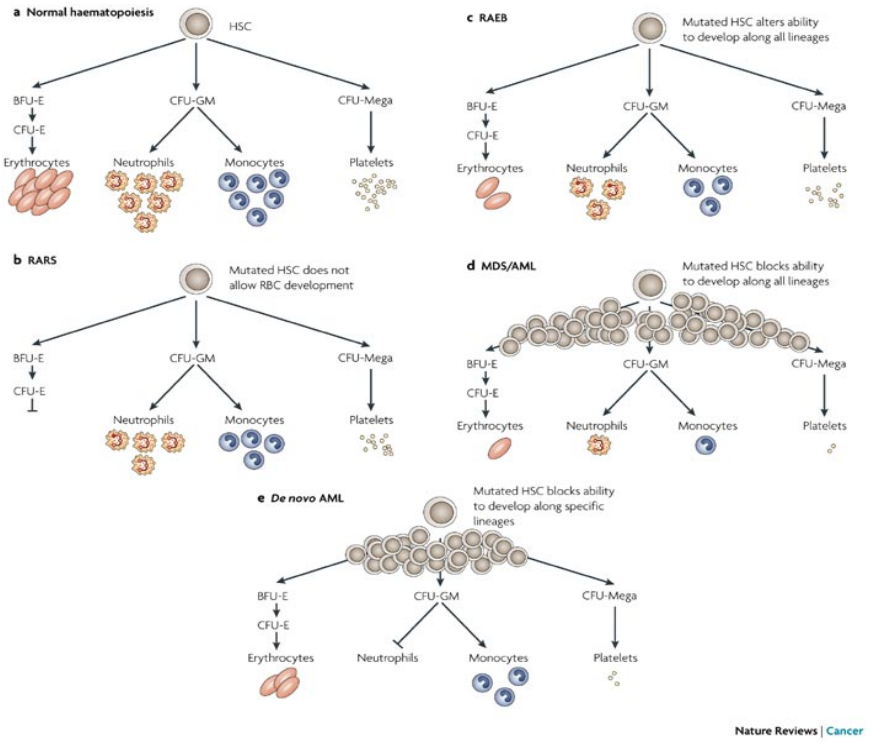


Figure 4 **MDS constitute a complex range of stem-cell diseases.** The myelodysplastic syndromes (MDS) cell clone can suppress normal hematopoiesis (**a**) directly or indirectly through stroma. Stem-cell defects can result in singlelineage deficiency (refractory anaemia and ringed sideroblasts (RARS; **b**)) or multiple-lineage deficiencies (refractory anemia with excess blasts (RAEB; **c**)). MDS stem-cell diseases (**d**) might seem like de novo acute myeloid leukemia (AML), however, the two are distinguishable. For example (**e**), cytopenias in de novo AML can be more restricted owing to a failure in differentiation. HSC, hematopoietic stem cell.

The evaluation of disease risk and outcome of patients with MDS is a critical point. In this context, multiple parameters like chromosomal changes, bone marrow blast cells number and the presence of multiple cytopenia appear to be useful in predicting the survival and transformation rate in MDS patients[48]. There are a number of disparate methods to evaluate the potential clinical outcomes for patients with MDS. Original assessment of prognostic factors has been suggested by the French–American– British Morphology group (FAB) in 1982[49] and, following that, at least six additional risk classification systems have been developed to predict survival and/or evolution to AML. In 1997, the International MDS Risk Analysis Workshop proposed the International Prognostic Scoring System (IPSS), which has compared favorably with the previous most widely used MDS risk evaluation systems[48]. According to IPPS, MDS patients can be classified into distinct prognostic subgroups based on medullary blast cell

count, number of cytopenias, and clonal chromosomal abnormalities. Based on these variables, four risk subgroups regarding survival and AML evolution were suggested: low-risk, 0; intermediate-1 risk (INT-1), 0.5–1.0; intermediate-2 risk (INT-2), 1.5–2.0; and high-risk, ≥2.5, with a median survival ranging from 5.7 years for low-risk MDS patients to 0.4 years in high-risk patients[50,51].

One of the paradoxes present in MDS is the presence of peripheral cytopenias and a hypercellular BM. All of the models suggest that the development of MDS might be due to a multi-step process that originates in the earliest progenitor cell, a pluripotent stem cell[52]. The initial assault or mutation occurs in the DNA of this cell, which adversely affects its differentiation and maturation. Three epidemiological studies have suggested that environmental factors (radiation, smoking, pesticides, organic solvents, heavy metals) contribute to the development of de novo MDS[53,54,55]. Recently, it has been hypothesized the relevance of immune-mediate pathway in stem cells depletion. The damaged cell, the clone, achieves a proliferation advantage that results in ineffective hematopoiesis. Apoptotic agents such as tumor necrosis factor-alpha (TNF-α), Fas/Fas-ligand, and a relative deficiency in hematopoietic growth factors result in the premature death of BM cells, and disease progression is associated with diminished immune response and a loss of tumor suppressor activity[52].

The clonal nature of MDS was confirmed by various techniques[56,57]. The abnormal clone can lead to an increase in cell proliferation in all hematopoietic cell lineages, but especially in the myeloid cell lineage[58]. However, the increased proliferation rate of the clonal population in the bone marrow is quickly equilibrated by an increase in apoptosis[56,58,59]. The clonal cells do not mature or differentiate and the majority of them remain in the BM. As a result, despite the high proliferation activity in the BM, the patient suffers from peripheral cytopenia. The majority of studies demonstrated that increased apoptosis, observed in patients with MDS compared to normal controls, was more pronounced in early stages of MDS than in advanced stages, and was largely restricted to CD34-positive cells[60,61]. Excessive cell death was largely restricted to the early MDS subtypes. Progression to AML was accompanied by a reduction in both

apoptosis and proliferation rates and may suggest that progression to AML is associated with a decreased susceptibility to apoptosis[61]. However, earlier data by Raza et al.[58] is in direct conflict with this, contending that apoptosis was maximal in advanced MDS and was restricted to more differentiated CD34-negative cells. As will be further discussed below, a T-cell attack on haemopoietic cells has been documented in MDS patients[62]. The 'T against the Clone' scenario includes an immune reaction operated by T cells, which in early stage MDS creates an apoptotic environment by the release of inhibitory cytokines and up-regulation of Fas expression on hemopoietic progenitors.

The acquisition of secondary genetic events is a model proposed to explain the stepwise progression of MDS to acute leukemia. These yet unrecognized epigenetic hits possibly perturb apoptotic cell-signal transduction pathways of the primitive clone, altering its apoptotic nature and offering it a growth or survival advantage. Alternatively, excessive apoptosis in early stage MDS could be interpreted as an immune response to antigens expressed by the clonal aberrant cells. This process probably delays the leukemic evolution, but also leads to BM failure. As the disease progresses immune reactions become ineffective in controlling the accumulatively damaged myeloid clone, thus allowing its expansion. Studies reporting increased Fas susceptibility and apoptosis of MDS cells with trisomy 8, but not with monosomy 7, support this perception[63,64]. In contrast, the autologous immune response against leukemic clones in patients with AML and chronic myeloid leukemia is less evident. The reasons for this failure include: insufficient antigen presentation by the malignant cells, immune suppression by soluble or cellular factor(s), and an insufficient number of specific lymphoid cells to react with the rapidly growing clone. (**Figure 5**)

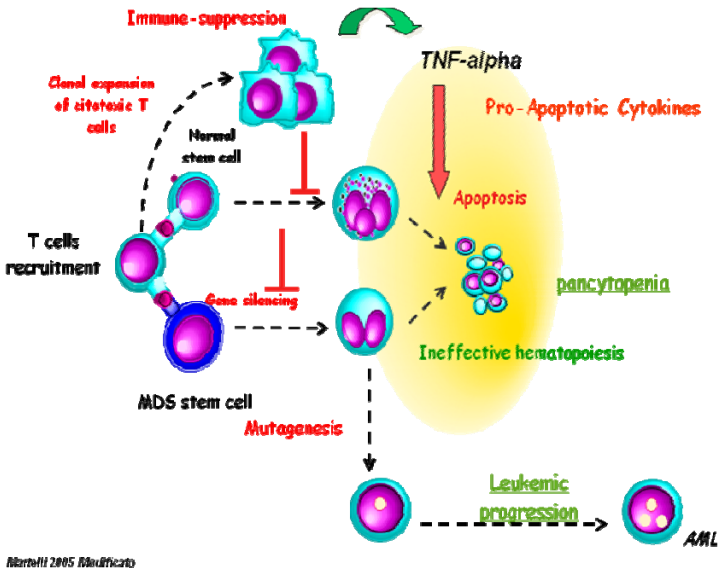


Figure 5. **Pathogenesis model of MDS**. A great number of mechanisms, causing the defective hematopoiesis and the expansion of dysplastic clones, have been proposed. The initial assault or mutation occurs in the DNA of hematopoietic stem cell, which adversely affects its differentiation and maturation. Environmental factors contribute to the development of de novo MDS. The damaged cell, the clone, achieves a proliferation advantage that results in ineffective hematopoiesis. In addition, selective pressure by effector cells could inhibit polyclonal hematopoiesis, favoring the selection/shaping of dysplastic clones. Moreover, mechanisms of gene silencing and oncogene activation could favor the neoplastic progression.

It is worth noting that a number of data refer significant increase of Treg cells in cancer patients[65]. This evidence suggests a deleterious role for Treg in suppressing the immune response against cancer as well as the ability of certain tumors to recruit or to expand Treg[66]. In this regard, some observations indicate that this increased frequency is due to active proliferation rather than redistribution from other compartments[67], while attraction of Treg cells via CCL22/CCR4[68], and their induction by Prostglandin E2 or H-ferritin have been proposed. Studies on Treg in hematological tumors are far to be complete. The presence of increased Treg cells has referred in Hodgkin Disease, in Chronic Lymphocytic Leukemia and in Multiple Myeloma patients[65]. Some of these studies need to be validated by using specific markers such FoxP3. Largely unknown appears the analysis of other regulatory populations like NKTi cells in hematological tumors as well as in non malignant haemopoietic disorders.

Aim of the study

This work got inside the biological mechanisms underlying the ongoing relationships between an expanding hematopoietic clone and the immune effectors seeding the BM. In this context, the study analyzed two hematological disorders, showing different clinical manifestations, in which defective or dysplastic clonal hematopoiesis develops through several lineages and replaces the normal counterpart (PNH and MDS). This clinical feature, suggesting an active BM failure condition, is expected to provide a useful model to investigate the potential involvement of T cell-mediated processes in pathogenesis of these two hematopoietic disorders.

In order to investigate whether an absolute growth vantage might underlie the expansion of GPI-defective compartments, in the first phase of the work the immune response in PNH patients has been characterized, with particular attention to functional differences between normal (GPI^+) and GPI-defective compartments. Then, the relevance of an altered regulation of immunological tolerance in the clonal emergence and expansion of GPI-defective clone has been investigated.

To this aim, the this part of the study addressed:

a) Functional analysis of innate and adaptive immune response in PNH patients:
   - Study of normal (GPI^+) and GPI-defective granulocyte cell compartments and their ability of bacterial-dependent intracellular ingestion with the consequent activation of oxidative burst.
   - Study of normal (GPI^+) and GPI-defective monocytes cell compartments and their ability to differentiate in vitro into functional Dendritic Cells (DC);
   - Characterization of (GPI^+) and GPI-defective T cells compartments:
b) Analysis of immunological tolerance regulation in PNH: Study of CD40-pathway
c) Analysis of a major susceptibility factor for autoimmunity: HLA class I and II genes;

The second part of the study has been investigated the biological mechanisms underlying the tolerance control and immune-dependent shaping of emerging/dominant clones in the BM of MDS patients. Thus, the correlation between the presence of a hyper-cellular, hypo-cellular BM environment, the stage of MDS progression and the immunological asset along MDS progression to AML, has been investigated.

This part of the study addressed:

a) Analysis of BM and peripheral blood immunological asset of MDS patients at different disease stages.
b) Analysis of BM recruitment of Treg and NKTi lymphocyte subsets in MDS patients along AML progression, as compared with healthy controls.

Methods

Patients and controls

Patient enrolment, classification and clinical management have been performed at the Division of Hematology, Department of Biochemistry and Medical Biotechnology of the University of Napoli "Federico II". BM and peripheral blood sample collection, karyotype analysis, chromosome and cytogenetic characterization of MDS patients have been performed at the same Institution. PNH diagnosis has been documented in all cases by flow cytometry analysis of peripheral blood, using labeled monoclonal antibodies (mAb) against GPI-linked molecules, as described[69,70]. Notably, the Division of Hematology represents one of the National Reference Centers for PNH, a very rare syndrome. MDS patient's clinical classification has been performed according to the WHO recommendations[71]. The stage of the disease has been evaluated following the IPSS criteria. None of the enrolled patients was receiving any medical treatment along the study. Informed consent has been obtained from individual patients before each blood sample collection. A group sex- and age-matched healthy donors were used as controls.

mAb, immunofluorescence, and flow cytometry

Fluorescein isothiocyanate (FITC), phycoerythrin (PE), cychrome, and allophycocyanin-labeled mAb against CD3, CD4, CD8, CD56, CD25, CD45, CD48, CD54, CD19, CD33, CD66b, CD14, human leukocyte antigen (HLA)-DR, CD154, interferon-gamma (IFN-γ), interleukin (IL)-4, TCRVα24Vβ11 and isotype-matched controls have been purchased from BD PharMingen (San Jose, CA). The PE-labeled CD48 mAb has been purchased from Serotec Ltd.(London, UK). The PE-labeled anti-human FoxP3 kit has been purchased from EBioscience (San Diego, USA) and used according to manufacturer's instruction. To analyze the production of IFN-γ and IL-4, intracellular staining with the specific mAb has been performed by using the fixing/permeabilization kit purchased from Caltag (Burlingame, CA) following the manufacturer's instructions. All phenotypes referred to flow cytometry analysis of the cell populations gated by using

forward-scatter and side-scatter parameters, as well as CD45 labeling. Flow cytometry, acquisition and data analysis have been performed by using a two-laser-equipped FACSCalibur apparatus and the CellQuest analysis software (Becton Dickinson, San Jose, CA).

PBMC, DC and resting T cell populations

Peripheral blood mononuclear cells (PBMC) have been isolated from patients and controls by centrifugation of peripheral blood over Lymphoprep (Nycomed, Oslo, Norway) gradient. To obtain a purified monocyte population, the cells collected from the plasma/Lymphoprep interface have been washed phosphate buffer saline (PBS) and incubated at 37°C in the presence of RPMI-1640 medium (Gibco-BRL, Grand Island, NY) in six-well plates (Falcon, Seattle,WA). After a 2-h incubation, the non-adherent cells have been removed, and each well has been washed three times with PBS. Adherent cells were cultured for a 7-day period in the presence of 80 ng/ml human recombinant granulocyte macrophage-colony stimulating factor (hrGM-CSF) and 1000 IU/ml hrIL-4 (Sigma-Aldrich), as described[66]. When indicated, lipopolysaccharide (LPS; 100 ng/ml, Sigma-Aldrich) or human trimeric CD40 ligand (CD40L) molecule (100 ng/ml, Bender MedSystems, Austria) has been added to the culture for an additional 16–24 h to induce terminal DC differentiation[72]. The purity of the DC population has been defined by immune fluorescence phenotype analysis. Only the populations showing less than 5% of lymphocyte contaminants have been used in the study. The ability of these DC populations to elicit antigen dependent T cell proliferation has been assessed by incubating DC with 10 μg/ml purified protein derivative (PPD; Staten Serum Institute, Copenhagen, Denmark) for 2 h at 37°C. After extensive washing, PPD-treated DC have been 30 Gy-irradiated and cultured for 5 days in the presence of autologous PBMC.

T lymphocytes were isolated from venous peripheral blood samples obtained from healthy controls as described previously[73]. Briefly, small, resting T cells have been purified from the PBMC population using a Percoll density gradient, after removing B cells and monocytes by plastic and nylon wool adherence. The recovered high buoyant density population

was always >97% CD3+CD56–. Purity of T cell preparation has been assessed using immunofluorescence and phytohemaglutinin stimulation.

Study of Phagocytosis: Analysis of Granulocyte Bacterial Ingestion and Respiratory Burst Induction

To study the phagocytic process in GPI-defective polymorphonuclear cells (PMN) of PNH patients, their effectiveness in ingesting opsonized bacterial particles by using FITC-labeled E. coli previously treated with human IgG immunoglobulins, as well as with serum- derived complement have been measured. Their specific bacterial intracellular ingestion has been measured as mean fluorescence intensity (MFI) in the green channel of the FSC–SSC gated PMN population, by using flow cytometry analysis. The evaluation has been performed after incubation at 37°C of the whole blood in the presence of the bacterial stimuli ($1x10^9$ bacteria/ ml), as indicated in the Result section. The use of a staining DNA solution allowed the identification of the diploid PMN population and the simultaneous exclusion of artifacts dependent on bacteria aggregates. To discriminate between attachment and ingestion of labeled bacteria, an appropriate quenching solution has been applied after the 37°C incubation of the whole blood. The percentage of granulocytes having performed phagocytosis has been detected on the FSC/SSC-defined region gate, also considering the DNA diploid content, as represented by the red fluorescence level of the PMN population[74]. To perform the kinetic analysis, the blood samples have been immediately put on ice after each incubation and erythrocytes have been removed by osmotic lysis. All the reagents have been purchased as Phagotest kit, from Orpegen Pharma, Heidelberg, Germany and used following the manufacturer's instructions.

The quantitative detection of PMN oxidative burst has been performed in heparinized whole blood samples by using the Phagoburst kit, purchased from Orpegen Pharma, and following the manufacturer's instructions.

To activate the respiratory burst, whole blood samples pretreated with the DHR 123 radical oxidant sensitive probe were incubated with the opsonized E. coli ($1x10^9$/ml), fMLP (5 mM), and phorbol 12-myristate 13-acetate (PMA, 8 mM) at 37°C, as indicated in the Result section. After the

activation with the above stimuli, samples were kept on ice, erythrocytes have been lysed and DNA staining solution added to identify the diploid PMN population and exclude aggregation-dependent artifacts. The percentage of granulocytes having performed the oxidative burst has been detected on the FSC/SSC-defined gate, also considering the DNA diploid content, as represented by the red fluorescence level of the PMN population. The quantitative assessment of the respiratory burst has been performed as a measure of the DHR 123 intracellular oxidation (MFI in the green emission channel of the PMN population). The effect of the PKC inhibitor bisindolylmaleimide (BDM), purchased from Sigma-Aldrich, St Louis, MO, on E. coli-triggered phagocytosis and Reacting Oxygen Species (ROS) production by PMN was also analyzed. Briefly, after a pretreatment of 30 min in the presence of 100 nM BDM, opsonized E. coli ($1x10^9$/ml) has been added for an additional 10 min incubation period. Bacteria uptake and ROS detection has been performed as previously described. These conditions have been described to allow specific PKC activity inhibition and were observed to completely block PMA-triggered PMN respiratory induction. No significant effects on PMN viability have been observed by Trypan blue and propidium iodide staining.

Analysis of lymphocyte proliferation

PBMC ($1x10^6$/ml) were cultured in 24 or 96 well, flat-bottomed plates (Falcon) with anti-CD3 mAb CLB-CD3/E (immunoglobulin E), a gift of Dr. R. van Lier (Central Laboratory of the Blood Transfusion Service, Amsterdam), phorbol 12-myristate 13-acetate (PMA), and ionomycin, all purchased from Sigma- Aldrich Italia (Milan). Cultures have been incubated for 3–5 days at 37°C in a humidified atmosphere containing 5% CO2 and pulsed with 0.5 μCi/well [3H] thymidine for the last 16 h. The incorporation of the labeled nucleotide has been determined by scintillation counting after automatic cell harvesting. All tests have been performed in the presence of RPMI-1640 medium supplemented with 5% heat-inactivated fetal calf serum (Gibco-BRL). Autologous serum was used for antigen-specific assays.

To analyze the proliferation of GPI^+ and GPI^- T lymphocytes, PBMC have been labeled with 5, 6-carboxyfluorescein-diacetate-succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) before the culture with the anti-CD3 CLB-CD3/E mAb. This technique has been described already as a reliable replacement of 3H thymidine incorporation for the evaluation of lymphocyte proliferation[75].

Indeed, CFSE fluorochrome spontaneously and irreversibly couples to intracellular proteins and is equally distributed to the daughter cells after mitosis. Proliferating cells can be tracked by flow cytometry, based on the sequential loss of fluorescence intensity. Furthermore, multi-parametric flow cytometry analysis allows the simultaneous assessment of normal and GPI-defective T cell subsets within the dividing cell population, also monitoring their phenotype changes associated with activation and cell division. All tests have been performed in the presence of RPMI-1640 medium supplemented with 5% heat-inactivated fetal calf serum (Gibco, Grand Island, NY).

Typing techniques

Low and high resolution typing for HLA-A, -B, -Cw and -DRB1 genes has been carried out by polymerase chain reaction with sequence specific primers (PCR-SSP). Commercial kits from Dynal A.S. (Oslo, Norway) have been used according to the manufacturer's instruction.

Statistical analysis

The statistical analysis for P calculation has been performed by using Student's t-test. Results have been considered significant when a P value < 0.05 was obtained. HLA Association has been tested by two-tailed Fisher's exact test with software InStat 3.0 (GraphPad Software Inc., San Diego, California, USA). For typing tests, the corrected p value (pc) were calculated by multiplying the p value by the number of the alleles showing at each locus a frequency $> 0.5\%$ in Italians.[76,77] (i.e.14 alleles at A locus with 3 specificities for A*02, 21 at B locus with 2 specificities for B*14, 13 at C locus with 2 specificities for Cw*08, 12 at DRB1 locus with 2 specificities for DRB1*01 and 3 for DRB1*15) (**Table I**).

Results

Study of GPI-defective compartment in PNH: GPI-defective granulocytes from PNH patients show significant increased ingestion effectiveness and a decreased oxidative burst induction.

To analyze the GPI-defective granulocyte effectiveness, we focused on their ability to ingest corpuscolate bacteria by using fluorescein- labeled opsonized E.coli bacteria. To avoid a possible interference of the isolation procedures on granulocyte activity, we selected for the study PNH patients whose percentage of GPI-defective PMN was > 95% . As shown in **Figure 6**, GPI-defective granulocytes from PNH patients exhibit a significant increase in their ability to ingest opsonized E. coli bacteria.

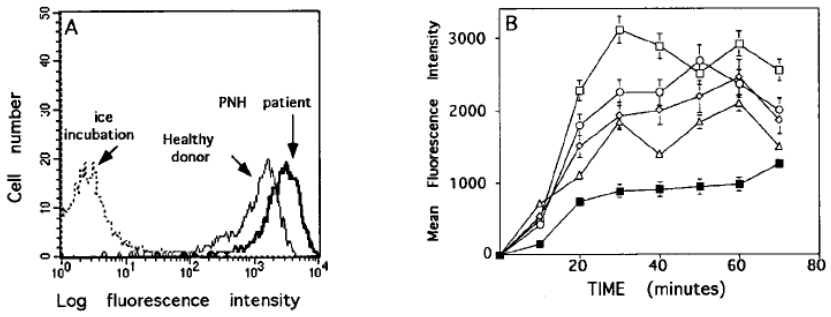


Figure 6. **Bacterial ingestion in PNH GPI-defective granulocytes**. Panel A shows the E. coli uptake levels in one representative experiment. Bold and light lines indicate staining profiles of PNH and normal PMN, respectively, after 20 min of incubation with fluorescent opsonized E. coli ($1x10^9$/ml). Dotted lines indicate control staining profiles obtained after on ice incubation. Panel B: black squares indicate mean MFI ± SD measured, at 10-min intervals, after incubation with $1x10^9$/ml FITC labeled opsonized E. coli, in granulocytes from 10 healthy controls. Empty symbols refer to mean MFI ± SD obtained in at least three independent experiments performed by using blood samples obtained from four PNH patients. All incubations were performed with whole blood samples. MFI was calculated in the FSC/SSC gate corresponding to the diploid PMN population.

To assess the generation of ROS inside normal and GPI-defective PMN under different triggering conditions, we performed flow cytometry analysis based on ROS dependent oxidation of the DHR 123 fluorescent indicator. **Figure 7** shows these that two independent bacterial stimuli (fMLP and E. coli) were unable to induce intracellular ROS production levels comparable to those observed in healthy controls in the GPI-defective granulocytes from PNH patients.

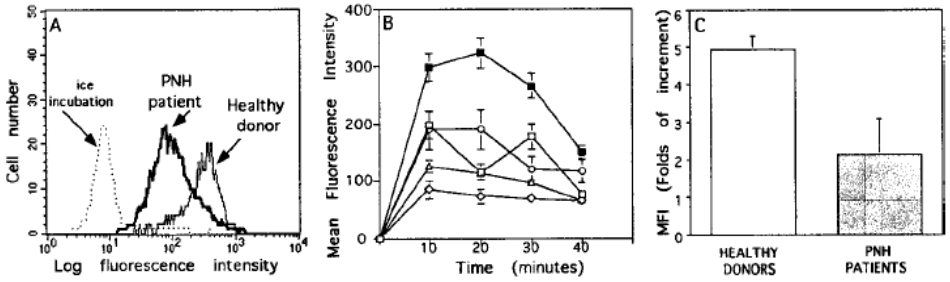


Figure 7. **Analysis of bacterial-induced respiratory burst in PMN population**. ROS production was measured as intracellular fluorescence level of oxidated DHR 123. Panel A shows DHR 123 staining levels in one representative experiment. Bold and light lines indicate staining profiles of PNH and control PMN after 20 min of incubation in the presence of $1x10^9$/ml opsonized E. coli. Dotted lines indicate control staining profiles obtained after sample incubation on ice. Panel B shows kinetic profiles of oxidated DHR 123 produced by PMN from 10 to 40 min incubation in the presence of opsonized unlabeled E. coli and measured as MFI emission in the green fluorescence channel. Black squares and empty symbols indicate mean MFI ± SD obtained in the healthy control group and in each PNH patient, respectively. For the patient population each value represents a mean of at least four concordant experiments. All incubations were performed with whole blood samples. MFI was calculated in the FSC/SSC gate corresponding to diploid PMN population. Panel C refers data obtained in the four PNH patients (gray column) and in the healthy controls (white column) after 10 min of incubation with fMLP. Histograms indicate the mean folds of increment of the green fluorescence levels in the PMN populations belonging to the two groups, as indicated.

To investigate whether the GPI-molecule defect could account for the altered PMN respiratory burst in PNH, we analyzed oxidative burst effectiveness in the presence of receptor-independent stimuli. In this context, we focused on PKC-dependent pathways already demonstrated to be critical for ROS generation in human PMN. Then, PKC-mediated ROS production was evaluated by analyzing DHR123 oxidation in response to treatment with PMA, a pharmacological agent mimicking diacylglycerol (DAG) and able to recruit and extensively activate intracellular PKC. (**Figure 8)**.

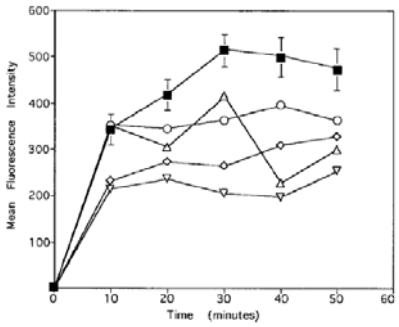


Figure 8. **Analysis of PMA-induced respiratory burst**. Black Squares and empty symbols show kinetic profiles of oxidated DHR 123 production in normal and PNH granulocytes after incubation with PMA, respectively. For the patient population each value represents a mean of at least four concordant experiments. All incubations were performed with whole blood samples. MFI was calculated in the FSC/SSC gate corresponding to the diploid PMN population.

Notably, the percentage of reduction of ROS level in the presence of PMA treatment was comparable to that observed in GPI-defective PMN triggered by E. coli. **Figure 9** shows that treatment with the BDM PKC inhibitor is able to differentially affect phagocytosis and respiratory burst induction dependent by E. coli triggering.

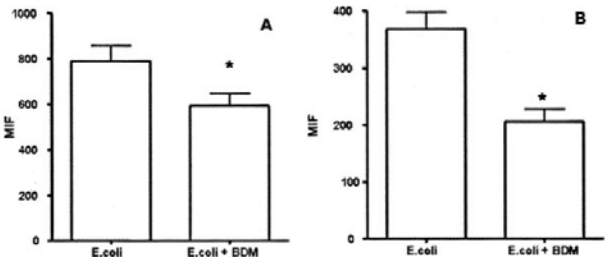


Figure 9. **Effect of PKC inhibition on E. coli uptake and respiratory burst induction of PMN**. Panel A shows mean fluorescence levels (MFI ± SD) observed in normal PMN after 10 min of incubation in the presence of fluorescent opsonized E. coli alone or in the presence of 100 nM BDM, as indicated in the material and method section. Panel B shows oxidated DHR 123 mean fluorescence levels (MFI ± SD) observed in normal PMN after 10 min of incubation in the presence of opsonized E. coli alone or in the presence of 100 nM BDM, as indicated in the material and method section. Columns refer to mean (MFI ± SD) data obtained by analyzing 10 healthy donors. MFI was calculated in the FSC/SSC gate corresponding to the diploid PMN population. (*) indicates the occurrence of significant differences between the data.

Thus, PKC-dependent pathways are differentially involved in the control of bacteria uptake and ROS production in normal PMN.

In conclusion, GPI-defective granulocytes from PNH patients show a significant increased ingestion effectiveness and a decreased oxidative burst induction.

Study of GPI-defective compartment in PNH: GPI-defective monocytes from PNH patients generate impaired in vitro DC differentiation.

To address the functional analysis of GPI-defective monocytes in PNH patients, we studied their ability to differentiate into DC in vitro. To minimize the possibility that procedures used to isolate GPI-defective monocytes from a mixed population could interfere with their differentiation, we tested only PNH patients showing more than 95% of GPI-defective monocytes in their peripheral blood. **Figure 10** shows the phenotype of GM-CSF- and rIL-4 treated monocytes; as indicated, a significant impairment of CD1a up-regulation as well as high expression of

CD86 molecule was detected. These data indicate that the DC derived in vitro from PNH monocytes showed significant phenotype alterations.

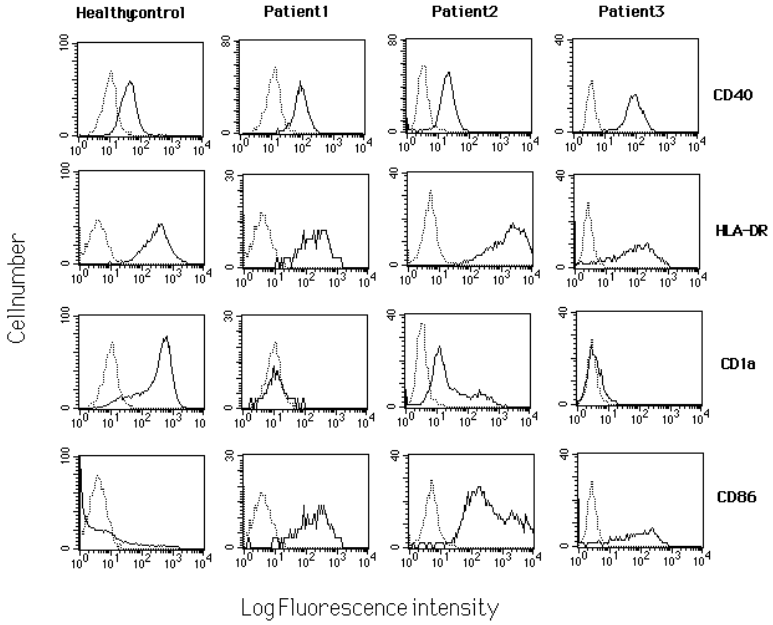


Figure 10. Phenotype analysis of DCs derived in vitro from PNH GPI-defective monocytes. Peripheral blood monocytes from one healthy donor and from three PNH patients were cultured for 7 days with GM-CSF and rIL-4 to obtain DC differentiation. At the end of the culture the cells were analyzed for the surface expression of CD40, HLA-DR, CD1a and CD86 molecules (as indicated). Lack of CD1a up-regulation and CD86 surface expression characterize the PNH patient DC phenotype.

The impaired maturation of DC obtained from GPI-defective monocytes significantly affects their cytokine production and co-stimulatory activity. Figure **11** shows that LPS (bold line peaks in C) and CD40L treatment (bold line peaks in D) induced a lesser TNF-α and IL12 production in PNH DCs if compared with the normal DC counterpart (A and B).

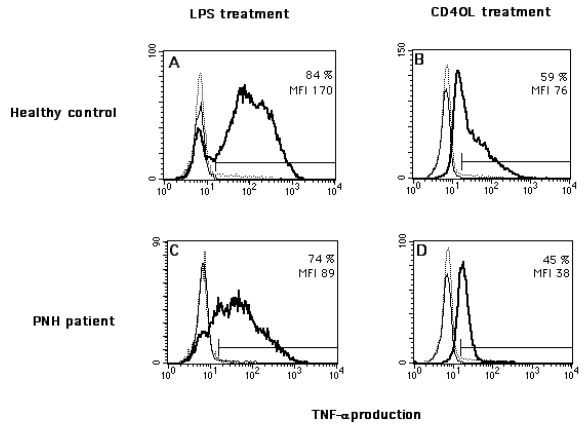


Figure 11. Flow cytometric analysis of TNF-α production by PNH GPI-defective DC Intracellular staining for TNF-α of monocyte-derived DC from one healthy control (Panels A and B)

and from one representative PNH patient (Panels C and D). Bold line peaks refer to TNF-α production after LPS (Panels A and C) and CD40L treatment (Panels B and D). Dotted line peaks indicate the cell incubation in medium alone (Panels A-D). A significant decrease in TNF-αproduction was observed in the PNH population. Data are representative of one of three independent experiments and were confirmed in all the PNH patients enrolled in this study.

It is interesting that deficiency in CD40-dependent cytokine production was observed in the presence of normal surface expression of CD40 on PNH monocytes (Fig. 1). Thereafter, we studied the ability of DC obtained from the GPI-defective monocytes to co-stimulate T cell receptor (TCR)-triggered T lymphocytes, as this function has been referred to LPS-treated DC already. **Figure 12A** shows that DC obtained in vitro from normal controls provided an optimal co-stimulation of TCR-triggered T cell proliferation at cell percentages as low as 2.5%, with a significant co-stimulatory activity maintained even at 0.5% DC. As shown, a strong impairment of PNH DC in providing accessory signals for TCR-triggered T cell proliferation was observed. To analyze the effectiveness of PNH DC in an antigen-dependent model, we also performed experiments by enrolling one patient and two healthy controls, referring recent bacillus Calmette-Guerin vaccination. Therefore, to assess the ability of PNH DC to take up, process, and present antigens to autologous T lymphocytes in comparison with the normal counterpart, we analyzed their ability to elicit T cell proliferation against a mixture of mycobacteral antigens, as represented by PPD preparation. PPD-treated PNH and control DCs were thus cultured with autologous T cells at saturating concentration ($2x10^4$/well). As shown (**Figure 12B**), PNH-DC exhibited a significantly decreased ability to trigger antigen-dependent T cell proliferation compared with the control DC populations.

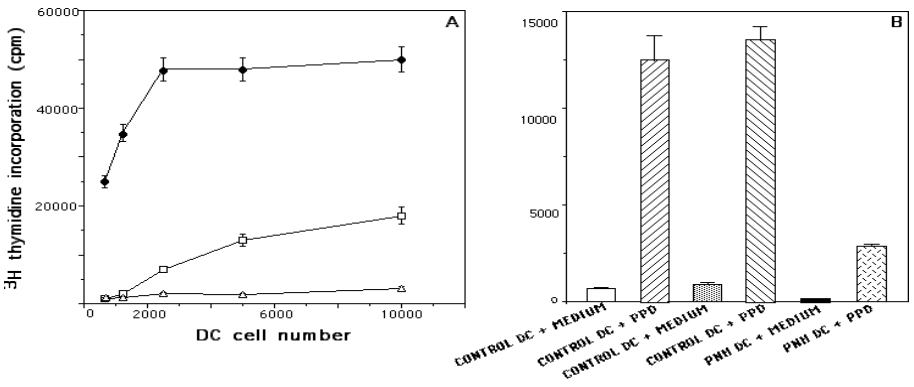


Figure 12. DC differentiation in vitro of PNH monocytes generates cells with impaired ability to deliver accessory signals for TCR-dependent T cell proliferation. Proliferation levels of resting T cells triggered with anti-CD3 mAb in the presence of an increasing number of irradiated LPS-treated

DCs (see materials and methods) from normal donors (filled circles) and from two PNH patients (open squares and triangles). The proliferation was measured by [^{3}H] thymidine incorporation after three days of incubation. Results are presented as mean cpm of triplicate cultures without background subtraction; SD was always <15%. No proliferation was observed after incubation of the T lymphocytes with or without anti-CD3 in the absence of irradiated DCs. Data are representative of the results obtained in four independent experiments.

Study of GPI-defective compartment in PNH: GPI-defective T lymphocytes show impaired functional effectiveness.

In order to investigate GPI-defective T lymphocytes, we analyzed their TCR-dependent ability to proliferate, to express activation surface molecules as well as to produce pro-inflammatory cytokines. The patients, chosen for the study, had GPI-defective T lymphocytes > 15%, as detected by double-labeling with anti-CD3 and anti-CD48 mAb, and showed a marked cytopenia. The deficiency of the GPI-linked CD48 molecule on defective T lymphocytes allowed a precise identification of the GPI$^+$ and GPI$^-$T cell subsets in the PNH-derived blood samples, maintaining the biological complexity of PNH and avoiding any functional interference as a result of the separation procedures. **Figure 13A** shows a significant impairment of the proliferative response of the GPI-defective (CD48$^-$) T cell compartment, revealed in a representative PNH patient. By contrast, the percentage of proliferating CD3$^+$CD48$^+$ T lymphocytes was increased significantly in comparison with the CD48$^-$ counterpart as well as with the healthy control T lymphocytes.

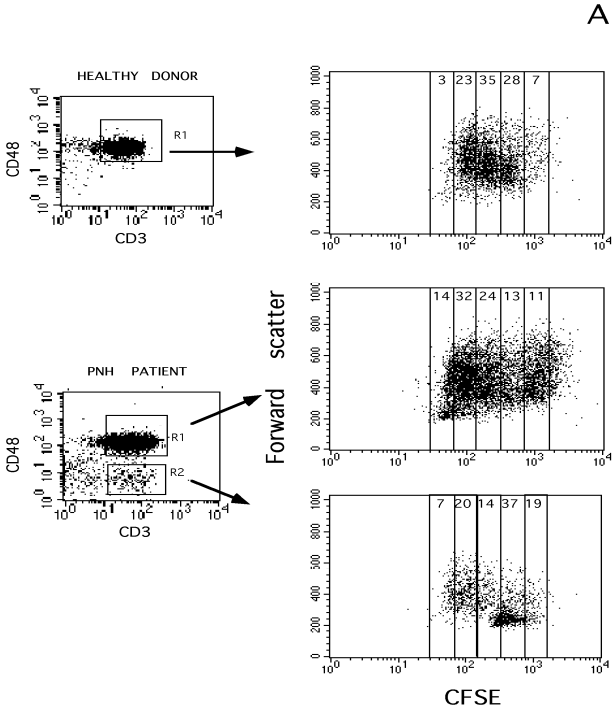


Figure 13. **Analysis of TCR-dependent activation of the GPI$^+$ and GPI$^-$ T cell compartments in PNH patients**. PBMC from one representative healthy control and one PNH patient were collected and analyzed after 72 hour of incubation with anti-CD3 mAb. Panel A shows the CFSE staining profiles as a measure of lymphocyte proliferative effectiveness (see Materials and Methods). GPI$^+$ and GPI$^-$ T cells were gated on FITC anti-CD48 and Cychrome anti-CD3 mAb fluorescences (R1 and R2, respectively). A defective proliferation of the CD3$^+$CD48$^-$ subset can be observed. Indeed, 56% of cells undergo none or only one replication cycle in comparison with 24% of the CD3$^+$CD48$^+$ population and 35% of the CD3$^+$CD48$^+$ healthy control derived counterpart. Notably, a significant increased proliferation of the CD3$^+$CD48$^+$ lymphocytes from PNH patients in comparison with the CD3$^+$CD48$^+$ healthy control derived subset can be observed. Indeed, 70% of CD3$^+$CD48$^+$ cells from PNH patient undergo two or more replication cycles in comparison with 61% of the CD3$^+$CD48$^+$ healthy control derived cells ($p<0.05$).

Similar data were obtained by analyzing the ability of the GPI-defective T lymphocytes to up-regulate CD25, HLA-DR, as well as CD54 (ICAM-1: intercellular adhesion molecule-1) molecules in comparison with the GPI^+ counterpart and the healthy, control-derived T cell population (**Fig. 13B**).
The comparison between PNH GPI^+ T cells and healthy donor lymphocytes revealed a significant alteration in all the parameters considered. Indeed, proliferation, CD25, CD54, and HLA-DR up-regulation were increased significantly.

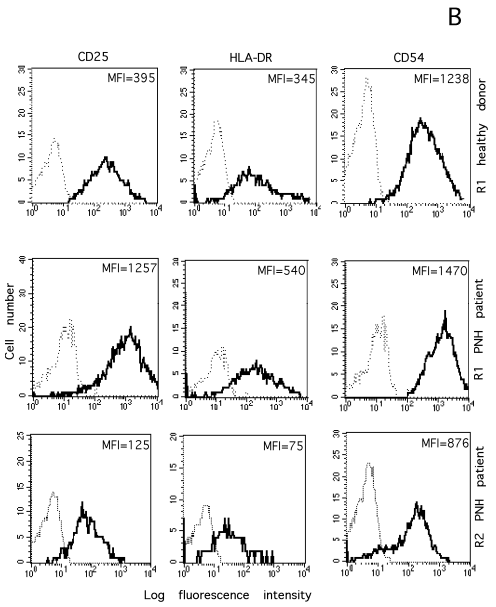


Figure 13B. **Analysis of TCR-dependent activation of the GPI^+ and GPI^- T cell compartments in PNH patients.** The graphs show the analysis of CD25, HLA-DR and CD54 surface levels, evaluated as mean fluorescence intensity (MFI) of the staining profiles obtained from the above populations. Bold lines indicate co-staining in the presence of the PE-labeled anti-CD25, HLA-DR and CD54 mAb; dotted lines refer to the staining in the presence of the isotype control mAb. A significant decrease of all parameters was revealed in the $CD3^+CD48^-$ population. Notably, increased expression of all these molecules was observed in the GPI^+ T cell compartment from the PNH patient. Data are representative of one of three independent experiments and were confirmed in all the PNH patients recruited in the study.

Figure 14 shows that a severe impairment of IFN-γ production can be revealed in the GPI-defective T cells A slight but consistent decrease of IL-4 production was also observed in the GPI-defective T lymphocytes, confirming their impaired activation ability (not shown). By contrast, the comparison of the GPI^+T cells from PNH patients with the healthy control population revealed a significant increase ($P<0.05$) in their IFN-γ production.

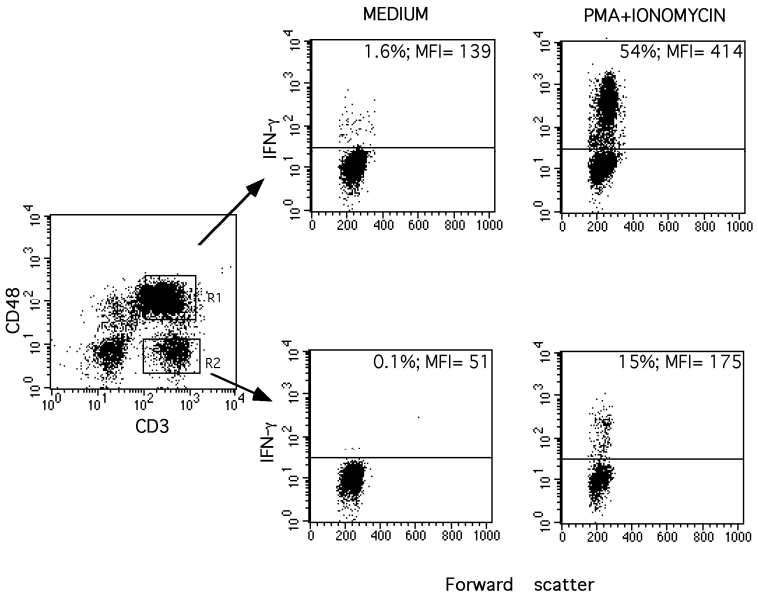


Figure 14. **Flow cytometry analysis of IFN- γ production by GPI$^+$ and GPI$^-$ T lymphocytes from a PNH patient**. Plots show the percentage of anti-IFN- γ mAb stained cells incubated with medium alone or with PMA + ionomycin. The GPI$^+$ and GPI$^-$ CD3 lymphocyte compartment was identified by gating on PE anti-CD48 and Cychrome anti-CD3 mAb fluorescences (R1 and R2, respectively). As shown, a significant decrease in the intracellular cytokine level was observed in the GPI-defective population. Data are representative of one of three independent experiments and were confirmed in all the PNH patients recruited in the study.

Taken in all, this results suggest that growth vantage of GPI-defective compartments is likely dependent on an increase of functional effectiveness of GPI-defective cells. Indeed, analysis of GPI$^-$ PMN, DC and T cells revealed an impairment in their biological and physiological functions. These data support the occurrence of an extrinsic selective pressure, favoring the expansion of GPI-defective clone, whose autoimmune origin need to be investigated.

Study of immunological tolerance regulation in PNH: TCR-triggered CD4$^+$CD48$^+$ lymphocytes from PNH patients show functional persistence of surface CD154 expression

Given the relevance of CD40 pathways in maintenance of immune tolerance[78], we investigate more deeply such pathway in PNH patients. Thus, the kinetics of CD154 induction occurring in GPI$^+$ and GPI$^-$T lymphocytes from PNH patients has been analyzed. As shown in **Figure 15**, after 8 h of incubation with anti-CD3 CLB-CD3/E mAb, a significant up-regulation of the CD154 molecule was observed in healthy control CD4$^+$ lymphocytes, as well as in the GPI$^+$ and GPI–defective compartments of

PNH patients. $CD48^+CD4^+$ cells, which represent the GPI^+ population in PNH, showed a significant increase in their surface CD154 expression when compared with the GPI-defective $CD4^+$ counterpart and the $CD4^+$ cells from the healthy controls.

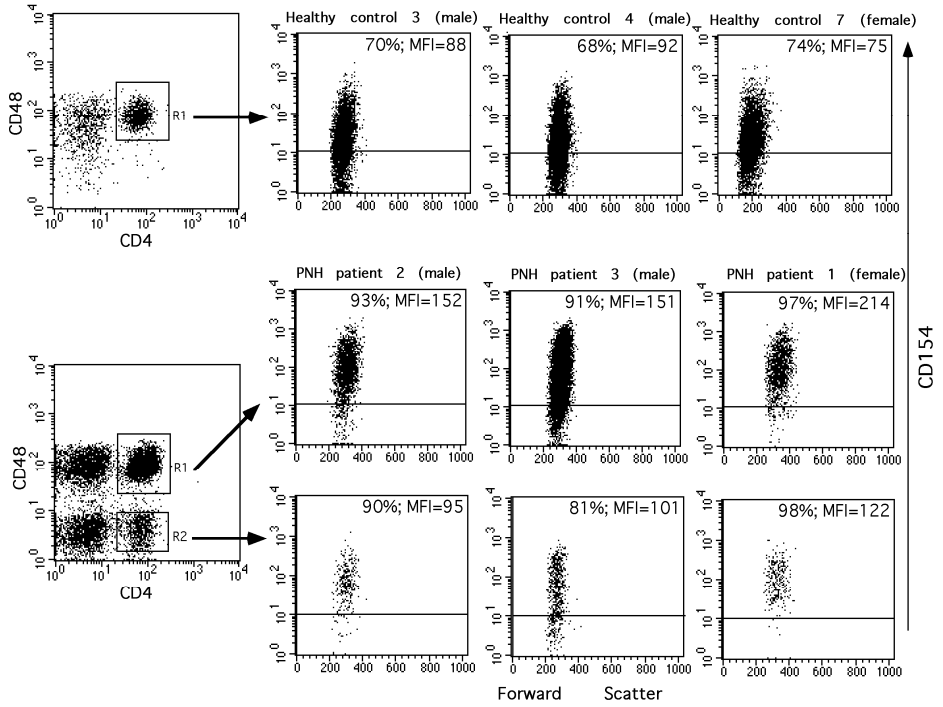


Figure 15. **Analysis of TCR-dependent CD154 expression on the GPI^+ and GPI^- $CD4^+$ population from two PNH patients, compared with healthy donors**. The plots refer to the staining of $CD48^+CD4^+$ and $CD48^-CD4^+$ (R1 and R2, respectively) with anti-CD154 mAb after 8 hour of incubation in the presence of anti-CD3 mAb. A significant impairment in CD154 induction was observed in the GPI-defective population, as compared with the GPI^+ counterpart. Notably, an increased surface level of this molecule was observed in the GPI^+ CD4 population from the PNH patients in comparison with the healthy controls. Data are representative of one of four concordant experiments.

To assess the functional relevance of the increased CD154 up-regulation on the surface of anti-CD3-treated T cells from PNH patients, we evaluated the ability of these populations to up-regulate the CD23 molecule on the surface of B lymphocytes from healthy controls. Indeed, the up-regulation of this molecule has been described as a relevant target for CD40 triggering[79]. Therefore, we co-cultured normal PBMC in the presence of irradiated healthy donor or PNH-derived PBMC, previously incubated with anti-CD3 mAb for 8 or 48 h. After overnight culture, the level of CD23 was measured on B lymphocytes, as identified by CD19 co-staining. As shown (**Fig. 16**), a

significantly increased up-regulation of this molecule was observed in the presence of PNH as compared with control PBMC collected after 8 h from CD3 triggering (P<0.05).

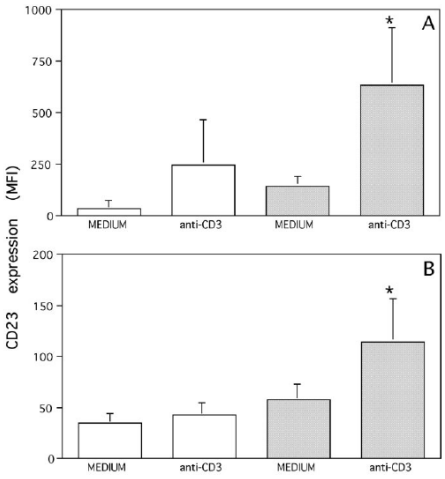


Figure 16. **CD3 triggered PBMC from PNH patients are able to up-regulate CD23 surface levels on control B cells**. The graph refers to the level of CD23 antigen expression on control B lymphocytes, identified by co-staining with anti-CD19 mAb, after overnight incubation in the presence of 30 Gy irradiated PNH (gray columns) or healthy control (white columns) PBMC, pre-treated with anti-CD3 mAb or medium, as indicated. PNH cells significantly up-regulated the CD23 molecule on control B cells both after 8 (Panel A) and 48 hours (Panel B) of CD3 triggering. Notably, healthy control derived PBMC showed significant ability to up-regulate B lymphocyte CD23 only after 8 hours of anti-CD3 incubation. Data refer to means of fluorescence levels (MFI) obtained in three independent experiments. Standard deviations are depicted on the top of each bar. The symbol * indicates the statistical significant differences (p<0.05), in comparison with normal control group.

Notably, also, the medium-cultured PNH cells, but not the healthy control, showed significant ability to up-regulate the CD23 molecule on B lymphocytes. Moreover, lymphocytes from PNH patients, collected after 48 h of anti-CD3 incubation (**Figure 16B**), revealed a persistent ability to up-regulate CD23. The healthy control cells, collected after the same culture period, were substantially unable to modify CD23 expression on the surface of the target population (P<0.05). The observed effects were partially (from 30% to 50%) inhibited by anti-CD40 mAb treatment of the target population, demonstrating the involvement of CD40-dependent pathways (data not shown). To assess whether the increased CD23 up-regulation on B cells could correlate with the expression of CD154 on the lymphocytes of PNH patients, we analyzed CD154 kinetic expression on the $CD4^+$population after incubation with the anti-CD3 mAb for 8, 24, and 48 h. As shown in **Figure 17**, a significant persistence of the CD154 molecule on the $CD48^+CD4^+$ cells from PNH patients was observed after 24 and 48 h from anti-CD3 treatment. In addition, a slight but consistent expression of

CD154 was observed after 48 h treatment with medium in the $CD48^+CD4^+$cells from the PNH patients.

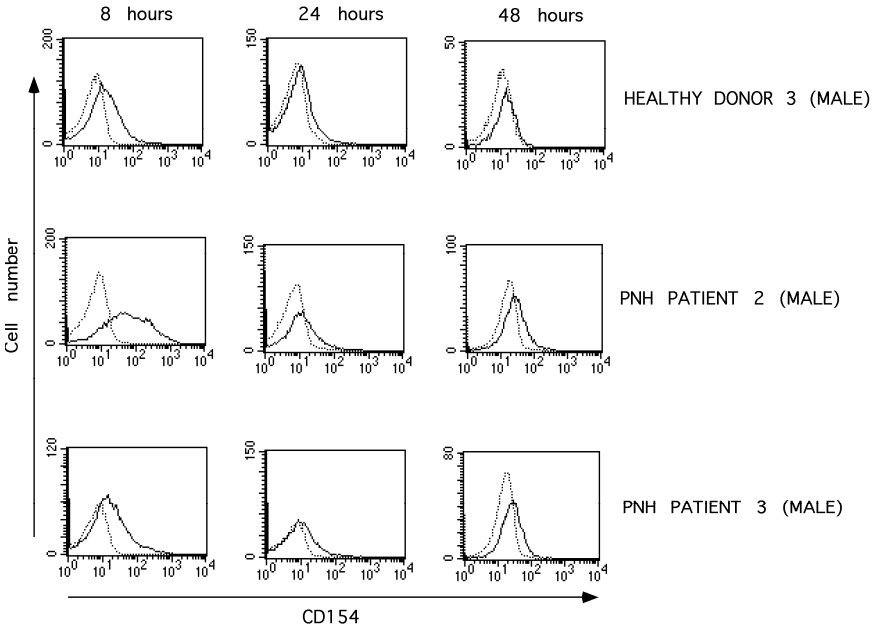


Figure 17. **Kinetics of TCR-dependent CD154 induction on $CD4^+CD48^+$ lymphocytes of one representative healthy control and two PNH patients**. Staining profile of anti-CD154 mAb on $CD4^+CD48^+$ lymphocytes. Plain and dotted lines indicate anti-CD3 and medium incubation, respectively. The CD154 up-regulation after 8 hours is accompanied by a consistent persistence of CD154 surface expression after 24 and 48 hours of anti-CD3 incubation in the PNH patients, but not in the representative healthy control. Data refer one of three concordant experiments and were confirmed in all the PNH patients recruited for the study.

A specific set of HLA alleles is significantly associated with the occurrence of PNH.

Since the relevance of HLA genes as a susceptibility factor in autoimmune diseases, we analyzed HLA class I and Class II alleles in our cohort of PNH patients. To this aim, molecular typing of HLA Class I and Class II genes in a homogeneous large group of Italian PNH patients compared with unrelated healthy controls with the same genetic background has been performed. Significant differences in the frequency of several Class I and Class II HLA alleles between PNH patients and controls are shown in Table I.

The frequency of HLA-A*0201 allele (52.5% vs. 32.2%; $pc<0.05$) is increased in our series of PNH patients. Moreover, we found an increased frequency of B*1402 (23.8% vs. 5.3%; $pc<0.001$) and Cw*0802 alleles (23.8% vs. 5.9%; $pc<0.005$).

The allele DRB1*1501, already associated with various autoimmune disorders and with PNH in the American and Japanese patients, was significantly increased in our series of Italian PNH patients (21.1% versus 5.3%; pc<0.01). In addition, the frequency of DRB1*01 (31.6% versus 10.9%; pc<0.05), is increased, but no specific association with the DRB1*0101 or DRB1*0102 allele has been found. Our study revealed that the haplotype B*1402, Cw*0802 (23.8 % versus 5.3%; p<0.0005) as part of the extended haplotype A*33, B*1402, Cw*0802, DRB1*0102 (10.5% versus 0.66%; p<0.005) were, respectively, 4 and 15 times more frequent in PNH patients than in controls.

Thus HLA alleles are associated with the occurrence of PNH in our cohort of PNH patients. It is intriguing that these associated alleles are arranged in an extended haplotype (A*33, B*1402, Cw*0802, DRB1*0102) whose Mediterranean origin has been described.

Immunological asset in MDS patients: Activated cytotoxic T cells recruitment in MDS bone marrow

The analysis of PNH patients revealed significant alterations of immune effectors compatible with an immune-mediated selective pressure able to account for the expansion of GPI-defective hematopoiesis. To evaluate the immunological asset in MDS patients, an immunophenotipic analysis of BM and peripheral blood samples has been performed, focusing on effector lymphocyte populations. As control, a group of healthy individuals has been analyzed. The study showed a significant recruitment (p=0,0255) of $CD3^+CD8^+$ CTL in MDS BM as compared with peripheral blood. No significant difference in $CD3^+CD4^+$ T cells has been observed between BM and blood (**Figure 18**). In addition, no significant difference has been revealed by comparing both $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells percentage between MDS patients and controls (data not shown).

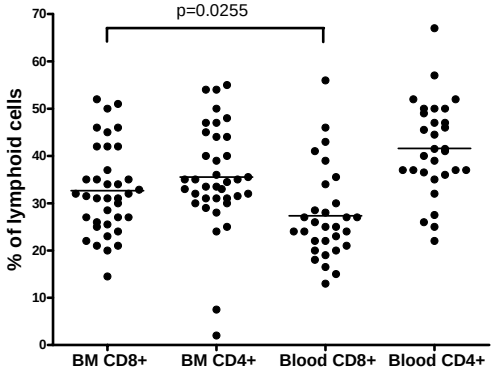


Figure 18. **BM recruitment of effector citotoxic T cells.** Multiparametric analysis of MDS BM and peripheral blood, performed by immunofluorescence and flow citometry, shows a significant increase of $CD3^+CD8^+$ T cell percentage in BM of MDS patients, compared to peripheral blood. (p=0,0255). No significant differences have been observed for $CD3^+CD4^+$ T cells between the two compartments. The analysis has been performed on lymphoid population.

In order to more deeply investigate the role of $CD3^+CD8^+$ CTL in MDS pathogenesis, these lymphocyte subsets have been analyzed in different prognostic groups: as shown in **figure 19** the percentage of BM $CD3^+CD8^+$ T cells was increased in comparison to peripheral blood in IPSS Low (p=0,042) e IPSS INT1 (p: 0,0027) groups. No significant difference has been observed in INT2 group.

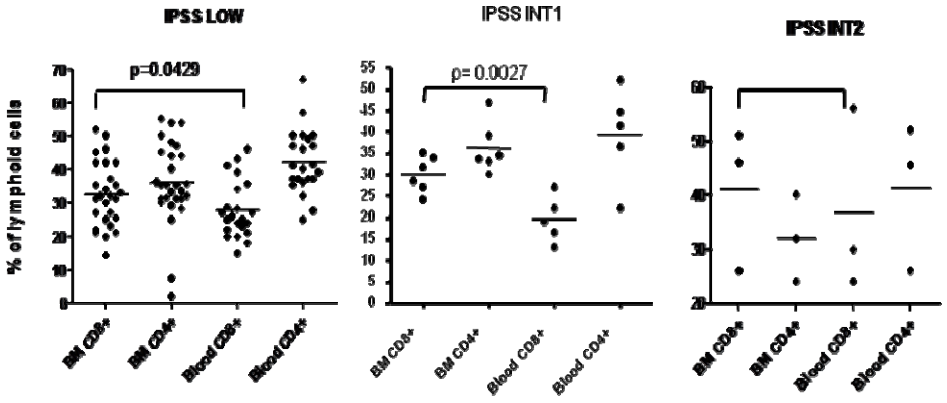


Figure 19. **BM recruitment of effector citotoxic T cells in MDS disease stages.** Statistical analysis of BM and peripheral blood effector T cells in different score groups. Difference between $CD3^+CD8^+$ citotoxic T cell percentage in BM and blood is significant in Low (p=0,042) and Int1 groups (p=0,0027). No significant difference is observed is INT2 group.

These data suggested a BM recruitment of effector CTL in MDS patients in first disease stages. To quantify the phenomena, the $CD4^+/CD8^+$ T cell ratio in BM and peripheral blood of MDS patients at different disease stages has been evaluated. The statistical analysis revealed a significant difference of

$CD4^+/CD8^+$ T cell ratio between MDS BM and peripheral blood in IPSS Low (p=0,0251) e INT1 (p=0 ,0042) groups. (**Figure 20**)

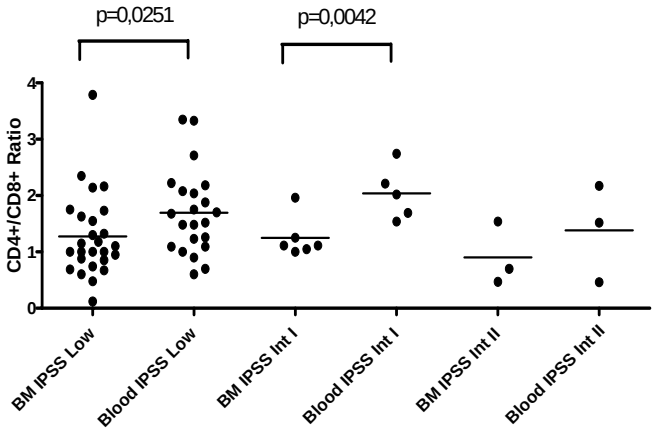


Figure 20: **Comparative analysis of CD4/CD8 T cell ratio in BM versus peripheral blood in MDS patients**. CD4/CD8 T cell ratio in BM and in peripheral blood of MDS patients, belonging to Low, Int-1 and Int-2 prognostic groups, are indicated. Statistical analysis of data revealed the occurrence of a significant decrease in BM CD4/CD8 T cell ratio in Low (p=0.02) and in Int-1 (p=0.004) prognostic groups, as compared with the peripheral blood. No difference in the BM versus peripheral blood distribution has been observed in the Int-2 MDS risk group.

To evaluate the role of these effector CTL in MDS progression, the expression levels of CD54 (intracellular-adhesion cell-1) on MDS $CD8^+$ and $CD4^+$ T cells has been evaluated. During activation, T cells up-regulate a great number of surface molecules, able to improve the functional effectiveness. The cytofluorimetric analysis of MDS bone marrow samples showed a significant decrease of CD54 expression on $CD8^+$ (p=0,01) and $CD4^+$ T cells (p=0,03) in High/Int2 score groups, compared to Low/Int1 MDS groups and healthy control (**Figure 21**). This data could suggest an involvement of activated effector T cell population in MDS progression to neoplastic disease (AML).

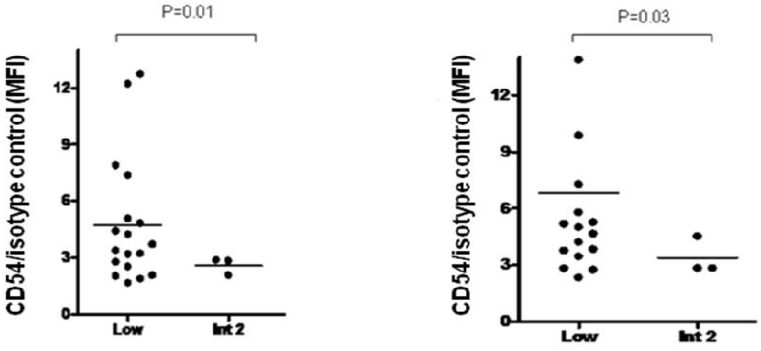


Figure 21: **Analysis of activation state of effector T cells.** CD3+CD4+ and CD3+CD8+ BM T cells show decreased CD54 expression in INT2 disease score groups in comparison to Low group. CD54 (ICAM-1) is an adhesion molecule, generally up-regulated after activation. Statistical analysis reveale a significant difference between two groups for two lymphocite populations, $CD8^+$ (p=0,01) and $CD4^+$ T cells (p=0,03)

Immune regulation in MDS: Regulatory T cells in different disease stage

Since the relevance of Treg cells $CD4^+CD25^+FoxP3^+$ in regulation of effector T cells functional effectiveness, as well as in tumor immune-surveillance control, the levels of this cell population in BM and peripheral blood of MDS patients has been also investigated. Preliminary data, obtained using immunofluorescence and flow cytometric analysis, revealed a significant increase of Treg cell percentage in the BM of High/Int2 disease score group, in comparison with Low/Int1 groups and healthy donor (**Fig. 22**). No significant difference has been observed between Treg cell levels in peripheral blood of different disease score MDS groups (not shown).

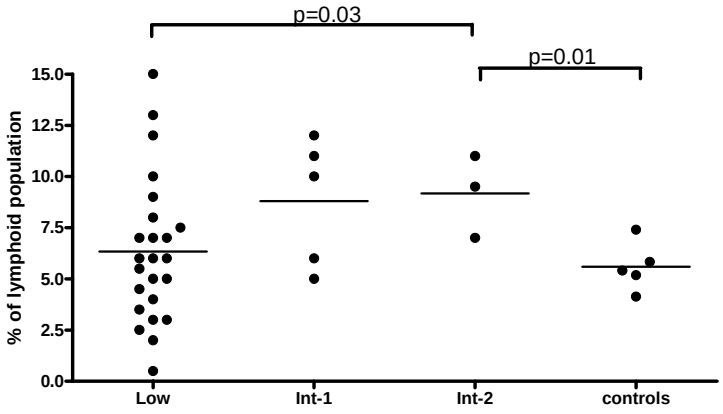


Figure 22: Analysis of Treg in BM of MDS patients. Treg percentage in BM of MDS patients belonging to Low, Int-1 and Int-2 groups, are indicated in comparison with healthy controls. As shown, statistical analysis of data revealed the significant increased levels of BM regulatory T cells in the Int-2 MDS group as compared with the Low risk and the controls (p=0.03 and 0.01, respectively).

Discussion

This study indicates that immune-mediated mechanisms can underlie the occurrence of hemopoietic disorders characterized by clonal expansion of defective and/or dysplastic clones in PNH and MDS patients. Indeed, functional defects characterize both the innate and adaptive GPI-defective immune compartments in PNH patients. In addition, significant increased frequency of HLA-DRB1*1501 allele ($p<0.01$) and of the B*1402, Cw*0802 haplotype ($p<0.0005$) as part of the extended Mediterranean haplotype A*33, B*1402, Cw*0802, DRB1*0102 ($p<0.005$) has been revealed in our cohort of PNH patients. Moreover, an altered CD40-dependent pathway and regulatory T cell tolerance control has been observed in PNH and MDS patients, respectively

Innate and adaptive GPI-defective compartments in PNH patients: the granulocytes.

This study suggests that GPI-defective granulocytes exhibit enhanced ingestion capability associated with impaired production of ROS in response to bacterial stimulation in PNH patients. This functional behavior seems to be specifically associated with PNH. Indeed, nor patients affected by Chronic Granulomatosis Disease (CGD), caused by a defect in the NADPH oxidase genes, with their heterozygous carriers, neither neutropenic individuals showed similar alterations.

The contact between opsonized bacteria and granulocytes is mainly mediated by specific receptors for Fc fragment of immunoglobulins and for complement fractions. The recruitment of both receptors has been described to generate synergistic effects[80], as compared with single receptor engagement. In addition, increased serum concentration of complement fractions has been associated with over-expression of the specific membrane receptors and enhancement of their affinity[81,82]. Indeed, increased binding of the third component of Complement (C3) on PNH granulocytes has been observed[83] suggesting the prevalent occurrence of a complement-mediated ingestion pathway in GPI-defective PMN.

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The ex vivo altered phagocytosis, observed in GPI$^-$ PMN from PNH patients, suggests the presence of a cell population altered in vivo. The absence of the GPI-linked CD55 and CD59 molecules, that mediate complement inactivation, increases the level of activated complement fractions in PNH. The availability of such activated complement components on the surface of GPI-defective PMN could act as a chronic triggering factor, as described for PNH platelets[84]. The increasing of activated C3 molecules, followed by over expression of higher affinity receptors on PNH granulocytes[81], may thus represent a chronic triggering element for the phagocytic process, likely able to mediate an enhanced ingestion ability by GPI$^-$ PMN.

The involvement of multiple pathways in regulating the engulfment processes points to the dispensable role for PKC in early phagocytosis processes, also suggesting the ability of integrin complement receptors to act as PKC depleting stimuli[85] in PNH patients. Our data confirm such observations suggesting the dominant role of PKC for respiratory burst induction with less relevant involvement in bacterial ingestion pathways. Moreover, our data indicate the occurrence of both increased ingestion ability and severe ROS production impairment in GPI-defective PMN. The analysis was performed by using two distinct bacterial stimuli, as represented by N-formil peptides and opsonized E. Coli recognized by specific receptors on PMN cell membrane. Defective ROS production was also observed after respiratory burst triggering by PMA, a DAG–homologous widely used as extensive PKC activator[86]. This biological behavior seems to characterize GPI-defective PMN in PNH, if compared with neutropenic or CG patients. On the basis of this observation, one could rule-out that increased engulfment could represent only a compensatory mechanism for the deficient burst induction.

The observation that two independent bacterial stimuli and PMA triggering were unable to induce normal ROS production in GPI-defective PMN might indicate the occurrence of decreased levels of intracellular PKC. In this regard, a possible relationship between enhanced C3 molecule-dependent phagocytosis, chronic intracellular PKC depletion and impaired efficiency of the respiratory burst in the GPI-defective granulocytes can be

hypothesized in PNH patients. Notably, the occurrence of activation-induced depletion of PKC, as a pathogenetic mechanism for impaired ROS production, has been recently demonstrated in septic patients[87].

Thus, the involvement of multiple intracellular pathways in the regulation of ingestion mechanisms[88] and the key role of PKS in mediating oxidative burst induction can account for the persistence of an increased ingestion ability in the presence of an impaired respiratory burst induction in human PNH granulocytes.

These alterations, together with the recently described defective adhesion and migration of PNH granulocytes, dependent on the absence of the GPI-linked molecules CD157[89] and GPI-80[90]points to the possibility that a decreased functional effectiveness of the GPI-defective PMN compartment could impair the innate immune response against pathogens, mainly compromised by cytopenia, in PNH patients. The clinical relevance of such functional defects needs more conclusive assessment.

Innate and adaptive GPI-defective compartments in PNH patients: the monocytes and dendritic cells.

Monocytes and granulocytes, the main effector populations of the innate compartment, play a key role in orchestrating the immune response. Indeed, they mediate essential effector activities (phagocytosis and the oxidative burst) to control pathogen survival and expansion[91] but are also involved in the activation of the adaptive immune response[92].

Recognition of the pathogens as well as the indirect sensing of infections trough inflammatory cytokines are involved in entering monocytes into an integrated developmental program called DC differentiation[93]. Monocyte-derived DC represents a key cell population for antigen-dependent activation of naive T lymphocytes[94] . Others and our data showed that a majority of granulocytes and monocytes in peripheral blood of PNH patients are GPI-defective.

Our data show that the treatment with GM-CSF and rIL-4 of GPI-defective monocytes was unable to generate in vitro functional DCs. GM-CSF and IL-4 receptors are expected to be functional in PNH patients, since both molecules do not need a GPI anchor to be expressed on the cell

membrane. Indeed, GM-CSF receptor-dependent medullar mobilization of GPI defective precursors was described in PNH patients.[95]

DCs are key regulators of the immune response and their differentiation reflects an ordered series of events involving specific gene expression patterns, intracellular protein targeting and organelle biogenesis leading to potent immuno-modulatory functions.[94,96]. The up-regulation of GPI-linked molecules, as represented by the CD48 antigen, has been already described as part of the DC differentiation program.[97] Therefore, it is possible that cell-to-cell functional interactions involving GPI-linked structures affect monocyte differentiation into DC. In addition, an involvement of GPI-linked structures in the molecular machinery underlying in vitro monocyte-DC differentiation could be hypothesized.

The lack of CD1a surface expression, the presence of CD86 and the mannose receptor expression are the hallmark of the DC population obtained in vitro from the PNH monocytes. CD1a represents a third class of MHC molecules able to associate with a wide range of lipids and glycolipidic antigens to be presented to $CD8^+$, $CD8^-CD4^-$ and γδ-T-lymphocytes.[98] In addition, the critical involvement of CD1 antigens in the DC-dependent activation of regulatory T and NKT lymphocytes in both mice and human models has been suggested.[99] Several observations indicate CD1 down-modulation, combined with a variable CD86 surface expression as a common feature of DC functional impairment occurring in cancer [100] as well as in immune-deficiency models[101].

Our data show a severe impairment of the DCs obtained from GPI-defective monocytes to deliver optimal accessory signals to TCR-triggered T lymphocytes, even in the presence of high levels of CD86 co-stimulatory molecule. In this respect, the observed lesser cytokine production and the availability of putative inhibitory molecules by DCs could account for such impairment in PNH. The level of co-stimulation depends on the expression of co-stimulatory molecules as well as on the stability of the immunological synapse[102]. In this context, the relevance of a correct recruitment of the lipid rafts, membrane domains rich in signaling molecules as well as in GPI-linked structures,[103] for the achievement of an optimal primary T-cell response has been established.[104]

The observation that GPI-defective monocytes generate impaired DC differentiation in vitro suggests the existence of a defect in the physiological cross talk between the innate and the adaptive immunity in PNH patients, whose monocyte compartment is completely GPI-defective. In addition to the frequent neutropenia and to the observed functional impairments of PNH neutrophils, the altered DC-differentiation of PNH monocytes may have also a role for the increased susceptibility to infections often observed in PNH patients.[105]

Innate and adaptive GPI-defective compartments in PNH patients: the T lymphocytes

This study reveals the occurrence of significant functional alterations in GPI-defective T cell compartment. Severe defects in TCR-dependent proliferation, CD25, CD54 and HLA-DR surface expression and cytokine production in the GPI-defective T cell population were demonstrated.

In this regard, the inability of healthy donor monocytes to overcome such impairment strongly supports the occurrence of some defects in the TCR-dependent activation machinery of the GPI-defective T lymphocytes. These data confirm and extend previous reports showing severe dysregulation of the immune system in a Pig-a^{-}/Rag$^{-/-}$ chimeric murine model[106], impaired lectin-triggered proliferation[107] and TCR-dependent signaling in GPI^{-} T cells[108].

In addition, a relationship between the lack of GPI-linked proteins and the occurrence of functional impairment of immune effectors, seem to be confirmed also in lymphoid compartment.

Several evidences suggest that lipid rafts regulate dynamic interactions between T cell signaling proteins[109]. Thus, raft micro-domains could represent key platforms that couple events outside the cell with signaling pathways inside the cell, also trough GPI-linked protein-dependent interactions.[110] Therefore, the involvement of the lack of GPI-linked proteins, observed in PNH, in the pathogenesis of the impaired TCR-triggered activation could be hypothesized.

Our observations, as a whole, strongly support the possibility that GPI-defective clones have to evolve under permissive conditions, in a

background that impairs normal haematopoieis. Indeed, all the GPI-defective immune-effectors by us analyzed showed significant functional impairment in PNH patients. Moreover, a number of data indicate that not mutated PIG-A$^+$ stem cells are markedly impaired in their growth in PNH patients[111].

The possibility that immune-mediated mechanisms might account for the clonal dominance of the PIG-A mutation and for the development of PNH has been already suggested[36,39,105,112]. In this regard, PNH patients showing a marked cytopenia, likely suggesting the occurrence of an active bone marrow failure process, are expected to represent optimal models to address such hypothesis. In order to address such issue, we analyzed GPI$^+$ T cell compartment in such a group of PNH patients. Our data indicate the occurrence of significant increase of TCR-dependent proliferation as well as of CD25, CD54 and HLA-DR surface expression. In these patients a possible defective regulation of T cell homeostasis, also affecting the functional asset of T lymphocytes, could be consequent to a perturbation of immune tolerance. Such condition could promote the generation of hyper-activated T cell effectors accounting for the increased response to TCR-triggering by us observed in vitro. In our experimental model the employment of multi-parametric immune fluorescence technique allows the analysis of GPI$^+$ compartment maintaining the biological complexity of PNH and avoiding any functional interference due to the separation procedures. Therefore, the increased activation level reached ex vivo by the GPI$^+$ T cell compartment of PNH patients might suggest the presence of a T cell population, which is altered even in vivo. In this context, the presence of a chronic stimulation, likely consequent to a defective control of activated complement components, as suggested for PNH platelets[84] cannot be ruled out.

Analysis of tolerance control mechanisms in PNH: the CD40-CD40 ligand pathway

Interactions between co-stimulatory ligands and their receptors are critical for activation of T lymphocytes, prevention of tolerance and development of T cell-dependent immunity. Moreover, only a few co-

stimulatory molecules are expressed on T cells in a constitutive fashion, whereas a majority is induced following cell activation subsequent to antigen recognition by TCR[113]. The CD40-dependent pathways have been described as relevant for the control of both innate and adaptive immune responses and for the achievement and maintenance of immune tolerance[114]. Given the relevance of these pathways, a tightly regulated expression of CD154, the CD40 ligand, could be necessary to maintain the antigen specificity of T cell activation.

This study shows a significant increase and the functional persistence of CD154 on TCR-triggered $CD4^+GPI^+$ lymphocytes from PNH patients. Our data indicate that 8 hour anti-CD3 treated lymphocytes from PNH patients showed increased ability to up-regulate surface CD23 levels on B cells, already demonstrated to represent a relevant target for CD154-dependent triggering of B lymphocytes[78]. Moreover, we observed that 48 hour anti-CD3 cultured lymphocytes from PNH patients, but not from healthy controls are able to up-regulate such molecule. These effects were significantly, but not completely inhibited by anti-CD40 mAb target treatment. The observed CD154 persistence on 48 hour anti-CD3 treated $CD4^+CD48^+$T cells from PNH patients are expected to likely account for such effect.

Altered persistent expression of CD154, the CD40 ligand, has been described in pathological conditions characterized by a defective control of autoreactive clones[115], as represented by SLE. Therefore, the persistence of a functional CD154 molecule on the TCR-triggered $CD48^+CD4^+$ lymphocytes could be suggested as relevant for the possible immune-mediated pathogenesis of the disease in PNH.

The observation that 8 hour medium-treated $CD48^+CD4^+$ lymphocytes from PNH patients show significant ability to up-regulate CD23 surface levels, in the absence of detectable CD154 expression, points to the involvement of both CD40-dependent and independent pathways in mediating such effects. These data, according with the presence of a skewed T cell repertoire in PNH[116] and AA patients[39] suggest the occurrence of dysregulation of the biological mechanisms underlying tolerance maintenance in PNH patients.

Looking for immune-mediated mechanisms in GPI-defective clone expansion in PNH: the HLA genes

The association between an auto-immune disease and certain HLA alleles has been attributed to the ability of these alleles to bind and present specific peptide auto-antigens to T cells with the appropriate receptors. [23,117] Our data suggest the involvement of a large shared peptide ligand, able to bind DRB1*1501 specificity, in all the populations of PNH patients till now analyzed[118,119]. In addition, the significant increase of the haplotype B*1402, Cw*0802, as part of the extended Mediterranean haplotype A*33, B*1402, Cw*0802, DRB1*0102 ($p<0.0005$ and $p<0.005$, respectively) suggest the specific involvement of other HLA-dependent factors in our Italian PNH population.

The described association between certain HLA types and a higher risk to develop PNH may provide further insight into the mechanism for selective damage to normal (GPI^+) hematopoiesis, which is responsible for the clonal expansion of GPI-defective hematopoiesis that ultimately gives rise to clinical PNH. Indeed, the possibility that critical target molecules could be identified in order to propose new innovative therapeutical approaches has to be considered.

Moreover, our findings might appear to be in conflict with recent work, showing that patients with different HLA types have expanded T-cell clones with the same or very similar TCR-β sequences[116], suggesting that the target auto-antigen may not be a peptide. However, other not mutually exclusive interpretations are possible:

- Since HLA genomic region contains many non-HLA genes with putative or proven immune functions, [117] the association here reported could be related to an increased ability to produce pro-inflammatory cytokines that may damage hematopoiesis. The fact that we have found a much stronger association with an extended haplotype than with any individual HLA allele is in keeping with this hypothesis.
- Peptides bound to certain HLA alleles may recruit T cells that, directly or indirectly, will in turn enhance the response to a non-peptide antigen.

- the target auto-antigen could be a peptide in some patients with PNH and a non-peptide in others.

Tolerance control dysregulation and expansion of dysplastic clones in MDS: BM regulatory T cells.

Taken in all, our data support the hypothesis that an immune-mediated attack against a normal Stem precursor could underlie the emergence and expansion of GPI-defective clone/s in PNH. Moreover, the analysis of MDS patients revealed the occurrence of significant alterations in the BM recruitment/activation state of immune effectors. Indeed, our analysis showed a significant BM recruitment of $CD8^+$ $CD3^+$ lymphocytes in the BM samples of MDS patients. The presence of such cytotoxic effectors was specifically observed in the BM of MDS patients belonging to the low/INT1 risk group, while no significant differences with the controls was observed in the high score disease group. Moreover, the analysis of the expression levels of CD54 (ICAM-1), whose expression has been related to the activation state of $CD4^+$ and $CD8^+$ effector T cells, showed similar distribution. This results support the hypothesis that BM immune effectors could have a role in the pathogenesis of MDS.

One of the model of MDS development proposes the occurrence of a multi-step process during which the neoplastic transformation of hematopietic stem cell could induce the expression of neoantigens, able to generate an immune-mediate attack. Moreover, the possibility that an autoimmune attack against normal stem precursor could occur needs to be investigated. Cytofluorimetric and molecular (spectratyping) data, showed the presence of oligoclonal T cell repertoire in MDS BM[120,121,122], while both RT-PCR and immunoistochemistry analysis revealed the increase of proinflammatory cytokines in MDS BM[123]. The possibility that such a proinflammatory microenvironment could represent the main cause of increased apoptosis of BM precursors, has been also suggested[124,125]. Indeed, Stifter et al. showed a close relationship between TNFα serum levels and anemia in AR and AREB MDS subtypes, indicating a relevant role of the cytokine with increased apoptosis of erythroid precursors in earlier state of disease[126]. Recently, microarray data, revealing the selective induction of

IFN-gamma-dependent genes in BM $CD34^+$ precursors in PNH and MDS, confirmed the critical relevance of a pro-inflammatory microenvironment in the pathogenesis of both diseases[127].

Our data, according with recent reports showing that immunosuppressive therapy with anti-thymocyte globulin and/or cyclosporine A can lead to lasting hematological responses and abrogation of pathogenetic T-cell clones in low-risk MDS[128,129], revealed an increase of CD3+CD8+ lymphocytes as well as of their CD54 surface levels in BM of Low/INT1 MDS patients. Thus the occurrence of CTL-dependent immune-mediated mechanisms in the earlier disease stages of MDS might be hypothesized.

Treg cells play an important role in the control of auto-reactive effectors as well as in the regulation of immune recognition of transformed clones. Moreover, recent data have been demonstrating that BM might harbor $CD4^+CD25^+$ Tregs and that can function as a reservoir for them[130], thus representing an important organ to fine tune T-cell immunity by modulating Treg trafficking.

Recent reports indicate the occurrence of defective Treg response in AA[131]and an increase of such regulatory population in the peripheral blood of MDS patients belonging to more advanced disease stage[132]. Our preliminary data revealed a significant increase of $CD4^+CD25^+Foxp3^+$ BM Treg cells in INT2/High score MDS groups, compared with the Low/INT1 patients. Therefore, the possibility that the occurrence of Tregs defects in BM may be associated, in low-risk MDS, with the emergence of auto-reactive T-cell clones, responsible for BM hypoplasia can be suggested. In addition, an expansion of this regulatory population in the late disease stages, could represent an effective escape mechanism of the dysplastic clone/s to immune-mediated control of transformation, as described in other malignant diseases.

Growth vantage and/or immune-mediated escape in hematopoietic disorder: the role of MHC molecules

A CASE REPORT

Since the great number of evidences that suggest the relevance of immune effectors in emergence of dysplastic clones, we investigated the pathogenesis of monocytosis and severe neutropenia in a MDS patient showing BM Natural Killer (NK) expansion.

A 73 year old MDS patient presenting mild anemia and neutropenia, showed $CD56^{+}CD3^{-}$ NK cell expansion in BM and peripheral blood with $CD14^{+}CD56^{+}$ monocytosis and neutropenia. In this context, to assess the possibility of clonal origin of expanded NK cell population, phenotypic analysis of these cells has been performed. The $CD56^{+}CD3^{-}$ NK cells present a random distribution of specific NK receptor both in BM and peripheral blood, suggesting polyclonality of these expanded cells. In order to investigate the functional effectiveness of NK cells, a cytotoxicity assay has been performed, using K562 cell lines as a target, as previously described[133]. The study revealed no significant differences between NK cells isolated from the MDS patient and from healthy donor. In this context, in order to address the possible role of NK cells in defective granulocyte maturation and expansion of $CD14^{+}CD56^{+}$ monocytes, a cytotoxicity assay has been performed against granulocytes and monocytes isolated from MDS patient and healthy control. A strong increase of NK-cell mediates lysis susceptibility has been observed for MDS granulocytes; no lysis has been observed against MDS monocytes and against healthy donor derived monocytes and granulocytes. This result suggested a possible role of these effector cells in neutropenia. To better understand the different NK-lysis susceptibility of MDS monocytes and granulocytes, MHC and MHC-associated molecules asset, able to regulate the cytotoxic function of these cells, has been investigated. Cytofluorimetric analysis showed a sever defect of non classic-MHC molecule HLAE on the surface of MDS patient monocytes and granulocytes, compared with healthy donor cells. It has been described that alteration of NK cell-target interactions, can be considered as

pathogenetic cofactor operating in vivo together with other immunological mechanisms, all contributing in giving the final pathological treat. In Celiac Disease, recent data, published by our group[134], revealed that gliadin could have a detrimental role in the regulation of NK cell-immature DC (iDC) cross-talk with consequences on the adaptive immunity activation. iDC gliadin treatment inhibits NK cell cytoxicity against iDC via the interaction between CD94/ NKG2A and HLA-E. Indeed, gliadin increases the HLA-E expression on iDC and the blocking of CD94/NKG2A receptor on NK cells restores their killing against gliadin-treated iDC. Moreover, the relevance of this molecule for the pathogenesis of hematopoietic diseases[135] has been suggested. Since the severe deficit of HLAE on the two different myeloid populations, the attention has been focused on the other MHC molecules; the analysis revealed the presence of decreased levels of HLA-class I expression on MDS granulocytes surface, compared to MDS monocytes and to healthy donor cells. These data could support the hypothesis that a different expression of classical MHC molecules could account for the different susceptibility of granulocytes and monocytes to NK lysis. In addition, phenotypic analysis of myeloid compartment showed abnormal expression of CD178 (Fas-Ligand) on $CD14^{+}CD56^{+}$ monocytes. To assess the functional relevance of the increased CD178 up-regulation on the surface of monocytes from this MDS patient, the ability of these cells to induce apoptosis in a CD95 positive (CD178 receptor) Jurkat T cells has been performed. After co-culture of Jurkat cell line and monocytes isolated from MDS patient and healthy control, the apoptosis levels, measured as Annexin V-positive cells, has been analyzed. , The percentage of positive cells in presence of MDS monocytes was significantly increased in comparison with healthy donor. This result could suggest that monocytes could contribute to defective granulopoiesis in this MDS patient.

Conclusions

This study analyzed two hematological disorders, showing different clinical manifestations, in which defective or dysplastic clonal hematopoiesis develops through several lineages and replaces the normal counterpart: PNH and MDS. In particular, the critical involvement of immune-mediated mechanisms for the pathogenesis of clonal dominance of the GPI-defective compartment and for selection/shaping of dysplastic clone has been investigated.

To this aim, the immunological asset of PNH patients has been analyzed, focusing on the functional difference between GPI^+ and GPI-defective compartments. The study revealed a critical functional impairment of GPI-defective cells, both in innate and adaptive compartments, indicating that the growth vantage of GPI-defective clone is relative. In addition, a significant increase in the functional effectiveness of PNH patients GPI^+ T cells has been revealed. Therefore, the increased activation level reached ex vivo by the GPI^+T cell compartment of PNH patients might suggest the presence of a T cell population, which is altered even in vivo. This data strongly support the possibility that GPI-defective clones could evolve under permissive conditions in a background that impairs normal hematopoieis. Since the relevance of CD40-dependent pathways for the control of both innate and adaptive immune responses and for the achievement and maintenance of immune tolerance, the study of CD40-dependent pathways in PNH patients has been addressed. The study revealed a functional persistence of CD154 (CD40-ligand) on GPI^+T cells, as previously described for other autoimmune disease. This evidence suggests that the alterations of CD40-dependent tolerance control could underlie an immune-mediated bone marrow failure likely associated with the expansion of the PIG-A defective clone. HLA represent a major susceptibility factor for autoimmune diseases. In this context, analysis of HLA alleles in PNH patients revealed an increased frequency of a specific set of HLA alleles in a large cohort of PNH patients. This result may provide further insight into the mechanism for selective damage to normal (GPI^+) hematopoiesis, which is responsible for the clonal expansion of GPI-defective hematopoiesis that ultimately gives rise to clinical PNH.

In order to investigate whether the growth advantage of the dysplastic, often clonal hematopoiesis, in MDS patients, could be dependent on damaging mechanisms preferentially affecting the normal counterpart in the bone marrow microenvironment, the study addressed the BM immunological asset in a group of MDS patients, at different disease stage. The analysis revealed a BM recruitment of $CD3^{+}CD8^{+}$ effector T cells in MDS patients in earlier stage of disease; in the same patients, these cells showed an increase of CD54 expression, whose critical relevance for CTL effector function has been largely described. These results confirm the hypothesis that in low-risk MDS, the emergence of auto-reactive T-cell clones could be responsible for BM hypoplasia. Moreover, the observed expansion of regulatory populations in the late disease stages, could represent an effective escape mechanism of the dysplastic clone/s to immune-mediated control of transformation, as described in other malignant diseases.

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APPENDIX: Table I. HLA allele frequency distribution in PNH patients versus healthy controls

HLA Genes	Controls [a] n (%)	PNH[a] n (%)	p[b]	Odd Ratio (95%CI)	
A*02	119 (39.5)	23 (54.8)	N.S	1.85	(0.96-3.54)
A*0201	97 (32.2)	21 (52,5)˜	0.03	2.32	(1,19-4,52)
A*0205	22 (7.3)	/			
A*0206	/	/			
A*33	7 (2.3)	4 (9.5)	N.S	4.42	(1.23-15.81)
B*14	18 (5.9)	10 (23.8)	0.01	4.91	(2.08-11.55)
B*1402	16 (5.3)	10 (23.8)	0.00064	5.56	(2.33-13.29)
Cw*08	18 (5.9)	10 (23.8)	0.009	4.91	(2.08-11.55)
Cw*0802	18 (5,9)	10 (23.8)	0.0014	4.91	(2.08-11.55)
DRB1*15	30 (9.9)	8 (21.1)	N.S	2.40	(1.01-5.73)
DRB1*1501	16 (5.3)	8 (21.1)	0.006	4.75	(1.87-12.02)
DRB1*01	33 (10.9)	12 (31.6)	0.01	3.74	(1.72-8.12)
DRB1*0101	16 (5.3)	6 (15.7)	0.05	3.34	(1.22-9.14)
DRB1*0102	17 (5.6)	5 (13.1)	N.S.	2.57	(0.87-7.31)
DRB1*07	82 (26.8)	4 (10.5)	N.S	0.31	(0.10-0.91)
HLA Haplotype					
B*1402, Cw*0802	16 (5.3)	10 (23.8)	0.00032	5.56	(2.33-13.29)
A*33, B*1402, Cw*0802, DRB1*0102	2 (0.66)	4 (10.5)	0.001	17.58	(3.10-99.65)

[a] data refer to 301 healthy controls and 42 or 38 PNH patients for HLA class I and class II respectively

[b] p was calculated by Fisher's two tailed exact test; Bonferroni correction of p values was performed for allele association by considering specificities with a frequency >0.5% in Italians (i.e. 14 alleles at A locus with 3 specificities for A*02, 21 at B locus with 2 specificities for B*14, 13 at C locus with 2 specificities for Cw*08, 12 at DRB1 locus with 2 specificities for DRB1*01 and 3 for DRB1*15)